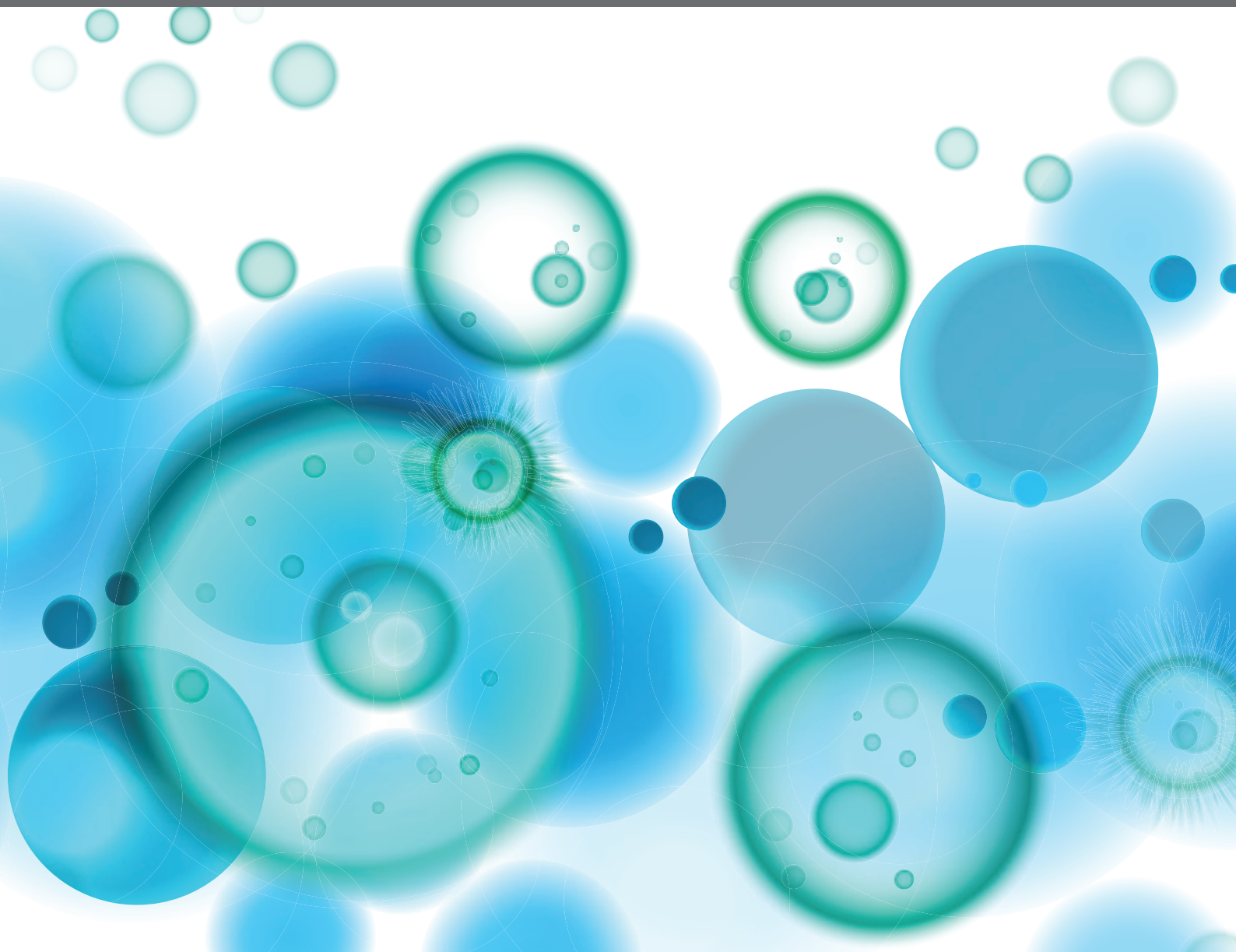


DIAGNOSTIC AND THERAPEUTIC APPLICATIONS OF PENTRAXIN AND PENTRAXIN-ASSOCIATED PROTEINS

EDITED BY: Kenji Daigo, Barbara Bottazzi and Richard H. Gomer
PUBLISHED IN: Frontiers in Immunology





frontiers

Frontiers eBook Copyright Statement

The copyright in the text of individual articles in this eBook is the property of their respective authors or their respective institutions or funders. The copyright in graphics and images within each article may be subject to copyright of other parties. In both cases this is subject to a license granted to Frontiers.

The compilation of articles constituting this eBook is the property of Frontiers.

Each article within this eBook, and the eBook itself, are published under the most recent version of the Creative Commons CC-BY licence.

The version current at the date of publication of this eBook is CC-BY 4.0. If the CC-BY licence is updated, the licence granted by Frontiers is automatically updated to the new version.

When exercising any right under the CC-BY licence, Frontiers must be attributed as the original publisher of the article or eBook, as applicable.

Authors have the responsibility of ensuring that any graphics or other materials which are the property of others may be included in the CC-BY licence, but this should be checked before relying on the CC-BY licence to reproduce those materials. Any copyright notices relating to those materials must be complied with.

Copyright and source acknowledgement notices may not be removed and must be displayed in any copy, derivative work or partial copy which includes the elements in question.

All copyright, and all rights therein, are protected by national and international copyright laws. The above represents a summary only. For further information please read Frontiers' Conditions for Website Use and Copyright Statement, and the applicable CC-BY licence.

ISSN 1664-8714

ISBN 978-2-88974-189-2

DOI 10.3389/978-2-88974-189-2

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.

Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

DIAGNOSTIC AND THERAPEUTIC APPLICATIONS OF PENTRAXIN AND PENTRAXIN-ASSOCIATED PROTEINS

Topic Editors:

Kenji Daigo, Cosmic Corporation Co., Ltd., Japan

Barbara Bottazzi, University of Milan, Italy

Richard H. Gomer, Texas A&M University, United States

Citation: Daigo, K., Bottazzi, B., Gomer, R. H., eds. (2022). Diagnostic and Therapeutic Applications of Pentraxin and Pentraxin-Associated Proteins. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88974-189-2

Table of Contents

- 05** *A Functional Genetic Variant at the C-Reactive Protein Promoter (rs3091244) Is Not Associated With Cancer Risk in a Chinese Population*
Ming-Yu Wang, Hai-Hong Zhou, Chun-Miao Zhang, Hai-Xiang Su, Shuo-Lei Li, Shang-Rong Ji, Enqi Liu and Yi Wu
- 11** *Conformationally Altered C-Reactive Protein Capable of Binding to Atherogenic Lipoproteins Reduces Atherosclerosis*
Asmita Pathak, Sanjay K. Singh, Douglas P. Thewke and Alok Agrawal
- 19** *Complement Activation by C-Reactive Protein Is Critical for Protection of Mice Against Pneumococcal Infection*
Sanjay K. Singh, Donald N. Ngwa and Alok Agrawal
- 26** *The Basic Characteristics of the Pentraxin Family and Their Functions in Tumor Progression*
Zeyu Wang, Xing Wang, Hecun Zou, Ziyu Dai, Songshan Feng, Mingyu Zhang, Gelei Xiao, Zhixiong Liu and Quan Cheng
- 42** *How C-Reactive Protein Structural Isoforms With Distinctive Bioactivities Affect Disease Progression*
Ibraheem M. Rajab, Peter C. Hart and Lawrence A. Potempa
- 54** *Treatment of Pneumococcal Infection by Using Engineered Human C-Reactive Protein in a Mouse Model*
Donald N. Ngwa, Sanjay K. Singh, Toh B. Gang and Alok Agrawal
- 69** *C-Reactive Protein and Cancer—Diagnostic and Therapeutic Insights*
Peter C. Hart, Ibraheem M. Rajab, May Alebraheem and Lawrence A. Potempa
- 86** *Pronounced Diurnal Pattern of Salivary C-Reactive Protein (CRP) With Modest Associations to Circulating CRP Levels*
Jonas Wetterö, Sarah von Löhneysen, Flordelyn Cobar, Margareta Kristenson, Peter Garvin and Christopher Sjöwall
- 97** *C-Reactive Protein-Based Strategy to Reduce Antibiotic Dosing for the Treatment of Pneumococcal Infection*
Donald N. Ngwa, Sanjay K. Singh and Alok Agrawal
- 105** *C-Reactive Protein Levels in Systemic Lupus Erythematosus Are Modulated by the Interferon Gene Signature and CRP Gene Polymorphism rs1205*
Helena Enocsson, Birgitta Gullstrand, Majja-Leena Eloranta, Jonas Wetterö, Dag Leonard, Lars Rönnblom, Anders A. Bengtsson and Christopher Sjöwall
- 111** *The Role of PTX3 in Mineralization Processes and Aging-Related Bone Diseases*
Umberto Tarantino, Chiara Greggì, Ida Cariati, Virginia Veronica Visconti, Monica Gasparini, Marco Cateni, Elena Gasbarra, Annalisa Botta, Antonietta Salustri and Manuel Scimeca
- 117** *Therapeutic Lowering of C-Reactive Protein*
Rachel V. Jimenez and Alexander J. Szalai

- 126 Identification of Neuronal Pentraxins as Synaptic Binding Partners of C1q and the Involvement of NP1 in Synaptic Pruning in Adult Mice**
 Réka Á. Kovács, Henrietta Vadászi, Éva Bulyáki, György Török, Vilmos Tóth, Dominik Mátyás, Judit Kun, Éva Hunyadi-Gulyás, Flóra Zsófia Fedor, Ádám Csincsi, Katalin Medzihradsky, László Homolya, Gábor Juhász, Katalin A. Kékesi, Mihály Józsi, Balázs A. Györffy and József Kardos
- 143 C-Reactive Protein Triggers Cell Death in Ischemic Cells**
 Ahmed Sheriff, Stefan Kayser, Patrizia Brunner and Birgit Vogt
- 151 Anti-Pentraxin Antibodies in Autoimmune Diseases: Bystanders or Pathophysiological Actors?**
 Benoit Brilland, Emeline Vinatier, Jean-François Subra, Pascale Jeannin, Jean-François Augusto and Yves Delneste
- 162 Endogenous Long Pentraxin 3 Exerts a Protective Role in a Murine Model of Pulmonary Fibrosis**
 Federica Maccarinelli, Mattia Bugatti, Ander Churrua Schuind, Sara Ganzerla, William Vermi, Marco Presta and Roberto Ronca
- 171 Increased Pentraxin 3 Levels Correlate With IVIG Responsiveness and Coronary Artery Aneurysm Formation in Kawasaki Disease**
 Toshiyuki Kitoh, Tsuyoshi Ohara, Taichiro Muto, Akihisa Okumura, Reizo Baba, Yusuke Koizumi, Yuka Yamagishi, Hiroshige Mikamo, Kenji Daigo and Takao Hamakubo
- 180 PTX3 Regulation of Inflammation, Hemostatic Response, Tissue Repair, and Resolution of Fibrosis Favors a Role in Limiting Idiopathic Pulmonary Fibrosis**
 Andrea Doni, Alberto Mantovani, Barbara Bottazzi and Remo Castro Russo
- 190 PTX3 Deficiency Promotes Enhanced Accumulation and Function of CD11c⁺CD11b⁺ DCs in a Murine Model of Allergic Inflammation**
 Jyoti Balhara, Latifa Koussih, Ashfaque Mohammed, Lianyu Shan, Bouchaib Lamkhioued and Abdelilah S. Gounni
- 202 A Novel Model Based on Serum Biomarkers to Predict Primary Non-Response to Infliximab in Crohn's Disease**
 Li Li, Rirong Chen, Yingfan Zhang, Gaoshi Zhou, Baili Chen, Zhirong Zeng, Minhu Chen and Shenghong Zhang



A Functional Genetic Variant at the C-Reactive Protein Promoter (rs3091244) Is Not Associated With Cancer Risk in a Chinese Population

Ming-Yu Wang^{1†}, Hai-Hong Zhou^{2†}, Chun-Miao Zhang¹, Hai-Xiang Su², Shuo-Lei Li¹, Shang-Rong Ji¹, Enqi Liu^{3*} and Yi Wu^{3,4*}

¹ Translational Medicine Research Center, MOE Key Laboratory of Cell Activities and Stress Adaptations, School of Life Sciences, Lanzhou University, Lanzhou, China, ² Children's Research Institute, Gansu Provincial Cancer Hospital, Lanzhou, China, ³ MOE Key Laboratory of Environment and Genes Related to Diseases, School of Basic Medical Sciences, Xi'an Jiaotong University, Xi'an, China, ⁴ The Affiliated Children's Hospital of Xi'an Jiaotong University, Xi'an, China

OPEN ACCESS

Edited by:

Kenji Daigo,
Nippon Medical School, Japan

Reviewed by:

Alok Agrawal,
East Tennessee State University,
United States
Alexander J. Szalai,
University of Alabama at Birmingham,
United States

*Correspondence:

Enqi Liu
liuengqi@mail.xjtu.edu.cn
Yi Wu
wuy@lzu.edu.cn

[†]These authors have contributed
equally to this work

Specialty section:

This article was submitted to
Molecular Innate Immunity,
a section of the journal
Frontiers in Immunology

Received: 06 March 2020

Accepted: 21 April 2020

Published: 14 May 2020

Citation:

Wang M-Y, Zhou H-H, Zhang C-M,
Su H-X, Li S-L, Ji S-R, Liu E and Wu Y
(2020) A Functional Genetic Variant at
the C-Reactive Protein Promoter
(rs3091244) Is Not Associated With
Cancer Risk in a Chinese Population.
Front. Immunol. 11:926.
doi: 10.3389/fimmu.2020.00926

Background: The association of genetically elevated levels of circulating C-reactive protein (CRP) with cancer risk has been extensively investigated in European populations; however, there are conflicting conclusions. The tri-allelic rs3091244 is a functionally validated genetic variant, and its allelic frequencies differ significantly between European and Asian populations. Here, we examined the association of rs3091244 with cancer risk in a Chinese population.

Methods: rs3091244 was genotyped by Sanger sequencing in 4,971 cancer cases and 2,485 controls. The rs1205 and rs2794521 gene variants were also genotyped using TaqMan assays in subgroups.

Results: No association was detected between the genotyped CRP variants and cancer risk, with or without distinguishing cancer types, suggesting that circulating CRP is not causally involved in tumorigenesis in Chinese populations.

Keywords: cancer, inflammation, C-reactive protein, genetic variants, cancer risk

INTRODUCTION

Genome instability and inflammation underlie the initiation and progression of cancer (1, 2). C-reactive protein (CRP) is a well-established circulating marker of inflammation (3) whose blood levels are positively associated with the risk and prognosis of several types of cancer (4, 5). Moreover, CRP has been shown to prevent the apoptosis of myeloma cells (6), to facilitate the invasiveness of breast cancer cells (7, 8), and to promote malignant properties of pancreatic neuroendocrine neoplasm cells (9). These findings raise the possibility that CRP, a putative soluble pattern recognition receptor acting in host defense and inflammation (10–16), might play a causal role in tumorigenesis.

A causal involvement suggests that genetically elevated levels of circulating CRP would affect cancer risk. However, large-scale genetic epidemiological studies (> 500 cases) fail to reach consistent conclusions, although European populations have mostly been examined (17–24). This might be explained by the fact that the examined genetic variants were not functionally linked to CRP expression. In addition, allelic frequencies of CRP variants usually differ among races. Therefore, examining whether functional CRP variants are associated with cancer risk in different populations might help to clarify the role of CRP in tumorigenesis.

Of the known CRP variants, only the promoter single-nucleotide polymorphism rs3091244, frequently observed in European and Asian populations, has been formally validated as a functional regulator of CRP expression (25–28). However, genotyping the tri-allelic rs3091244 is not trivial using the regular TaqMan assay. Thus, we genotyped this variant using Sanger sequencing and examined its association with the risk of any and specific types of cancer in a Chinese population. Our results revealed that rs3091244 and another promoter variant, rs2794521, were not associated with cancer risk, arguing for a noncausal role of circulating CRP in tumorigenesis.

MATERIALS AND METHODS

Participants

Control and cancer cases are all Han Chinese. The types of cancer were diagnosed according to the criteria of WHO Classification of Tumors. Genomic DNA samples from cancer cases were obtained from the Tissue Bank of Gansu Cancer Hospital

(diagnosed during 2015–2017). Genomic DNA samples from controls were obtained from individuals receiving health checks at Gansu Cancer Hospital (2016–2017). Informed consent for blood sampling was obtained by all participants. Their clinical characteristics are shown in **Table 1** and **Table S1**. The study was approved by the Ethics Committee of the Gansu Provincial Cancer Hospital (A201307050027) and Xi'an Jiaotong University (2016-065) and was performed in accordance with relevant guidelines and regulations of the Ethics Committee.

Genotyping

The tri-allelic single-nucleotide polymorphism rs3091244 was genotyped by Sanger sequencing with specific primers (forward: 5' -AGGGGGGAGGGATAGCATTAGAA-3'; reverse: 5' -CGTCCTGCTGCCAGTGATACAAG-3') (BGI, Shenzhen, China). The bi-allelic single-nucleotide polymorphisms rs1205 and rs2794521 were genotyped using the TaqMan assay (Thermo Fisher Scientific, Rockford, IL; catalog number: c_7479334_10/c_318207_10; lot number: p151028-003).

TABLE 1 | Characteristics of controls and cancer cases.

	Control (2485)	All cancer (4971)		Gastric cancer (1557)		Breast cancer (1153)	
		Cases	P*	Cases	P*	Cases	P*
Female sex, <i>n</i>	1125	2162	0.1513	361	<0.0001	1144	<0.0001
(%, total)	(45.27, 2485)	(43.49, 4971)		(23.19, 1557)		(99.22, 1153)	
Age, years, median	32	58	<0.0001	58	<0.0001	49	<0.0001
(IQR, total)	(27–39, 2485)	(49–65, 4971)		(50–65, 1557)		(43–56, 1153)	
BMI, kg/m ² , median	22.98	22.26	<0.0001	21.25	<0.0001	23.82	0.0062
(IQR, total)	(20.69–25.47, 1552)	(20.07–24.76, 4635)		(19.33–23.73, 1439)		(21.77–26.07, 1114)	
CRP, mg/L, median	0.44	1.54	<0.0001	1.6	<0.0001	0.57	<0.0001
(IQR, total)	(0.15–0.95, 2046)	(0.40–9.08, 4778)		(0.38–8.94, 1489)		(0.20–1.52, 1127)	
Prior radio/chemotherapy, <i>n</i>		1124		220		364	
(%, total)		(40.23, 2794)		(29.69, 741)		(44.55, 817)	
Tumor Stage 0–2, <i>n</i>		1506		340		569	
(%, total)		(42.84, 3515)		(31.60, 1076)		(59.83, 951)	
	Control (2485)	Lung cancer (1016)		Esophagus cancer (688)		Colorectal cancer (498)	
		Cases	P*	Cases	P*	Cases	P*
Female sex, <i>n</i>		324	<0.0001	98	<0.0001	201	0.0481
(%, total)		(31.89, 1016)		(14.24, 688)		(40.36, 498)	
Age, years, median		60	<0.0001	63	<0.0001	59	<0.0001
(IQR, total)		(52–67, 1016)		(58–69, 688)		(50–66, 498)	
BMI, kg/m ² , median		22.54	0.0071	21.3	<0.0001	22.4	0.0047
(IQR, total)		(20.51–24.74, 964)		(19.37–23.63, 641)		(20.06–24.91, 435)	
CRP, mg/L, median		5.95	<0.0001	2.46	<0.0001	2.08	<0.0001
(IQR, total)		(1.12–24.65, 978)		(0.57–12.64, 662)		(0.57–10.27, 474)	
Prior radio-chemotherapy, <i>n</i>		295		128		112	
(%, total)		(54.63, 540)		(36.06, 355)		(34.67, 323)	
Tumor Stage 0–2, <i>n</i>		132		295		167	
(%, total)		(22.88, 577)		(56.08, 526)		(45.26, 396)	

**p* values were determined by Fisher's exact test (for sex) or Wilcoxon's Rank Sums test (for age and BMI). IQR represents interquartile range. *p* value for CRP levels was determined with age correction by Scheirer-Ray-Hare test.

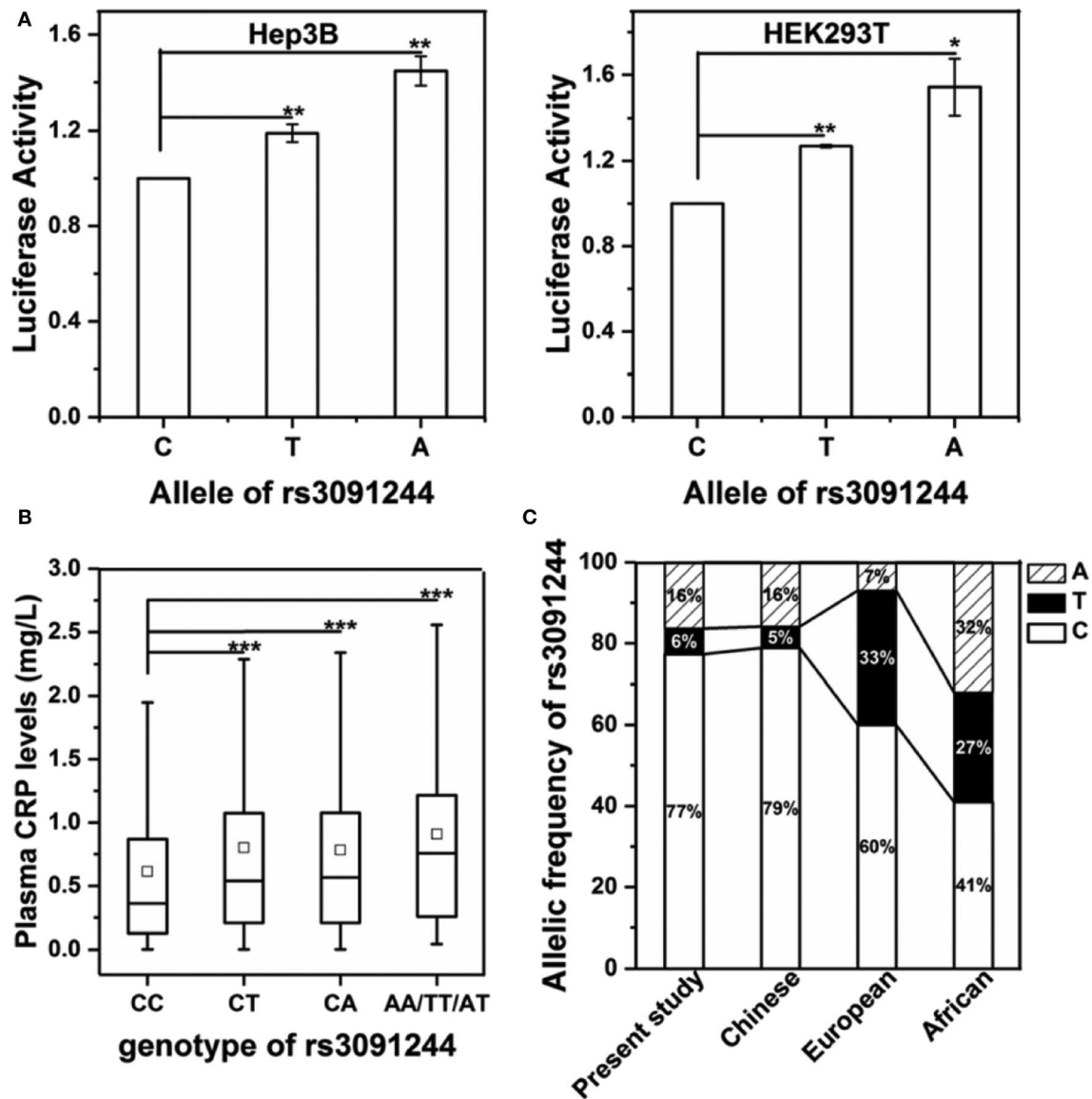


FIGURE 1 | Validation of the functional impact of rs3091244 on CRP expression. **(A)** The effects of different rs3091244 alleles on CRP promoter activities in Hep3B (left) and HEK293 cells (right). The A- and T-alleles show higher luciferase reporter activities than the C-allele. **(B)** Serum levels of CRP in healthy controls with different genotypes of rs3091244 (CC: 1250, CT: 199, CA: 500, AA/TT/AT: 97). **(C)** Allelic frequencies of rs3091244 in the present study versus those in Chinese, European, and African populations obtained from the 1000 genomes project database. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

rs3091244	Change in CRP levels %	All cancers		Gastric cancer		Breast cancer		Lung cancer		Esophagus cancer		Colorectal cancer	
		Co/Ca	OR	Co/Ca	OR	Co/Ca	OR	Co/Ca	OR	Co/Ca	OR	Co/Ca	OR
CC	0	1484/2720		1484/852		1484/686		1484/558		1484/361		1484/226	
CA/CT	27.42	880/1543		880/509		880/381		880/301		880/212		880/123	
AA/TT/AT	43.55	121/245		121/83		121/48		121/55		121/34		121/24	
p-trend		0.9713		0.4168		0.2599		0.9361		0.7144		0.8326	

FIGURE 2 | Risk of cancer by rs3091244 genotypes. p values (two-sided) are from a test for the trend of ORs across rs3091244 genotypes with increasing levels of circulating CRP. Black squares indicate ORs, and error bars indicate 95 % confidence intervals (CI). The numbers of control (Co) and cancer cases (Ca) are indicated.

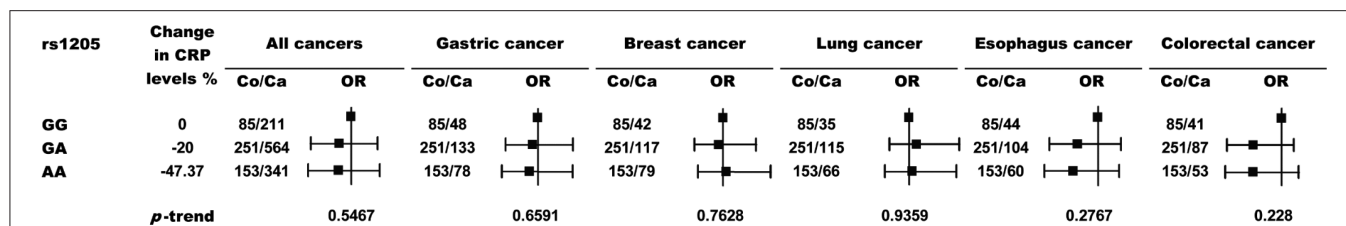


FIGURE 3 | Risk of cancer by rs1205 genotypes. *P* values (two-sided) are from a test for the trend of ORs across genotypes with increasing levels of circulating CRP. Black squares indicate ORs, and error bars indicate 95 % confidence intervals (CI). The numbers of control (Co) and cancer cases (Ca) are indicated.

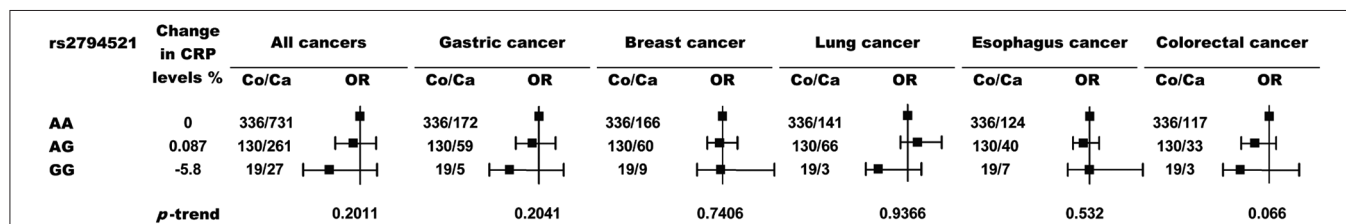


FIGURE 4 | Risk of cancer by rs2794521 genotypes. *P* values (two-sided) are from a test for the trend of ORs across genotypes with increasing levels of circulating CRP. Black squares indicate ORs, and error bars indicate 95 % confidence intervals (CI). The numbers of control (Co) and cancer cases (Ca) are indicated.

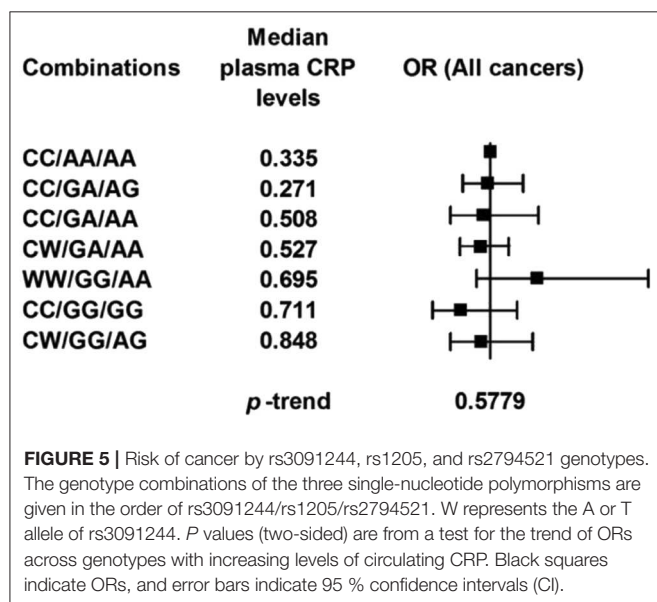


FIGURE 5 | Risk of cancer by rs3091244, rs1205, and rs2794521 genotypes. The genotype combinations of the three single-nucleotide polymorphisms are given in the order of rs3091244/rs1205/rs2794521. W represents the A or T allele of rs3091244. *P* values (two-sided) are from a test for the trend of ORs across genotypes with increasing levels of circulating CRP. Black squares indicate ORs, and error bars indicate 95 % confidence intervals (CI).

Luciferase Reporter Assay

The promoter fragment of CRP (−533~+103 bp) was cloned into the PGL4.10 (luc2) vector (Promega, Madison, WI; catalog number: E6651). Hep3B or HEK293T cells were transfected with 1.5 μg of PGL4.10 CRP reporter vector and 0.075 μg of phRL-TK (Promega; catalog number: E6241) using the X-treme GENE 9 DNA Transfection Reagent (Roche, Basel, Switzerland; catalog number: 06365787001; lot number: 23644700). After 48 h of transfection, luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega; catalog number: E1960; lot number: 0000201344) on a Synergy HTX Multi-Mode Microplate Reader (BioTek, Winooski, VT). Firefly

luciferase activities were normalized to that of co-transfected Renilla luciferase.

Statistical Analysis

Hardy-Weinberg equilibrium was checked in healthy controls using the chi-squared test. Clinical characteristics between cases and controls were compared using Fisher's exact test, Wilcoxon signed rank test or Scheirer-Ray-Hare test. The activity of different alleles in the luciferase assay was tested using analysis of variance (ANOVA). The association of genotypes with circulating CRP levels was tested using a Kruskal-Wallis ANOVA. The crude odds ratio (OR) was estimated using a conditional logistic regression model to assess associations between CRP genotypes and cancer risk. OR trends among genotypes were calculated using the Cochran-Armitage trend test. *P* values below 0.05 were considered significant. Statistical analyses were conducted using SAS 9.3 (SAS Institute, Cary, NC, USA) or R package 3.6.0.

RESULTS

We first examined the direct effects of rs3091244 on the expression of human CRP using a luciferase reporter assay (Figure 1A). The promoter activity of human CRP increased in order with the rs3091244 C-, T- and A-alleles in both human hepatic Hep3B and renal HEK293 cells. This order also corresponded to levels of circulating CRP in healthy controls with different rs3091244 genotypes (Figure 1B). Overall, our results are in line with previous findings that rs3091244 functionally influences CRP expression (18, 25–27).

Next, we genotyped the rs3091244 variant in 2485 healthy controls and 4971 cancer patients (Table 1 and Table S1). rs3091244 allelic frequencies in healthy controls were in Hardy-Weinberg equilibrium and were comparable to those in Asian

populations, but differed from those in European populations (Figure 1C). Although the rs3091244 T- and A- alleles were associated with higher baseline levels of circulating CRP, they, either alone or in combination, showed no association with the risk of any type of cancer examined (Figure 2).

We also genotyped the most frequently examined CRP variant, rs1205, and another CRP promoter variant, rs2794521, in a subgroup of 489 healthy controls and 1116 cancer patients using TaqMan assays. However, these two variants (Figures 3, 4) or their combination with rs3091244 (Figure 5) again showed no association with cancer risk. Thus, we propose that circulating CRP is unlikely to be casually involved in tumorigenesis in Chinese populations.

DISCUSSION

There have been several large-scale studies investigating the associations between genetic CRP variants and cancer risk in European (17–21, 24), American (22) and Chinese populations (23). One study claimed that CRP variants are not associated with the overall cancer risk (18), while others reported the opposite (19) and also identified risk associations with certain cancer types (17, 19, 20, 22). Confusingly, the identified variants can be associated with increased cancer risk but with decreased levels of circulating CRP (17, 22). Such a negative association is also observed for a different cancer type, i.e., breast cancer, in the present study albeit statistically insignificant. Nevertheless, these were difficult to reconcile with the established positive association between cancer risk and circulating CRP levels. Moreover, the same CRP variant rs1205 has been found to be associated with an increased risk of colon cancer in one study (22) but with decreased risk in another (20).

Regarding these inconsistencies, it should be noted that a clear functional association with CRP expression has not been demonstrated for most examined variants (17–23). The only functional variant, rs3091244 (25–27) was only examined in one study in the general population of Denmark (18). This might partly be due to the tri-allelic nature of rs3091244, which is difficult to be genotyped using the regular TaqMan assay (18). The present study used the more accurate Sanger sequencing to genotype rs3091244 in a Chinese population, and reached the same conclusion as the Denmark study (18). The lack of a significant association between rs3091244 and cancer risk suggests that circulating CRP does not play a causal role in tumorigenesis.

The noncausal involvement of circulating CRP in cancer was not entirely unexpected. Our recent work has revealed that tissue-localized CRP may be predominantly produced *in situ* rather than transported from the circulation (29). This

would imply that instead of liver-produced, circulating CRP, it is locally-produced CRP that potentially plays an etiological role in tumorigenesis. We have further shown that malignant cell-derived CRP can conversely be transported to the circulation (29), likely contributing to the risk-associated subtle elevations of circulating CRP. However, such contributions might be obscured in genetic association studies due to confounding factors including tissue-specific effects of genetic variants on CRP expression, and their profound modulation on circulating CRP levels. Whether extrahepatic tissue-derived CRP is causally involved in tumorigenesis remains to be investigated.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethic Committee of the Gansu Provincial Cancer Hospital (A201307050027) and Xi'an Jiaotong University (2016-065). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

YW, EL, and S-RJ designed the research. M-YW, H-HZ, C-MZ, H-XS, and S-LL performed the research. YW, EL, M-YW, and H-HZ analyzed the data and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

FUNDING

This work was supported by grants from the National Natural Science Foundation of China (grant numbers: 31671339, 31570749, and 31960141).

ACKNOWLEDGMENTS

We thank the Core Facility of the School of Life Sciences, Lanzhou University for technical and instrumental support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2020.00926/full#supplementary-material>

REFERENCES

1. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. (2011) 144:646–74. doi: 10.1016/j.cell.2011.02.013
2. Grivninkov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. *Cell*. (2010) 140:883–99. doi: 10.1016/j.cell.2010.01.025
3. Pepys MB, Hirschfield GM. C-reactive protein: a critical update. *J Clin Invest*. (2003) 111:1805–12. doi: 10.1172/JCI2003.18921
4. Allin KH, Nordestgaard BG. Elevated C-reactive protein in the diagnosis, prognosis, and cause of cancer. *Crit Rev Clin Lab Sci*. (2011) 48:155–70. doi: 10.3109/10408363.2011.599831

5. Hajishengallis G, Reis ES, Mastellos DC, Ricklin D, Lambris JD. Novel mechanisms and functions of complement. *Nat Immunol.* (2017) 18:1288–98. doi: 10.1038/ni.3858
6. Yang J, Wezeman M, Zhang X, Lin P, Wang M, Qian J, et al. Human C-reactive protein binds activating Fcγ receptors and protects myeloma tumor cells from apoptosis. *Cancer Cell.* (2007) 12:252–65. doi: 10.1016/j.ccr.2007.08.008
7. Kim ES, Cha Y, Ham M, Jung J, Kim SG, Hwang S, et al. Inflammatory lipid sphingosine-1-phosphate upregulates C-reactive protein via C/EBPβ and potentiates breast cancer progression. *Oncogene.* (2014) 33:3583–93. doi: 10.1038/ncr.2013.319
8. Kim ES, Kim SY, Koh M, Lee HM, Kim K, Jung J, et al. C-reactive protein binds to integrin α2 and Fcγ receptor I, leading to breast cell adhesion and breast cancer progression. *Oncogene.* (2018) 37:28–38. doi: 10.1038/ncr.2017.298
9. Schimmack S, Yang Y, Felix K, Herbst M, Li Y, Schenk M, et al. C-reactive protein (CRP) promotes malignant properties in pancreatic neuroendocrine neoplasms. *Endocr Connect.* (2019) 8:1007–1019. doi: 10.1530/EC-19-0132
10. Du Clos TW. Pentraxins: structure, function, and role in inflammation. *ISRN Inflamm.* (2013). (2013) 2013:379040. doi: 10.1155/2013/379040
11. Bottazzi B, Doni A, Garlanda C, Mantovani A. An integrated view of humoral innate immunity: pentraxins as a paradigm. *Annu Rev Immunol.* (2010) 28:157–83. doi: 10.1146/annurev-immunol-030409-101305
12. Wu Y, Potempa LA, El Kebir D, Filep JG. C-reactive protein and inflammation: conformational changes affect function. *Biol Chem.* (2015) 396:1181–97. doi: 10.1515/hsz-2015-0149
13. McFadyen JD, Kiefer J, Braig D, Loseff-Silver J, Potempa LA, Eisenhardt SU, et al. Dissociation of C-reactive protein localizes and amplifies inflammation: evidence for a direct biological role of c-reactive protein and its conformational changes. *Front Immunol.* (2018) 9:1351. doi: 10.3389/fimmu.2018.01351
14. Sproston NR, Ashworth JJ. Role of C-reactive protein at sites of inflammation and infection. *Front Immunol.* (2018) 9:754. doi: 10.3389/fimmu.2018.00754
15. Ngwa DN, Agrawal A. Structure-function relationships of c-reactive protein in bacterial infection. *Front Immunol.* (2019) 10:166. doi: 10.3389/fimmu.2019.00166
16. Pathak A, Agrawal A. Evolution of C-Reactive protein. *Front Immunol.* (2019) 10:943. doi: 10.3389/fimmu.2019.00943
17. Siemes C, Visser LE, Coebergh JW, Splinter TA, Witteman JC, Uitterlinden AG, et al. C-reactive protein levels, variation in the C-reactive protein gene, and cancer risk: the Rotterdam Study. *J Clin Oncol.* (2006) 24:5216–22. doi: 10.1200/JCO.2006.07.1381
18. Allin KH, Nordestgaard BG, Zacho J, Tybjaerg-Hansen A, Bojesen SE. C-reactive protein and the risk of cancer: a mendelian randomization study. *J Natl Cancer Inst.* (2010) 102:202–6. doi: 10.1093/jnci/djp459
19. Heikkilä K, Silander K, Salomaa V, Jousilahti P, Koskinen S, Pukkala E, et al. C-reactive protein-associated genetic variants and cancer risk: findings from FINRISK 1992, FINRISK. 2011, 1997 and Health 2000 studies. *Eur J Cancer.* (2010) 47:404–12. doi: 10.1016/j.ejca.2010.07.032
20. Nimptsch K, Aleksandrova K, Boeing H, Janke J, Lee YA, Jenab M, et al. Association of CRP genetic variants with blood concentrations of C-reactive protein and colorectal cancer risk. *Int J Cancer.* (2015) 136:1181–92. doi: 10.1002/ijc.29086
21. Markt SC, Rider JR, Penney KL, Schumacher FR, Epstein MM, Fall K, et al. Genetic variation across C-reactive protein and risk of prostate cancer. *Prostate.* (2014) 74:1034–42. doi: 10.1002/pros.22820
22. Slattery ML, Curtin K, Poole EM, Duggan DJ, Samowitz WS, Peters U, et al. Genetic variation in C-reactive protein in relation to colon and rectal cancer risk and survival. *Int J Cancer.* (2011) 128:2726–34. doi: 10.1002/ijc.25721
23. Wen WQ, Cai QY, Xiang YB, Xu WH, Ruan ZX, Cheng JR, et al. The modifying effect of C-reactive protein gene polymorphisms on the association between central obesity and endometrial cancer risk. *Cancer.* (2008) 112:2409–16. doi: 10.1002/cncr.23453
24. Wang X, Dai JY, Albanes D, Arndt V, Berndt SI, Bezieau S, et al. Mendelian randomization analysis of C-reactive protein on colorectal cancer risk. *Int J Epidemiol.* (2019) 48:767–80. doi: 10.1093/ije/dyy244
25. Szalai AJ, Wu J, Lange EM, McCrory MA, Langefeld CD, Williams A, et al. Single-nucleotide polymorphisms in the C-reactive protein (CRP) gene promoter that affect transcription factor binding, alter transcriptional activity, and associate with differences in baseline serum CRP level. *J Mol Med (Berl).* (2005) 83:440–7. doi: 10.1007/s00109-005-0658-0
26. Kim HA, Chun HY, Kim SH, Park HS, Suh CH. C-reactive protein gene polymorphisms in disease susceptibility and clinical manifestations of Korean systemic lupus erythematosus. *J Rheumatol.* (2009) 36:2238–43. doi: 10.3899/jrheum.090243
27. Chang SN, Tsai CT, Wu CK, Lee JK, Lai LP, Huang SW, et al. A functional variant in the promoter region regulates the C-reactive protein gene and is a potential candidate for increased risk of atrial fibrillation. *J Int Med.* (2012) 272:305–15. doi: 10.1111/j.1365-2796.2012.02531.x
28. Zhang SC, Wang MY, Feng JR, Chang Y, Ji SR, Wu Y. Reversible promoter methylation determines fluctuating expression of acute phase proteins. *Elife.* (2020) 9:51317. doi: 10.7554/eLife.51317
29. Li HY, Liu XL, Liu YT, Jia ZK, Filep JG, Potempa LA, et al. Matrix sieving-enforced retrograde transcytosis regulates tissue accumulation of C-reactive protein. *Cardiovasc Res.* (2019) 115:440–52. doi: 10.1093/cvr/cvy181

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Wang, Zhou, Zhang, Su, Li, Ji, Liu and Wu. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Conformationally Altered C-Reactive Protein Capable of Binding to Atherogenic Lipoproteins Reduces Atherosclerosis

Asmita Pathak[†], Sanjay K. Singh[†], Douglas P. Thewke and Alok Agrawal*

Department of Biomedical Sciences, James H. Quillen College of Medicine, East Tennessee State University, Johnson City, TN, United States

OPEN ACCESS

Edited by:

Kenji Daigo,
Nippon Medical School, Japan

Reviewed by:

Tomohide Takaya,
Shinshu University, Japan
Lawrence Albert Potempa,
Roosevelt University, United States

*Correspondence:

Alok Agrawal
agrawal@etsu.edu

[†]These authors have contributed
equally to this work

Specialty section:

This article was submitted to
Molecular Innate Immunity,
a section of the journal
Frontiers in Immunology

Received: 21 May 2020

Accepted: 03 July 2020

Published: 11 August 2020

Citation:

Pathak A, Singh SK, Thewke DP and
Agrawal A (2020) Conformationally
Altered C-Reactive Protein Capable of
Binding to Atherogenic Lipoproteins
Reduces Atherosclerosis.
Front. Immunol. 11:1780.
doi: 10.3389/fimmu.2020.01780

The aim of this study was to test the hypothesis that C-reactive protein (CRP) protects against the development of atherosclerosis and that a conformational alteration of wild-type CRP is necessary for CRP to do so. Atherosclerosis is an inflammatory cardiovascular disease and CRP is a plasma protein produced by the liver in inflammatory states. The co-localization of CRP and low-density lipoproteins (LDL) at atherosclerotic lesions suggests a possible role of CRP in atherosclerosis. CRP binds to phosphocholine-containing molecules but does not interact with LDL unless the phosphocholine groups in LDL are exposed. However, CRP can bind to LDL, without the exposure of phosphocholine groups, if the native conformation of CRP is altered. Previously, we reported a CRP mutant, F66A/T76Y/E81A, generated by site-directed mutagenesis, that did not bind to phosphocholine. Unexpectedly, this mutant CRP, without any more conformational alteration, was found to bind to atherogenic LDL. We hypothesized that this CRP mutant, unlike wild-type CRP, could be anti-atherosclerotic and, accordingly, the effects of mutant CRP on atherosclerosis in atherosclerosis-prone LDL receptor-deficient mice were evaluated. Administration of mutant CRP into mice every other day for a few weeks slowed the progression of atherosclerosis. The size of atherosclerotic lesions in the aorta of mice treated with mutant CRP for 9 weeks was ~40% smaller than the lesions in the aorta of untreated mice. Thus, mutant CRP conferred protection against atherosclerosis, providing a proof of concept that a local inflammation-induced structural change in wild-type CRP is a prerequisite for CRP to control the development of atherosclerosis.

Keywords: atherosclerosis, C-reactive protein, inflammation, low-density lipoprotein, phosphocholine

INTRODUCTION

Atherosclerosis is a chronic inflammatory disease whose development begins with the dysfunction of the endothelium of the arteries. Endothelial dysfunction leads to infiltration of plasma low-density lipoprotein (LDL) and monocytes in the arterial wall. LDL is subsequently deposited, modified and engulfed by monocyte-derived macrophages. Lipid-laden macrophage foam cells are proinflammatory which enhances the process of the formation of atherosclerotic lesions (1, 2). The inflammatory microenvironment of atherosclerotic lesions is characterized by macrophage activation, hypoxia, and lactate and proton generation, resulting in an acidic extracellular pH (3–7).

C-reactive protein (CRP) is a plasma protein produced by the liver in inflammatory states (8). CRP is also present, co-localized with modified LDL and macrophages, at atherosclerotic lesions in both humans and experimental animals (9, 10). CRP is composed of five identical subunits arranged in a pentameric symmetry. The molecular weight of each subunit is ~23 kDa and there are 206 amino acid residues in each subunit. CRP binds to molecules with exposed phosphocholine (PCh) groups in a Ca^{2+} -dependent manner. There are five PCh-binding sites in the CRP pentamer, one site per subunit (11, 12).

Oxidized LDL (ox-LDL), enzymatically-modified LDL (E-LDL) and acetylated LDL (ac-LDL) are different forms of modified atherogenic LDL that are used in *in vitro* experiments (1, 2, 13). In the presence of Ca^{2+} , native or recombinant wild-type (WT) CRP interacts with E-LDL due to the exposure of PCh groups on E-LDL, but does not interact with ox-LDL and ac-LDL (14–16). In the absence of Ca^{2+} , WT CRP does not interact with any form of atherogenic LDL (16–19). The native pentameric structure of CRP is altered in response to a variety of experimental conditions (16–23). It has been shown that the pentameric structure of WT CRP is subtly altered by acidic pH and by oxidation, and conformationally altered CRP is capable of binding to atherogenic LDL independent of the PCh-binding site (17–19). Employing E-LDL, it has also been shown that if CRP is bound to atherogenic LDL, it prevents the formation of lipid-laden macrophage foam cells (24). Due to the presence of CRP at atherosclerotic lesions and due to the binding capability of CRP for atherogenic LDL under certain conditions, CRP has been implicated in the development of atherosclerosis (25).

We have previously reported a CRP mutant, F66A/T76Y/E81A, in which Phe⁶⁶, Thr⁷⁶, and Glu⁸¹ (three critical amino acid residues in the PCh-binding site) were substituted with Ala, Tyr, and Ala, respectively, to abolish the PCh-binding activity of CRP (26). Mutant CRP did not bind to PCh and was used as a tool to investigate the importance of the PCh-binding site in CRP-mediated protection of mice against pneumococcal infection (26). Biochemical characterization of this mutant CRP, in comparison to that of WT CRP, has been published previously (26). The overall structure of mutant CRP was pentameric and the mutation did not affect the stability of the protein *in vivo*. Mutant CRP circulated freely in the mouse serum and its rate of clearance *in vivo* was similar to that of WT CRP (26).

Further analysis of F66A/T76Y/E81A mutant CRP, presented here, revealed that mutant CRP had inadvertently gained the ability to bind to any protein that was immobilized on microtiter plates, including atherogenic forms of LDL, without the need for any further inflammatory milieu-dependent or acidic pH-induced structural change. We, therefore, hypothesized that mutant CRP might show an atheroprotective effect in murine models of atherosclerosis. We reasoned that even if there was no acidic pH around injected mutant CRP in the available murine models, mutant CRP would be able to recognize and bind atherogenic LDL and exert a protective effect on the development

of atherosclerosis. Accordingly, in this study, we evaluated the effects of mutant CRP (F66A/T76Y/E81A) on the development of atherosclerosis employing LDL receptor-deficient (*Ldlr*^{-/-}) mice, an animal model commonly used to investigate molecules involved in human atherosclerosis.

MATERIALS AND METHODS

Preparation of F66A/T76Y/E81A Mutant CRP

The construction of F66A/T76Y/E81A mutant CRP cDNA used in this study has been reported previously (26). Mutant CRP was expressed in CHO cells using the ExpiCHO Expression System (Thermo Fisher Scientific), according to manufacturer's instructions. As described previously (26), mutant CRP was purified from cell culture supernatant by Ca^{2+} -dependent affinity chromatography on a phosphoethanolamine-Sepharose column, since this CRP mutant does not bind to PCh. Mutant CRP was further purified by gel filtration on a Superose12 column. Eluted mutant CRP was immediately dialyzed against 10 mM Tris-HCl, 150 mM NaCl, pH 7.2 (TBS), containing 2 mM CaCl_2 , stored at 4°C, and was used within a week. The purity of CRP was confirmed by using denaturing SDS-PAGE. For *in vivo* experiments, purified mutant CRP was treated with the Detoxi-Gel Endotoxin Removing Gel (Thermo Fisher Scientific) according to manufacturer's instructions. The removal of endotoxin from mutant CRP preparations was confirmed by using the Limulus Amebocyte Lysate kit QCL-1000 (Lonza) according to manufacturer's instructions.

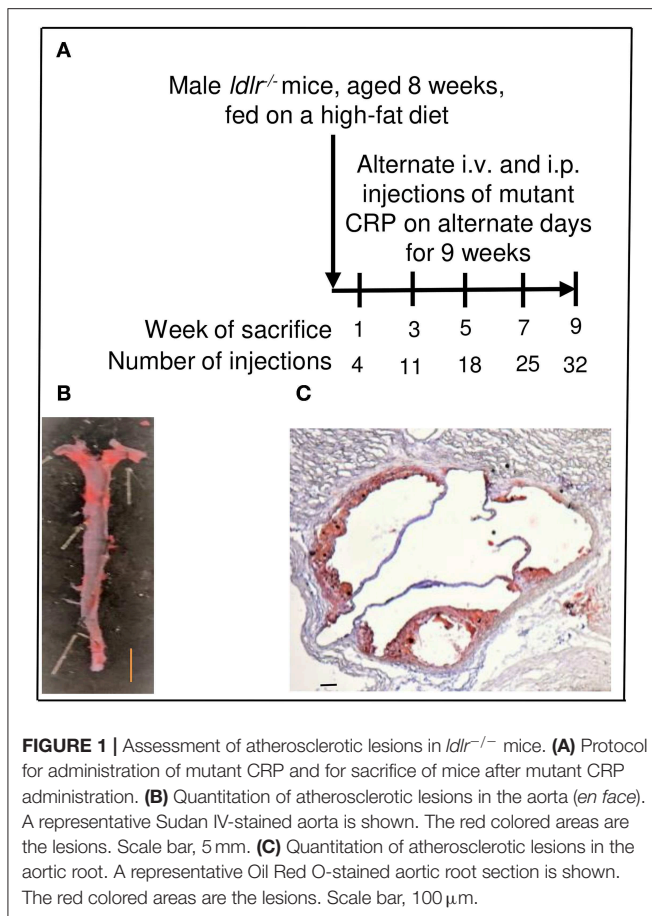
Solid Phase Ligand-Binding Assay

The solid phase ligand-binding assay was used to determine the binding of mutant CRP to immobilized proteins, as described earlier (17–19). Ox-LDL, E-LDL, ac-LDL, factor H (Complement Technology) and amyloid β peptide 1-42 (Bachem, H-1368) were used as protein ligands. Ox-LDL, E-LDL, and ac-LDL were prepared as described previously (17–19, 24). Briefly, microtiter wells were coated with protein ligands (10 $\mu\text{g}/\text{ml}$) diluted in TBS and incubated overnight at 4°C. The unreacted sites in the wells were blocked with TBS containing 0.5% gelatin. Freshly purified mutant CRP was diluted in TBS-Ca (TBS containing 2 mM CaCl_2 , 0.1% gelatin and 0.02% Tween 20), added to the wells, and incubated overnight at 4°C. Bound mutant CRP was detected by using a polyclonal rabbit anti-human CRP antibody (Millipore Sigma, 235752). HRP-conjugated donkey anti-rabbit IgG (GE Healthcare) was used as the secondary antibody. Color was developed using ABTS as the substrate and the OD₄₀₅ was read in a plate reader.

Atherosclerosis Protection Experiments

Eight-week-old C57BL/6 male *Ldlr*^{-/-} mice (Jackson Lab, 002207) were used in experiments according to protocols approved by and conducted in accordance with the guidelines administered by the Institutional Animal Care and Usage Committee of East Tennessee State University. Sixty mice were fed on a high-fat western-type diet (21% fat, 0.2% cholesterol), purchased from Envigo (TD.88137), for 10 weeks. After 1 week

Abbreviations: CRP, C-reactive protein; LDL, Low-density lipoprotein; *Ldlr*^{-/-}, LDL receptor-deficient; PCh, Phosphocholine; WT, wild-type.



on high-fat diet, mice were divided into two groups of 30 mice in each group: untreated group and mutant CRP-treated group. Mice were injected with either TBS (untreated group) or mutant CRP (50 μ g/injection) on alternate days for 9 weeks, via alternating intravenous and intraperitoneal routes (**Figure 1A**). Blood, heart and aorta were collected at five different time points (weeks 1, 3, 5, 7, and 9), as described previously (27). Briefly, six mice from each group, at each time point, were sacrificed, blood was collected by cardiac puncture, and the plasma was separated and stored frozen. After collecting blood, the heart was perfused with 10% formalin, followed by removing fat from the entire aorta. Heart was excised from the aorta, was cut into two halves, and the upper half with the aortic root was mounted using OCT medium and stored at -80°C . Following heart excision, aorta was excised and stored in 10% formalin at 4°C . The whole experiment was repeated once more employing another 60 mice and a separate, freshly purified batch of mutant CRP.

Measurement of the Size of Atherosclerotic Lesions

To measure the size of atherosclerotic lesions in the whole aorta (*en face*), aorta was cut open longitudinally, stained with Sudan IV, and digitally photographed, as described previously (27). A total of 120 aortae (60 mice for untreated group and 60 mice

for mutant CRP-treated group; 12 mice per time point) were processed, stained and photographed. A representative Sudan IV-stained aorta is shown in **Figure 1B**.

To measure the size of atherosclerotic lesions in the aortic root, 8 μ m cross-sections of frozen OCT-embedded heart were collected from the appearance of the aortic valve leaflets to their disappearance (48–72 sections per heart). Every other cross-section of the entire aortic root was stained with Oil Red O for lipids, counterstained with hematoxylin, and digitally photographed, as described previously (27). A total of 120 hearts (60 mice for untreated group and 60 mice for mutant CRP-treated group; 12 mice per time point) were processed. A representative Oil Red O-stained aortic root section is shown in **Figure 1C**.

Digital photographs were acquired with an Olympus BX41 microscope equipped with a CCD color camera (QImaging). The stained lesion areas were quantified in digital images using the ImageJ software (28). Quantification of the lesion areas in the photographs was performed by two observers blinded to the experimental protocol. Non-parametric test (Mann-Whitney test) using GraphPad Prism software was employed to calculate the *p*-values.

Immunostaining of CRP

Sudan IV-stained aorta was first processed to remove the stain, as described earlier (29). Immunostaining of CRP was performed using Vectastain ABC Elite kit (Vector laboratories, PK-6100) according to manufacturer's instructions. CRP was detected by using a polyclonal rabbit anti-human CRP antibody (Millipore Sigma, 235752). Biotinylated goat-anti rabbit IgG was used as the secondary antibody. Color was developed using DAB (Vector laboratories, ImmPACT DAB, SK-4105) as the substrate, according to manufacturer's instructions.

Measurement of Lipoproteins in the Plasma

The concentrations of high-density lipoprotein (HDL) and LDL in the plasma were measured using Cholesterol Assay Kit-HDL and LDL/VLDL (Abcam; ab65390) according to manufacturer's instructions. Lipoprotein levels were measured in the pooled plasma samples collected at weeks 1, 3, 5, 7, and 9 (12 mice per time point). Unpaired student *t*-test was employed to calculate the *p*-values.

RESULTS

All experiments were performed three times, unless otherwise mentioned, and comparable results were obtained each time. Results of a representative experiment are shown in the figures where the raw data (OD_{405}) were used to plot the curves.

Mutant CRP Binds to Atherogenic LDL

As shown in **Figure 2**, mutant CRP bound to all three forms of atherogenic LDL immobilized on microtiter wells. The binding occurred at physiological conditions, that is, in the absence of any protein structure-modifying agent, such as acidic pH. Since mutant CRP does not bind to PCh (26), the binding of mutant

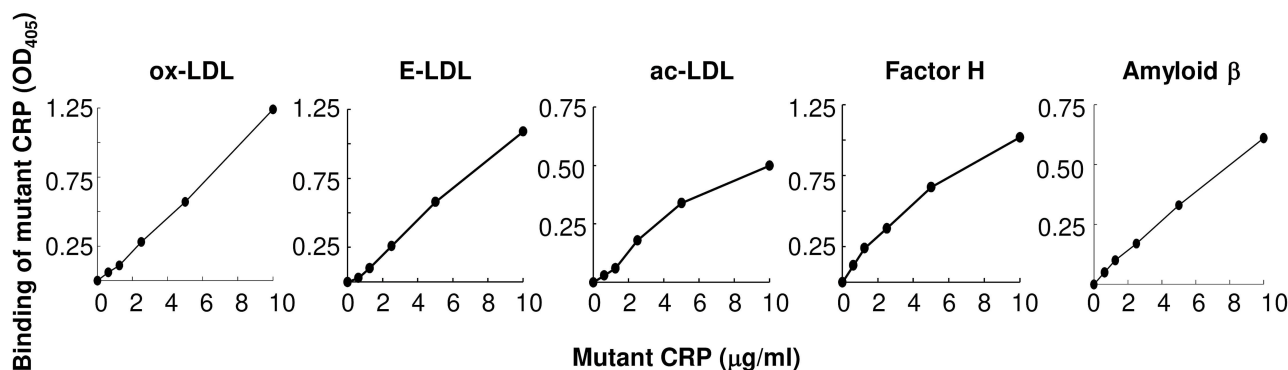


FIGURE 2 | Binding of F66A/T76Y/E81A mutant CRP to immobilized proteins including atherogenic LDL at physiological pH. Microtiter wells were coated with protein ligands as shown. After blocking the unreacted sites in the wells, mutant CRP diluted in TBS-Ca was added to the wells and incubated for 2 h at 37°C. Bound mutant CRP was detected by using a rabbit anti-human CRP antibody and HRP-conjugated donkey anti-rabbit IgG. Color was developed and the OD was read at 405 nm. A representative of three experiments are shown.

CRP to either ox-LDL, E-LDL, or ac-LDL was independent of the PCh groups present in atherogenic LDL. In addition to testing the binding of mutant CRP to atherogenic LDL, we also included two other proteins, factor H and amyloid β peptide, in the binding assay. Mutant CRP also bound to factor H and amyloid β peptide in a concentration-dependent manner, similar to its binding to atherogenic LDL. These results suggest that mutant CRP did not recognize immobilized atherogenic LDL *per se*, but it recognized a pattern, as yet undefined, on immobilized proteins in general.

Mutant CRP Reduces Atherosclerotic Lesions in the Whole Aorta

The total size of all lesion areas in the whole aorta (*en face*) was measured. **Figure 3** shows the combined results of two separate experiments with 12 mice in each group, for each time point. There was no effect of mutant CRP on the *en face* lesions for the first 5 weeks. The effect of mutant CRP on the lesion size was visible once the disease had progressed further. In comparison to untreated mice, the lesion area in mutant CRP-treated mice was 30.9% less after 7 weeks and 42% less after 9 weeks of CRP administration. As shown, in mutant CRP-treated mice, the lesion area did not increase after 5 weeks unlike in the untreated group where the lesion area kept increasing for another 2 weeks. Similar results were seen when the data from each of the two experiments (6 mice/group/time point/experiment) were analyzed separately (**Supplemental Figure 1**). We did not determine the specific stages of atherosclerosis at any time point.

The Effects of Mutant CRP Are Not Visible at the Aortic Root Lesions

The total size of all lesion areas in the aortic root was measured. **Figure 4** shows the combined results of two separate experiments using 12 mice in each group, for each time point. As shown, the disease progressed for the entire duration of 9 weeks in untreated mice. However, the administration of mutant CRP did not affect the lesion size at any time point. There was no statistically significant difference in the lesion size at any time

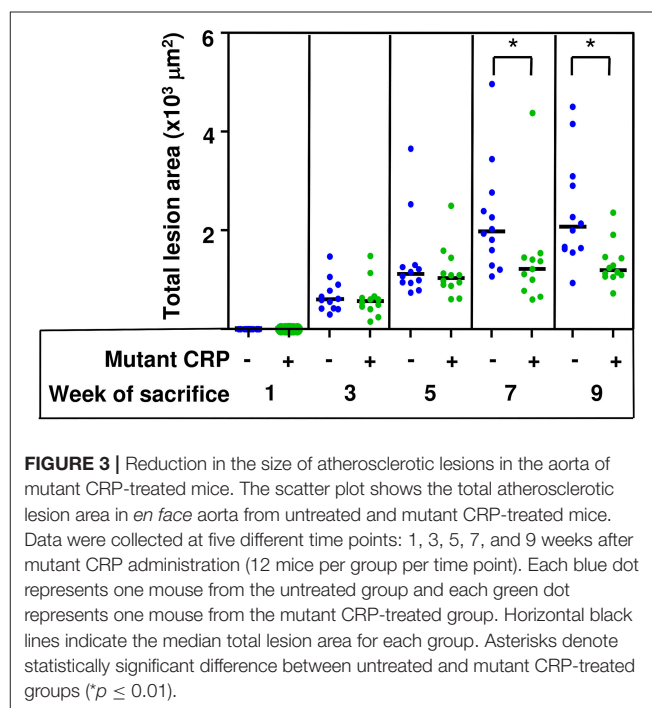


FIGURE 3 | Reduction in the size of atherosclerotic lesions in the aorta of mutant CRP-treated mice. The scatter plot shows the total atherosclerotic lesion area in *en face* aorta from untreated and mutant CRP-treated mice. Data were collected at five different time points: 1, 3, 5, 7, and 9 weeks after mutant CRP administration (12 mice per group per time point). Each blue dot represents one mouse from the untreated group and each green dot represents one mouse from the mutant CRP-treated group. Horizontal black lines indicate the median total lesion area for each group. Asterisks denote statistically significant difference between untreated and mutant CRP-treated groups (* $p \leq 0.01$).

point between untreated and mutant CRP-treated groups of mice. Similar results were seen when the data from each of the two experiments (6 mice/group/time point/experiment) were analyzed separately (**Supplemental Figure 2**).

Administered Mutant CRP Is Present in the Aorta

Immunostaining of CRP in the aorta isolated from mice treated with mutant CRP for seven weeks was performed to confirm the presence of administered mutant CRP at the *en face* atherosclerotic lesions in mutant CRP-treated mice. As shown (**Figure 5**), the lesions in the aorta of mutant CRP-treated mice

were stained while the lesions in the aorta of untreated mice did not stain for CRP. The polyclonal anti-human CRP antibody used in this study does not react with purified murine CRP (data not shown), consistent with the previously published report that anti-human CRP antibodies do not react with murine CRP (30).

Mutant CRP Does Not Alter the Concentration of Lipoproteins in the Plasma

Injecting a total of 1.6 mg of mutant CRP into mouse, in 32 injections over a period of 9 weeks, did not alter the

concentration of lipoproteins in the circulation. As shown in **Table 1**, the concentrations of LDL and HDL in the plasma of untreated and mutant CRP-treated mice were comparable. There was no statistically significant difference in the levels of either LDL or HDL in the plasma at any time point between untreated and mutant CRP-treated groups of mice. Administration of mutant CRP did not affect the body weight. Body weights of untreated and mutant CRP-treated mice were similar at each time point (data not shown).

DISCUSSION

In this study, we investigated the effects of mutant CRP F66A/T76Y/E81A, capable of binding to atherogenic LDL but incapable of binding to PCh, on the development of atherosclerosis in male *ldlr*^{-/-} mice. Our major finding was that the administration of mutant CRP for 7 weeks slowed the progression of atherosclerosis. The total size of atherosclerotic lesions in the whole aorta of mice treated with mutant CRP on

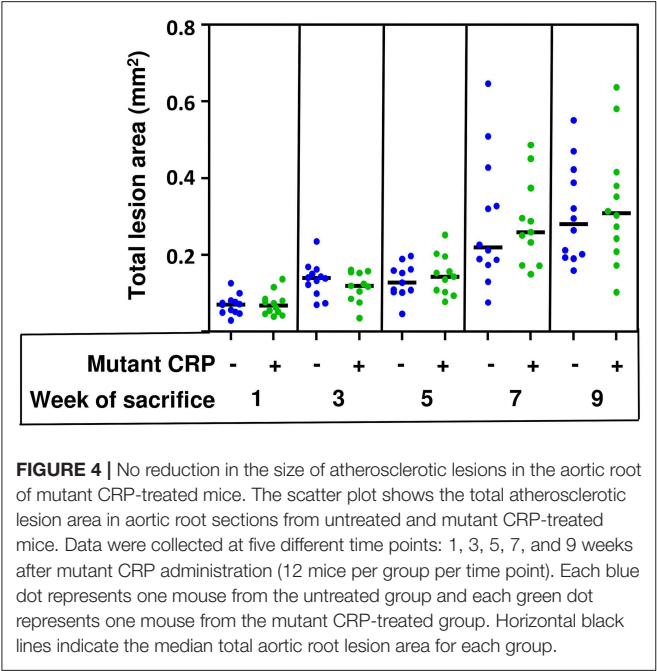
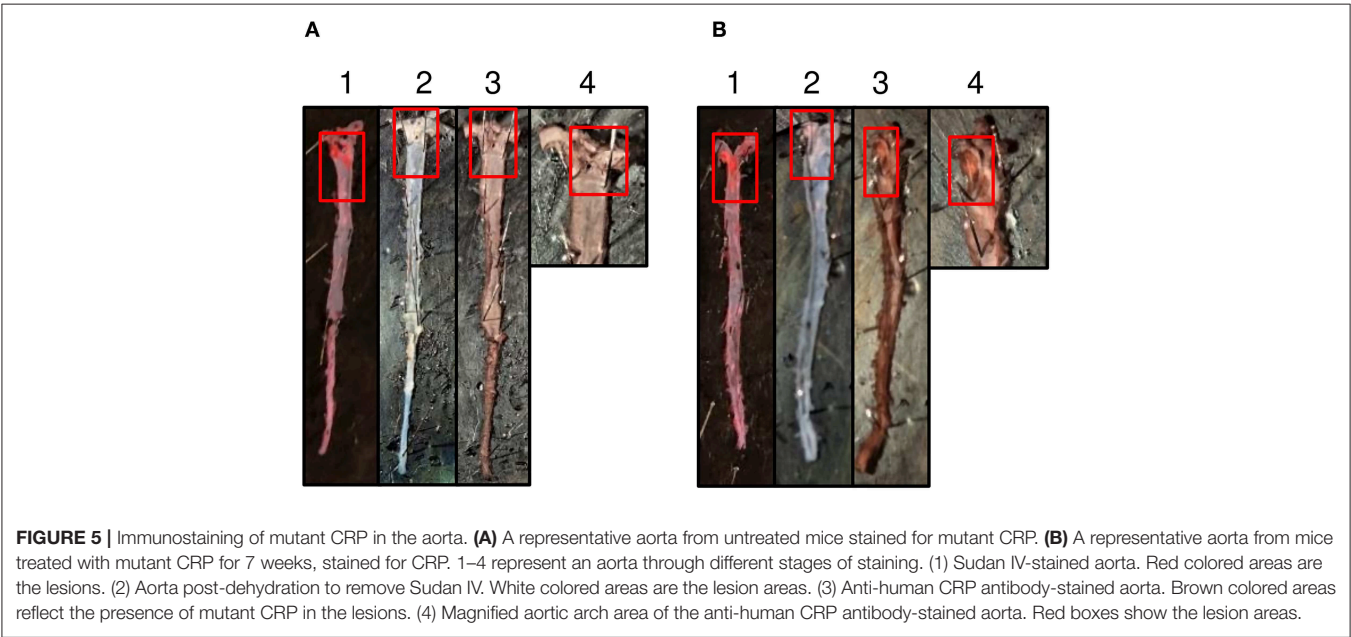


TABLE 1 | Concentration of lipoproteins in the plasma.

Week of sacrifice	HDL (mg/dl)		LDL (mg/dl)	
	Untreated	CRP-treated	Untreated	CRP-treated
1	61 ± 2	85 ± 19	864 ± 16	713 ± 31
3	91 ± 15	71 ± 19	838 ± 231	786 ± 246
5	78 ± 4	74 ± 12	754 ± 118	834 ± 185
7	131 ± 1	107 ± 15	932 ± 86	1,086 ± 23
9	80 ± 3	105 ± 2	1,054 ± 3	1,103 ± 19

Plasma HDL and LDL levels were analyzed in samples pooled from all 12 mice in each group. Results are expressed as mean ± SEM.



alternate days for 7 weeks and beyond was significantly smaller (~40%) than the lesions in the aorta of untreated mice.

Previously, the role of WT CRP in atherosclerosis had been explored, employing a variety of experimental strategies, and 12 papers have been published using CRP from man, mouse and rabbit in both murine and rabbit models of atherosclerosis (31–42). In these studies, both, normal mice and CRP-deficient mice have been employed. Both, normal rabbits and rabbits in which CRP was inhibited by using anti-sense technology have been employed. Both, passively administered CRP and transgenic human CRP have been employed. Three different types of atherosclerosis-prone mice, *apoE*^{−/−}, *ldlr*^{−/−}, and *apoB*^{100/100} *ldlr*^{−/−}, have been used in these studies. Two of the 12 papers indicated that both human and murine CRP might be playing an atheroprotective role: human CRP was shown to slow the development of atherosclerosis in the *apoB*^{100/100} *ldlr*^{−/−} mice (36), and the lesion size in CRP-deficient mice on *apoE*^{−/−} or *ldlr*^{−/−} background was either equivalent or increased compared to that in normal mice (41). In the other 10 papers, regardless of the experimental strategy used, WT CRP from all species did not show any effect on the development of atherosclerosis in animals, suggesting that WT CRP is neither pro-atherogenic nor anti-atherogenic (25, 43).

The most logical explanation for the observations of zero or nominal effects of WT CRP (31–42) on atherosclerosis in animal models is that the animal models of atherosclerosis do not possess the required inflammatory microenvironment that is needed by CRP to change its structure and be able to interact with atherogenic LDL (10, 18, 25). WT CRP shows no effect because pH near the lesions may not be acidic in animal models and, therefore, the structure of administered or endogenous WT CRP remains unchanged (10, 18, 25). WT CRP treated with acidic pH *in vitro* was unsuitable for administration into blood circulation of animals since the acidic pH-induced conformational alteration in the pentameric structure of CRP was found to be reversible at physiological pH (17). Therefore, we hypothesized that an *in vitro*-generated mutant CRP, capable of binding to atherogenic LDL without the requirement of any structural modification *in vivo*, would be suitable to investigate the mechanism of action of CRP on the development of the disease in animal models (10, 16, 18, 25). Indeed, in one study, monomeric CRP, that is also capable of binding to atherogenic LDL, was employed and the results showed that monomeric CRP was protective against atherosclerosis in *apoE*^{−/−} mice (34). Thus, our current findings using mutant CRP and previous findings using monomeric CRP, both molecules capable of binding to atherogenic LDL, indicate that structurally altered CRP protects against atherosclerosis; it is just that WT CRP does not exert a protective effect in most animal models (25, 44).

The effect of mutant CRP on the size of atherosclerotic lesions in the *en face* aorta was obvious since we found that administered mutant CRP had reached the aorta. The staining of aortic roots for the presence of administered mutant CRP provided inconclusive results (data not shown). It is assumed that if mutant CRP reached the aorta, it also reached the aortic root, and if this assumption is correct, then the effect of mutant CRP was site-specific. Site-specific effects of experimental manipulations have been observed in other studies using *ldlr*^{−/−} mice where the

disease developed differently at various lesion-prone sites (45–50). Since the effects of mutant CRP were still observed after week 9 in the *en face* aorta, it is unlikely that anti-CRP antibodies were produced in response to intravenous administration of mutant CRP that could have inhibited its functions.

The topology of the LDL-binding site and the number of LDL-binding sites on mutant CRP remain undefined. Two possible mechanisms have been proposed for the interaction between conformationally altered CRP and atherogenic LDL. The intrinsically disordered region present in CRP has been shown to participate in the binding of monomeric CRP and atherogenic LDL (51). We proposed that the loosening of the CRP pentamer contributed to the formation of the LDL-binding site (16–18, 25). Recently, it has been suggested that the pentameric assembly of CRP harbors a pronounced plasticity in inter-subunit interactions, which may form the basis for a reversible activation of CRP in inflammation (52). However, it is unknown whether the intrinsically disordered region was exposed or the pentamer was loosened in mutant CRP employed in this study. We speculate that there is only one LDL-binding site per CRP pentamer. However, for the protection of mice against atherosclerosis, it is possible that both sites, the PCh-binding site and the LDL-binding site, participate. The search for a new mutant CRP which can bind to both PCh and to atherogenic LDL is in progress. Any possible similarity between mutant CRP used in this study and previously reported conformationally altered pentameric forms of CRP, pCRP*, and mCRPm (20, 21), is unknown.

Based on the data obtained from a single regimen for mutant CRP treatment, we conclude that one of the functions of CRP is to confer protection against atherosclerosis. We propose, again, that there is no need to stop the biosynthesis of CRP, and that a drug that can lower cholesterol level but not CRP levels could be superior to statins which reduce both CRP and cholesterol levels (53, 54). These suggestions are supported by the fact that, in rabbits, the inhibition of plasma CRP did not affect the development of atherosclerosis (42). It has also been previously postulated that the deposition of CRP at the atherosclerotic lesions may be independent of the CRP levels in the circulation and that CRP-mediated lipoprotein removal likely underlies the regression of early lesions which occurs continuously throughout life and that CRP should be considered as an anti-atherosclerotic protein (10, 13). A long-term goal should be the discovery and design of small-molecule compounds to aid endogenous native CRP in capturing atherogenic LDL, as proposed earlier (14, 25). Another goal could be to investigate the possible protective effects of mutant CRP used in this study in animal models of other inflammatory diseases.

Our data also provide a proof of concept that a local inflammation-induced structural change in native CRP is a prerequisite for CRP to control the development of atherosclerosis. An appropriate inflammatory microenvironment at the site of LDL deposition seems to be critical for CRP to prevent atherosclerosis. One function of inflammation could be to change the structure of proteins, including CRP. Inflammation is not a silent killer, perhaps, as has been suggested (55).

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by University Committee on Animal Care, East Tennessee State University.

AUTHOR CONTRIBUTIONS

AP, SS, and DT performed the experiments. AP and AA analyzed the data. AA conceived and designed the experiments and wrote

the paper. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by National Institutes of Health Grant AR068787.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2020.01780/full#supplementary-material>

REFERENCES

- Libby P, Buring JE, Badimon L, Hansson GK, Deanfield J, Bittencourt MS, et al. Atherosclerosis. *Nat Rev Dis Primers*. (2019) 5:56. doi: 10.1038/s41572-019-0106-z
- Galkina E, Ley K. Immune and inflammatory mechanisms of atherosclerosis. *Annu Rev Immunol*. (2009) 27:165–97. doi: 10.1146/annurev.immunol.021908.132620
- Leake DS. Does an acidic pH explain why low-density lipoprotein is oxidized in atherosclerotic lesions? *Atherosclerosis*. (1997) 129:149–57. doi: 10.1016/S0021-9150(96)00635-2
- Björnheden T, Levin M, Evaldsson M, Wiklund O. Evidence of hypoxic areas within the arterial wall in vivo. *Arterioscler Thromb Vasc Biol*. (1999) 19:870–6. doi: 10.1161/01.ATV.19.4.870
- Naghavi M, John R, Naguib S, Siadaty MS, Grasu R, Kurian KC, et al. pH heterogeneity of human and rabbit atherosclerotic plaques; a new insight into detection of vulnerable plaque. *Atherosclerosis*. (2002) 164:27–35. doi: 10.1016/S0021-9150(02)00018-7
- Sneck M, Kovanen PT, Öörni K. Decrease in pH strongly enhances binding of native, proteolyzed, lipolyzed, and oxidized low density lipoprotein particles to human aortic proteoglycans. *J Biol Chem*. (2005) 280:37449–54. doi: 10.1074/jbc.M508565200
- Haka AS, Grosheva I, Chiang E, Buxbaum AR, Baird BA, Pierini LM, et al. Macrophages create an acidic extracellular hydrolytic compartment to digest aggregated lipoproteins. *Mol Biol Cell*. (2009) 20:4932–40. doi: 10.1091/mbc.09-07-0559
- Gabay C, Kushner I. Acute-phase proteins and other systemic responses to inflammation. *N Engl J Med*. (1999) 340:448–54. doi: 10.1056/NEJM199902113400607
- Sun H, Koike T, Ichikawa T, Hatakeyama K, Shiomi M, Zhang B, et al. C-reactive protein in atherosclerotic lesions: Its origin and pathophysiological significance. *Am J Pathol*. (2005) 167:1139–48. doi: 10.1016/S0002-9440(10)61202-3
- Agrawal A, Hammond DJ Jr, Singh SK. Atherosclerosis-related functions of C-reactive protein. *Cardiovasc Haematol Disord Drug Targets*. (2010) 10:235–40. doi: 10.2174/187152910793743841
- Volanakis JE, Kaplan MH. Specificity of C-reactive protein for choline phosphate residues of pneumococcal C-polysaccharide. *Proc Soc Exp Biol Med*. (1971) 136:612–4. doi: 10.3181/00379727-136-35323
- Thompson D, Pepys MB, Wood SP. The physiological structure of human C-reactive protein and its complex with phosphocholine. *Structure*. (1999) 7:169–77. doi: 10.1016/S0969-2126(99)80023-9
- Torzewski M. Enzymatically modified LDL, atherosclerosis and beyond: paving the way to acceptance. *Front Biosci*. (2018) 23:1257–71. doi: 10.2741/4642
- Bhakdi S, Torzewski M, Klouche M, Hemmes M. Complement and atherogenesis: binding of CRP to degraded, nonoxidized LDL enhances complement activation. *Arterioscler Thromb Vasc Biol*. (1999) 19:2348–54. doi: 10.1161/01.ATV.19.10.2348
- Chang M-K, Binder CJ, Torzewski M, Witztum JL. C-reactive protein binds to both oxidized LDL and apoptotic cells through recognition of a common ligand: phosphorylcholine of oxidized phospholipids. *Proc Natl Acad Sci USA*. (2002) 99:13043–8. doi: 10.1073/pnas.192399699
- Agrawal A, Gang TB, Rusiñol AE. Recognition functions of pentameric C-reactive protein in cardiovascular disease. *Mediators Inflamm*. (2014) 2014:319215. doi: 10.1155/2014/319215
- Hammond DJ Jr, Singh SK, Thompson JA, Beeler BW, Rusiñol AE, Pangburn MK, et al. Identification of acidic pH-dependent ligands of pentameric C-reactive protein. *J Biol Chem*. (2010) 285:36235–44. doi: 10.1074/jbc.M110.142026
- Singh SK, Thirumalai A, Hammond DJ Jr, Pangburn MK, Mishra VK, Johnson DA, et al. Exposing a hidden functional site of C-reactive protein by site-directed mutagenesis. *J Biol Chem*. (2012) 287:3550–8. doi: 10.1074/jbc.M111.310011
- Singh SK, Thirumalai A, Pathak A, Ngwa DN, Agrawal A. Functional transformation of C-reactive protein by hydrogen peroxide. *J Biol Chem*. (2017) 292:3129–36. doi: 10.1074/jbc.M116.773176
- Ji S-R, Wu Y, Zhu L, Potempa LA, Sheng F-L, Lu W, et al. Cell membranes and liposomes dissociate C-reactive protein (CRP) to form a new, biologically active structural intermediate: mCRPm. *FASEB J*. (2007) 21:284–94. doi: 10.1096/fj.06-6722com
- Braig D, Nero TL, Koch H-G, Kaiser B, Wang X, Thiele JR, et al. Transitional changes in the CRP structure lead to the exposure of proinflammatory binding sites. *Nat Commun*. (2017) 8:14188. doi: 10.1038/ncomms14188
- Li S-L, Feng J-R, Zhou H-H, Zhang C-M, Lv G-B, Tan Y-B, et al. Acidic pH promotes oxidation-induced dissociation of C-reactive protein. *Mol Immunol*. (2018) 104:47–53. doi: 10.1016/j.molimm.2018.09.021
- McFadyen JD, Zeller J, Potempa LA, Pietersz GA, Eisenhardt SU, Peter K. C-reactive protein and its structural isoforms: an evolutionary conserved marker and central player in inflammatory diseases and beyond. *Subcell Biochem*. (2020) 94:499–520. doi: 10.1007/978-3-030-41769-7_20
- Singh SK, Suresh MV, Prayther DC, Moorman JP, Rusiñol AE, Agrawal A. C-reactive protein-bound enzymatically modified low-density lipoprotein does not transform macrophages into foam cells. *J Immunol*. (2008) 180:4316–22. doi: 10.4049/jimmunol.180.6.4316
- Singh SK, Agrawal A. Functionality of C-reactive protein for atheroprotection. *Front Immunol*. (2019) 10:1655. doi: 10.3389/fimmu.2019.01655
- Gang TB, Hammond DJ Jr, Singh SK, Ferguson DA Jr, Mishra VK, Agrawal A. The phosphocholine-binding pocket on C-reactive protein is necessary for initial protection of mice against pneumococcal infection. *J Biol Chem*. (2012) 287:43116–25. doi: 10.1074/jbc.M112.427310
- Netherland CD, Pickle TG, Bales A, Thewke DP. Cannabinoid receptor type 2 (CB2) deficiency alters atherosclerotic lesion

- formation in hyperlipidemic Ldlr-null mice. *Atherosclerosis*. (2010) 213:102–8. doi: 10.1016/j.atherosclerosis.2010.07.060
28. Schneider CA, Rasband WS, Eliceiri KW. NIH image to imageJ: 25 years of image analysis. *Nat Methods*. (2012) 9:671–5. doi: 10.1038/nmeth.2089
 29. Fu Z, Yan K, Rosenberg A, Jin Z, Crain B, Athas G, et al. Improved protein extraction and protein identification from archival formalin-fixed paraffin-embedded human aortas. *Proteomics Clin Appl*. (2013) 7:217–24. doi: 10.1002/prca.201200064
 30. Maudsley S, Pepys MB. Immunochemical cross-reactions between pentraxins of different species. *Immunology*. (1987) 62:17–22.
 31. Paul A, Ko KWS, Li L, Yechoor V, McCrory MA, Szalai AJ, et al. C-reactive protein accelerates the progression of atherosclerosis in apolipoprotein E-deficient mice. *Circulation*. (2004) 109:647–55. doi: 10.1161/01.CIR.0000114526.50618.24
 32. Hirschfield GM, Gallimore JR, Kahan MC, Hutchinson WL, Sabin CA, Benson GM, et al. Transgenic human C-reactive protein is not proatherogenic in apolipoprotein E-deficient mice. *Proc Natl Acad Sci USA*. (2005) 102:8309–14. doi: 10.1073/pnas.0503202102
 33. Reifenberg K, Lehr H-A, Baskal D, Wiese E, Schaefer SC, Black S, et al. Role of C-reactive protein in atherogenesis: can the apolipoprotein E knockout mouse provide the answer? *Arterioscler Thromb Vasc Biol*. (2005) 25:1641–6. doi: 10.1161/01.ATV.0000171983.95612.90
 34. Schwedler SB, Amann K, Wernicke K, Krebs A, Nauck M, Wanner C, et al. Native C-reactive protein increases whereas modified C-reactive protein reduces atherosclerosis in apolipoprotein E-knockout mice. *Circulation*. (2005) 112:1016–23. doi: 10.1161/CIRCULATIONAHA.105.556530
 35. Trion A, de Matt MPM, Jukema JW, van der Laarse A, Mass MC, Offerman EH, et al. No effect of C-reactive protein on early atherosclerosis development in apolipoprotein E*3-leiden/human C-reactive protein transgenic mice. *Arterioscler Thromb Vasc Biol*. (2005) 25:1635–40. doi: 10.1161/01.ATV.0000171992.36710.1e
 36. Kovacs A, Tornvall P, Nilsson R, Tegné J, Hamsten A, Björkegren J. Human C-reactive protein slows atherosclerosis development in a mouse model with human-like hypercholesterolemia. *Proc Natl Acad Sci USA*. (2007) 104:13768–73. doi: 10.1073/pnas.0706027104
 37. Tennent GA, Hutchinson WL, Kahan MC, Hirschfield GM, Gallimore JR, Lewin J, et al. Transgenic human CRP is not pro-atherogenic, pro-atherothrombotic or pro-inflammatory in apoE^{-/-} mice. *Atherosclerosis*. (2008) 196:248–55. doi: 10.1016/j.atherosclerosis.2007.05.010
 38. Torzewski M, Reifenberg K, Cheng F, Wiese E, Küpper I, Crain J, et al. No effect of C-reactive protein on early atherosclerosis in LDLR^{-/-}/human C-reactive protein transgenic mice. *Thromb Haemost*. (2008) 99:196–201. doi: 10.1160/TH07-10-0595
 39. Koike T, Kitajima S, Yu Y, Nishijima K, Zhang J, Ozaki Y, et al. Human C-reactive protein does not promote atherosclerosis in transgenic rabbits. *Circulation*. (2009) 120:2088–94. doi: 10.1161/CIRCULATIONAHA.109.872796
 40. Ortiz MA, Campana GL, Woods JR, Boguslawski G, Sosa MJ, Walker CL, et al. Continuously-infused human C-reactive protein is neither proatherosclerotic nor proinflammatory in apolipoprotein E-deficient mice. *Exp Biol Med*. (2009) 234:624–31. doi: 10.3181/0812-RM-347
 41. Teupser D, Weber O, Rao TN, Sass K, Thiery J, Fehling HJ. No reduction of atherosclerosis in C-reactive protein (CRP)-deficient mice. *J Biol Chem*. (2011) 286:6272–9. doi: 10.1074/jbc.M110.161414
 42. Yu Q, Liu Z, Waqar AB, Ning B, Yang X, Shiomi M, et al. Effects of antisense oligonucleotides against C-reactive protein on the development of atherosclerosis in WHHL rabbits. *Mediators Inflamm*. (2014) 2014:979132. doi: 10.1155/2014/979132
 43. Badimon L, Peña E, Arderiu G, Padró T, Slevin M, Vilahur G, et al. C-reactive protein in atherothrombosis and angiogenesis. *Front Immunol*. (2018) 9:430. doi: 10.3389/fimmu.2018.00430
 44. Boncler M, Wu Y, Watala C. The multiple faces of C-reactive protein - physiological and pathophysiological implications in cardiovascular disease. *Molecules*. (2019) 24:2062. doi: 10.3390/molecules24112062
 45. Babaev VR, Patel MB, Semenkovich CF, Fazio S, Linton MF. Macrophage lipoprotein lipase promotes foam cell formation and atherosclerosis in low density lipoprotein receptor-deficient mice. *J Biol Chem*. (2000) 275:26293–9. doi: 10.1074/jbc.M002423200
 46. Schiller NK, Kubo N, Boisvert WA, Curtiss LK. Effect of γ -irradiation and bone marrow transplantation on atherosclerosis in LDL receptor-deficient mice. *Arterioscler Thromb Vasc Biol*. (2001) 21:1674–80. doi: 10.1161/hq1001.096724
 47. King VL, Szilvassy SJ, Daugherty A. Interleukin-4 deficiency decreases atherosclerotic lesion formation in a site-specific manner in female LDL receptor^{-/-} mice. *Arterioscler Thromb Vasc Biol*. (2002) 22:456–61. doi: 10.1161/hq0302.104905
 48. VanderLaan PA, Reardon CA, Getz GS. Site specificity of atherosclerosis: Site-selective responses to atherosclerotic modulators. *Arterioscler Thromb Vasc Biol*. (2004) 24:12–22. doi: 10.1161/01.ATV.0000105054.43931.f0
 49. Goel R, Schrank BR, Arora S, Boylan B, Fleming B, Miura H, et al. Site-specific effects of PECAM-1 on atherosclerosis in LDL receptor-deficient mice. *Arterioscler Thromb Vasc Biol*. (2008) 28:1996–2002. doi: 10.1161/ATVBAHA.108.172270
 50. Melgar-Lesmes P, Sánchez-Herrero A, Lozano-Juan F, de la Torre Hernández JM, Montell E, Jiménez W, et al. Chondroitin sulphate attenuates atherosclerosis in apoE knockout mice involving cellular regulation of the inflammatory response. *Thromb Haemost*. (2018) 118:1329–39. doi: 10.1055/s-0038-1657753
 51. Li H-Y, Wang J, Meng F, Jia Z-K, Su Y, Bai Q-F, et al. An intrinsically disordered motif mediates diverse actions of monomeric C-reactive protein. *J Biol Chem*. (2016) 291:8795–804. doi: 10.1074/jbc.M115.695023
 52. Lv J-M, Chen J-Y, Liu Z-P, Yao Z-Y, Wu Y-X, Tong C-S, et al. Cellular folding determinants and conformational plasticity of native C-reactive protein. *Front Immunol*. (2020) 11:583. doi: 10.3389/fimmu.2020.00583
 53. Voleti B, Agrawal A. Statins and nitric oxide reduce C-reactive protein production while inflammatory conditions persist. *Mol Immunol*. (2006) 43:891–6. doi: 10.1016/j.molimm.2005.06.045
 54. Agrawal A. CRP after 2004. *Mol Immunol*. (2005) 42:927–30. doi: 10.1016/j.molimm.2004.09.028
 55. McDade TW. Early environments and the ecology of inflammation. *Proc Natl Acad Sci USA*. (2012) 109:17281–8. doi: 10.1073/pnas.1202244109

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Pathak, Singh, Thewke and Agrawal. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Complement Activation by C-Reactive Protein Is Critical for Protection of Mice Against Pneumococcal Infection

Sanjay K. Singh[†], Donald N. Ngwa[†] and Alok Agrawal^{*}

Department of Biomedical Sciences, James H. Quillen College of Medicine, East Tennessee State University, Johnson City, TN, United States

OPEN ACCESS

Edited by:

Kenji Daigo,
Nippon Medical School, Japan

Reviewed by:

Christine Gaboriaud,
UMR5075 Institut de Biologie
Structurale (IBS), France
Kenneth Reid,
University of Oxford, United Kingdom

*Correspondence:

Alok Agrawal
agrawal@etsu.edu

[†]These authors have contributed
equally to this work

Specialty section:

This article was submitted to
Molecular Innate Immunity,
a section of the journal
Frontiers in Immunology

Received: 11 May 2020

Accepted: 07 July 2020

Published: 13 August 2020

Citation:

Singh SK, Ngwa DN and Agrawal A
(2020) Complement Activation by
C-Reactive Protein Is Critical for
Protection of Mice Against
Pneumococcal Infection.
Front. Immunol. 11:1812.
doi: 10.3389/fimmu.2020.01812

C-reactive protein (CRP), a component of the innate immune system, is an antipneumococcal plasma protein. Human CRP has been shown to protect mice against infection with lethal doses of *Streptococcus pneumoniae* by decreasing bacteremia. *in vitro*, CRP binds to phosphocholine-containing substances, such as pneumococcal C-polysaccharide, in a Ca²⁺-dependent manner. Phosphocholine-complexed human CRP activates the complement system in both human and murine sera. The mechanism of antipneumococcal action of CRP *in vivo*, however, has not been defined yet. In this study, we tested a decades-old hypothesis that the complement-activating property of phosphocholine-complexed CRP contributes to protection of mice against pneumococcal infection. Our approach was to investigate a CRP mutant, incapable of activating murine complement, in mouse protection experiments. We employed site-directed mutagenesis of CRP, guided by its three-dimensional structure, and identified a mutant H38R which, unlike wild-type CRP, did not activate complement in murine serum. Substitution of His³⁸ with Arg in CRP did not affect the pentameric structure of CRP, did not affect the binding of CRP to pneumococci, and did not decrease the stability of CRP in mouse circulation. Employing a murine model of pneumococcal infection, we found that passively administered H38R CRP failed to protect mice against infection. Infected mice injected with H38R CRP showed no reduction in bacteremia and did not survive longer, as opposed to infected mice treated with wild-type CRP. Thus, the hypothesis that complement activation by phosphocholine-complexed CRP is an antipneumococcal effector function was supported. We can conclude now that complement activation by phosphocholine-complexed CRP is indeed essential for CRP-mediated protection of mice against pneumococcal infection.

Keywords: C-reactive protein, acute phase response, complement, inflammation, pneumococcal infection

INTRODUCTION

C-reactive protein (CRP) is a multifunctional component of the acute phase response and innate host defense machinery (1, 2). CRP is composed of five identical subunits arranged as a cyclic pentamer (3, 4). Each subunit has a phosphocholine (PCh)-binding site through which CRP binds to PCh-containing substances such as C-polysaccharide (PnC) of the cell wall of *Streptococcus*

pneumoniae, in a Ca^{2+} -dependent manner (3–6). After complexing with a ligand such as PnC, CRP activates the complement system (7, 8). Human CRP activates complement in both human and murine sera (9, 10). In human serum, CRP binds to C1q and activates the classical pathway of complement (7). Since human CRP does not interact with murine C1q, it is not known which pathway is utilized by human CRP to activate murine complement (9).

The C1q-binding site of CRP is formed in and around a cleft that is located on the opposite side of the PCh-binding site of the CRP pentamer (3, 4). The amino acid residues which contribute to the formation of the C1q-binding site of CRP are His³⁸, Glu⁸⁸, Asp¹¹², Asn¹⁵⁸, and Tyr¹⁷⁵ from one subunit and Lys¹¹⁴ from the neighboring subunit. Mutational analysis of these amino acids revealed that His³⁸, Asp¹¹², and Tyr¹⁷⁵ were critical for binding to C1q and activating complement in human serum (11, 12). Asp¹¹² and Tyr¹⁷⁵ appeared to be the C1q contact residues. Three CRP mutants, H38R, D112N, and Y175A have been previously identified as the mutants which displayed reduced binding to C1q and did not activate complement in human serum (12).

Human CRP has been shown to protect mice against lethal pneumococcal infection (13–16). Although a functioning complement system is required for full CRP-mediated protection, the exact mechanism of action of CRP in protecting mice against pneumococcal infection is not known (17–19). Decades ago, it was hypothesized that complement activation by CRP complexed with PCh was responsible for CRP-mediated protection of mice against pneumococcal infection (20). This hypothesis could not be tested experimentally at the time due to the unavailability of a CRP mutant which would bind to pneumococci but would not activate complement in murine serum.

Previously, we tested the Y175A CRP mutant for activation of murine complement. We reported that Y175A CRP did not activate human complement but activated murine complement (9). Other CRP mutants, H38R and D112N, that did not activate human complement were not tested for murine complement activation earlier. Here, we report that the CRP mutant H38R does not activate murine complement either. The availability of H38R CRP provided us with the needed tool to test the hypothesis that complement activation by PCh-complexed CRP is critical for CRP to protect mice against pneumococcal infection.

MATERIALS AND METHODS

Construction and Expression of CRP Mutants

The construction of H38A and H38R CRP mutants has been described earlier (12). CRP mutants were expressed in CHO cells using the ExpiCHO Expression System (ThermoFisher Scientific) according to manufacturer's instructions. In brief, non-adherent ExpiCHO-S cells (Gibco) were cultured in a shaker flask at 37°C with 5% CO₂. Cells (6×10^6 cells/ml) were then transfected with mutant CRP cDNA (1 µg) using Expifectamine reagent (3.2

µl/ml). Transfected cells were cultured for 20 h at 37°C with 5% CO₂. At 20 h post-transfection, ExpiCHO enhancer (6 µl/ml) and ExpiCHO feed (240 µl/ml) were added to the transfected cells and the culture was then transferred to 32°C with 8% CO₂. The culture media containing expressed CRP mutants were harvested 14 days post-transfection.

Purification of CRP

WT CRP was purified exactly as described previously, and the same method was used to purify CRP mutants H38A and H38R (21). In brief, CRP was purified by Ca^{++} -dependent affinity chromatography on a PCh-Sepharose column (Pierce), followed by gel filtration on a Superose12 column (GE Healthcare) using the Biologic Duo Flow Protein Purification System (Bio-Rad). Purified CRP was stored in TBS (10 mM Tris-HCl, pH 7.2, containing 150 mM NaCl) containing 2 mM CaCl₂ at 4°C and was used within 10 days. The purity and pentameric structure of CRP mutants were determined by SDS-PAGE and gel filtration.

For use in mice, purified CRP was treated with the Detoxi-Gel Endotoxin Removing Gel (ThermoFisher Scientific) according to manufacturer's instructions. The concentration of endotoxin in all CRP preparations, as determined by using the Limulus Amebocyte Lysate kit QCL-1000 (Lonza), was <2.2 endotoxin units per 25 µg CRP.

Murine Complement Activation Assay

First, poly-L-lysine-PnC (P-PnC) was synthesized, as described previously (22), with some modifications. Briefly, 200 µl of 1 mg/ml PnC (Statens Serum Institute, 3459) was slowly added to 10 ml of 10 mM NaOH. Then, 10 mg of cyanuric chloride (Sigma, C95501) was added, followed by the addition of 2 ml of poly-L-lysine (200 µg/ml in H₂O), to the mixture. After adjusting the pH to 8.2 using NaOH, the mixture was incubated for 2 h at 4°C with occasional stirring. The resulting P-PnC (poly-L-lysine ~20 µg/ml and PnC ~100 µg/ml) was stored at 4°C; a 1:4 dilution of this preparation was used to coat microtiter wells for the following assays.

Binding of CRP to the PCh ligand P-PnC was evaluated as follows: Microtiter wells were coated with P-PnC in 100 µl TBS, overnight at 4°C. The unreacted sites were blocked with TBS containing 0.5% gelatin for 1 h at room temperature. CRP, diluted in TBS containing 2 mM CaCl₂, 0.1% gelatin and 0.02 % Tween 20 (TBS-Ca), was then added in duplicate wells and incubated for 2 h at 37°C. After washing the wells with TBS-Ca, bound CRP was detected by using anti-CRP monoclonal antibody HD2.4 diluted in TBS-Ca. HRP-conjugated goat anti-mouse IgG diluted in TBS-Ca was used as the secondary antibody. Color was developed using ABTS substrate and the OD was read at 405 nm in a plate reader.

Murine complement activation was assessed by measuring the deposition of activated murine C3 on P-PnC-complexed CRP, as follows: Microtiter wells were coated with P-PnC in 100 µl 10 mM phosphate buffer saline, pH 7.2 (PBS), overnight at 4°C. The unreacted sites were blocked with PBS containing 1% BSA for 1 h at room temperature, followed by rinsing the wells with buffer A (PBS containing 0.1% BSA and 1 mM CaCl₂). CRP diluted in buffer B (buffer A containing 0.01%

Abbreviations: CRP, C-reactive protein; PCh, phosphocholine; PnC, pneumococcal C-polysaccharide; P-PnC, poly-L-lysine-conjugated PnC; WT, wild-type.

Tween 20) was then added in duplicate wells and incubated for 1 h at 37°C. The wells were washed with buffer B and then with buffer C (PBS containing 1% BSA, 0.15 mM CaCl₂ and 0.5 mM MgCl₂). Normal mouse serum (Innovative Research IGMSC57SER), diluted 1/30 in chilled buffer C, was added to each well and incubated for 30 min at 37°C, followed by washing with buffer C. Goat anti-mouse C3 antibody (Cappel), diluted 1/750 in buffer C, was added to each well. After 1 h at 37°C, the wells were washed, and developed with HRP-conjugated bovine anti-goat IgG (Santa Cruz Biotechnology). Color was developed using ABTS substrate and the OD was read at 405 nm in a plate reader.

Pneumococcus Binding Assay

Pneumococcus binding assay was performed exactly as described previously (16, 23). Briefly, microtiter wells were coated with 10⁷ CFU of pneumococci overnight at 4°C. The unreacted sites in the wells were blocked with TBS containing 0.5% gelatin. CRP, diluted in TBS-Ca, was then added to the wells for 2 h at 37°C. After washing the wells with TBS-Ca, bound CRP was detected by using anti-CRP monoclonal antibody HD2.4. HRP-conjugated goat-anti mouse IgG was used as the secondary antibody. Color was developed using ABTS substrate and the OD was read at 405 nm in a plate reader.

Clearance of H38R CRP From Mouse Circulation

The clearance rate of H38R CRP from the mouse blood was determined as described previously (23). Briefly, five mice were injected i.v. with 50 µg of H38R CRP in 100 µl TBS containing 2 mM CaCl₂ through the tail vein. Blood samples were collected from the tip of the tail after 12, 16, 20, and 24 h, and sera were separated. The concentration of CRP in the sera was measured by ELISA.

Mice

Male C57BL/6J mice, 8–10 weeks old, were purchased from Jackson Laboratories and used in the protection experiments. All animal studies have been reviewed and approved by the University Committee on Animal Care.

Pneumococci

Virulent *S. pneumoniae* type 3, strain WU2 (obtained from Dr. David Briles, University of Alabama, Birmingham, AL), was cultured as described previously (23). Single use bacterial aliquot (1 ml) of virulent stock was prepared and stored at –80°C. For each experiment, an aliquot of frozen pneumococci was thawed in 50 ml Todd-Hewitt broth containing 0.5% yeast extract and incubated at 37°C with shaking at 125 rpm for 3 h and collected from mid-log phase cultures. The culture was centrifuged at 7,500 rpm for 15 min. The bacterial pellet was washed and resuspended in 10 ml normal saline and the volume adjusted to an absorbance A₆₀₀ = 0.29 (3.5 × 10⁸ CFU/ml). The concentration, purity, and viability of pneumococci was confirmed by plating on sheep blood agar plates.

Mouse Protection Experiments

Mouse protection experiments were performed exactly as described previously (24). In brief, mice were injected i.v. with 25 µg CRP. After 30 min, 100 µl of 3.4 × 10⁸ CFU/ml of pneumococci was injected. Survival of mice was recorded three times per day for 7 days. To determine bacteremia (CFU/ml), blood samples were collected from each surviving mouse twice daily for the first 3 days, followed by once daily for next 2 days. Blood was diluted and plated on blood agar plates and incubated for 18 h at 37°C before the colonies were counted. The plotting and statistical analyses of the data were done using the GraphPad Prism 4 software. Statistical significance for survival among the groups was determined by Log-rank test and differences in bacteremia were analyzed by Mann-Whitney test.

RESULTS

All experiments were performed three times, unless otherwise mentioned, and comparable results were obtained each time. Results of a representative experiment are shown in the figures where the raw data (OD₂₈₀ or A₄₀₅) were used to plot the curves.

H38R CRP Does Not Activate Murine Complement

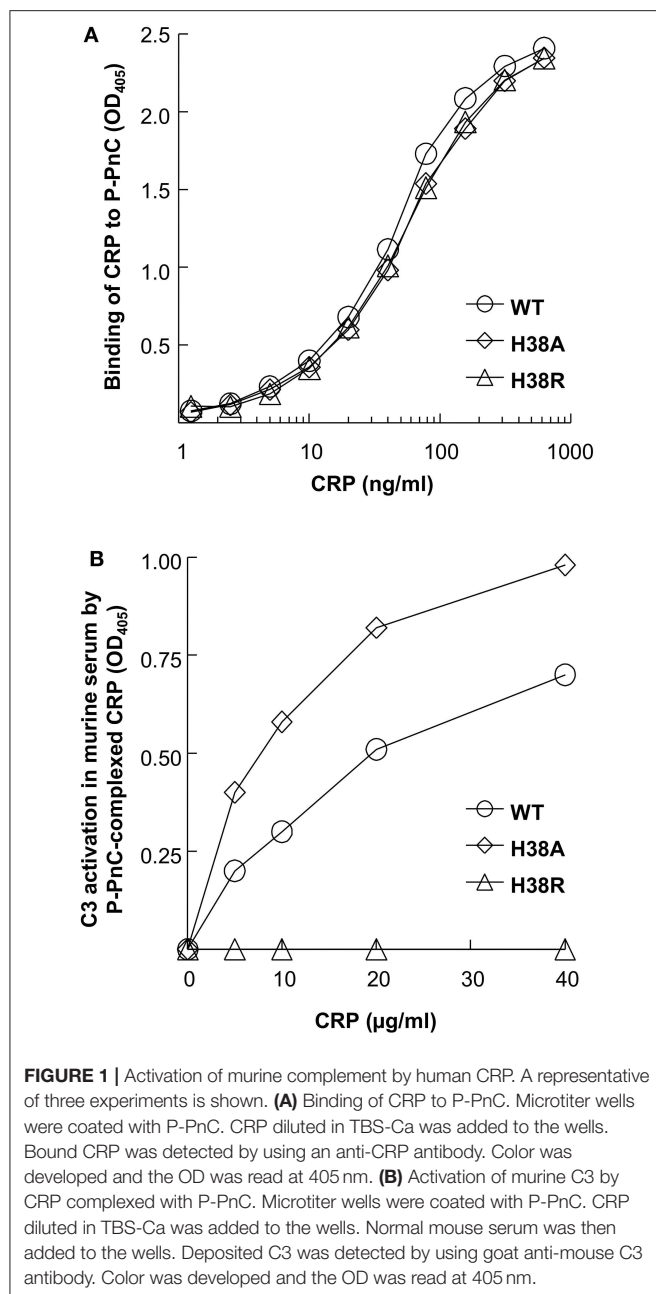
Previously, for murine C3 deposition assays, we used CRP-PnC complexes to activate complement (9). However, we failed to generate a reliable murine C3 deposition assay using commercially available batch of PnC at this time. Instead of using CRP-PnC complexes, we used CRP-P-PnC complexes for murine C3 activation. As shown (Figure 1A), H38A and H38R CRP mutants bound to P-PnC as well as WT CRP did. In the C3 deposition assay (Figure 1B), WT CRP activated murine C3 in a CRP concentration-dependent manner. Like WT CRP, H38A CRP also activated murine C3 in a CRP concentration-dependent manner. Even if the binding of WT CRP and H38A CRP to P-PnC did not differ from each other, H38A CRP was more efficient than WT CRP in activating murine C3. However, H38R CRP did not result in any C3 deposition on CRP-P-PnC, suggesting that H38R CRP was unable to activate murine complement.

H38R CRP Is Pentameric and Binds to Pneumococci

The elution volume of H38R CRP from the gel filtration column was identical to that of WT CRP (Figure 2A), indicating that the molecular weight of H38R CRP was same as WT CRP. SDS-PAGE analysis (Figure 2B) of H38R CRP confirmed the purity of the preparation and showed that there was no difference in the molecular weight of the subunits of WT and H38R CRP. Thus, H38R CRP was pentameric. Also, the Ca²⁺-dependent binding of H38R CRP to pneumococci was similar to that of WT CRP (Figure 2C). We have reported previously that the Ca²⁺-dependent binding of H38R CRP to PnC and PCh-conjugated BSA was also similar to that of WT CRP (12).

H38R CRP Is Stable *in vivo*

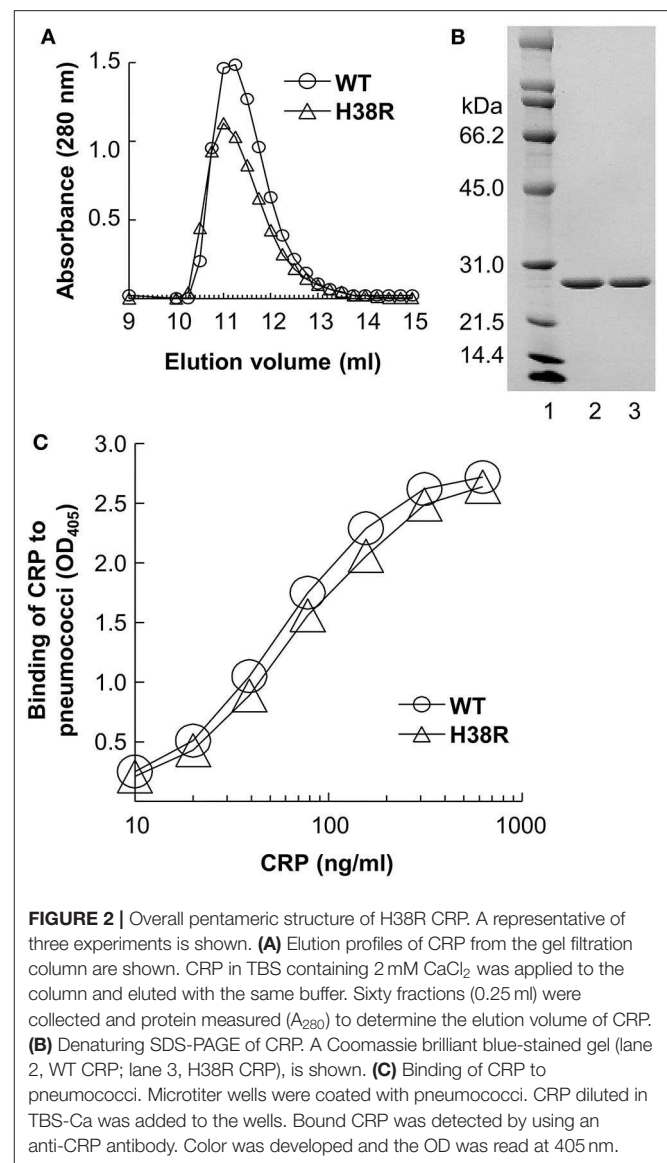
We have reported previously that the rate of clearance of WT CRP from mouse circulation was 0.67 µg/ml/h (23). To



determine the dose of H38R CRP for *in vivo* use, we evaluated the rate of clearance of H38R CRP from mouse circulation (Figure 3). The clearance rate of H38R CRP was found to be 0.20 $\mu\text{g/ml/h}$, suggesting that the clearance of H38R CRP was not faster than that of WT CRP and that the substitution of His³⁸ with Arg did not reduce the stability of H38R CRP *in vivo*.

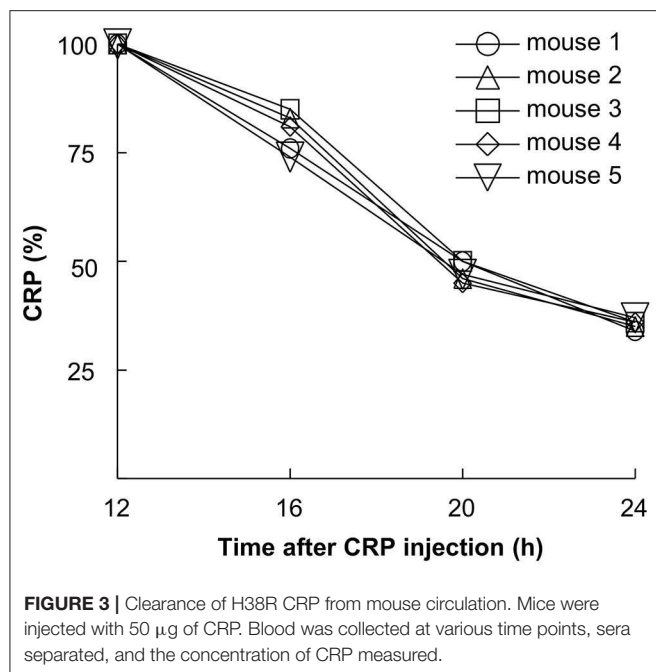
H38R CRP Does Not Protect Mice Against Pneumococcal Infection

Figure 4 shows the combined results from two separate mouse protection experiments. H38A CRP, which was not different from WT CRP in activating murine complement, was included as a



control in the experiment. The median survival time (MST, the time taken for the death of 50% of mice) for mice injected with bacteria alone was 56 h. The MST for mice injected with H38R CRP was 72 h. There was no statistically significant difference between mice receiving H38R CRP and mice not receiving any CRP. The MST for mice injected with either WT CRP or H38A CRP could not be determined since >50% mice survived in both groups. There was no statistically significant difference between mice receiving either WT or H38A CRP.

Next, we determined bacteremia in each surviving mouse (Figure 5). In mice receiving H38A CRP, bacteremia decreased, like in WT CRP-treated mice. There was no statistically significant difference in bacteremia in WT CRP-treated and H38A CRP-treated mice. However, bacteremia continued to increase in H38R CRP-treated mice, like in untreated mice, and mice died once bacteremia was $>10^8$ CFU/ml. There was no

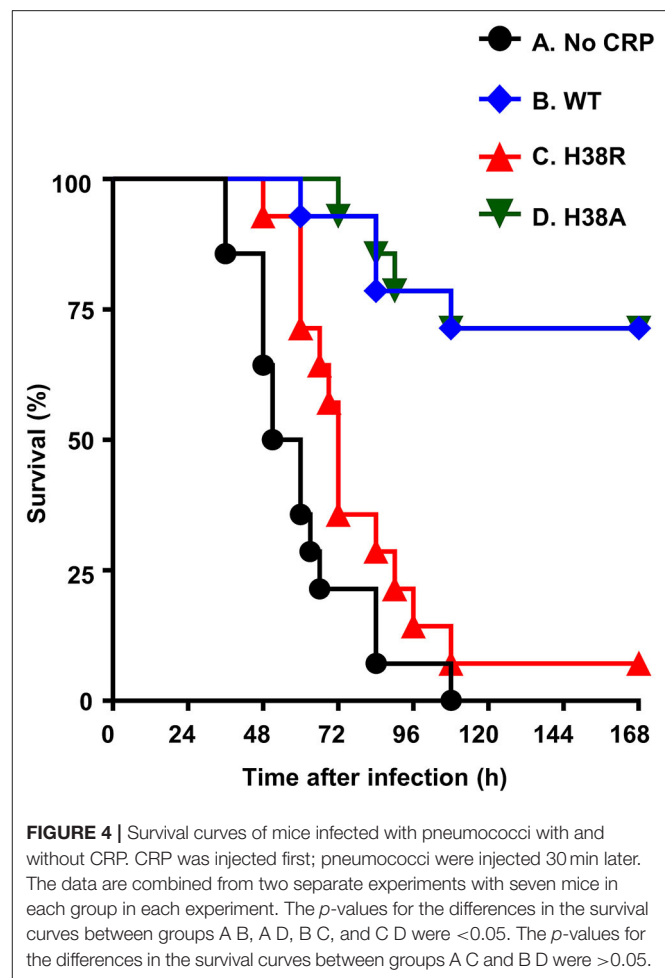


statistically significant difference in bacteremia in untreated and H38R CRP-treated mice. Combined data from survival of mice and bacteremia suggested that H38R CRP was not protective against pneumococcal infection and that the lethality of H38R CRP-treated mice was due to the inability of H38R CRP to decrease bacteremia.

DISCUSSION

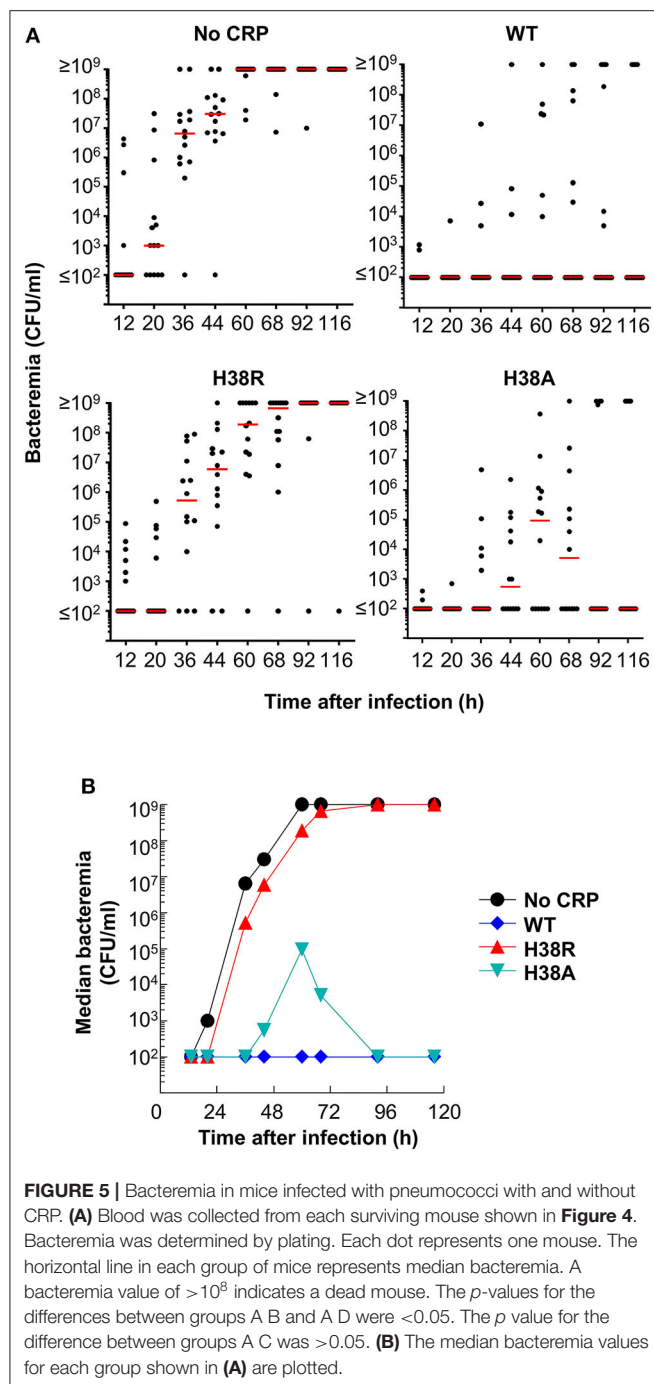
Our major findings in this study were: 1. H38R CRP, which did not activate complement in human serum as reported previously (12), did not activate complement in murine serum either. 2. H38R CRP incapable of activating murine complement failed to protect mice against lethal pneumococcal infection. The inability of H38R CRP to protect mice against pneumococcal infection was solely due to its inability to activate the complement system since the H38R mutation did not reduce the stability of CRP; H38R CRP was more stable than WT CRP *in vivo*. Our findings confirm that complement activation by CRP-PCh complexes constitute the mechanism of CRP-mediated protection (decrease in bacteremia and increase in survival time) of mice against lethal pneumococcal infection.

Previously, we investigated the role of the PCh-binding site of CRP in protection of mice against pneumococcal infection employing a CRP triple mutant, F66A/T76Y/E81A, incapable of binding to PCh and pneumococci. Different mouse models provided different results (23, 24). Surprisingly, in one mouse model, CRP triple mutant protected mice against infection despite being unable to bind to PCh, suggesting that complement activation by CRP-PCh complexes was not required for protection (24). Later, we found out that CRP triple mutant, like acidic pH-treated WT CRP, had inadvertently gained the capability to bind to any protein that was immobilized



on a polystyrene surface, including complement factor H (unpublished observations) (25, 26). Factor H is an inhibitor of complement activation and pneumococci recruit factor H to escape complement-dependent killing (27). Our current finding that complement activation by CRP-PCh complexes is absolutely required for protection suggests that in the previously published protection experiments involving CRP triple mutant (incapable of binding to PCh but capable of binding to immobilized factor H), at some point during the decrease in bacteremia, endogenous murine CRP might have participated in protection by binding to PCh on pneumococci and activating the murine complement system (24).

Human CRP activates complement in both human and murine sera (9, 10). Human CRP binds to C1q and activates the classical pathway of complement in human serum (7). It is not known whether the classical pathway is the only pathway through which human CRP can activate human complement. Human CRP does not interact with murine C1q and, therefore, the activation of murine complement by human CRP is not through the classical pathway (9). The pathway through which human CRP activates murine C3 remains undefined (9, 10). Based on the known crosstalk among CRP, lectins, ficolins, and pneumococci, it was proposed earlier that human CRP



REFERENCES

1. Kushner I. The phenomenon of the acute phase response. *Ann N Y Acad Sci.* (1982) 389:39–48. doi: 10.1111/j.1749-6632.1982.tb22124.x
2. Simons JP, Loeffler JM, Al-Shawi R, Ellmerich S, Hutchinson WL, Tennent GA, et al. C-reactive protein is essential for innate resistance to pneumococcal infection. *Immunology.* (2014) 142:414–20. doi: 10.1111/imm.12266
3. Shrive AK, Cheetham GMT, Holden D, Myles DAA, Turnell WG, Volanakis JE, et al. Three dimensional structure of human C-reactive protein. *Nat Struct Biol.* (1996) 3:346–54. doi: 10.1038/nsb0496-346

can activate complement through the lectin pathway also (9, 10, 28, 29). Irrespective of the pathway through which human CRP activates murine complement, our data suggest that the cleft on CRP, that accommodates the binding site for human C1q, is critical for human CRP to activate murine complement. However, all three amino acid residues, His³⁸, Asp¹¹², and Tyr¹⁷⁵, critical for the formation of the binding site for human C1q and for activation of human complement, are not critical for murine complement activation. The Y175A CRP does not activate human complement but activates murine complement. The H38R does not activate complement in both human and murine sera. The D112N CRP does not activate human complement and has not been tested for murine complement activation yet. The role of the other amino acid residues, Glu⁸⁸ and Asn¹⁵⁸, present in the CRP cleft in activating murine complement is also unknown.

Despite several unanswered questions regarding the mechanisms of complement activation by human CRP in human and murine sera, we conclude that CRP cannot protect against pneumococcal infection if CRP is unable to activate the complement system. Also, since endogenous murine CRP has been shown to be protective against pneumococcal infection in another mouse model (2), we propose that the experiments on structure-function relationships of CRP in pneumococcal infection employing human CRP mutants should always be conducted employing CRP knockout mice (30).

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article.

ETHICS STATEMENT

All animal studies have been reviewed and approved by the East Tennessee State University Committee on Animal Care.

AUTHOR CONTRIBUTIONS

SS and DN performed the experiments. AA conceived and designed the experiments. SS, DN, and AA analyzed the data and drafted the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by National Institutes of Health Grants AR068787, AI117730, and AI151561.

7. Kaplan MH, Volanakis JE. Interaction of C-reactive protein complexes with the complement system. I. Consumption of human complement associated with the reaction of C-reactive protein with pneumococcal C-polysaccharide and with the choline phosphatides, lecithin and sphingomyelin. *J Immunol.* (1974) 112:2135–47.
8. Haapasalo K, Meri S. Regulation of the complement system by pentraxins. *Front Immunol.* (2019) 10:1750. doi: 10.3389/fimmu.2019.01750
9. Suresh MV, Singh SK, Ferguson DA Jr, Agrawal A. Role of the property of C-reactive protein to activate the classical pathway of complement in protecting mice from pneumococcal infection. *J Immunol.* (2006) 176:4369–74. doi: 10.4049/jimmunol.176.7.4369
10. Agrawal A, Suresh MV, Singh SK, Ferguson DA Jr. The protective function of human C-reactive protein in mouse models of *Streptococcus pneumoniae* infection. *Endocr Metab Immune Disord Drug Targets.* (2008) 8:231–7. doi: 10.2174/187153008786848321
11. Agrawal A, Volanakis JE. Probing the C1q-binding site on human C-reactive protein by site-directed mutagenesis. *J Immunol.* (1994) 152:5404–10.
12. Agrawal A, Shrive AK, Greenhough TJ, Volanakis JE. Topology and structure of the C1q-binding site on C-reactive protein. *J Immunol.* (2001) 166:3998–4004. doi: 10.4049/jimmunol.166.6.3998
13. Yother J, Volanakis JE, Briles DE. Human C-reactive protein is protective against fatal *Streptococcus pneumoniae* infection in mice. *J Immunol.* (1982) 128:2374–6.
14. Mold C, Nakayama S, Holzer TJ, Gewurz H, Du Clos TW. C-reactive protein is protective against *Streptococcus pneumoniae* infection in mice. *J Exp Med.* (1981) 154:1703–8. doi: 10.1084/jem.154.5.1703
15. Szalai AJ, Briles DE, Volanakis JE. Human C-reactive protein is protective against fatal *Streptococcus pneumoniae* infection in transgenic mice. *J Immunol.* (1995) 155:2557–63.
16. Suresh MV, Singh SK, Ferguson DA Jr, Agrawal A. Human C-reactive protein protects mice from *Streptococcus pneumoniae* infection without binding to pneumococcal C-polysaccharide. *J Immunol.* (2007) 178:1158–63. doi: 10.4049/jimmunol.178.2.1158
17. Horowitz J, Volanakis JE, Briles DE. Blood clearance of *Streptococcus pneumoniae* by C-reactive protein. *J Immunol.* (1987) 138:2598–603.
18. Mold C, Rodic-Polic B, Du Clos TW. Protection from *Streptococcus pneumoniae* infection by C-reactive protein and natural antibody requires complement but not Fc γ receptors. *J Immunol.* (2002) 168:6375–81. doi: 10.4049/jimmunol.168.12.6375
19. Szalai AJ, Briles DE, Volanakis JE. Role of complement in C-reactive protein-mediated protection of mice from *Streptococcus pneumoniae*. *Infect Immun.* (1996) 64:4850–3. doi: 10.1128/IAI.64.11.4850-4853.1996
20. Volanakis JE. Complement activation by C-reactive protein complexes. *Ann N Y Acad Sci.* (1982) 389:235–50. doi: 10.1111/j.1749-6632.1982.tb22140.x
21. Thirumalai A, Singh SK, Hammond DJ Jr, Gang TB, Ngwa DN, Pathak A, et al. Purification of recombinant C-reactive protein mutants. *J Immunol Methods.* (2017) 443:26–32. doi: 10.1016/j.jim.2017.01.011
22. Messina JP, Hickox PG, Lepow ML, Pollara B, Venezia RA. Modification of a direct enzyme-linked immunosorbent assay for the detection of immunoglobulin G and M antibodies to pneumococcal capsular polysaccharide. *J Clin Microbiol.* (1985) 21:390–4. doi: 10.1128/JCM.21.3.390-394.1985
23. Gang TB, Hammond DJ Jr, Singh SK, Ferguson DA Jr, Mishra VK, Agrawal A. The phosphocholine-binding pocket on C-reactive protein is necessary for initial protection of mice against pneumococcal infection. *J Biol Chem.* (2012) 287:43116–25. doi: 10.1074/jbc.M112.427310
24. Gang TB, Hanley GA, Agrawal A. C-reactive protein protects mice against pneumococcal infection via both phosphocholine-dependent and phosphocholine-independent mechanisms. *Infect Immun.* (2015) 83:1845–52. doi: 10.1128/IAI.03058-14
25. Hammond DJ Jr, Singh SK, Thompson JA, Beeler BW, Rusinol AE, Pangburn MK, et al. Identification of acidic pH-dependent ligands of pentameric C-reactive protein. *J Biol Chem.* (2010) 285:36235–44. doi: 10.1074/jbc.M110.142026
26. Agrawal A, Gang TB, Rusinol AE. Recognition functions of pentameric C-reactive protein in cardiovascular disease. *Mediators Inflamm.* (2014) 2014:319215. doi: 10.1155/2014/319215
27. Zipfel PF, Hallström T, Hammerschmidt S, Skerka C. The complement fitness factor H: Role in human diseases and for immune escape of pathogens, like pneumococci. *Vaccine.* (2008) 26:67–74. doi: 10.1016/j.vaccine.2008.11.015
28. Ng PML, Le Saux A, Lee CM, Tan NS, Lu J, Thiel S, et al. C-reactive protein collaborates with plasma lectins to boost immune response against bacteria. *EMBO J.* (2007) 26:3431–40. doi: 10.1038/sj.emboj.76.01762
29. Vassal-Stermann E, Lacroix M, Gout E, Laffly E, Pedersen CM, Martin L, et al. Human L-ficolin recognizes phosphocholine moieties of pneumococcal teichoic acid. *J Immunol.* (2014) 193:5699–708. doi: 10.4049/jimmunol.1400127
30. Ngwa DN, Agrawal A. Structure-function relationships of C-reactive protein in bacterial infection. *Front Immunol.* (2019) 10:166. doi: 10.3389/fimmu.2019.00166

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Singh, Ngwa and Agrawal. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



The Basic Characteristics of the Pentraxin Family and Their Functions in Tumor Progression

Zeyu Wang^{1,2†}, Xing Wang^{3†}, Hecun Zou⁴, Ziyu Dai¹, Songshan Feng¹, Mingyu Zhang¹, Gelei Xiao^{1*}, Zhixiong Liu^{1,2*} and Quan Cheng^{1,2,4**}

¹ Department of Neurosurgery, Xiangya Hospital, Central South University, Changsha, China, ² National Clinical Research Center for Geriatric Disorders, Changsha, China, ³ Department of Neurosurgery, West China Hospital, Sichuan University, Chengdu, China, ⁴ Department of Clinical Pharmacology, Xiangya Hospital, Central South University, Changsha, China

OPEN ACCESS

Edited by:

Silvano Sozzani,
Sapienza University of Rome, Italy

Reviewed by:

Kenji Daigo,
Nippon Medical School, Japan
Barbara Bottazzi,
University of Milan, Italy

*Correspondence:

Quan Cheng
chengquan@csu.edu.cn
Zhixiong Liu
zhixiongliu@csu.edu.cn
Gelei Xiao
xiaogelei@csu.edu.cn

[†]These authors have contributed
equally to this work and share first
authorship

*ORCID:

Quan Cheng
orcid.org/0000-0003-2401-5349

Specialty section:

This article was submitted to
Cytokines and Soluble Mediators in
Immunity,
a section of the journal
Frontiers in Immunology

Received: 16 March 2020

Accepted: 30 June 2020

Published: 18 August 2020

Citation:

Wang Z, Wang X, Zou H, Dai Z,
Feng S, Zhang M, Xiao G, Liu Z and
Cheng Q (2020) The Basic
Characteristics of the Pentraxin Family
and Their Functions in Tumor
Progression.
Front. Immunol. 11:1757.
doi: 10.3389/fimmu.2020.01757

The pentraxin is a superfamily of proteins with the same domain known as the pentraxin domain at C-terminal. This family has two subgroups, namely; short pentraxins (C-reactive protein and serum amyloid P component) and long pentraxins (neuronal pentraxin 1, neuronal pentraxin 2, neuronal pentraxin receptor, pentraxin 3 and pentraxin 4). Each group shares a similar structure with the pentameric complexes arranged in a discoid shape. Previous studies revealed the functions of different pentraxin family members. Most of them are associated with human innate immunity. Inflammation has commonly been associated with tumor progression, implying that the pentraxin family might also participate in tumor progression. Therefore, we reviewed the basic characteristics and functions of the pentraxin family and their role in tumor progression.

Keywords: C-reactive protein, serum amyloid P component, the long pentraxins, pentraxin 3, tumor

INTRODUCTION

The pentraxin family is a superfamily of protein that share the same domain and are made from monomers arranged in pentameric structures with a discoid shape (1). The members of the family are characterized by a 205 amino acids (AA) long conserved sequence located at C-terminal called the pentraxin domain. Members of the pentraxin family share a similar 8 AA (His-x-Cys-x-Ser/Thr-Trp-x-Ser, in which x represent any AA) long conserved sequence called the pentraxin signature within the pentraxin domain (2). Based on the length of the protein sequence, the pentraxin family can be classified into two subfamilies: the short and long pentraxins. The short pentraxins are comprised of C-reactive protein (CRP) and serum amyloid P component (SAP), whereas the long pentraxins are composed of neuronal pentraxin 1 (NPTX1), neuronal pentraxin 2 (NPTX2), neuronal pentraxin receptor (NPTXR), pentraxin 3 (PTX3), and pentraxin 4 (PTX4) (3). The long pentraxins are approximately twice the size of the short pentraxins with an unrelated long N-terminal sequence. This structure variability of family members could explain their function difference.

In the past decades, studies have revealed the functions of specific members of the pentraxin family. For example, the member of the short pentraxin family, CRP, and SAP were previously reported as mediators in human immune system regulation (4–6). Their functions in immune regulation include acting against pathogen invasion, removing mutant cells, and triggering inflammation. The neuronal pentraxins are involved in the development of the central nervous system and neurodegenerative diseases (7). PTX3 not only participates in immune system activation but also affects tumor progression (8, 9).

Chronic inflammations such as chronic atrophic gastritis and cervical intraepithelial neoplasia have been recognized as precancerous lesions (10). Therefore, inflammation is closely associated with tumor progression, including tumorigenesis, metastasis. Reactive oxygen/nitrogen species (ROS/RNS) produced by immune cells and epithelial cells fight against microbial invasion and eliminate the mutant cell. However, they can cause cell dysfunction and promote tumorigenesis (11, 12). Tumors induce inflammation by either producing antibodies or rejecting immunocytes infiltration to avoid immune system surveillance (13). Additionally, the tumor microenvironment facilitates the growth of cancer cells, metastasis, and enhances drug resistance (14). It is necessary to explore the mechanisms through which inflammation and tumor microenvironment enhances tumor progression.

Several reports have also been documented on the role of the pentraxin family in tumor progression. Likewise, several receptors and pathways have been proposed that could be associated with the mechanisms employed by the pentraxin family in mediating tumor progression. Most members of the pentraxin family can activate the PI3K/AKT/mTOR pathways, thereby interfering with the normal cell cycle. The neuronal pentraxins and PTX3 possess specific unique receptors that mediate tumor progression (15). In this review, we have summarized the basic characteristics and functions of each pentraxin family member and highlighted the existing connection between their structures and specific roles in tumor progression.

C-REACTIVE PROTEIN

The human C-reactive protein (CRP) gene is located on the chromosome 1q23.2 (16), and has a length of 1.8-kb. It consists of 0.1 kb at untranslated region (UTR) in the 5' terminal and a 1.2 kb pair UTR region in the 3' terminal, with two exons separated by an intron. The first exon encodes 18 AA signal peptide and the first two amino acids, whereas, the second exon encodes the rest AA (16). The X-ray derived structures of CRP are pentameric with five subunits arranged in a discoid shape. Each unit contains 206 AA with two anti-parallel β -sheets appearing as a flattened β -barrel with a jellyroll topology. The two sides of its discoid have distinct functions. The Ca^{2+} binds to the "A" side and activates the classical complement pathway and phagocytosis by interacting with C1q and Fc γ receptor, respectively (17, 18). The "B" side, CRP recognizes phosphocholine (PCh), a bacterial cell wall component, and eliminates the pathogen (19). The CRP also binds to soluble control protein factor H regulating the alternative-pathway amplification and C3 convertase (18). The secretion of CRP by hepatocytes can be stimulated by the IL6 and IL1 (20), which enhances innate immunity by triggering inflammation and neutralizing pathogen (18, 21).

CRP and Tumor

CRP activates various signaling pathways by binding to the Fc γ receptor (22, 23), which links it with inflammation (17). The PI3K/AKT/mTOR signaling pathway is associated with tumor cell proliferation, metabolic reprogramming, apoptosis,

and metastasis (24). For instance, the CRP arrest cell-cycle at the sub G1 phase by negatively regulating the PI3K/AKT/mTOR signaling pathway in myeloid leukemia (25) and tongue squamous cell carcinoma (26) thus promoting tumor progression. It has been reported that interaction between CRP and Fc γ receptor I facilitate tumor cell metastasis in breast cancer (23). Furthermore, CRP targets to the p38/MAPK pathway causing lytic bone lesions (27) and activates the PI3K/AKT/mTOR and the ERK/NF- κ B pathway thereby inhibiting tumor cell apoptosis via the Fc γ receptor II in multiple myeloma (22).

Previous reports showed that CRP is a clinical marker for infection and has a regulatory role in innate immunity (17, 28). The IL-6/JAK/STAT signaling pathway has been reported to enhance CRP expression in glioblastoma (29), clear cell renal cell carcinoma (30) and gastroesophageal cancers (31). Moreover, the pathway enhances the formation of CRP-mediated tumor microenvironment by activating tumor-associated macrophages (30) and tumor angiogenesis (32, 33). CRP regulates cell apoptosis and cell-cycle in clear cell renal cell cancer (34). Notably, inhibiting CRP expression by targeting IL-1 can prolong overall survival time for patients with multiple myeloma (35).

Multiple studies have, therefore, confirmed that CRP could be used as a prognosis factor (36–38). This has been reported in different tumors including breast cancer (39), prostate cancer (40), non-small cell lung cancer (41), hepatocellular carcinoma (42), cervical cancer (43), head and neck squamous cell carcinoma (44), diffuse large B-cell lymphoma (45) and osteosarcoma (46). The specific mechanisms involved are still lacking. Controversies have also emerged in determining the role of CRP in pancreatic cancer (47, 48) and colorectal cancer (49–51). Considering that CRP also affects tumor progression, more effort in explicating its role in the tumor might be beneficial in understanding the connection between tumor and human innate immunity (Figure 1).

SERUM AMYLOID P COMPONENT

The serum amyloid P component (SAP) gene, identified as a close CRP paralog, is also localized on the chromosome 1q23.2 and shares the same gene architecture (52). The gene is approximately 1.1 kb long with 0.1 kb 5' UTR and 0.15 kb 3' UTR. The SAP structure is similar to CRP except that its subunits consist of 204 AA and has a slight difference at the calcium-binding site (4, 53, 54). In the absence of calcium, SAP form decamers composed of two pentamers facing each other (4). The two ligands of SAP, deoxyadenosine 5'-monophosphate (dAMP), and the 4,6-pyruvate acetal of β -D-galactose (Mo β DG), bind calcium and amyloid fibrils, respectively (55). To distinguish structure and gene sequence of short pentraxins, we archived data on the 3D structure for CRP and SAP from the PDB web portal (<https://www.rcsb.org/>, Figures 2A,B). The prediction of their domain was inferred from the Pfam database (<http://pfam.xfam.org/>, Figure 3) and the Genecard database (<https://www.genecards.org/>, Table 1).

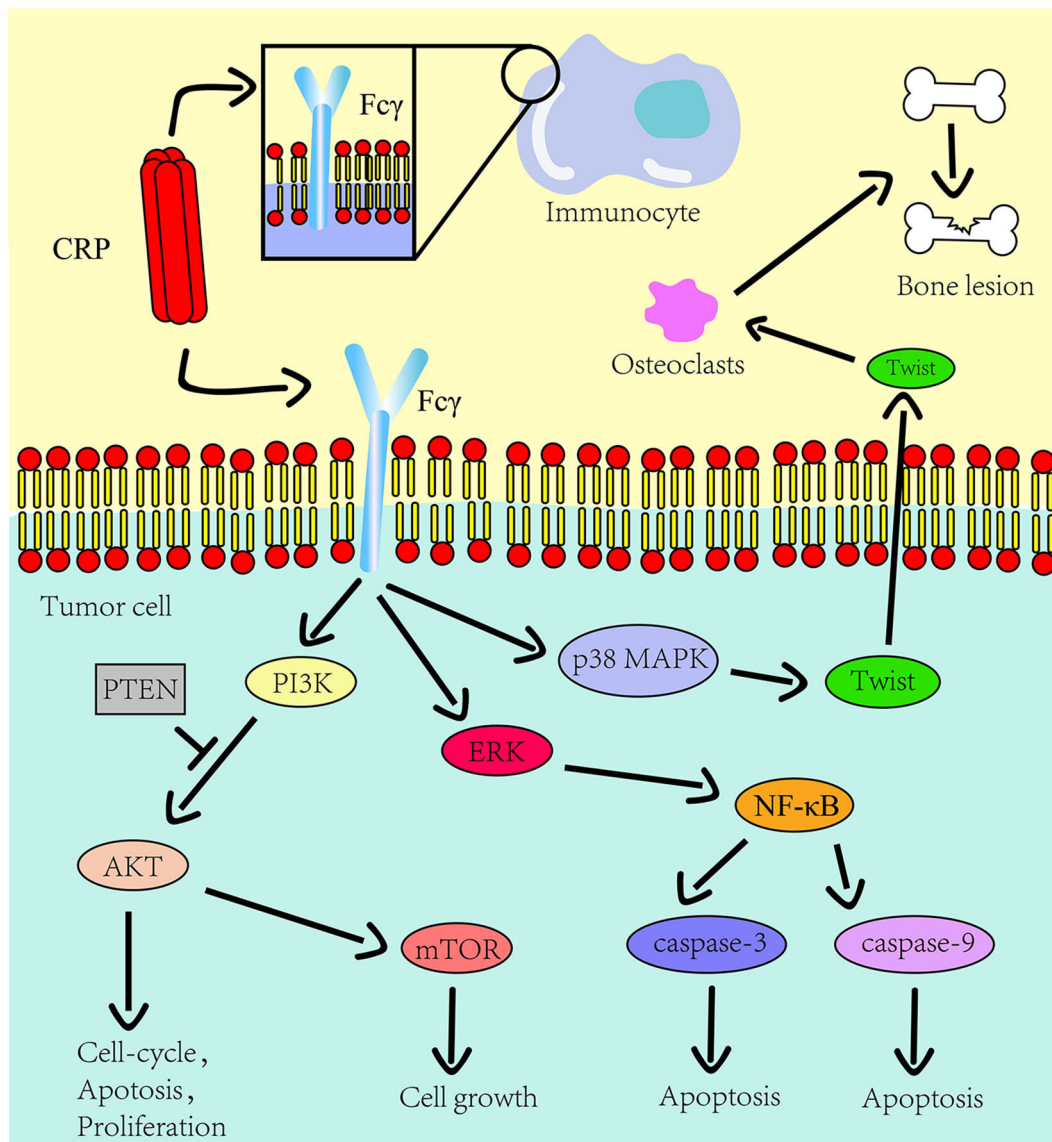


FIGURE 1 | The role CRP on the innate immune system and tumor progression. The Fc γ receptor is expressed on the cell surface of immunocyte and multiple myeloma cells. CRP binds to Fc γ receptor to promote inflammation and tumor progression. The downstream pathways of this receptor in tumors include the PI3K/AKT/mTOR pathway, the ERK/NF- κ B pathway and the p38/MAPK pathway. CRP regulates the expression of osteolytic cytokines in myeloma cells through p38 MAPK-Twist signaling.

Like CRP, SAP is also secreted by hepatocytes and mediates innate immunity by interacting with the complement system and the Fc γ receptor (17). This decreases neutrophil adhesion, inhibits neutrophil spreading, regulates macrophage activation (56), and inhibits fibrocyte differentiation (57). Besides, SAP is involved in immunological tolerance by binding to DNA or chromatin resulting from necrosis and apoptosis cell (58). Furthermore, SAP binds to amyloid fibrils through Mo β DG, thereby causing amyloidosis disease (4–6). SAP is also associated with tuberculosis (59) and sickle cell disease (60), but the specific mechanisms are unknown. Despite limited reports on the role of SAP in tumors, SAP is considered a prognosis factor in non-small

cell lung cancer (61). The highly structural homology between SAP and CRP suggests that SAP has the potential to mediate tumor progression through the Fc γ receptor.

THE NEURONAL PENTRAXINS

Neuronal pentraxin 1 (NPTX1 or NP1) is a 47–50 kDa secreted glycoprotein mainly expressed in neurons. The NPTX1 gene is located on chromosome 17q25.3. Its cDNA clones sequence is made up of a 150 bp 5'UTR, a 1.3 kb coding region, and a 3.6 kb 3'UTR with four introns (62). NPTX1 contains three main domains: a putative ligand- and calcium-binding site, the

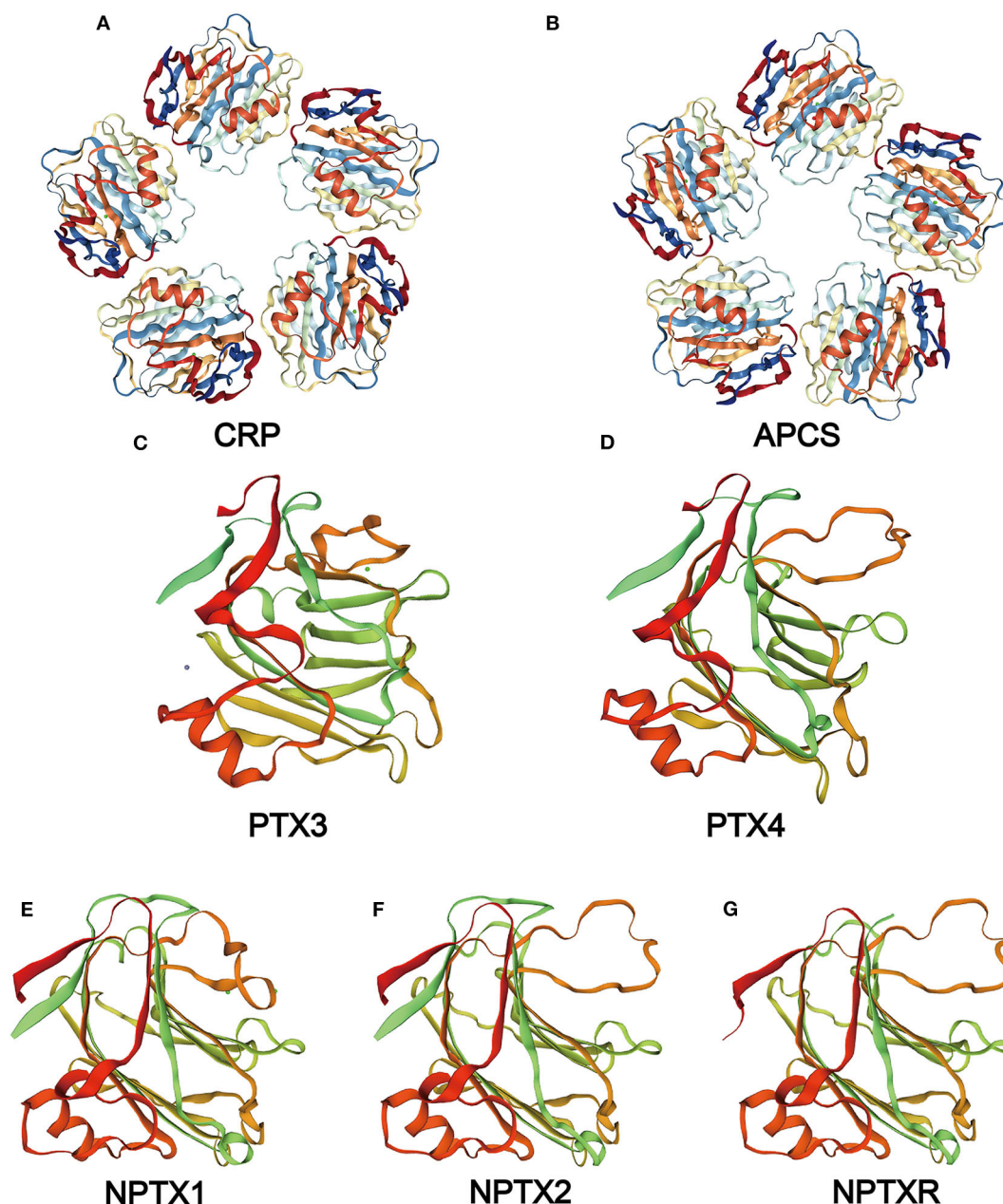


FIGURE 2 | Structure of the pentraxin family members. Protein structure of CRP (A) and SAP (B) from the PDB website and the hypothetical structure of the full monomer (C-terminal domain and N-terminal domain) of PTX3 (C), PTX4 (D), NPTX1 (E), NPTX2 (F), NPTXR (G) from the Swiss database.

pentraxin domain, and an Asn-linked glycosylation site (62). Three NPTX1 domains, including the Pentraxin-related domain, the Pentraxin_CS (Pentraxin, conserved site), and glucanase domain superfamily (ConA-like_dom_sf) are speculated from the Genecard database.

Neuronal pentraxin 2 (NPTX2 or NP2, also known as apelin/p50 in guinea pig or narp in rat), is a ~47 kDa secretory glycoprotein with 431 AA. It is expressed in various tissues of the brain, testicle, pancreas, and skeletal muscle (1). The human NPTX2 gene is located on chromosome 7q22.1. Its cDNA

sequence is made up of a 1.3 kb coding region and 1.2 kb 3'-UTR with four introns (1). Domains of NPTX2 are the same as those of NPTX1, according to the speculation from the Genecard website.

Neuronal pentraxin receptor (NPTXR or NPR) is an ~53 kDa type-II transmembrane protein with 500 AA and is mostly expressed in the brain. It is the only pentraxin family member anchored to the cell membrane by a putative N-terminal transmembrane domain. The receptor binds tightly to its ligands, such as taipoxin, TCBP49, NPTX1, and NPTX2, and activates different downstream signal transduction processes

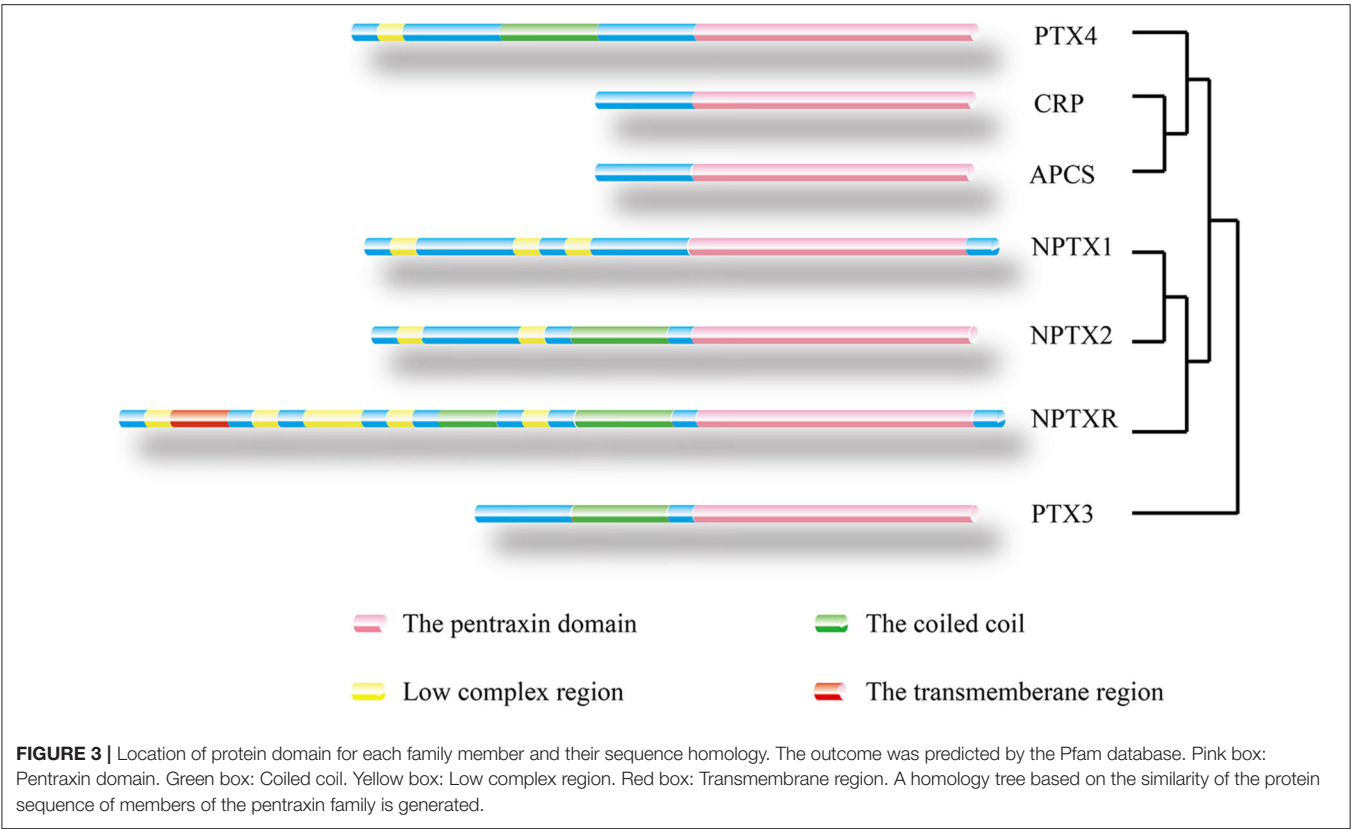


TABLE 1 | The basic characteristics of two short pentraxins, CRP and SAP.

Characteristics	CRP	SAP
Genomic location	1q23.2	1q23.2
Gene sequence	1.8 kb in length, 0.1 kb of 5' UTR, 1.2 kb of 3' UTR	1.1 kb in length, 0.1 kb of 5' UTR, 0.15 kb of 3' UTR
Domain structures	Pentraxin-related, ConA-like_dom_sf, Pentaxin_CS	Pentraxin-related, ConA-like_dom_sf, Pentaxin_CS
Top tissue expression	Liver	Liver
Signaling pathway	PI3K/Akt signaling pathway (25), JAK/STAT signaling pathway (35)	PI3K/Akt/ERK signaling pathway (56)

CRP, C-reactive protein; SAP, serum amyloid P component; UTR, un-translated region; The domain structures were obtained from the Genecard database.

(63). In the human genome, the NPTXR gene is located on chromosome 22q13.1 and has the longest cDNA clones sequence containing a 3.9kb 3' UTR and a 1.5kb open reading frame (63). From the Gencard database, this protein consists of two main domains, Pentraxin-related domain and glucanase domain superfamily (ConA-like_dom_sf). The N-terminal structure of the neuronal pentraxins is unrelated to other known human protein structures (64). Therefore, multiple online databases were used to generated detailed information about the structures of

the neuronal pentraxins. The 3D structures of monomer were referenced from the Swiss database (<https://www.swissmodel.expasy.org/>, **Figures 2E–G**), and their domains were projected from the Pfam (**Figure 3**) and the Genecard databases (**Table 2**).

The Function of the Neuronal Pentraxins

The neuronal pentraxins have different functions in the development of the central nervous system (7) such as mediation of neural differentiation (69), synaptogenesis (75) and synapse plasticity (76, 77). Abnormal expression of the neuronal pentraxins has been reported in some mental diseases such as bipolar disorder (78), central precocious puberty (79), anxiety (80), depression (80), childhood-onset mood disorders (81) and schizophrenia (82). The neuronal pentraxins are associated with neurodegenerative diseases, including Alzheimer’s disease (AD) (83, 84) and Parkinson’s disease (PD) (85, 86). Researchers concluded that NPTXR protein in cerebrospinal fluid is a novel potential biomarker of AD progression and could have important utility in assessing treatment success in clinical trials (83), and NPTX1 could significantly contribute to the pathogenesis of PD (87). Moreover, only NPTX1 among all the neuronal pentraxins participates in inflammation by inducing mitochondria dysfunction (65, 67). Despite the neuronal pentraxins contains the pentraxin domain like the short pentraxins, few studies have classified their roles in human innate immunity which might result from the difference in their tertiary structure.

TABLE 2 | The basic characteristics of the long pentraxins.

Characteristics	NPTX1	NPTX2	NPTXR	PTX3	PTX4
Genomic location	17q25.3	7q22.1	22q13.1	3q25.32	16p13.3
Sequence features	150 bp of 5' UTR, 1.3 kb coding sequence, 3.6 kb 3' UTR	1.3 kb coding sequence, 1.2 kb 3'-UTR	5.5 kb in length, 3.9 kb of 3' UTR, 1.5 kb open reading frame	68 bp of 5' UTR, 650 bp of 3' UTR,	–
Domain structures	Pentraxin-related, Pentraxin_CS, ConA-like_dom_sf	Pentraxin-related, Pentraxin_CS, ConA-like_dom_sf	Pentraxin-related, ConA-like_dom_sf, N-terminal transmembrane domain	Pentraxin-related, Pentraxin_CS, ConA-like_dom_sf	Pentraxin-related, Pentraxin_CS, ConA-like_dom_sf
Top tissue expression	Brain	Brain, liver, testis, skeletal muscle, heart, pancreas	Brain	Monocytes, macrophage, fibroblasts, epithelial cells	Thymus, spleen, small intestine, liver
Signaling pathway	HIF-1 signaling pathway (65), IRS-1/PI3K/Akt signaling pathway (66), JNK and GSK3 signaling pathways (67), Rb/E2F pathway (68), Nodal and BMP signaling pathway (69)	Wnt/ β -catenin signaling pathway (70), p53/PTEN/Akt/NF- κ B signaling pathway (71)	–	Akt/NF- κ B signaling pathway (9), JNK signaling pathway (72), IL-6/Stat3 signaling pathway (73), PI3K signaling pathway (74)	–

NPTX1, neuronal pentraxin 1; NPTX2, neuronal pentraxin 2; NPTXR, neuronal pentraxin receptor; PTX3, pentraxin 3; PTX4, pentraxin 4; UTR, un-translated region; Domain structure were obtained from the Genecard database.

The Neuronal Pentraxins in Cancer

Several pathways were identified as potential mechanisms through which the neuronal pentraxins promote tumor progression. NPTX1 and NPTX2 were reported to cause dysfunction of the PI3K/AKT/mTOR pathway thereby affecting tumor progression in glioma (66), gastrointestinal stromal tumors (GIST) (88) and subependymal giant cell astrocytoma (89). Additionally, NPTX2 promotes tumor cell proliferation and metastasis by activating the NF- κ B pathway (71, 90) and the Wnt/ β -catenin pathway (70). It also induces tissue edema via an independent pathway from the classical VEGF-related pathway (91). The dysfunction of the PI3K/AKT/mTOR pathway interferes with the normal cell-cycle and causes tumorigenesis. Similarly, NPTX1 and NPTX2 can inhibit cyclin A2 and CDK2 through the Rb/E2F signaling pathway (68), respectively, thus inducing G0/G1 arrest in pancreatic cancer (92, 93). Overexpression of NPTX2 has been identified as a prognosis factor in clear cell renal cell carcinoma, and its interaction with AMPA-selective glutamate receptor-4 affects tumor cell viability and metastasis (94, 95).

Abnormal expression of the neuronal pentraxins has been reported in different tumors such as cervical carcinoma (96), primary lung cancer (97), Ewing sarcoma (98), neuroblastoma (99), small cell lung cancer (100) and neuroblastoma (99). Another study has reported an increase in NPTX1 expression in pancreatic cancer after treatment with metformin and aspirin (101). On the contrary, a low level of NPTX2 showed better response to neoadjuvant chemoradiation (CRT) treatment in rectal adenocarcinomas (102).

The neuronal pentraxins are crucial in the central nervous system development when they interact with the AMPA receptor. The AMPA receptor has been proved to be associated with tumors (103, 104). Therefore, the AMPA receptor is a

potential linkage between the neuronal pentraxins and tumor development as opposed to other members of the pentraxin family. Conclusively, the above-reviewed studies did not reveal the explicit mechanism through which the neuronal pentraxins affect tumor progression; however, they form the basis for in-depth studies on the existing association between the two.

PENTRAXIN 3 AND PENTRAXIN 4

Pentraxin 3 (PTX3) and pentraxin 4 (PTX4) are characterized as long pentraxins. The PTX3 gene is located on chromosome 3q25 and has three exons and two introns (105). The three exons encode the leader peptide, an N-terminal domain, and the pentraxin domain, respectively (106). The N-terminal domain of PTX3 and PTX4 is not related to any known protein. However, based on previous research, PTX3 has a putative N-terminal domain that shows structural similarity to the mannose-binding protein and the surfactant proteins (107). PTX3 is secreted by different cell types that include dendritic cells, macrophages, and fibroblasts (108).

PTX4 gene is located on chromosome 16p13.3 and consists of three exons. However, the human PTX4 cDNA sequence and its first exon failed to amplify because it was different from the sequences in the various database (109). The sequence analysis of the protein showed a highly structural homology between PTX4 and the short pentraxins (**Figure 2D**). This indicates that PTX4 might be playing specific roles in innate immunity or tumor progression. To compare the PTX3 and PTX4 structures, their 3D structures were predicted from the Swiss database (<https://www.swissmodel.expasy.org/>, **Figures 2C,D**), and their domain predicted from the Pfam (**Figure 3**) and the Genecard databases (**Table 2**).

Functions of PTX3 and PTX4

From the previous study, an increase in blood PTX3 concentration serves as a monitor of inflammation initiation. The maximal PTX3 level increased slightly earlier than the CRP level (110), suggesting that PTX3 could be a highly sensitive inflammation-related factor. The interaction between PTX3 and the complement system has broad implications in host defense against microbial infections, regulation of the inflammatory reaction, and removal of dead cells. PTX3 is actively involved in the complement pathways activation (111). For example, the classical activation cascade may be initiated when PTX3 binds to C1q via the Fcγ receptor III once the latter is bound on a microbial surface (64, 112, 113). However, the process can be inhibited if the interaction occurs in the fluid-phase (114).

Furthermore, the N-terminal domain of PTX3 enhances tissue repair and remodeling functions (115). A study reported that PTX3 inhibited interstitial fibrosis in acute renal injury (73), indicating its role in an extracellular matrix formation. The N-terminal domain also combines with FGF2 to mediate angiogenic activity (116). For PTX4, there is currently no evidence supporting its functions in innate immunity or tumor progression.

PTX3 and Cancer

PTX3 interacts with the PI3K/AKT/mTOR signaling pathway to induce tumor cell proliferation, apoptosis and metastasis in lung cancer (9), head and neck squamous cell carcinoma (74) and breast cancer (117). PTX3 also inhibits cell proliferation and tumor metastasis by modulating the expression of protein related to the G2/M phase cell-cycle in cervical cancer (118). Furthermore, it arrests cell-cycle at the G0/G1 phase by stimulating the secretion of p21 protein in glioma (119).

Notably, we reported that PTX3 interacts with the fibroblast growth factor-2 (FGF2)/FGF receptor (FGFR) system that mediates the epithelial-mesenchymal transition (EMT) through its N-terminal domain (120). Through this system, PTX3 inhibits tumor metastasis, tumor growth and tumor angiogenesis in melanoma (8), breast cancer (121), prostate cancer (122) and multiple myeloma (123). Besides, PTX3 binds to fibroblast growth factor-8b receptor (FGF8b) and inhibits tumor cell proliferation in steroid hormone-regulated tumors (124). Compared to other pentraxin family members, the PTX3 is highly associated with the FGFR system.

Abnormal PTX3 expression was also observed in different tumors, including glioma (125), esophageal squamous cell carcinoma (126), pancreatic cancer (127), gastric cancer (128), colorectal cancer (129), leiomyosarcoma and desmoid tumors (130). Previous studies have shown that the activation of Fcγ receptor promotes tumor progression (131, 132). Of note, Fcγ receptor expression on NK cells modulates tumor response to immunotherapy (133), and PTX3 can exert its function in human immunity by interacting with this receptor. However, no current research has bridged PTX3 and tumor progression through the Fcγ receptor. Therefore, the multifaceted role of PTX3 in cancer requires further comprehensive study.

OVERALL SURVIVAL ANALYSIS PREDICTION

We conducted the overall survival analysis of the pentraxin family to establish the relationship between the tumor and the pentraxin family members using the Gepia web portal (<http://gepia.cancer-pku.cn/>, **Supplementary Table**).

For short pentraxins, low expression of CRP showed better survival outcomes in kidney renal papillary cell carcinoma (KIRP) than high CRP expression (**Figure 4A**). There was no significant difference in survival outcome between the high and low expression of SAP. The expression levels of PTX4, with similar protein sequence as short pentraxins, showed a significant difference in survival outcome in adrenocortical carcinoma (ACC) and head and neck squamous cell carcinoma (HNSC) (**Figures 4B,C**).

We revealed that low NPTX1 expression improved the survival outcomes in patients with adrenocortical carcinoma (ACC), urothelial bladder carcinoma (BLCA), kidney renal papillary cell carcinoma (KIRP), stomach adenocarcinoma (STAD) and uveal melanoma (UVM), (**Figures 4D–H**). Patients that showed high expression of NPTX2 frequently exhibited worse survival outcomes for glioblastoma multiforme (GBM), kidney renal papillary cell carcinoma (KIRP), lung squamous cell carcinoma (LUSC) and uveal melanoma (UVM), (**Figures 4I–L**). On the other hand, overexpression of NPTXR predicted worse survival outcomes for colon adenocarcinoma (COAD), mesothelioma (MESO), and pancreatic adenocarcinoma (PAAD) and better survival outcome in uterine corpus endometrial carcinoma (UCEC) (**Figures 4M–P**).

Therefore, PTX3 is considered a promoter in tumor progression since its overexpression resulted in worse survival outcomes in invasive breast carcinoma (BRCA), cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC), head and neck squamous cell carcinoma (HNSC), kidney renal clear cell carcinoma (KIRC), kidney renal papillary cell carcinoma (KIRP), brain lower-grade glioma (LGG), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), mesothelioma (MESO), stomach adenocarcinoma (STAD), thyroid carcinoma (THCA) and uterine corpus endometrial carcinoma (UCEC), (**Figure 5**).

The protein structure variability sheds light on the prognosis prediction ability of members of the pentraxin family. The expression of PTX3 affects multiple tumor types compared to other members of the pentraxin family, implying a potential association between its putative N-terminal domain and tumor progression. PTX4 shares a similar protein structure with the short pentraxins, and they are both poor predictors of survival outcomes of patients suggesting that they mainly concentrate on monitoring inflammation. However, this deduced relationship between protein structure and their prognosis capability requires further confirmation.

CONCLUSIONS

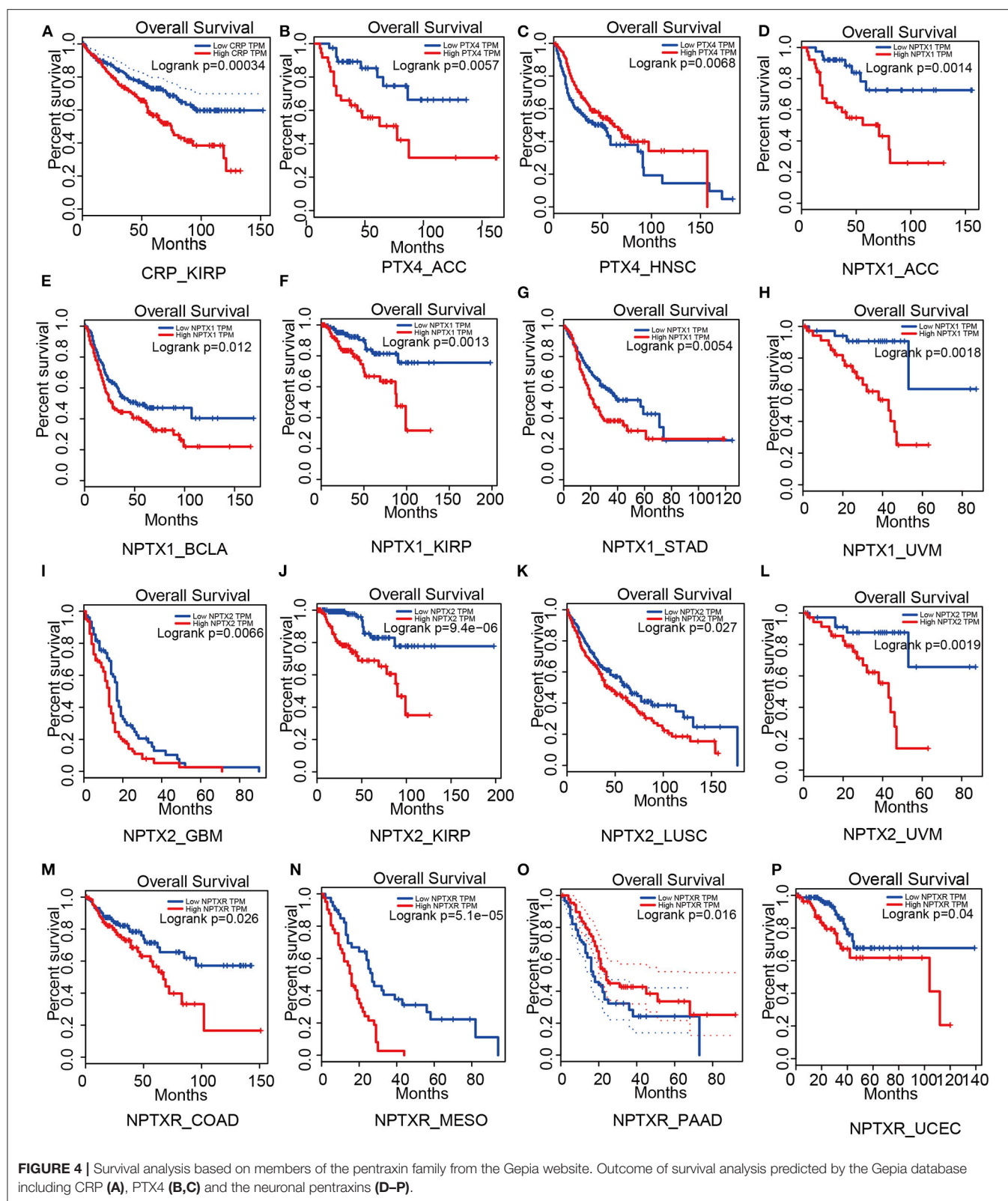
Emerging evidence confirms that the pentraxin family is associated with tumor progression by affecting tumor

TABLE 3 | Role of pentraxins in the control of various tumors.

Tumor	Gene	Expression	References	Mechanism
Glioma	PTX3	Differ from low and high grade	(134)	Arrest cell cycle at the G0/G1 phase to affect glioma proliferation and metastasis.
Glioma	NPTX1	Increased	(66)	Promote tumor proliferation and metastasis via the IRS-1/PI3K/AKT signaling pathway
Glioma	NPTX2	Increased	(91)	Induce tumor tissue edema independent of the classical VEGF-related pathway
Glioblastoma	CRP	Increased	(29)	Stimulate microglial cells to secrete IL-1 β which could induce tumor angiogenesis.
Glioblastoma	NPTX2	Decreased	(90)	Increase survival ratio through reducing NF- κ B activity via inhibiting AKT by p53/PTEN-dependent pathway
Neuroblastoma	NPTX2	Increased	(92)	NPTX2 antagonist could reduce tumor progression
Neuroblastoma	NPTXR	Increased	(92)	Give NPTXR antagonist inhibit tumor progress
Subependymal giant cell astrocytoma	NPTX1	Decreased	(89)	After inhibiting the mTOR signaling pathway not only decrease tumor volume but also increase the expression of NPTX1
Colorectal cancer	NPTX2	Increased	(70)	Combine to frizzled class receptor 6(FZD6) which activate the Wnt/ β -catenin signaling pathway to promote tumor growth and metastasis
Rectal adenocarcinomas	NPTX2	Decreased	(102)	Low level expression improve response to neoadjuvant chemoradiation (CRT) treatment
Colorectal cancer	NPTX1	Decreased	(68)	Inhibit cell proliferation by influence the combination of cyclin A2 and CDK2 and the Rb-E2F signaling pathway
Lung cancer	PTX3	Increased	(9)	Deglycosylated PTX3 observed suppress tumor migration via inactivating the PI3K/AKT and the NF- κ B signaling pathway.
Non-small Cell Lung Cancer	SAP	Increased	(61)	SAP contributes to the clearance of apoptotic cells.
Breast cancer	CRP	Increased	(23)	Binds to Fc γ receptor I and to promote tumor metastasis.
Breast cancer	PTX3	Decreased	(124)	PTX3 inhibit tumor progression via combining to receptor of fibroblast growth factor-8b (FGF8b) and metastasis by activating the EMT process.
Clear cell renal cell cancer	NPTX2	Increased	(94)	Promote tumor viability and invasion via binding to AMPA-selective glutamate receptor-4
Clear cell renal cell cancer	CRP	Increased	(34)	Up-regulate the expression of ATG9B gene to inhibit tumor cell apoptosis and the formation of tumor microenvironment.
Head and neck cancer	PTX3	Increased	(135)	Affect tumor metastasis via the PI3K/AKT and the NF- κ B signaling pathway.
Head and neck squamous cell carcinoma	CRP	Increased	(44)	Promote tumor cell proliferation, metastasis and angiogenesis through the PI3K/AKT signaling pathway.
Multiple myeloma	CRP	Increased	(25)	Binds to Fc γ receptor II to protect tumor and cause lytic bone lesions
Multiple myeloma	PTX3	Increased	(123)	Promote tumor cell proliferation, metastasis and angiogenesis through the PI3K/AKT signaling pathway.
Melanoma	CRP	Increased	(136)	Inhibit tumor angiogenesis, via FGF2/FGFR system, proliferation and apoptosis.
Melanoma	PTX3	Increased	(8)	Cause vitamin D deficiency.
Cervical cancer	PTX3	Increased	(118)	Inhibit tumor metastasis via FGF2/FGFR system which impair the EMT process
Gastroesophageal cancer	CRP	Increased	(31)	Modulate the G2/M phase cell-cycle related protein expression to affect cell proliferation.
Hepatocellular carcinoma	CRP	Increased	(137)	Promote tumor angiogenesis via influencing certain cytokines.
Myeloid leukemia	CRP	Increased	(25)	Its expression level is parallel with HBV activity while silence could promote tumor progression.
Pancreatic cancer	NPTX2	Decreased	(92)	Inhibits cell proliferation through the PI3K/AKT signaling pathway.
Prostate cancer	PTX3	Decreased	(122)	Inhibit tumor progression and migration decrease via inducing cell G0–G1 arrest and inhibiting cell apoptosis

Data from merely abnormal expression or with lack of specific relationships are not mentioned.

CRP, C-reactive protein; SAP, serum amyloid P component; NPTX1, neuronal pentraxin 1; NPTX2, neuronal pentraxin 2; NPTXR, neuronal pentraxin receptor; PTX3, pentraxin 3; PTX4, pentraxin 4.



proliferation, mediating tumor cell apoptosis, inducing tumor metastasis, and promoting tumor tissue edema (Table 3, Figure 6). The tumor microenvironment is an extremely

complex network consisting of cancer-associated fibroblasts, adipose cells, immunocytes, new-born vessels, and extracellular matrix. The pentraxin family promotes the formation of tumor

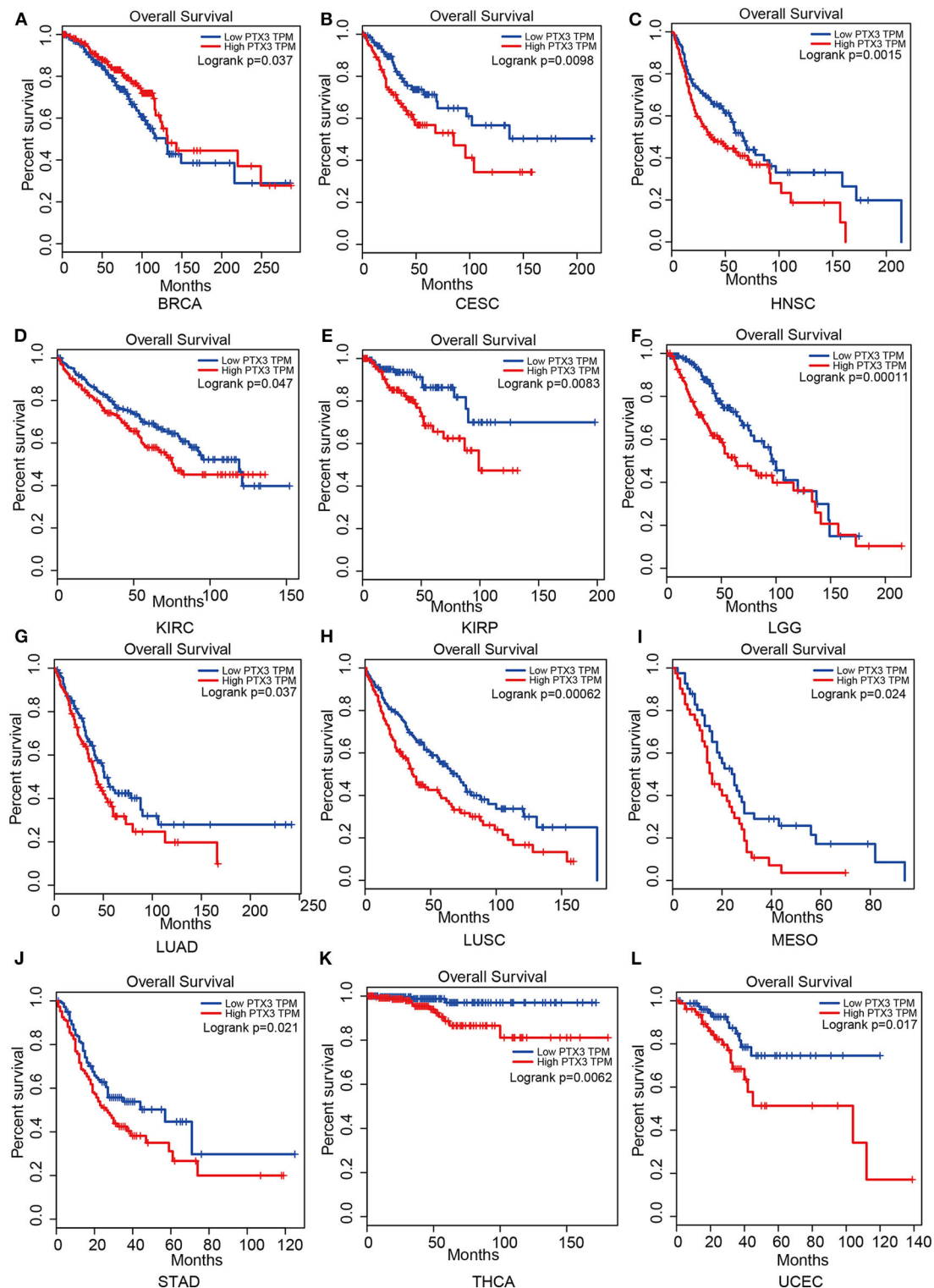


FIGURE 5 | Impact of PTX3 on overall survival and tumor phenotypes based on the Gepia dataset. **(A)** Breast invasive carcinoma. **(B)** Cervical squamous cell carcinoma and endocervical adenocarcinoma. **(C)** Head and Neck squamous cell carcinoma. **(D)** Kidney renal clear cell carcinoma. **(E)** Kidney renal papillary cell carcinoma. **(F)** Brain Lower Grade Glioma. **(G)** Lung adenocarcinoma. **(H)** Lung squamous cell carcinoma. **(I)** Mesothelioma. **(J)** Stomach adenocarcinoma. **(K)** Thyroid carcinoma. **(L)** Uterine Corpus Endometrial Carcinoma. The overall survival of tumor that cannot be predicted by PTX3 expression is not listed.

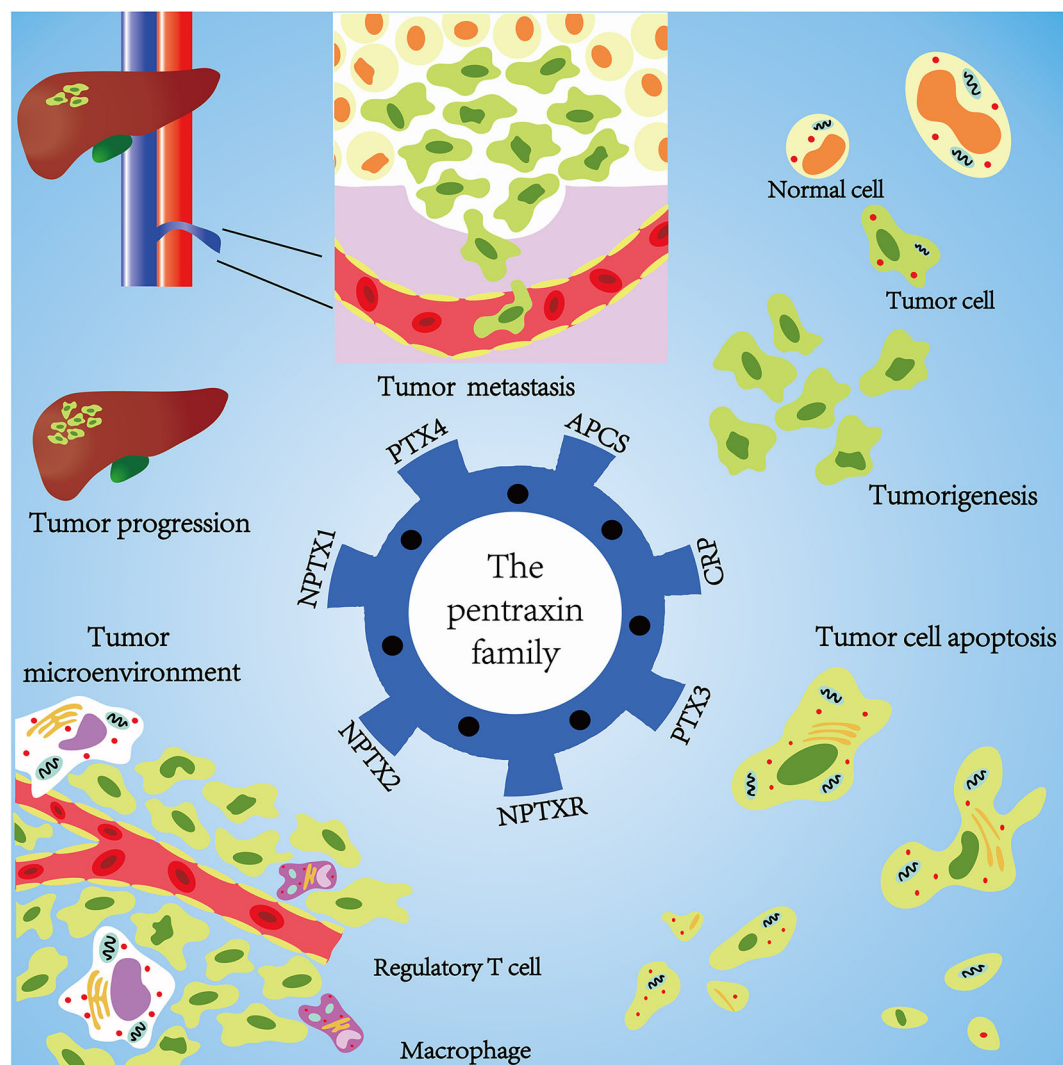


FIGURE 6 | Mechanisms of pentraxin family in tumor progression. At the core is the pentraxin family and its seven members. The pentraxin family mainly participates in tumor metastasis, tumorigenesis, tumor cell apoptosis, tumor cell proliferation, and regulation of tumor microenvironment.

microenvironment by facilitating macrophage infiltration and stimulating cytokines secretion. The pentraxin family, therefore, plays an essential role in tumor progression, although the exact mechanisms are still unknown and require further in-depth studies.

Protein structure of short pentraxins and their role in the immune system has highly been explored compared to the long pentraxins. It has been reported that PTX3 and NPTXR, for example, have an additional N-terminal domain other than the known pentraxin domain and exhibit different functions. Each pentraxin family member has their specific unique functions, for instance, SAP binds to amyloid fibrils and the neuronal pentraxins and extensively participates in the central nervous system development. Furthermore, the pentraxin family affects tumor progression through their unique receptors and pathways. Therefore, an in-depth analysis of the protein structure of the pentraxins family and their underlying mechanisms using

advanced electron microscopy technologies, such as cryo-electron microscopy, is important for future research.

The PI3K/AKT/mTOR pathway is commonly associated with the pentraxin family in inducing tumor progression. Nevertheless, each pentraxin family member poses a specific receptor linked with tumor progression. Short pentraxins bind with the Fcγ receptor to activate different pathways. Studies have proposed that the AMPA receptor bridges the neuronal pentraxins and tumor by mediating intracellular free concentration of calcium known to be vital for various downstream pathways. PTX3 modulates tumor cell adhesion and metastasis by interacting with the FGFR system. SAP and PTX4 share highly structural homology with CRP, but limited research has focused on their association with the tumor, similarly with the neuronal pentraxins. Tumor microenvironment and inflammation are two crucial components that influence tumor progression through active communication with each other (138,

139). The pentraxin family can both initiate inflammation and promote tumor progression.

AUTHOR CONTRIBUTIONS

QC, ZL, and GX: offered the idea of this review, and acted as the mentors and guarantors of the review. ZW and XW: wrote the review. ZW, XW, and ZD: modified the review. ZW: drew the figures. HZ, MZ, and SF: offered modification advice and checked grammar. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the National Nature Science Foundation of China (Nos. 81703622 and 81803582); the

China Postdoctoral Science Foundation (No. 2018M63302); Hunan Provincial Natural Science Foundation of China (Nos. 2018JJ3838, 2019JJ50949, and 2020JJ8111); Grants from Xiangya Hospital Central South University postdoctoral foundation; Grants from Clinical medical technology innovation guidance program of Hunan Province (No. 2017SK50109); and grants from the research projects of Hunan Provincial Health Commission of China (Nos. C2019186 and B2019187).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2020.01757/full#supplementary-material>

REFERENCES

- Hsu YC, Perin MS. Human neuronal pentraxin II (NPTX2): conservation, genomic structure, and chromosomal localization. *Genomics*. (1995) 28:220–7. doi: 10.1006/geno.1995.1134
- Liu Q, Tu T, Bai Z, Liu Z, Zhou S. Elevated plasma pentraxin 3: a potential cardiovascular risk factor? *Med Hypotheses*. (2011) 77:1068–70. doi: 10.1016/j.mehy.2011.09.004
- Gonzalez OA, Kirakodu S, Novak MJ, Stromberg AJ, Orraca L, Gonzalez-Martinez J, et al. Comparative analysis of microbial sensing molecules in mucosal tissues with aging. *Immunobiology*. (2018) 223:279–87. doi: 10.1016/j.imbio.2017.10.034
- Thompson D, Pepys MB, Tickle I, Wood S. The structures of crystalline complexes of human serum amyloid P component with its carbohydrate ligand, the cyclic pyruvate acetal of galactose. *J Mol Biol*. (2002) 320:1081–6. doi: 10.1016/S0022-2836(02)00514-4
- Richards DB, Cookson LM, Berges AC, Barton SV, Lane T, Ritter JM, et al. Therapeutic clearance of amyloid by antibodies to serum amyloid P component. *N Engl J Med*. (2015) 373:1106–14. doi: 10.1056/NEJMoa1504942
- Richards DB, Cookson LM, Barton SV, Liefwaard L, Lane T, Hutt DF, et al. Repeat doses of antibody to serum amyloid P component clear amyloid deposits in patients with systemic amyloidosis. *Sci Transl Med*. (2018) 10:eaa3128. doi: 10.1126/scitranslmed.aan3128
- Farhy-Tselnick I, van Casteren ACM, Lee A, Chang VT, Aricescu AR, Allen NJ. Astrocyte-secreted glypican 4 regulates release of neuronal pentraxin 1 from axons to induce functional synapse formation. *Neuron*. (2017) 96:428–45.e13. doi: 10.1016/j.neuron.2017.09.053
- Ronca R, Di Salle E, Giacomini A, Leali D, Alessi P, Coltrini D, et al. Long pentraxin-3 inhibits epithelial-mesenchymal transition in melanoma cells. *Mol Cancer Ther*. (2013) 12:2760–71. doi: 10.1158/1535-7163.MCT-13-0487
- Ahmed B, Khan MN, Nisar MA, Kampo S, Zheng Q, Li Y, et al. Tunicamycin enhances the suppressive effects of cisplatin on lung cancer growth through PTX3 glycosylation via AKT/NF-kappaB signaling pathway. *Int J Oncol*. (2019) 54:431–42. doi: 10.3892/ijo.2018.4650
- Landskron G, De la Fuente M, Thuwajit P, Thuwajit C, Hermoso MA. Chronic inflammation and cytokines in the tumor microenvironment. *J Immunol Res*. (2014) 2014:149185. doi: 10.1155/2014/149185
- Duan Y, Li Z, Cheng S, Chen Y, Zhang L, He J, et al. Nasopharyngeal carcinoma progression is mediated by EBER-triggered inflammation via the RIG-I pathway. *Cancer Lett*. (2015) 361:67–74. doi: 10.1016/j.canlet.2015.02.037
- Murata M. Inflammation and cancer. *Environ Health Prev Med*. (2018) 23:50. doi: 10.1186/s12199-018-0740-1
- Joyce JA, Fearon DT. T cell exclusion, immune privilege, and the tumor microenvironment. *Science*. (2015) 348:74–80. doi: 10.1126/science.aaa6204
- Wang M, Zhao J, Zhang L, Wei F, Lian Y, Wu Y, et al. Role of tumor microenvironment in tumorigenesis. *J Cancer*. (2017) 8:761–73. doi: 10.7150/jca.17648
- Ronca R, Tamma R, Coltrini D, Ruggieri S, Presta M, Ribatti D. Fibroblast growth factor modulates mast cell recruitment in a murine model of prostate cancer. *Oncotarget*. (2017) 8:82583–92. doi: 10.18632/oncotarget.19773
- Woo P, Korenberg JR, Whitehead AS. Characterization of genomic and complementary DNA sequence of human C-reactive protein, and comparison with the complementary DNA sequence of serum amyloid P component. *J Biol Chem*. (1985) 260:13384–8.
- Lu J, Marjon KD, Mold C, Du Clos TW, Sun PD. Pentraxins and Fc receptors. *Immunol Rev*. (2012) 250:230–8. doi: 10.1111/j.1600-065X.2012.01162.x
- Vilahrur G, Badimon L. Biological actions of pentraxins. *Vascul Pharmacol*. (2015) 73:38–44. doi: 10.1016/j.vph.2015.05.001
- Clark SE, Weiser JN. Microbial modulation of host immunity with the small molecule phosphorylcholine. *Infect Immun*. (2013) 81:392–401. doi: 10.1128/IAI.01168-12
- Ridker PM. From C-reactive protein to interleukin-6 to interleukin-1: moving upstream to identify novel targets for atheroprotection. *Circ Res*. (2016) 118:145–56. doi: 10.1161/CIRCRESAHA.115.306656
- Zhang YA, Salinas I, Li J, Parra D, Bjork S, Xu Z, et al. IgT, a primitive immunoglobulin class specialized in mucosal immunity. *Nat Immunol*. (2010) 11:827–35. doi: 10.1038/ni.1913
- Yang J, Wezeman M, Zhang X, Lin P, Wang M, Qian J, et al. Human C-reactive protein binds activating Fcgamma receptors and protects myeloma tumor cells from apoptosis. *Cancer Cell*. (2007) 12:252–65. doi: 10.1016/j.ccr.2007.08.008
- Kim ES, Kim SY, Koh M, Lee HM, Kim K, Jung J, et al. C-reactive protein binds to integrin alpha2 and Fcgamma receptor I, leading to breast cell adhesion and breast cancer progression. *Oncogene*. (2018) 37:28–38. doi: 10.1038/ncr.2017.298
- Aoki M, Fujishita T. Oncogenic roles of the PI3K/AKT/mTOR axis. *Curr Top Microbiol Immunol*. (2017) 407:153–89. doi: 10.1007/82_2017_6
- Chen W, Pilling D, Gomer RH. C-reactive protein (CRP) but not the related pentraxins serum amyloid P and PTX3 inhibits the proliferation and induces apoptosis of the leukemia cell line Mono Mac 6. *BMC Immunol*. (2017) 18:47. doi: 10.1186/s12865-017-0230-z
- Du J, Hu W, Yang C, Wang Y, Wang X, Yang P. C-reactive protein is associated with the development of tongue squamous cell carcinoma. *Acta Biochim Biophys Sin*. (2018) 50:238–45. doi: 10.1093/abbs/gmy004

27. Yang J, Liu Z, Liu H, He J, Yang J, Lin P, et al. C-reactive protein promotes bone destruction in human myeloma through the CD32-p38 MAPK-Twist axis. *Sci Signal.* (2017) 10:eaan6282. doi: 10.1126/scisignal.aan6282
28. Morrison L, Laukkanen JA, Ronkainen K, Kurl S, Kauhanen J, Toriola AT. Inflammatory biomarker score and cancer: a population-based prospective cohort study. *BMC Cancer.* (2016) 16:80. doi: 10.1186/s12885-016-2115-6
29. Nijaguna MB, Schroder C, Patil V, Shwetha SD, Hegde AS, Chandramouli BA, et al. Definition of a serum marker panel for glioblastoma discrimination and identification of Interleukin 1beta in the microglial secretome as a novel mediator of endothelial cell survival induced by C-reactive protein. *J Proteomics.* (2015) 128:251–61. doi: 10.1016/j.jprot.2015.07.026
30. Nakayama T, Saito K, Kumagai J, Nakajima Y, Kijima T, Yoshida S, et al. Higher serum C-reactive protein level represents the immunosuppressive tumor microenvironment in patients with clear cell renal cell carcinoma. *Clin Genitourin Cancer.* (2018) 16:e1151–8. doi: 10.1016/j.clgc.2018.07.027
31. Xu BB, Lu J, Zheng ZF, Xie JW, Wang JB, Lin JX, et al. The predictive value of the preoperative C-reactive protein-albumin ratio for early recurrence and chemotherapy benefit in patients with gastric cancer after radical gastrectomy: using randomized phase III trial data. *Gastric Cancer.* (2019) 22:1016–28. doi: 10.1007/s10120-019-00936-w
32. Krzystek-Korpacka M, Matusiewicz M, Diakowska D, Grabowski K, Blachut K, Kustrzeba-Wojcicka I, et al. Acute-phase response proteins are related to cachexia and accelerated angiogenesis in gastroesophageal cancers. *Clin Chem Lab Med.* (2008) 46:359–64. doi: 10.1515/CCLM.2008.089
33. Kuribayashi K, Motoyama S, Sasaki T, Sato Y, Yoshino K, Wakita A, et al. C-reactive protein reduces the relative number of tumor-associated M2 macrophages and intratumoral angiogenesis in mice. *Tohoku J Exp Med.* (2014) 233:249–55. doi: 10.1620/tjem.233.249
34. Ma Z, Qi Z, Shan Z, Li J, Yang J, Xu Z. The role of CRP and ATG9B expression in clear cell renal cell carcinoma. *Biosci Rep.* (2017) 37:BSR20171082. doi: 10.1042/BSR20171082
35. Lust JA, Lacy MQ, Zeldin SR, Witzig TE, Moon-Tasson LL, Dinarello CA, et al. Reduction in C-reactive protein indicates successful targeting of the IL-1/IL-6 axis resulting in improved survival in early stage multiple myeloma. *Am J Hematol.* (2016) 91:571–4. doi: 10.1002/ajh.24352
36. Saito K, Tatokoro M, Fujii Y, Iimura Y, Koga F, Kawakami S, et al. Impact of C-reactive protein kinetics on survival of patients with metastatic renal cell carcinoma. *Eur Urol.* (2009) 55:1145–53. doi: 10.1016/j.eururo.2008.10.012
37. Xia WK, Wu X, Yu TH, Wu Y, Yao XJ, Hu H. Prognostic significance of lymphocyte-to-monocyte ratio and CRP in patients with nonmetastatic clear cell renal cell carcinoma: a retrospective multicenter analysis. *Onco Targets Ther.* (2016) 9:2759–67. doi: 10.2147/OTT.S101458
38. Dalpiaz O, Luef T, Seles M, Stotz M, Stojakovic T, Pummer K, et al. Critical evaluation of the potential prognostic value of the pretreatment-derived neutrophil-lymphocyte ratio under consideration of C-reactive protein levels in clear cell renal cell carcinoma. *Br J Cancer.* (2017) 116:85–90. doi: 10.1038/bjc.2016.393
39. Kaur RP, Rubal, Banipal RPS, Vashistha R, Dhiman B, Munshi A. Association of elevated levels of C-reactive protein with breast cancer, breast cancer subtypes, and poor outcome. *Curr Probl Cancer.* (2019) 43:123–9. doi: 10.1016/j.cuprob.2018.05.003
40. Liu ZQ, Chu L, Fang JM, Zhang X, Zhao HX, Chen YJ, et al. Prognostic role of C-reactive protein in prostate cancer: a systematic review and meta-analysis. *Asian J Androl.* (2014) 16:467–71. doi: 10.4103/1008-682X.123686
41. Muller DC, Larose TL, Hodge A, Guida F, Langhammer A, Grankvist K, et al. Circulating high sensitivity C reactive protein concentrations and risk of lung cancer: nested case-control study within Lung Cancer Cohort Consortium. *BMJ.* (2019) 364:k4981. doi: 10.1136/bmj.k4981
42. Kinoshita A, Onoda H, Imai N, Iwaku A, Oishi M, Tanaka K, et al. The C-reactive protein/albumin ratio, a novel inflammation-based prognostic score, predicts outcomes in patients with hepatocellular carcinoma. *Ann Surg Oncol.* (2015) 22:803–10. doi: 10.1245/s10434-014-4048-0
43. He X, Li JP, Liu XH, Zhang JP, Zeng QY, Chen H, et al. Prognostic value of C-reactive protein/albumin ratio in predicting overall survival of Chinese cervical cancer patients overall survival: comparison among various inflammation based factors. *J Cancer.* (2018) 9:1877–84. doi: 10.7150/jca.23320
44. Park MJ, Roh JL, Kim SB, Choi SH, Nam SY, Kim SY. Prognostic value of circulating biomarker score in advanced-stage head and neck squamous cell carcinoma. *Eur J Cancer.* (2018) 92:69–76. doi: 10.1016/j.ejca.2018.01.069
45. Qin W, Yuan Q, Wu J, Yu H, Wang Y, Chen Q. Prognostic value of pre-therapy C-reactive protein level in diffuse large B-cell lymphoma: a meta-analysis. *Leuk Lymphoma.* (2019) 60:358–66. doi: 10.1080/10428194.2018.1482540
46. Li W, Luo X, Liu Z, Chen Y, Li Z. Prognostic value of C-reactive protein levels in patients with bone neoplasms: A meta-analysis. *PLoS ONE.* (2018) 13:e0195769. doi: 10.1371/journal.pone.0195769
47. Grote VA, Kaaks R, Nieters A, Tjonneland A, Halkjaer J, Overvad K, et al. Inflammation marker and risk of pancreatic cancer: a nested case-control study within the EPIC cohort. *Br J Cancer.* (2012) 106:1866–74. doi: 10.1038/bjc.2012.172
48. Szkandera J, Stotz M, Absenger G, Stojakovic T, Samonigg H, Kornprat P, et al. Validation of C-reactive protein levels as a prognostic indicator for survival in a large cohort of pancreatic cancer patients. *Br J Cancer.* (2014) 110:183–8. doi: 10.1038/bjc.2013.701
49. Chan AT, Ogino S, Giovannucci EL, Fuchs CS. Inflammatory markers are associated with risk of colorectal cancer and chemopreventive response to anti-inflammatory drugs. *Gastroenterology.* (2011) 140:799–808, quiz e711. doi: 10.1053/j.gastro.2010.11.041
50. Nimptsch K, Aleksandrova K, Boeing H, Janke J, Lee YA, Jenab M, et al. Association of CRP genetic variants with blood concentrations of C-reactive protein and colorectal cancer risk. *Int J Cancer.* (2015) 136:1181–92. doi: 10.1002/ijc.29086
51. Farsad-Naeimi A, Alizadeh M, Esfahani A, Darvish Aminabad E. Effect of fisetin supplementation on inflammatory factors and matrix metalloproteinase enzymes in colorectal cancer patients. *Food Funct.* (2018) 9:2025–31. doi: 10.1039/C7FO01898C
52. Xi D, Luo T, Xiong H, Liu J, Lu H, Li M, et al. SAP: structure, function, and its roles in immune-related diseases. *Int J Cardiol.* (2015) 187:20–6. doi: 10.1016/j.ijcard.2015.03.179
53. Emsley J, White HE, O'Hara BP, Oliva G, Srinivasan N, Tickle IJ, et al. Structure of pentameric human serum amyloid P component. *Nature.* (1994) 367:338–45. doi: 10.1038/367338a0
54. Mikolajek H, Kolstoe SE, Pye VE, Mangione P, Pepys MB, Wood SP. Structural basis of ligand specificity in the human pentraxins, C-reactive protein and serum amyloid P component. *J Mol Recognit.* (2011) 24:371–7. doi: 10.1002/jmr.1090
55. Hind CR, Collins PM, Renn D, Cook RB, Caspi D, Baltz ML, et al. Binding specificity of serum amyloid P component for the pyruvate acetal of galactose. *J Exp Med.* (1984) 159:1058–69. doi: 10.1084/jem.159.4.1058
56. Zhang W, Xu W, Xiong S. Macrophage differentiation and polarization via phosphatidylinositol 3-kinase/Akt-ERK signaling pathway conferred by serum amyloid P component. *J Immunol.* (2011) 187:1764–77. doi: 10.4049/jimmunol.1002315
57. Cox N, Pilling D, Gomer RH. Serum amyloid P: a systemic regulator of the innate immune response. *J Leukoc Biol.* (2014) 96:739–43. doi: 10.1189/jlb.1MR0114-068R
58. Bickerstaff MC, Botto M, Hutchinson WL, Herbert J, Tennent GA, Bybee A, et al. Serum amyloid P component controls chromatin degradation and prevents antinuclear autoimmunity. *Nat Med.* (1999) 5:694–7. doi: 10.1038/9544
59. Jacobs R, Tshelha E, Malherbe S, Kriel M, Loxton AG, Stanley K, et al. Host biomarkers detected in saliva show promise as markers for the diagnosis of pulmonary tuberculosis disease and monitoring of the response to tuberculosis treatment. *Cytokine.* (2016) 81:50–6. doi: 10.1016/j.cyto.2016.02.004
60. Chantrathammachart P, Mackman N, Sparkenbaugh E, Wang JG, Parise LV, Kirchhofer D, et al. Tissue factor promotes activation of coagulation and inflammation in a mouse model of sickle cell disease. *Blood.* (2012) 120:636–46. doi: 10.1182/blood-2012-04-424143
61. Zhao J, Chi A, Mao R, Hu G, Ji M. Serum amyloid P-component level may be a biomarker for lung toxicities and overall survival after thoracic

- radiotherapy for non-small cell lung cancer. *Clin Lab.* (2016) 62:2183–90. doi: 10.7754/Clin.Lab.2016.160411
62. Omeis IA, Hsu YC, Perin MS. Mouse and human neuronal pentraxin 1 (NPTX1): conservation, genomic structure, and chromosomal localization. *Genomics.* (1996) 36:543–5. doi: 10.1006/geno.1996.0503
 63. Dodds DC, Omeis IA, Cushman SJ, Helms JA, Perin MS. Neuronal pentraxin receptor, a novel putative integral membrane pentraxin that interacts with neuronal pentraxin 1 and 2 and taipoxin-associated calcium-binding protein 49. *J Biol Chem.* (1997) 272:21488–94. doi: 10.1074/jbc.272.34.21488
 64. Du Clos TW. Pentraxins: structure, function, and role in inflammation. *ISRN Inflamm.* (2013) 2013:379040. doi: 10.1155/2013/379040
 65. Thatipamula S, Al Rahim M, Zhang J, Hossain MA. Genetic deletion of neuronal pentraxin 1 expression prevents brain injury in a neonatal mouse model of cerebral hypoxia-ischemia. *Neurobiol Dis.* (2015) 75:15–30. doi: 10.1016/j.nbd.2014.12.016
 66. Huo L, Wang B, Zheng M, Zhang Y, Xu J, Yang G, et al. miR-128-3p inhibits glioma cell proliferation and differentiation by targeting NPTX1 through IRS-1/PI3K/AKT signaling pathway. *Exp Ther Med.* (2019) 17:2921–30. doi: 10.3892/etm.2019.7284
 67. Guzeloglu-Kayisli O, Basar M, Shapiro JP, Semerci N, Huang JS, Schatz F, et al. Long-acting progestin-only contraceptives enhance human endometrial stromal cell expressed neuronal pentraxin-1 and reactive oxygen species to promote endothelial cell apoptosis. *J Clin Endocrinol Metab.* (2014) 99:E1957–66. doi: 10.1210/jc.2014.1770
 68. Peng X, Pan K, Zhao W, Zhang J, Yuan S, Wen X, et al. NPTX1 inhibits colon cancer cell proliferation through down-regulating cyclin A2 and CDK2 expression. *Cell Biol Int.* (2018) 42:589–97. doi: 10.1002/cbin.10935
 69. Boles NC, Hirsch SE, Le S, Corneo B, Najm F, Minotti AP, et al. NPTX1 regulates neural lineage specification from human pluripotent stem cells. *Cell Rep.* (2014) 6:724–36. doi: 10.1016/j.celrep.2014.01.026
 70. Xu C, Tian G, Jiang C, Xue H, Kuerbanjiang M, Sun L, et al. NPTX2 promotes colorectal cancer growth and liver metastasis by the activation of the canonical Wnt/beta-catenin pathway via FZD6. *Cell Death Dis.* (2019) 10:217. doi: 10.1038/s41419-019-1467-7
 71. Shukla S, Pia Patric IR, Thinnagarajan S, Srinivasan S, Mondal B, Hegde AS, et al. A DNA methylation prognostic signature of glioblastoma: identification of NPTX2-PTEN-NF-kappaB nexus. *Cancer Res.* (2013) 73:6563–73. doi: 10.1158/0008-5472.CAN-13-0298
 72. Liu H, Jiang Q, Ju Z, Guan S, He B. Pentraxin 3 promotes cardiac differentiation of mouse embryonic stem cells through JNK signaling pathway. *Cell Biol Int.* (2018) 42:1556–63. doi: 10.1002/cbin.11049
 73. Xiao Y, Yang N, Zhang Q, Wang Y, Yang S, Liu Z. Pentraxin 3 inhibits acute renal injury-induced interstitial fibrosis through suppression of IL-6/Stat3 pathway. *Inflammation.* (2014) 37:1895–901. doi: 10.1007/s10753-014-9921-2
 74. Chan SH, Tsai JP, Shen CJ, Liao YH, Chen BK. Oleate-induced PTX3 promotes head and neck squamous cell carcinoma metastasis through the up-regulation of vimentin. *Oncotarget.* (2017) 8:41364–78. doi: 10.18632/oncotarget.17326
 75. Sia GM, Beique JC, Rumbaugh G, Cho R, Worley PF, Haganir RL. Interaction of the N-terminal domain of the AMPA receptor GluR4 subunit with the neuronal pentraxin NP1 mediates GluR4 synaptic recruitment. *Neuron.* (2007) 55:87–102. doi: 10.1016/j.neuron.2007.06.020
 76. Cummings DM, Benway TA, Ho H, Tedoldi A, Fernandes Freitas MM, Shahab L, et al. Neuronal and peripheral pentraxins modify glutamate release and may interact in blood-brain barrier failure. *Cereb Cortex.* (2017) 27:3437–48. doi: 10.1093/cercor/bbx046
 77. Lee SJ, Wei M, Zhang C, Maxeiner S, Pak C, Calado Botelho S, et al. Presynaptic neuronal pentraxin receptor organizes excitatory and inhibitory synapses. *J Neurosci.* (2017) 37:1062–80. doi: 10.1523/JNEUROSCI.2768-16.2016
 78. Rajkumar AP, Christensen JH, Mattheisen M, Jacobsen I, Bache I, Pallesen J, et al. Analysis of t(9;17)(q33.2;q25.3) chromosomal breakpoint regions and genetic association reveals novel candidate genes for bipolar disorder. *Bipolar Disord.* (2015) 17:205–11. doi: 10.1111/bdi.12239
 79. Liu H, Kong X, Chen F. Mkrn3 functions as a novel ubiquitin E3 ligase to inhibit Nptx1 during puberty initiation. *Oncotarget.* (2017) 8:85102–9. doi: 10.18632/oncotarget.19347
 80. Li T, Mamillapalli R, Ding S, Chang H, Liu ZW, Gao XB, et al. Endometriosis alters brain electrophysiology, gene expression and increases pain sensitization, anxiety, and depression in female mice. *Biol Reprod.* (2018) 99:349–59. doi: 10.1093/biolre/boy035
 81. Strauss J, McGregor S, Freeman N, Tiwari A, George CJ, Kovacs M, et al. Association study of early-onset genes in childhood-onset mood disorders and suicide attempt. *Psychiatry Res.* (2012) 197:49–54. doi: 10.1016/j.psychres.2011.11.022
 82. Manchia M, Piras IS, Huentelman MJ, Pinna F, Zai CC, Kennedy JL, et al. Pattern of gene expression in different stages of schizophrenia: down-regulation of NPTX2 gene revealed by a meta-analysis of microarray datasets. *Eur Neuropsychopharmacol.* (2017) 27:1054–63. doi: 10.1016/j.euroneuro.2017.07.002
 83. Begcevic I, Tsolaki M, Brinc D, Brown M, Martinez-Morillo E, Lazarou I, et al. Neuronal pentraxin receptor-1 is a new cerebrospinal fluid biomarker of Alzheimer's disease progression. *F1000Res.* (2018) 7:1012. doi: 10.12688/f1000research.15095.1
 84. Goetzl EJ, Abner EL, Jicha GA, Kapogiannis D, Schwartz JB. Declining levels of functionally specialized synaptic proteins in plasma neuronal exosomes with progression of Alzheimer's disease. *FASEB J.* (2018) 32:888–93. doi: 10.1096/fj.201700731R
 85. Charbonnier-Beaupel F, Malerbi M, Alcacer C, Tahiri K, Carpentier W, Wang C, et al. Gene expression analyses identify Narp contribution in the development of L-DOPA-induced dyskinesia. *J Neurosci.* (2015) 35:96–111. doi: 10.1523/JNEUROSCI.5231-13.2015
 86. Li J, Sun Y, Chen J. Transcriptome sequencing in a 6-hydroxydopamine rat model of Parkinson's disease. *Genes Genet Syst.* (2019) 94:61–9. doi: 10.1266/ggs.18-00036
 87. Qu Z, D'Mello SR. Proteomic analysis identifies NPTX1 and HIP1R as potential targets of histone deacetylase-3-mediated neurodegeneration. *Exp Biol Med.* (2018) 243:627–38. doi: 10.1177/1535370218761149
 88. Zook P, Pathak HB, Belinsky MG, Gersz L, Devarajan K, Zhou Y, et al. Combination of imatinib mesylate and AKT inhibitor provides synergistic effects in preclinical study of gastrointestinal stromal tumor. *Clin Cancer Res.* (2017) 23:171–80. doi: 10.1158/1078-0432.CCR-16-0529
 89. Tyburczy ME, Kotulska K, Pokarowski P, Mieczkowski J, Kucharska J, Grajkowska W, et al. Novel proteins regulated by mTOR in subependymal giant cell astrocytomas of patients with tuberous sclerosis complex and new therapeutic implications. *Am J Pathol.* (2010) 176:1878–90. doi: 10.2353/ajpath.2010.090950
 90. Skiriute D, Vaitkiene P, Asmoniene V, Steponaitis G, Deltuva VP, Tamasauskas A. Promoter methylation of AREG, HOXA11, hMLH1, NDRG2, NPTX2 and Tes genes in glioblastoma. *J Neurooncol.* (2013) 113:441–9. doi: 10.1007/s11060-013-1133-3
 91. Carlson MR, Pope WB, Horvath S, Braunstein JG, Nghiemphu P, Tso CL, et al. Relationship between survival and edema in malignant gliomas: role of vascular endothelial growth factor and neuronal pentraxin 2. *Clin Cancer Res.* (2007) 13:2592–8. doi: 10.1158/1078-0432.CCR-06-2772
 92. Zhang L, Gao J, Li L, Li Z, Du Y, Gong Y. The neuronal pentraxin II gene (NPTX2) inhibit proliferation and invasion of pancreatic cancer cells in vitro. *Mol Biol Rep.* (2011) 38:4903–11. doi: 10.1007/s11033-010-0632-y
 93. Zhang L, Gao J, Li Z, Gong Y. Neuronal pentraxin II (NPTX2) is frequently down-regulated by promoter hypermethylation in pancreatic cancers. *Dig Dis Sci.* (2012) 57:2608–14. doi: 10.1007/s10620-012-2202-8
 94. von Roemeling CA, Radisky DC, Marlow LA, Cooper SJ, Grebe SK, Anastasiadis PZ, et al. Neuronal pentraxin 2 supports clear cell renal cell carcinoma by activating the AMPA-selective glutamate receptor-4. *Cancer Res.* (2014) 74:4796–810. doi: 10.1158/0008-5472.CAN-14-0210
 95. Schrodtter S, Braun M, Syring I, Klumper N, Deng M, Schmidt D, et al. Identification of the dopamine transporter SLC6A3 as a biomarker for patients with renal cell carcinoma. *Mol Cancer.* (2016) 15:10. doi: 10.1186/s12943-016-0495-5
 96. Yang N, Eijssink JJ, Lendvai A, Volders HH, Klip H, Buikema HJ, et al. Methylation markers for CCNA1 and C13ORF18 are strongly associated with high-grade cervical intraepithelial neoplasia and cervical cancer in cervical scrapings. *Cancer Epidemiol Biomarkers Prev.* (2009) 18:3000–7. doi: 10.1158/1055-9965.EPI-09-0405

97. Zhou C, Qin Y, Xie Z, Zhang J, Yang M, Li S, et al. NPTX1 is a novel epigenetic regulation gene and associated with prognosis in lung cancer. *Biochem Biophys Res Commun.* (2015) 458:381–6. doi: 10.1016/j.bbrc.2015.01.124
98. Alholle A, Brini AT, Gharanei S, Vaiyapuri S, Arrigoni E, Dallol A, et al. Functional epigenetic approach identifies frequently methylated genes in Ewing sarcoma. *Epigenetics.* (2013) 8:1198–204. doi: 10.4161/epi.26266
99. Bartolini A, Di Paolo D, Noghero A, Murgia D, Sementa AR, Cilli M, et al. The neuronal pentraxin-2 pathway is an unrecognized target in human neuroblastoma, which also offers prognostic value in patients. *Cancer Res.* (2015) 75:4265–71. doi: 10.1158/0008-5472.CAN-15-0649
100. Poulsen TT, Pedersen N, Perin MS, Hansen CK, Poulsen HS. Specific sensitivity of small cell lung cancer cell lines to the snake venom toxin taipoxin. *Lung Cancer.* (2005) 50:329–37. doi: 10.1016/j.lungcan.2005.06.011
101. Yue W, Wang T, Zachariah E, Lin Y, Yang CS, Xu Q, et al. Transcriptomic analysis of pancreatic cancer cells in response to metformin and aspirin: an implication of synergy. *Sci Rep.* (2015) 5:13390. doi: 10.1038/srep13390
102. Karagkounis G, Thai L, DeVecchio J, Gantt GA, Duraes L, Pai RK, et al. NPTX2 is associated with neoadjuvant therapy response in rectal cancer. *J Surg Res.* (2016) 202:112–7. doi: 10.1016/j.jss.2015.12.042
103. de Groot J, Sontheimer H. Glutamate and the biology of gliomas. *Glia.* (2011) 59:1181–9. doi: 10.1002/glia.21113
104. Radin DP, Patel P. A current perspective on the oncopreventive and oncolytic properties of selective serotonin reuptake inhibitors. *Biomed Pharmacother.* (2017) 87:636–9. doi: 10.1016/j.biopha.2017.01.024
105. Breviario F, d'Aniello EM, Golay J, Peri G, Bottazzi B, Bairoch A, et al. Interleukin-1-inducible genes in endothelial cells. Cloning of a new gene related to C-reactive protein and serum amyloid P component. *J Biol Chem.* (1992) 267:22190–7.
106. Lee GW, Lee TH, Vilcek J. TSG-14, a tumor necrosis factor- and IL-1-inducible protein, is a novel member of the pentaxin family of acute phase proteins. *J Immunol.* (1993) 150:1804–12.
107. Presta M, Camozzi M, Salvatori G, Rusnati M. Role of the soluble pattern recognition receptor PTX3 in vascular biology. *J Cell Mol Med.* (2007) 11:723–38. doi: 10.1111/j.1582-4934.2007.00061.x
108. Moalli F, Jaillon S, Inforzato A, Sironi M, Bottazzi B, Mantovani A, et al. Pathogen recognition by the long pentraxin PTX3. *J Biomed Biotechnol.* (2011) 2011:830421. doi: 10.1155/2011/830421
109. Martinez de la Torre Y, Fabbri M, Jaillon S, Bastone A, Nebuloni M, Vecchi A, et al. Evolution of the pentraxin family: the new entry PTX4. *J Immunol.* (2010) 184:5055–64. doi: 10.4049/jimmunol.0901672
110. Mantovani A, Garlanda C, Doni A, Bottazzi B. Pentraxins in innate immunity: from C-reactive protein to the long pentraxin PTX3. *J Clin Immunol.* (2008) 28:1–13. doi: 10.1007/s10875-007-9126-7
111. Ma YJ, Doni A, Hummelshoj T, Honore C, Bastone A, Mantovani A, et al. Synergy between ficolin-2 and pentraxin 3 boosts innate immune recognition and complement deposition. *J Biol Chem.* (2009) 284:28263–75. doi: 10.1074/jbc.M109.009225
112. Bottazzi B, Vouret-Craviari V, Bastone A, De Gioia L, Matteucci C, Peri G, et al. Multimer formation and ligand recognition by the long pentraxin PTX3. Similarities and differences with the short pentraxins C-reactive protein and serum amyloid P component. *J Biol Chem.* (1997) 272:32817–23. doi: 10.1074/jbc.272.52.32817
113. Inforzato A, Peri G, Doni A, Garlanda C, Mantovani A, Bastone A, et al. Structure and function of the long pentraxin PTX3 glycosidic moiety: fine-tuning of the interaction with C1q and complement activation. *Biochemistry.* (2006) 45:11540–51. doi: 10.1021/bi0607453
114. Nauta AJ, Bottazzi B, Mantovani A, Salvatori G, Kishore U, Schwaeble WJ, et al. Biochemical and functional characterization of the interaction between pentraxin 3 and C1q. *Eur J Immunol.* (2003) 33:465–73. doi: 10.1002/immu.200310022
115. Bottazzi B, Inforzato A, Messa M, Barbagallo M, Magrini E, Garlanda C, et al. The pentraxins PTX3 and SAP in innate immunity, regulation of inflammation and tissue remodelling. *J Hepatol.* (2016) 64:1416–27. doi: 10.1016/j.jhep.2016.02.029
116. Presta M, Foglio E, Churrua Schuind A, Ronca R. Long pentraxin-3 modulates the angiogenic activity of fibroblast growth factor-2. *Front Immunol.* (2018) 9:2327. doi: 10.3389/fimmu.2018.02327
117. Thomas C, Henry W, Cuiffo BG, Collmann AY, Marangoni E, Benhamo V, et al. Pentraxin-3 is a PI3K signaling target that promotes stem cell-like traits in basal-like breast cancers. *Sci Signal.* (2017) 10:eah4674. doi: 10.1126/scisignal.aah4674
118. Ying TH, Lee CH, Chiou HL, Yang SF, Lin CL, Hung CH, et al. Knockdown of Pentraxin 3 suppresses tumorigenicity and metastasis of human cervical cancer cells. *Sci Rep.* (2016) 6:29385. doi: 10.1038/srep29385
119. Pfeifer GP. Defining driver DNA methylation changes in human cancer. *Int J Mol Sci.* (2018) 19:1166. doi: 10.3390/ijms19041166
120. Giacomini A, Ghedini GC, Presta M, Ronca R. Long pentraxin 3: A novel multifaceted player in cancer. *Biochim Biophys Acta Rev Cancer.* (2018) 1869:53–63. doi: 10.1016/j.bbcan.2017.11.004
121. Kampo S, Ahmmed B, Zhou T, Owusu L, Anabiah TW, Doudou NR, et al. Scorpion venom analgesic peptide, BmK AGAP inhibits stemness, and epithelial-mesenchymal transition by down-regulating PTX3 in breast cancer. *Front Oncol.* (2019) 9:21. doi: 10.3389/fonc.2019.00021
122. Ronca R, Alessi P, Coltrini D, Di Salle E, Giacomini A, Leali D, et al. Long pentraxin-3 as an epithelial-stromal fibroblast growth factor-targeting inhibitor in prostate cancer. *J Pathol.* (2013) 230:228–38. doi: 10.1002/path.4181
123. Basile A, Moschetta M, Ditunno P, Ria R, Marech I, De Luisi A, et al. Pentraxin 3 (PTX3) inhibits plasma cell/stromal cell cross-talk in the bone marrow of multiple myeloma patients. *J Pathol.* (2013) 229:87–98. doi: 10.1002/path.4081
124. Leali D, Alessi P, Coltrini D, Ronca R, Corsini M, Nardo G, et al. Long pentraxin-3 inhibits FGF8b-dependent angiogenesis and growth of steroid hormone-regulated tumors. *Mol Cancer Ther.* (2011) 10:1600–10. doi: 10.1158/1535-7163.MCT-11-0286
125. Locatelli M, Ferrero S, Martinelli Boneschi F, Boiocchi L, Zavanone M, Maria Gaini S, et al. The long pentraxin PTX3 as a correlate of cancer-related inflammation and prognosis of malignancy in gliomas. *J Neuroimmunol.* (2013) 260:99–106. doi: 10.1016/j.jneuroim.2013.04.009
126. Ma D, Zong Y, Zhu ST, Wang YJ, Li P, Zhang ST. Inhibitory role of pentraxin-3 in esophageal squamous cell carcinoma. *Chin Med J.* (2016) 129:2233–40. doi: 10.4103/0366-6999.189921
127. Zambirinis CP, Pushalkar S, Saxena D, Miller G. Pancreatic cancer, inflammation, and microbiome. *Cancer J.* (2014) 20:195–202. doi: 10.1097/PPO.0000000000000045
128. Choi B, Lee EJ, Park YS, Kim SM, Kim EY, Song Y, et al. Pentraxin-3 silencing suppresses gastric cancer-related inflammation by inhibiting chemotactic migration of macrophages. *Anticancer Res.* (2015) 35:2663–8. Available online at: <http://ar.iiarjournals.org/content/35/5/2663.long>
129. Tsuji S, Midorikawa Y, Seki M, Takayama T, Sugiyama Y, Aburatani H. Network-based analysis for identification of candidate genes for colorectal cancer progression. *Biochem Biophys Res Commun.* (2016) 476:534–40. doi: 10.1016/j.bbrc.2016.05.158
130. Bonavita E, Gentile S, Rubino M, Maina V, Papait R, Kunderfranco P, et al. PTX3 is an extrinsic oncosuppressor regulating complement-dependent inflammation in cancer. *Cell.* (2015) 160:700–14. doi: 10.1016/j.cell.2015.01.004
131. Dahan R, Segal E, Engelhardt J, Selby M, Korman Alan J, Ravetch Jeffrey V. FcγRs modulate the anti-tumor activity of antibodies targeting the PD-1/PD-L1 axis. *Cancer Cell.* (2015) 28:285–95. doi: 10.1016/j.ccell.2015.08.004
132. Brandsma AM, Bondza S, Evers M, Koutstaal R, Nederend M, Jansen JHM, et al. Potent Fc receptor signaling by IgA leads to superior killing of cancer cells by neutrophils compared to IgG. *Front Immunol.* (2019) 10:704. doi: 10.3389/fimmu.2019.00704
133. Sanseviero E. NK cell-Fc receptors advance tumor immunotherapy. *J Clin Med.* (2019) 8:1667. doi: 10.3390/jcm8101667
134. Liu Q, Wang XY, Qin YY, Yan XL, Chen HM, Huang QD, et al. SPOCD1 promotes the proliferation and metastasis of glioma cells by up-regulating PTX3. *Am J Cancer Res.* (2018) 8:624–35. Available online at: <http://www.ajcr.us/files/ajcr0067117.pdf>
135. Chang WC, Wu SL, Huang WC, Hsu JY, Chan SH, Wang JM, et al. PTX3 gene activation in EGF-induced head and neck cancer cell metastasis. *Oncotarget.* (2015) 6:7741–57. doi: 10.18632/oncotarget.3482
136. Fang S, Sui D, Wang Y, Liu H, Chiang YJ, Ross MI, et al. Association of vitamin D levels with outcome in patients with melanoma after

- adjustment for C-reactive protein. *J Clin Oncol.* (2016) 34:1741–7. doi: 10.1200/JCO.2015.64.1357
137. She S, Xiang Y, Yang M, Ding X, Liu X, Ma L, et al. C-reactive protein is a biomarker of AFP-negative HBV-related hepatocellular carcinoma. *Int J Oncol.* (2015) 47:543–54. doi: 10.3892/ijo.2015.3042
 138. Huang A, Cao S, Tang L. The tumor microenvironment and inflammatory breast cancer. *J Cancer.* (2017) 8:1884–91. doi: 10.7150/jca.17595
 139. Xie X, Yang M, Ding Y, Chen J. Microbial infection, inflammation and epithelial ovarian cancer. *Oncol Lett.* (2017) 14:1911–9. doi: 10.3892/ol.2017.6388

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Wang, Wang, Zou, Dai, Feng, Zhang, Xiao, Liu and Cheng. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



How C-Reactive Protein Structural Isoforms With Distinctive Bioactivities Affect Disease Progression

*Ibraheem M. Rajab**, *Peter C. Hart* and *Lawrence A. Potempa**

Roosevelt University College of Pharmacy, Schaumburg, IL, United States

OPEN ACCESS

Edited by:

Kenji Daigo,
Institute for Advanced Medical
Sciences, Japan

Reviewed by:

Blanca Molins,
Institut de Recerca Biomèdica August
Pi i Sunyer (IDIBAPS), Spain
Karlheinz Peter,
Baker Heart and Diabetes
Institute, Australia

*Correspondence:

Ibraheem M. Rajab
irajab@roosevelt.edu
Lawrence A. Potempa
lpotempa01@roosevelt.edu

Specialty section:

This article was submitted to
Molecular Innate Immunity,
a section of the journal
Frontiers in Immunology

Received: 16 June 2020

Accepted: 05 August 2020

Published: 10 September 2020

Citation:

Rajab IM, Hart PC and Potempa LA
(2020) How C-Reactive Protein
Structural Isoforms With Distinctive
Bioactivities Affect Disease
Progression.
Front. Immunol. 11:2126.
doi: 10.3389/fimmu.2020.02126

C-reactive protein (CRP) is a widely known, hepatically synthesized protein whose blood levels change rapidly and pronouncedly in response to any tissue damaging event associated with an inflammatory response. The synthesis and secretion of CRP is stimulated by interleukin-6, an early pleiotropic cytokine released by macrophages, endothelial, and other cells that are activated when localized normal tissue structures are compromised by trauma or disease. Serum CRP levels can change rapidly and robustly from 10-100-fold within 6-72 h of any tissue damaging event. Elevated blood levels correlate with the onset and extent of both activated inflammation and the acute phase biochemical response to the tissue insult. Because its functional bioactivity as the prototypic acute phase reactant has eluded clear definition for decades, diagnosticians of various conditions and diseases use CRP blood levels as a simple index for ongoing inflammation. In many pathologies, which involves many different tissues, stages of disease, treatments, and responses to treatments, its interpretive diagnostic value requires a deeper understanding of the localized tissue processes and events that contribute signals which regulate protective or pathological host defense bioactivities. This report presents concepts that describe how local tissue activation events can lead to a non-proteolytic, conformational rearrangement of CRP into a unique isoform with distinctive solubility, antigenicity, binding reactivities and bioactivities from that protein widely known and measured in serum. By describing factors that control the expression, tissue localization, half-life and pro-inflammatory amplification activity of this CRP isoform, a unifying explanation for the diagnostic significance of CRP measurement in disease is advanced.

Keywords: CRP - C-reactive protein, MCRP, inflammation, conformational isoforms, blood kinetics, modified/monomeric (mCRP)

INTRODUCTION

C-reactive protein (CRP) is a key protein of innate immunity. Structurally, it is a non-glycosylated, non-covalently associated protein of five homologous globular subunits arranged in discoid symmetry (1). Each subunit has a calcium-regulated shallow binding pocket for ligands expressing phosphocholine (PC) moieties with all five PC binding pockets expressed on the same face of the flattened pentameric disc. CRP's bioactivities as an effector molecule of innate immunity involves its role as a pattern recognition ligand for exposed PC groups as may be expressed in teichoic acid

of Gram positive bacteria or on activated cell membranes where phospholipid bilayers buckle in response to activation signals such as Phospholipase A2 or lipid acyl chain oxidation (2, 3). When bound, CRP is reported to activate endothelial cells, platelets, leukocytes and the complement system, and influence the overall inflammatory response that involves the earliest acute phase host defense response to any inciting threats to tissue homeostasis (4, 5). While CRP has been widely studied as a key protein of the acute phase response (APR), its role in activating and/or regulating host defense processes has remained undefined.

While CRP blood levels change rapidly and pronouncedly with any host defense response to tissue damaging events that triggers inflammation, the lack of understanding of its true function has limited the diagnostic interpretation of its blood levels. On the other hand, assays to quantify CRP in blood have evolved to be rapid, economical and sensitive. Due to its recently availability and accessibility as point-of-care measurements, CRP has replaced the Erythrocyte Sedimentation Rate as the best diagnostic index for ongoing inflammation in an individual. However, CRP blood levels can vary 10-to-100 fold within 6–72 h of the initial tissue insult, its levels are not only used as general identifier of ongoing inflammation but as an index of the extent to which the APR and inflammation are actively stimulated and for how prolonged the inflammation persists. Extreme elevations in blood levels of CRP that persist or recur over time are generally perceived to be a bad omen for disease progression.

One particularly confounding disease in understanding of diagnostic relevance of CRP is cancer. Cancer disease can be variable in that it involves different tissues and pathological stages, can persist for years and can be in progression or remission. Despite these disparate conditions, many publications include discussions of CRP blood levels without elaborating on disease conditions when CRP blood levels were taken. Indeed, reported CRP levels vary from high sensitivity levels (i.e., CRP values $<10\mu\text{g/ml}$), to conventional levels (i.e., values above $10\mu\text{g/ml}$), and in certain instances up to more than $200\mu\text{g/ml}$. A consensus understanding of what divergent CRP levels indicate in individual cancer cases as related to both the disease and the progression of disease has been lacking. Exhaustive reviews in the literature (6–9) have revealed that (1) CRP levels above $10\mu\text{g/ml}$ are indicative of an ongoing tissue damaging inflammatory response in a patient; (2) CRP levels progressively increasing from $10\mu\text{g/ml}$ to more than $100\mu\text{g/ml}$ are indicative of progressively more active inflammation and disease progression; (3) CRP levels above $100\mu\text{g/ml}$ are prognostic of a poor outcome and/or a failure to respond to therapy; (4) CRP and other inflammatory markers are not useful tools to diagnose the presence of cancer. Because CRP levels increase and decrease rapidly (within days), changing without a memory response, it is important to assess the diagnostic relevance of CRP as a function of when measurements are made, as a function of disease stage, and as a function of patient responses to any treatment. A potential strategy was hypothesized by Coventry et al. (10) to monitor the sequential CRP values in a defined time frame and correlate the CRP levels increase with cancer disease progression;

and reciprocal decrease of the sequential CRP values during cancer remission.

This report introduces a unifying explanation for the quantitative and temporal appearance of CRP in blood and its innate bioactivities as the prototypic acute phase reactant. With the newly appreciated understanding that CRP is a dynamic protein that can undergo *in situ* a non-proteolytic conformational change into a unique, distinctive isoform, its role in amplifying and regulating inflammation is described. The protein found and quantified in blood is the pentameric discoid protein (i.e., abbreviated “pCRP” for “pentameric CRP”). When pCRP binds to an activated membrane, biochemical forces contribute to dissociation of the pentamer which induces a pronounced structural rearrangement exposing a cryptic binding site on the dissociated CRP subunits for cholesterol molecules found in lipid rafts. Dissociated CRP subunits are described as “monomeric, modified” CRP (i.e., abbreviated as “mCRP”) (11–16). The mCRP isoform is antigenically distinctive from the pCRP isoform and has significantly reduced aqueous solubility. When mCRP enters into lipid rafts, it triggers intracellular signaling pathways that strongly enhance pro-inflammatory activities generally known to be activated as part of the earliest phases of the acute phase response (17). By carefully evaluating the distinctive bioactivities of pCRP and mCRP, it is now established that pCRP has weak anti-inflammatory bioactivity while mCRP has strong pro-inflammatory bioactivity (4). By introducing and interpreting these novel concepts of the distinctive structural isoforms of CRP, a unified hypothesis for the diagnostic and therapeutic role of CRP is advanced. This report presents concepts of two structural isoforms of CRP with distinctive bioactivities as a relevant index to assess disease progression, and how the natural host defenses respond to disease.

HISTORICAL REFLECTIONS ON CRP AND ITS ROLE IN HEALTH AND DISEASE

As an evolutionarily conserved protein found in almost all lower species (18), it is tempting to speculate on the fundamental role that CRP may have in regulating innate defenses. Studies have appeared noting that CRP blood levels do increase in children with protein calorie malnutrition (19–21). This observation suggests that even during periods where hepatic gluconeogenesis is activated to supply metabolic fuel, the need for CRP supersedes the need to supply metabolic energy.

Even though the CRP response is associated early during innate immunity, its bioactivities have also been linked to binding immunoglobulin Fc receptors [reviewed in (4)]. Thus, CRP contribute to effector mechanisms with crossover activity between innate and adaptive immunity. However, CRP blood levels fluctuate independently of immunoglobulin concentrations (22) and a CRP response has been measured in agammaglobulinemic patients (23) indicating at least part of its biofunction occurs in the absence of antibody-mediated effector responses.

The extent to which blood levels of CRP increases above 10 µg/ml has been used to differentially diagnose certain diseases (24, 25). For example, CRP levels increase more pronouncedly in rheumatoid arthritis compared to systemic lupus (26). CRP levels are also found higher in bacterial infections than those caused by a virus, being helpful in directing the use of antibiotic therapy (27). CRP levels are also elevated during acute bronchitis compared to episodes of asthma (28). Most fundamentally, the more severe the tissue damage and stimulated inflammatory response, the higher the CRP levels. Higher CRP values are reported to correlate with poorer prognoses in any disease where CRP is measured. By focusing on the relationship of CRP blood levels to tissue damage rather than inflammation, a consensus can be made that CRP levels > 50–100 µg/ml are indicative of tissue damage that is so severe as to threaten survival.

Surprisingly, even though there is a clear association of CRP levels with the inflammatory response that occurs following any tissue damaging event, CRP is not found to selectively localize to injured tissue sites (29). Using intravenously injected ¹²⁵I-labeled CRP in human turnover studies, CRP was found to be a blood protein with a mean plasma half-life of 19 h. Its plasma clearance rate was similar in both normal control and various patient groups including patients with active autoimmune diseases, localized infections and localized cancers. While on the surface the fact that CRP does not locally sequester to the site of injury or disease would appear to be counterintuitive, these results call to question its true relationship as a mediator of host defense processes activated at sites of injured tissues.

USE OF CRP AS A DIAGNOSTIC MARKER IN DISEASE

The US Department of Health and Human Services guidelines for interpreting the diagnostic significance of CRP values defines a difference between “Conventional CRP” values and “High Sensitivity CRP” values (30). FDA guidance is only given for conventional CRP levels, defined as being > 10 µg/ml. While hsCRP levels (i.e., <10 µg/ml) are an area of great research interest, the FDA warns that such values are non-specific and should only be interpreted in combination with a full clinical evaluation. In multicenter studies involving tens of thousands of CRP measurements, the significance of hsCRP levels as having predictive clinical value has been questioned (31, 32).

One explanation for differences in baseline hsCRP levels has been attributed to genetic polymorphisms in the promoter region of the transcribed CRP gene. The gene for CRP is located on Chromosome 1 locus q23.2, the largest human chromosome, having about 8% of total DNA in human cells. Chromosome 1 is reported to have 249×10^6 nucleotide base pairs and > 4,300 genes. The CRP gene is comprised of 2 exons separated by a single intron of 278 nucleotides that includes a dinucleotide GT repeat sequence. Exon 1 encodes for an 18 amino acid leader sequence and the first 2 (of 206) amino acids in the mature CRP subunit and exon 2 codes for the remaining 204 amino acids (33). While genetic polymorphism has been

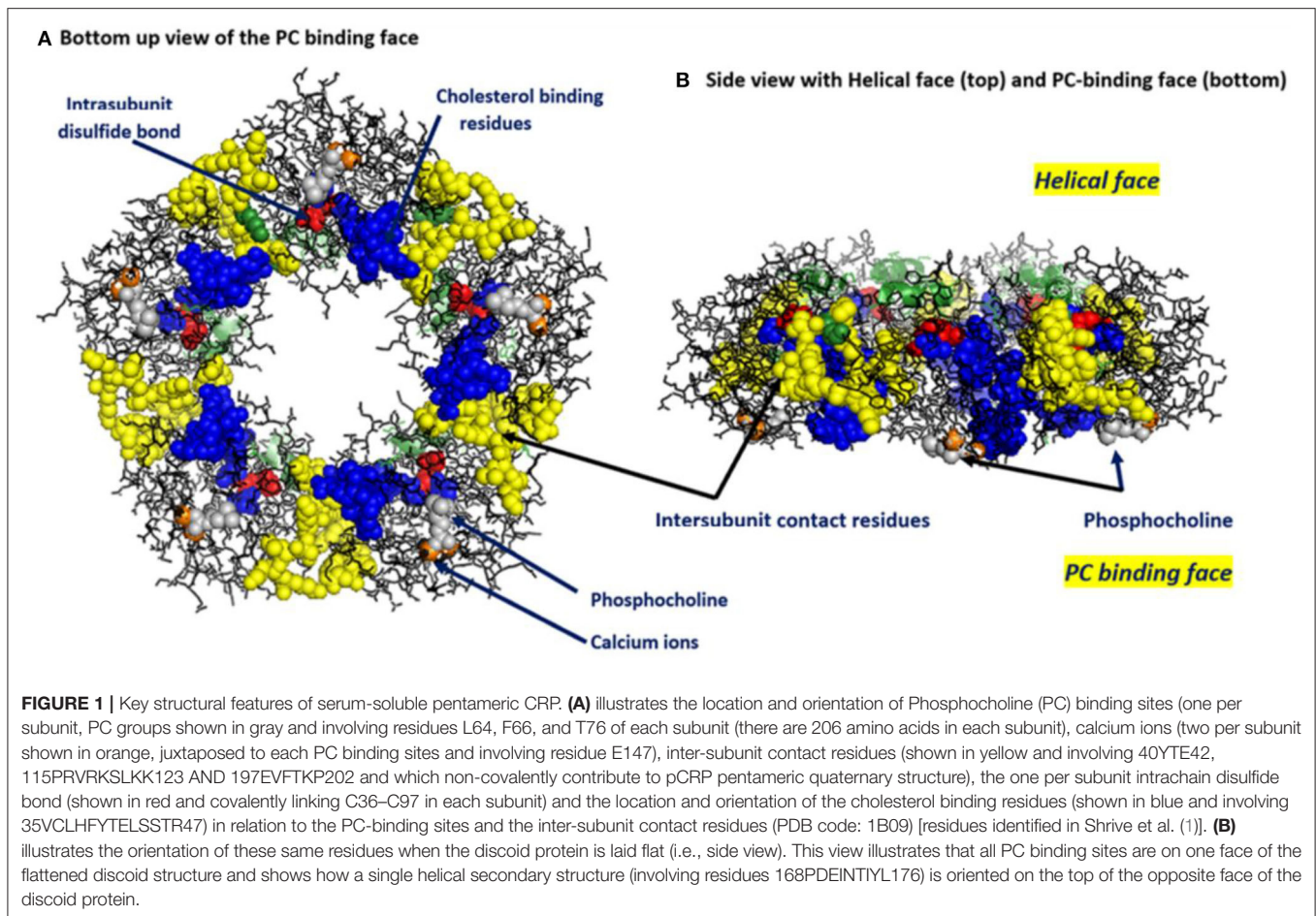
associated with slight changes in circulating CRP levels (34), there is no known significance of such polymorphism on disease risk (35).

In focusing on specific patient ethnicities, genders and general activities rather than disease, differing interpretations and explanations for the diagnostic value of hsCRP have emerged (36, 37). hsCRP levels were found to be higher in women and black ethnicities and were found to decrease in Hispanic men engaged in average to above average physical activity. Of note, vigorous exercise by black and white men appeared to lower hsCRP levels. In this report, no interpretation is given to any CRP level < 10 µg/ml (i.e., hsCRP). Emphasis is instead placed on understanding the relationship of conventional CRP levels as an index for and persistence of tissue damage associated with disease and disease progression. The widely accepted association of CRP with inflammation is not challenged. However, since inflammation is a natural response to tissue damage, readers are encouraged to broaden their understanding to associate CRP levels with the presence and extent of tissue damage. In doing so, and by understanding that CRP is not a single, unchanging/rigid structural entity (as outlined below), a common theme for the bioactivities of CRP emerges, defining the understanding of its fundamental role as a key protein of the acute phase host defense response.

STRUCTURAL ISOFORMS OF C-REACTIVE PROTEIN (CRP) EXHIBIT DISTINCT ANTIGENICITY AND BIOACTIVITY

CRP is widely recognized as a key acute phase reactant found in blood, primarily produced hepatically in response to tissue damage that elicits an inflammatory response (38–44). CRP is a protein of the innate (natural) immune system, providing baseline protection as a pattern recognition molecule, and as a regulator of host defense responses involving tissue barriers, vascular activation, phagocytic responses, and amplification mechanisms. As these host defenses feed into and direct certain responses of the acquired (specific) immune system (3, 45), CRP has been broadly studied as a molecule that can contribute to both positive and negative immune responses to essentially all disease etiologies. While the consequences of an impaired CRP response are unknown, the consequences of an impaired innate immune response include changes in natural resistance to pathogens and the development of autoimmune diseases. While CRP has been recognized for over 70 years as a blood protein whose levels change correlate with ongoing active inflammatory responses, its biological role during such processes remained undefined and controversial with conflicting reports concluding it both promoted and inhibited inflammation (46).

CRP is a highly soluble protein comprised of five non-glycosylated globular subunits arranged in cyclic symmetry (Figure 1). Its three-dimensional structural conformation has been resolved using X-ray crystallographic analyses (47, 48), as well as by deduction from the X-ray crystallographic analysis of the Serum Amyloid P component (SAP), which shares 51%



amino acid sequence homology with CRP and 59% nucleotide sequence identity (49, 50).

CRP levels in blood change markedly within hours to days of any event that involves tissue damaging pathology. While some variability exists in laboratory methods used to establish baseline levels, healthy individuals are found to have blood levels between 1 and 3 $\mu\text{g/ml}$ (25). In response to tissue trauma, blood levels increase within 6–48 h to levels generally related to the extent of the tissue damage. As inflammation is a natural host defense response to tissue damage, CRP levels have historically been diagnostically associated with inflammation rather than tissue damage. More directly, CRP levels change in response to the presence and extent of tissue damage which in turn activates the acute phase inflammatory response to the injury. Acute inflammation, occurring within minutes to hours of the tissue damaging event, is generally regarded as beneficial to host defense response. Chronic inflammation, which can persist for days, weeks, months, or years, can lead to healthy tissue damage and disruption, weakened host defenses, and problematic pain responses. This distinction is of relevance to a discussion of CRP because CRP is now known to exist in at least two isomeric structural forms with opposing activities on the inflammatory response (see below). The CRP isoform

recognized and measured in blood is the highly soluble, non-covalently associated cyclic pentamer (described as pentameric or “pCRP”). pCRP has recently been shown to have weak anti-inflammatory bioactivities. When the pentamer is coerced to dissociate into individual subunits, however, it undergoes a non-proteolytic, substantial conformational change into a structurally, antigenically and biologically distinctive molecule described as modified, monomeric CRP (i.e., “mCRP”). mCRP is a short-lived isoform with potent pro-inflammatory, acute phase amplification bioactivities that are associated with the earliest phases of the acute, beneficial inflammatory response (4).

pCRP is a substrate for the formation of mCRP. At local sites of tissue damage, pCRP can be induced to convert into mCRP by binding to and interacting with membrane lipids [initially using its calcium dependent binding specificity for ligands expressing phosphocholine (PC)] (51). Membrane PC groups are most accessible for pCRP binding after Phospholipase A2 cleaves an acyl chain from a phospholipid, creating the detergent-like lipid, monoacyl (Lyso)-PC. Membrane bound pCRP is brought into juxtaposition with apolar regions of the membrane, which contributes biochemical energies needed to dissociate the pentamer. Furthermore, the structural change of each CRP subunit that accompanies membrane interaction, exposes a

new binding site (only expressed on the mCRP isoform) for cholesterol found in lipid rafts (2, 17, 52) (**Figure 2**), membrane microdomains that regulate cellular signaling pathways of importance in health and disease (53).

The expression of mCRP must be tightly regulated and controlled; its half-life must be short and localized. As pCRP is a substrate for the formation of mCRP, the relative level of pCRP measured in blood will depend in part on the rate at which pCRP is converted into mCRP. During the earliest minutes of tissue trauma, the conversion of pCRP into mCRP is efficient and rapid. Expressed mCRP, which has potent proinflammatory bioactivities, then upregulates and amplifies the acute phase of the inflammatory response. Over the next minutes to hours as the immediate threat to tissue homeostasis is controlled, the conversion of pCRP to mCRP slows, leading to an increased measurement of pCRP in blood. Only the pCRP antigen is measured and interpreted as a diagnostic marker in blood. As the mCRP antigen forms and quickly sequesters into membranes and tissues involved in the inflammatory response, aqueous solubility is reduced and antigenicity is masked, making it a difficult antigen to detect in body fluids. As the pCRP isoform has weak anti-inflammatory activities while the mCRP isoform has strong pro-inflammatory activities, the level of pCRP that is soluble and easily quantified in blood reflects on the extent of a weakened inflammatory, unamplified acute phase response. As pCRP hepatic synthesis is regulated by cytokines released by damaged endothelial cells (38, 54), persistently elevated synthesis and plasma levels of CRP is more likely directly related to tissue damaging pathologies rather than the inflammation. Chronic (unamplified) inflammation could persist in such a situation, exacerbating tissue destructive processes and impairing wound healing and repair to reestablish health homeostasis.

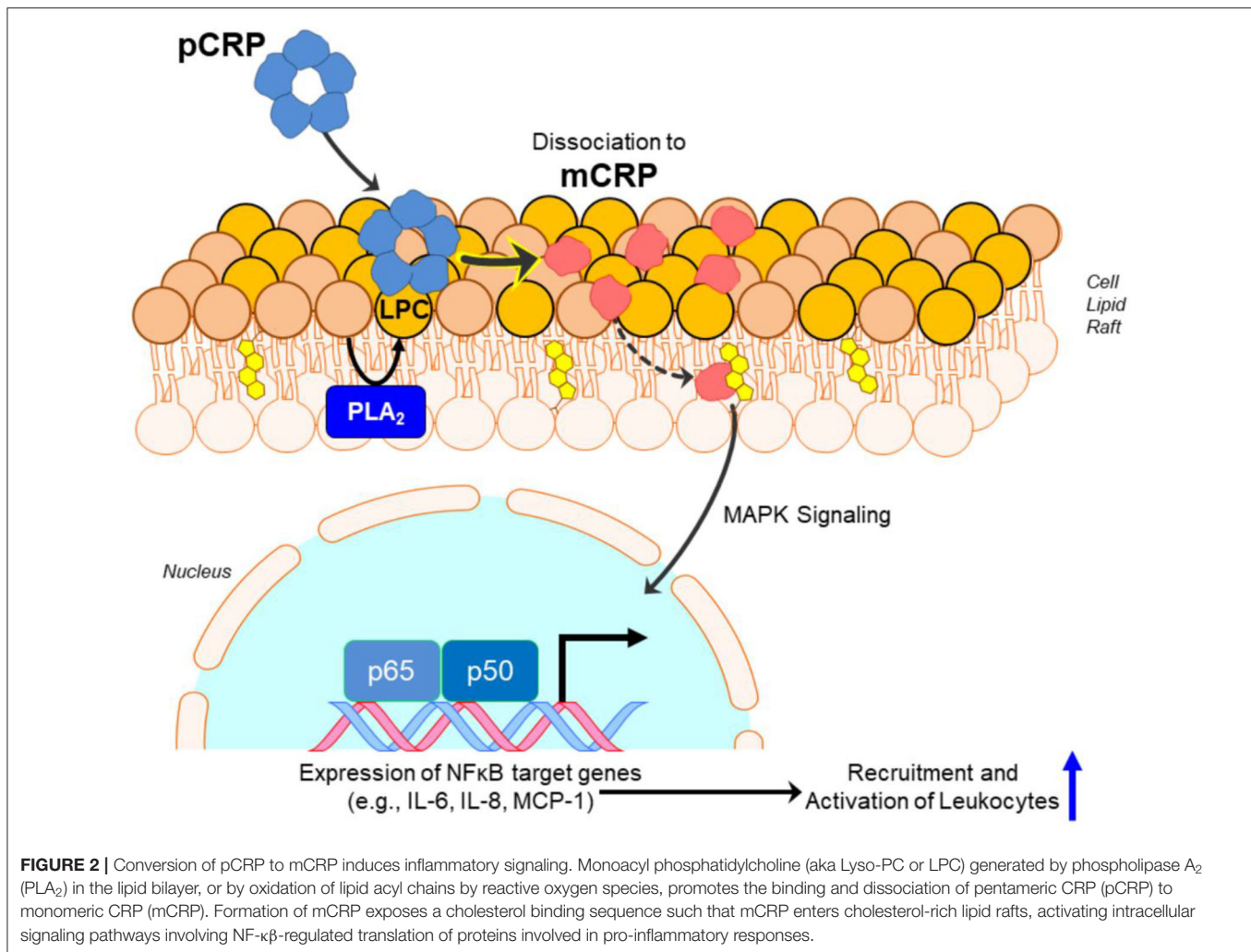
Taken together, pCRP levels that remain elevated at and above values generally considered to be diagnostic of ongoing inflammatory responses reflect not on the presence of generalized inflammation, but rather the degree to which acute, amplified (beneficial) inflammation is stimulated. Higher pCRP levels can be an index for inefficient or inhibited processes by which pCRP converts into mCRP. If mCRP is not formed, inflammation cannot be amplified to its acute phase, resulting in a persistent chronic response and pathological complications.

KINETICS OF THE CRP RESPONSE

Plasma CRP is synthesized and released by hepatocytes in response to IL-6, IL1 β , IL-17 and stress signals that accompany vascular stimulation associated with tissue damage (54, 55). Prior to receiving stress signals, hepatocytes will slowly release basal levels of CRP that were pre-synthesized and stored in intracellular vesicles. The secretory rate of CRP increases 6-fold in response to stress signals (43), which also initiates new protein synthesis. In healthy individuals, the normal synthesis rate is 1.5 $\mu\text{g/kg-hr}$; in diseased states, its synthesis rate is reported to range from 43.3 to 103.4 $\mu\text{g/kg-h}$ (i.e., a 30-70-fold increase). These values calculate such that an average person will synthesize 2.4 mg of CRP/day, increasing up to 174 mg/day

in response to an exacerbating event (29). CRP's fractional catabolic rate (i.e., its rate of consumption) is reported to be independent of its plasma concentration indicating changes in CRP blood levels during an acute inflammatory response reflect on an increased synthesis rate and not on an increased rate at which it is utilized. These observations appear to confound the understanding of CRP as the prototypic acute phase reactant which would be expected to be involved with and consumed by inflammatory processes.

While synthesis and hepatic release rates of CRP in response to cytokine stimulation have been reported (29, 41, 43, 56), measured increases in CRP blood level appear to involve a lag of 6–12 h after stimulation (40, 57, 58). Its level will continue to increase between 6 and 72 h as a result of both increased synthesis and release from hepatocytes. The exact kinetics of CRP appearance in blood during the first minutes to hours of an acute phase stimulation requires additional, focused experimentation. In reflecting on published quantification kinetics and the immediate, 6-fold increase in CRP secretion rate during the first moments of a stimulating cause, and by taking into account the propensity for CRP to dissociate into an isoform with distinctive structural, solubility and antigenicity differences from that CRP molecule measured in blood (4, 17, 59–63), the apparent lag phase was interpreted to represent a physiologically relevant finding in the bioactivity of CRP. If CRP is released from hepatocytes within minutes of acute phase stimulation but is not detected in blood for hours, such data are consistent with a rapid conversion of soluble, pentameric CRP into its distinctive structural and antigenic mCRP isoform during these early moments. Indeed, at the site of tissue damage, as reactions of acute inflammation are activated, dissociation of pentameric CRP into conformationally unique mCRP would represent an important regulatory signal that functionally contributes a potent pro-inflammatory amplification signal to immediately and aggressively respond to the threat to tissue integrity. As the need to maintain acute inflammation remains, pCRP localizes and converts to mCRP to maintain and amplify the acute phase response. When amplified, acute inflammation subsides and the rate of pCRP conversion to mCRP slows, pCRP is no longer consumed, which results in increased levels appearing in blood (i.e., after a 6–12-h lag). These concepts involving kinetic changes of hepatic secretion of pCRP during early and late acute inflammation, and the hypothetical impact of the timing and rate of conversion of pCRP to mCRP are illustrated in **Figure 3**. This illustration depicts the potential relationship between CRP production and conversion into the mCRP isoform during the early phases of acute inflammation, while accounting for the latent increase in significantly elevated levels of pCRP in blood. Conformational activation is a basic tenet of biochemical signaling control (64, 65). Herein, we discuss CRP as a protein requiring conformational changes to elicit and control key host defense bioactivities. Furthermore, the widely held notion that CRP is the prototypic acute phase reactant needs to be refined to more specifically associate the mCRP isoform (rather than the pCRP isoform measured in blood) as the true pro-inflammatory “acute” reactant.



THE PRO- OR ANTI-INFLAMMATORY ACTIVITY OF CRP IS DEFINED BY ITS CONFORMATION

Over many decades, CRP has been studied as both a contributor to and protector from the pathologies associated with disease. A definitive activity remained elusive for many years as conflicting reports described CRP as both pro- and anti-inflammatory activities, and as both pro- and anti-thrombotic [reviewed in (4)]. Because of this mechanistic uncertainty, CRP could only be described and used as a diagnostic marker of clinical situations involving some level of inflammation with no true understanding of its mechanism(s) of action. Because its blood levels increase and decrease rapidly over days it has only been of limited use in helping devise treatment strategies or in understanding disease pathologies.

The confusion concerning CRP's bioactivities only recently began to clarify when it was recognized that the serum soluble non-covalently associated pentameric CRP structure could be induced to dissociate into monomeric subunits. When separated, the biochemical energies involved in the folding and packaging of

each subunit both individually and as a non-covalently associated pentameric multiyear, rapidly and irreversibly redistribute such that the subunits modify into a unique, non-proteolyzed isoform which is described as modified, monomeric CRP (i.e., mCRP). mCRP can be expressed from pCRP under physiological conditions that occur at sites of where endothelial cells are activated. At such sites, pCRP will bind as a function of calcium to its primary ligand – phosphocholine (PC), which only become more exposed when diacyl phosphatidylcholine lipids are hydrolyzed by phospholipase enzymes, producing monoacyl (Lyso) phosphatidyl choline (i.e., Lyso-PC) (2). PC bound pCRP is brought into close proximity to surface membrane charges and to apolar lipid regions which contribute the biochemical energies needed to dissociate pCRP subunits which then spontaneously rearrange to express mCRP structure and its distinctive immunological and biochemical attributes (**Figure 2**).

By recognizing that CRP can exist as more than a stagnant, unchanging cyclic pentameric discoid protein, but also as a dynamic structure that can be induced to undergo conformational activation, a reason for its appearance and pharmacokinetic changes in blood levels, and its bioactivity

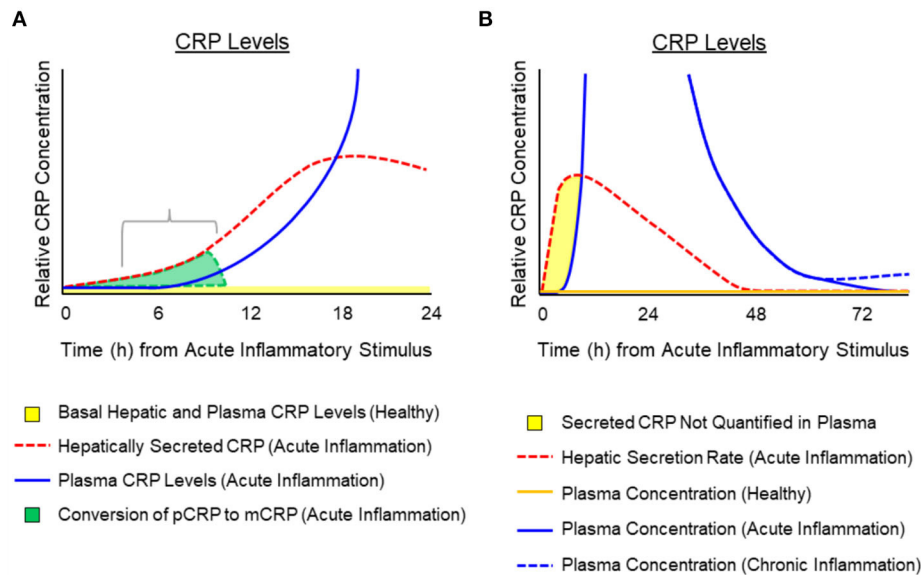
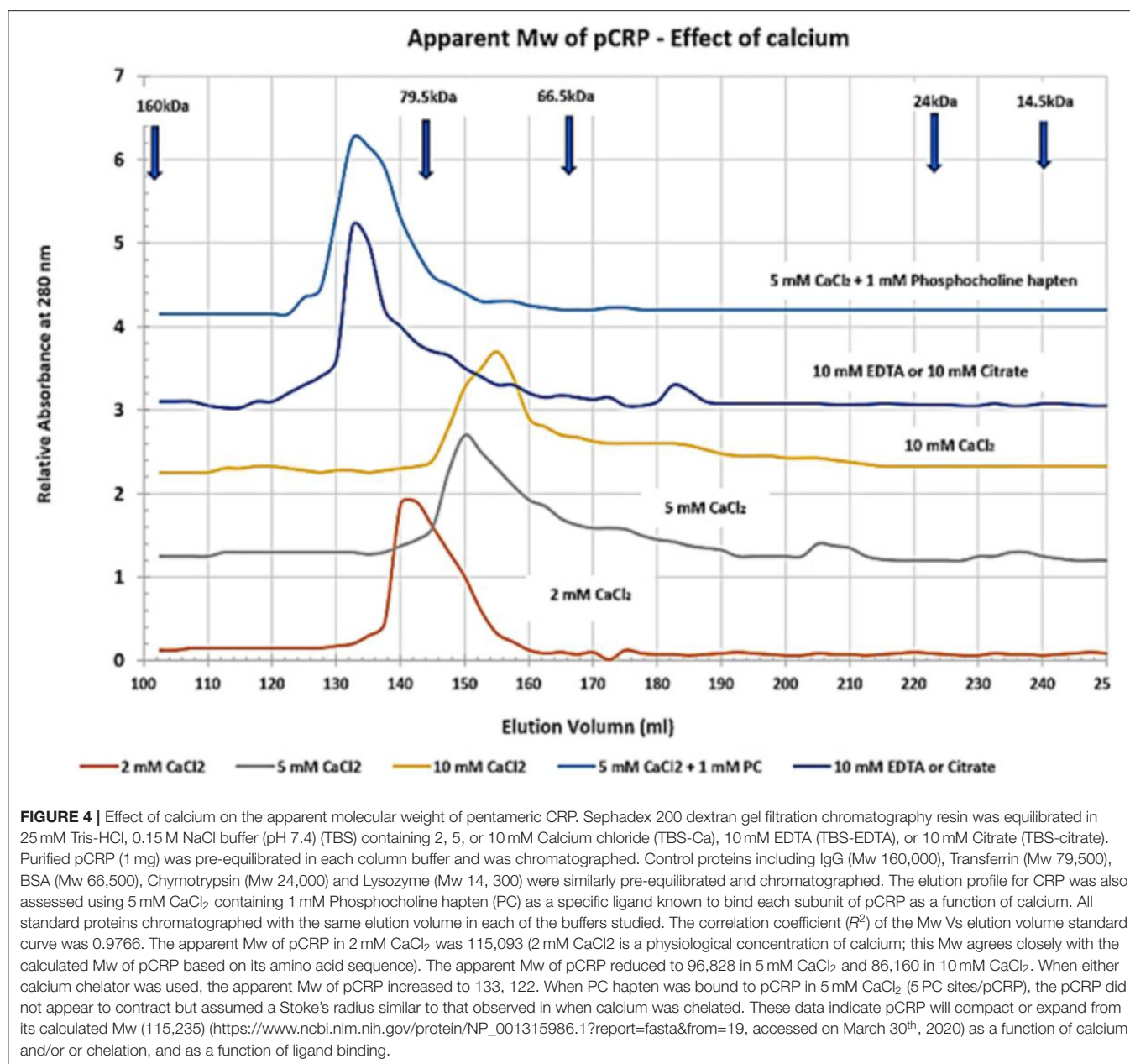


FIGURE 3 | Temporal depiction of the hypothesized conversion of pCRP into mCRP and the appearance of pCRP in blood after activation or an acute phase response. Schematic representation for the relative amounts of pCRP released from hepatocytes in response to cytokine signals (e.g., IL-6) and its appearance in the blood. **(A)** Depicts that while pCRP is hepatically secreted within minutes of acute phase response signaling (dotted red line) measured plasma levels of CRP display a lag (i.e., 6–12 h) (blue solid line). Hepatically released CRP can be from both pre-synthesized CRP stored in vesicles, or from *de novo* synthesis. In the first 12 h. of an inciting stimulus, the pCRP that is released from hepatocytes, but which is not quantified in blood is converted at the site of tissue damage into mCRP (gray shaded area). **(B)** Depicts a generalized time course for the hepatic secretion of pCRP (red dotted line), the relative amount of pCRP measured in blood (solid blue line as may occur during acute inflammation associated with major tissue damage; dotted blue line as may occur with low grade, chronic inflammation with lesser tissue damage). Plasma concentration of pCRP in healthy individuals (i.e., baseline CRP levels of < 10 $\mu\text{g/ml}$) is shown as a solid orange line. The shaded yellow area depicts a time period in which pCRP is secreted but rapidly consumed so that it is not quantified in plasma. The consumption of pCRP into mCRP would involve membrane binding to activated cells (e.g., endothelial cells, platelets, leukocytes) and entry into lipid rafts where mCRP activates signaling pathways to stimulate immediate, amplified pro-inflammatory responses to the threat.

during an inflammatory acute phase response begins to unfold. The concept of conformational activation of proteins is a widely accepted for many biochemical systems including allosteric signaling (66), enzyme catalysis (67) and ion-gated channel activities (68). CRP, as a tightly packed non-covalently associated pentameric protein with identical non-glycosylated globular subunits, would be a strong candidate for such an activation mechanism as it will express five stimulatory proteins from one serum-delivered substrate that forms on activated endothelial tissues. The tight packing of pCRP involves the binding of two calcium ions per subunit (1). Calcium has direct effect on CRP packing as summarized in **Figure 4**, which shows how the apparent size of pCRP (i.e., its Stoke's radius) changes as a function of the amount of calcium included in CRP solutions. In 2 mM calcium chloride, a physiological concentration of calcium in blood, pCRP migrates with an apparent molecular weight of 115 kDa – in close agreement with its calculated molecular weight based on its primary protein sequence. As calcium is increased to 5 and 10 mM, however, pCRP appears as a smaller (more compacted) protein (smaller Stoke's radius). In the tightly packed configuration, pCRP has an electron microscopy average diameter of $10.42 \pm 0.08 \text{ nm}$ (69) and is resistant to proteolysis (70). When calcium is chelated using citrate or EDTA in chromatographic buffers, pCRP appears to

“swell,” having an apparent molecular weight of 125–140 kD (i.e., a larger Stoke's radius). In early studies establishing isolation protocols for CRP from blood, many chromatographic steps used 0.05 M citrate buffers (71). CRP's larger apparent size led the authors to conclude that at least some CRP could exist as a hexameric protein.

Calcium has long been known to regulate CRP binding reactivity for PC; the calcium binding sites of each CRP subunit frame its PC binding pocket (see **Figure 1**). These data show calcium can also affect pCRP packing and proteolytic susceptibility, and suggest CRP has a capacity to change its three-dimensional protein structural packing (i.e., its conformation). Swanson et al. (72) reported that calcium could affect CRP structure in a way to regulate binding of CRP-specific monoclonal antibodies. Furthermore, the physiological relevance of structural changes in CRP was demonstrated in electron microscopy and adsorption studies of pCRP onto Lyso-PC containing monolayers. Binding pCRP to the PC-containing lipid monolayers required calcium and led to a two-stage alteration in the pentameric CRP structure. Initially, membrane bound pCRP changed conformation into a hybrid molecule called membrane-associated-mCRP (or mCRP_m). With lengthened incubation times, the second alteration occurred, completely converting the protein into its mCRP isoform (17, 73). Additional studies



have reported on calcium-dependent pCRP binding to Lyso-PC activated platelets and neutrophils, producing a structurally altered CRP hybrid molecule described as pCRP* (5, 12). In each instance, potent pro-inflammatory bioactivities of CRP are expressed correlate with the structural changes of pCRP into mCRP.

The mCRP isoform has significantly different solubility and antigenicity compared to the pCRP isoform. mCRP enters and associates with membrane lipids, into cholesterol rich lipid rafts (52, 73) and is not freely soluble in aqueous phase, only being found in body fluids associated with micro-vesicles that are sloughed off activated endothelial cells as part of the activated inflammatory response (74, 75).

Monoclonal antibody reagents were developed to clearly define and differentiate the pCRP antigen from the mCRP antigen (61, 62). Furthermore, by carefully identifying conditions which contribute to the conversion of pCRP into mCRP, and by using reagents that certifiably distinctive as predominantly pCRP and mCRP, key physiological distinctions between each isoform were identified.

While the pCRP antigen is mainly found in blood, it can occasionally be found weakly associated with vascular surfaces and easily eluted with washing. The mCRP antigen, in contrast, is found as a naturally occurring molecule within a wide variety of normal tissues, in particular at the intima, media and adventitia of healthy blood vessels and in fibrous tissues of the skin (76–78).

While the pCRP antigen is resistant to proteolysis, the mCRP antigen can be proteolyzed by a variety of neutrophil-derived peptidases (79–81). Peptides derived from the proteolysis of mCRP have been shown to inhibit activation of platelets and neutrophils (82), downregulating the potent pro-inflammatory activities of the intact mCRP protein. Hence, there is a direct feedback mechanism elicited by neutrophil-derived enzymatic proteolysis that can quickly reverse the pro-inflammatory bioactivity of mCRP.

For decades, CRP has been appreciated solely as a diagnostic marker for ongoing inflammation. With the now recognized appreciation that CRP is a dynamic protein that can undergo a pronounced structural and functional change, and by understanding the conditions that contribute to such change *in situ*, insights can now also be made into the therapeutic relevance of CRP in health and disease. Notably, pCRP will change into mCRP by interacting with activated membranes (83) using a process that involves an intermediate form of CRP described as mCRP_m (17) or pCRP* (63). The intermediate structure represents initial stages of subunit dissociation in which the still pentameric protein begins expressing antigenic and functional attributes now known to be characteristic of mCRP. As the pentamer fully dissociates into its monomeric form (i.e., mCRP), it inserts into cholesterol rich membranes (lipid rafts) and supplies an activation signal for pro-inflammatory pathways associated with the acute phase inflammatory response. Activated membranes are known to slough microvesicles (aka microparticles), which have been reported to contain mCRP (63, 74, 75, 84, 85). To date, while a reliable, direct, quantitative blood-based assay for mCRP remains elusive, when developed, it will provide a new perspective on the role CRP has as a regulator of inflammation, not only as a diagnostic marker but as a therapeutic parameter in deciding disease conditions and treatment options.

By influencing or controlling the conversion of pCRP to mCRP, it may be possible to therapeutically control the rate and extent of anti- and pro-inflammatory responses. Indeed, Caprio et al. (2), have discussed using inhibitors of Phospholipase A₂ (PLA₂) to mitigate the production of lyso-PC which is a critical step in binding pCRP and bringing it into juxtaposition to the apolar membrane zone (83). Pepys et al. (86) designed a small molecule bi-valent compound having phosphocholine groups on opposite ends of a linking group. As PC is the primary ligand for CRP, and since the PC binding sites are all the same face of the pentameric structure (see **Figure 1**), this bis-PC compound would link to two pentamers face to face, essentially forming a CRP decamer. By occupying 10 PC sites, and by complexing 2 CRP pentamers, CRP would be unable to bind membrane associated PC and interact with apolar membrane regions that

contribute biochemical energies needed to conformationally change pCRP into mCRP. Braig et al. (63) described how this bis-PC compound influenced CRP, pCRP* and mCRP reactivities with microvesicles. All these examples offer evidence of some strategies being studied and developed to control inflammation by controlling, not the quantity of CRP in blood, but in the way and extent that pCRP converts into mCRP.

SUMMARY AND CONCLUSION

Readers are referred to the following manuscripts that detail the expression of mCRP from pCRP and describe its potent pro-inflammatory bioactivities (4, 5, 11, 12, 63, 73, 87–91). The focus of this report is to provide a new paradigm to understanding what diagnostic CRP levels indicate and in introducing the connection between CRP and inflammation-associated tissue damage. By understanding that the pCRP protein is fundamentally a serum soluble, precursor protein with significant stored potential energies, its diagnostic utility begins to take on a new meaning. The potential energy needed to stimulate and amplify acute inflammation gets released by the spontaneous conformational change that occurs simply when the pentamer is dissociated. The mCRP isoform of CRP is, in fact, the “prototypic acute phase reactant.” Its proinflammatory activities are potent, localized and short lived. Any pCRP that is present during the earliest phases of the host defense response to tissue damage, will be readily converted to mCRP. When expression of mCRP slows or stops, or when mCRP is destroyed by proteases, acute inflammation slows down to chronic inflammation. pCRP, which is synthesized and secreted by hepatocytes is the substrate for the formation of mCRP. It will only start to accumulate in blood after the conversion to mCRP slows down (e.g., after 6–10 h. of the inciting cause). Measured elevation in blood pCRP is more diagnostic of a chronic inflammatory response, which, if prolonged or severe enough, will lead to exacerbated tissue damage caused by prolonged neutrophil stimulated, non-specific effector responses involving secretion of reactive oxygen species and hydrolytic enzymes into affected areas.

AUTHOR CONTRIBUTIONS

IR, PH, and LP equally contributed to the concepts of writing of and editing of this manuscript. LP developed concepts, reagents to study isoforms, and especially relevant to the mCRP isoform. PH contributed to inflammation concepts and produced figures presented. IR contributed extensively to summarizing literature reports including data. All authors contributed to the article and approved the submitted version.

REFERENCES

- Shrive AK, Cheetham GMT, Holden D, Myles DAA, Turnell WG, Volanakis JE, et al. Three-dimensional structure of human C-reactive protein. *Nat Struct Biol.* (1996) 3:346–54. doi: 10.1038/nsb0496-346
- Caprio V, Badimon L, Di Napoli M, Fang W-H, Ferris GR, Guo B, et al. pCRP-mCRP dissociation mechanisms as potential targets for the development of small-molecule anti-inflammatory chemotherapeutics. *Front Immunol.* (2018) 9:1089–96. doi: 10.3389/fimmu.2018.01089

3. Du Clos TW, Mold C. C-reactive protein: an activator of innate immunity and a modulator of adaptive immunity. *Immunol Res.* (2004) 30:261–77. doi: 10.1385/IR.30:3:261
4. Wu Y, Potempa LA, Kebir DE, Filep JG. C-reactive protein and inflammation: conformational changes affect function. *Biol Chem.* (2015) 396:1181–97. doi: 10.1515/hsz-2015-0149
5. McFadyen J, Kiefer J, Loeffler-Silver J, Braig D, Potempa LA, Eisenhardt SU, et al. Dissociation of C-reactive protein localizes and amplifies inflammation: Evidence for a direct biological role of CRP and its conformational changes. *Front Immunol.* (2018) 9:1351. doi: 10.3389/fimmu.2018.01351
6. Allin KH, Nordestgaard BG. Elevated C-reactive protein in the diagnosis, prognosis, and cause of cancer. *Crit Rev Clin Lab Sci.* (2011) 48:155–70. doi: 10.3109/10408363.2011.599831
7. Shrotriya S, Walsh D, Bennani-Baiti N, Thomas S, and Lorton C. C-reactive protein is an important biomarker for prognosis tumor recurrence and treatment response in adult solid tumors: a systematic review. *PLoS ONE.* (2015) 10:e0143080. doi: 10.1371/journal.pone.0143080
8. Shrotriya S, Walsh D, Nowacki AS, Lorton C, Aktas A, Hullihen B, et al. Serum C-reactive protein is an important and powerful prognostic biomarker in most adult solid tumors. *PLoS ONE.* (2018) 13:e0202555. doi: 10.1371/journal.pone.0202555
9. Watson J, Salisbury C, Banks J, Whiting P, Hamilton W. Predictive value of inflammatory markers for cancer diagnosis in primary care: a prospective cohort study using electronic health records. *Br J Cancer.* (2019) 120:1045–51. doi: 10.1038/s41416-019-0458-x
10. Coventry BJ, Ashdown ML, Quinn MA, Markovic SN, Yatomi-Clarke SL, Robinson AP. CRP identifies homeostatic immune oscillations in cancer patients: a potential treatment targeting tool? *J Transl Med.* (2009) 7:102. doi: 10.1186/1479-5876-7-102
11. Thiele JR, Zeller J, Bannasch H, Stark GB, Peter K, Eisenhardt SU. Targeting C-reactive protein in inflammatory disease by preventing conformational changes. *Mediat Inflamm.* (2015) 2015:372432. doi: 10.1155/2015/372432
12. Thiele JR, Zeller J, Kiefer J, Braig D, Kreuzaler S, Lenz Y, et al. A conformational change in C-reactive protein enhances leukocyte recruitment and reactive oxygen species generation in ischemia/reperfusion injury. *Front Immunol.* (2018) 9:675. doi: 10.3389/fimmu.2018.00675
13. Wang J, Tang B, Liu X, Wu X, Wang H, Xu D, et al. Increased monomeric CRP levels in acute myocardial infarction: a possible new and specific biomarker for diagnosis and severity assessment of disease. *Atherosclerosis.* (2015) 2:343–9. doi: 10.1016/j.atherosclerosis.2015.01.024
14. Yao Z, Zhang Y, Potempa LA, Rajab I, Ji L, Lv J, et al. A redox sensitivity-based method to quantify both pentameric and monomeric C-reactive protein in a single assay. *J Immunol Methods.* (2019) 470:40–5. doi: 10.1016/j.jm.2019.04.009
15. Zhang L, Li H-Y, Li W, Shen Z-Y, Wang Y-D, Ji S-R, et al. An ELISA assay for quantifying monomeric C-reactive protein in plasma. *Front Immunol.* (2019) 9:511. doi: 10.3389/fimmu.2018.00511
16. Williams RD, Moran JA, Fryer AA, Littlejohn JR, Williams HM, Greenhough TJ, et al. Monomeric C-reactive protein in serum with markedly elevated CRP levels shares common calcium-dependent ligand binding properties with an *in vitro* dissociated form of C-reactive protein. *Front Immunol.* (2020) 11:115. doi: 10.3389/fimmu.2020.00115
17. Ji SR, Wu Y, Zhu L, Potempa LA, Sheng FL, Wei L, et al. Cell membranes and liposomes dissociate C-reactive protein. (CRP) to form a new, biologically active structural intermediate: mCRP_m. *FASEB J.* (2007) 21:284–94. doi: 10.1096/fj.06-6722com
18. Pathak A, Agrawal A. Evolution of C-reactive protein. *Front Immunol.* (2019) 10:00943. doi: 10.3389/fimmu.2019.00943
19. Cunningham-Rundles S. Effects of nutritional status on immunological function. *Am J Clin Nutr.* (1982) 35:1202–10. doi: 10.1093/ajcn/35.5.1202
20. Sauerwein RW, Mulder JA, Mulder L, Lowe B, Peshu N, Demacker PN, et al. Inflammatory mediators in children with protein-energy malnutrition. *Am J Clin Nutr.* (1997) 65:1534–9. doi: 10.1093/ajcn/65.5.1534
21. Reid M, Badaloo A, Forrester T, Morlese JE, Heird WC, Jahoor F. The acute-phase protein response to infection in edematous and nonedematous protein-energy malnutrition. *Am J Clin Nutr.* (2002) 6:1409–15. doi: 10.1093/ajcn/76.6.1409
22. Kogure T, Itoh T, Shimada Y, Shintani T, Ochiai H, Terasawa K. Detection of serum soluble markers of immune activation in rheumatoid arthritis. *Mediat Inflamm.* (1996) 5:262–5. doi: 10.1155/S0962935196000373
23. Vieira DG, Costa-Carvalho BT, Hix S, da Silva R, Correia MSG, Sarni ROS. Higher cardiovascular risk in common variable immunodeficiency and x-linked agammaglobulinemia patients. *Ann Nutr Metab.* (2015) 66:237–41. doi: 10.1159/000435818
24. Morley JJ, Kushner I. Serum C-reactive protein levels in disease. *Ann NY Acad Sci.* (1982) 389:406–18. doi: 10.1111/j.1749-6632.1982.tb22153.x
25. Antonelli M, Kushner I. It's time to redefine inflammation. *FASEB J.* (2017) 31:1787–91. doi: 10.1096/fj.201601326R
26. Vogt B, Führer B, Müller R, Sheriff A. CRP and the disposal of dying cells: consequences for systemic lupus erythematosus and rheumatoid arthritis. *Autoimmunity.* (2007) 40:295–8. doi: 10.1080/08916930701358925
27. Butler CC, Gillespie D, White P, Bates J, Lowe R, Thomas-Jones E, et al. C-reactive protein antibiotics prescribing for COPD exacerbations. *NEJM.* (2019) 381:111–20. doi: 10.1056/NEJMoa1803185
28. Andreeva E, Melbye H. Usefulness of C-reactive protein testing in acute cough/respiratory tract infection: an open cluster-randomized clinical trial with C-reactive protein testing in the intervention group. *BMC Family Pract.* (2014) 15:80. doi: 10.1186/1471-2296-15-80
29. Vigushin DM, Pepys MB, Hawkins PN. Metabolic and scintigraphic studies of radioiodinated human C-reactive protein in health and disease. *J Clin Invest.* (1993) 91:1351–7. doi: 10.1172/JCI116336
30. FDA. U.S. Department of Health and Human Services. Guidance for Industry and FDA Staff. Review Criteria for Assessment of C-reactive protein (CRP), High Sensitivity C-Reactive Protein (hsCRP) and Cardiac C-Reactive Protein (cCRP) Assays. (2005).
31. Yousuf O, Mohanty BD, Martin SS, Joshi PH, Blaha MJ, Nasir K, et al. High-sensitivity C-reactive protein and cardiovascular disease: a resolute belief or an elusive link? *J Am Coll Cardiol.* (2013) 62:397–408. doi: 10.1016/j.jacc.2013.05.016
32. Elliott P, Chambers JC, Zhang W, Clarke R, Hopewell JC, Peden JF, et al. Genetic loci associated with C-reactive protein levels and risk of coronary heart disease. *JAMA.* (2009) 302:37–48. doi: 10.1001/jama.2009.954
33. Hage HG, Szalai AJ. C-reactive protein gene polymorphisms, C-reactive protein blood levels, and cardiovascular disease risk. *J Am Coll Cardiol.* (2007) 50:1115–22. doi: 10.1016/j.jacc.2007.06.012
34. Carlson CS, Aldred SF, Lee PK, Tracy RP, Schwartz SM, Reider M, et al. Polymorphisms within the C-reactive protein (CRP) promoter region are associated with plasma CRP levels. *Am J Hum Genet.* (2005) 77:64–77. doi: 10.1086/431366
35. Wang M-Y, Zhou H-H, Zhang C-M, Su H-X, Li S-L, Ji S-R, et al. A functional genetic variant at C-reactive protein promoter (rs3091244) is not associated with cancer risk in a Chinese population. *Front Immunol.* (2020) 11:926. doi: 10.3389/fimmu.2020.00926
36. Majka DS, Chang RW, Vu T-H, Palmas W, Geffken DF, Ouyang P, et al. Physical activity and high-sensitivity C-reactive protein. The multi-ethnic study of atherosclerosis. *Am J Prev Med.* (2009) 36:56–62. doi: 10.1016/j.amepre.2008.09.031
37. Fonseca FA, Izar MC. High-sensitivity c-reactive protein and cardiovascular disease across countries and ethnicities. *Clinics.* (2016) 71:235–242. doi: 10.6061/clinics/2016(04)11
38. Ganapathy MK, Rzewnicki D, Samols D, Jiang S-L, Kushner I. Effect of combinations of cytokines and hormones on synthesis of serum amyloid A and C-reactive protein in Hep 3B cells. *J Immunol.* (1991) 147:1261–5.
39. Ganter U, Arcone R, Toniatti C, Morrone G, Ciliberto G. Dual control of C-reactive protein gene expression by interleukin-1 and interleukin-6. *EMBO J.* (1989) 8:3773–9. doi: 10.1002/j.1460-2075.1989.tb08554.x
40. Kushner I, Feldman G. Control of the acute phase response. Demonstration of C-reactive protein synthesis and secretion by during acute inflammation in the rabbit. *J Exp Med.* (1978) 148:466–77. doi: 10.1084/jem.148.2.466
41. Macintyre SS. Regulated export of a secretory protein from the ER of the hepatocyte: a specific binding site retaining C-reactive protein within the ER is downregulated during the acute phase response. *J Cell Biol.* (1992) 118:253–65. doi: 10.1083/jcb.118.2.253
42. Macintyre SS, Schultz D, Kushner I. Biosynthesis of C-reactive protein. *NY Acad Sci.* (1982) 389:76–87. doi: 10.1111/j.1749-6632.1982.tb22126.x

43. Macintyre SS, Kushner I, Samols D. Secretion of C-reactive protein becomes more efficient during the course of the acute phase response. *J Biol Chem.* (1985) 260:4169–73.
44. Yap SH, Moshage HJ, Hazenberg BPC, Roelofs HMJ, Bijzet J, Limburg PC, et al. Tumor necrosis factor (TNF) inhibits interleukin (IL)-1 and/or IL-6 stimulated synthesis of C-reactive protein (CRP) and serum amyloid A (SAA) in primary cultures of human hepatocytes. *Biochim Biophys Acta.* (1991) 1091:405–8. doi: 10.1016/0167-4889(91)90207-E
45. Suffredini AF, Fantuzzi G, Badolato R, Oppenheim JJ, O'Grady NP. New insights into the biology of the acute phase response. *J Clin Immunol.* (1999) 19:203–14. doi: 10.1023/A:1020563913045
46. Allin KH, Bojesen SE, Nordestgaard BG. Baseline C - reactive protein is associated with incident cancer and survival in patients with cancer. *J Clin Oncol.* (2009) 27:213:2217–24. doi: 10.1200/JCO.2008.19.8440
47. Thompson D, Pepys MB, Wood SP. The physiological structure of human C-reactive protein and its complex with phosphocholine. *Structure Feb.* (1999) 7:169–77. doi: 10.1016/S0969-2126(99)80023-9
48. Ramadan MAM, Shrive AK, Holden D, Myles DAA, Volanakis JE, DeLucas L, et al. The three-dimensional structure of calcium-depleted human C-reactive protein from perfectly twinned crystals. *Acta Cryst D.* (2002) 58:992–1001. doi: 10.1107/S0907444902005693
49. Srinivasan N, White HE, Emsley J, Wood SP, Pepys MB, Blundell TL. Comparative analyses of pentraxins: Implications for protomer assembly and ligand binding. *Structure.* (1994) 2:1017–27. doi: 10.1016/S0969-2126(94)00105-7
50. Emsley J, White HE, O'Hara BP, Oliva G, Srinivasan N, Tickle IJ, et al. Structure of pentameric human serum P component. *Nature.* (1994) 367:338–45. doi: 10.1038/367338a0
51. Alnaas AA, Moon CL, Alton M, Reed SM, Knowles MK. Conformational changes in C-reactive protein affect binding to curved membranes in a lipid bilayer model of the apoptotic cell surface. *J Phys Chem B.* (2017) 121:2631–9. doi: 10.1021/acs.jpcc.6b11505
52. Wang M-Y, Ji S-R, Bai C-J, El Kebir D, Li H-Y, Shi J-M, et al. A redox switch in C-reactive protein modulates activation of endothelial cells. *FASEB J.* (2011) 25:3186–96. doi: 10.1096/fj.11-182741
53. Mollinedo F, Gajate C. Lipid rafts as major platforms for signaling regulation in cancer. *Adv Biol Regul.* (2015) 57:130–46. doi: 10.1016/j.jbior.2014.10.003
54. Eklund CM. Proinflammatory cytokines in CRP baseline regulation. *Adv Clin Chem.* (2009) 48:111–36. doi: 10.1016/S0065-2423(09)48005-3
55. Kang S, Tanaka T, Narazaki M, Kishimoto T. Targeting interleukin-6 signaling in clinic. *Immunity.* (2019) 50:1007–23. doi: 10.1016/j.immuni.2019.03.026
56. Kushner I, Broder ML, Karp D. Control of the acute phase response. Serum C-reactive protein kinetics after acute myocardial infarction. *J Clin Invest.* (1978) 61:235–42. doi: 10.1172/JCI108932
57. Colley CM, Fleck A, Goode AW, Muller BR, Myers MA. Early time course of the acute phase protein response in man. *J Clin Pathol.* (1983) 36:203–7. doi: 10.1136/jcp.36.2.203
58. Nordgreen J, Munsterhjelm C, Aae F, Popova A, Boysen P, Ranheim B, et al. The effect of lipopolysaccharide (LPS) on inflammatory markers in blood and brain and on behavior in individually housed pigs. *Physiol Behav.* (2018) 195:98–111. doi: 10.1016/j.physbeh.2018.07.013
59. Potempa LA, Maldonado BA, Laurent P, Zemel ES, Gewurz H. Antigenic, electrophoretic and binding alteration of human C-reactive protein modified selectively in the absence of calcium. *Mol Immunol.* (1983) 20:1165–75. doi: 10.1016/0161-5890(83)90140-2
60. Potempa LA, Siegel JN, Fiedel BA, Potempa RT, Gewurz H. Expression, detection and assay of a neoantigen (neo-CRP) associated with a free, human C-reactive protein subunit. *Mol Immunol.* (1987) 24:531–41. doi: 10.1016/0161-5890(87)90028-9
61. Ying SC, Gewurz H, Kinoshita CM, Potempa LA, Siegel JN. Identification and partial characterization of multiple native and neoantigenic epitopes of human C-reactive protein using monoclonal antibodies. *J Immunol.* (1989) 143:221–8.
62. Ying SC, Shephard E, deBeer FC, Siegel JN, Harris D, Gewurz BE, et al. Localization of sequence-determined neoepitopes and neutrophil digestion fragments of C-reactive protein utilizing monoclonal antibodies and synthetic peptides. *Mol Immunol.* (1992) 29:677–87. doi: 10.1016/0161-5890(92)90205-C
63. Braig D, Nero TL, Koch HG, Kaiser B, Wang X, Thiele JR, et al. Characterization of transitional changes in the CRP structure leading to the exposure of pro-inflammatory binding sites. *Nat Commun.* (2017) 8:14188. doi: 10.1038/ncomms14188
64. Burger VM, Nolasco DO, Stultz CM. Expanding the range of protein function at the far end of the order-structure continuum. *J Biol Chem.* (2016) 291:6706–13. doi: 10.1074/jbc.R115.692590
65. Lv JM, Chen JY, Liu ZP, Yao ZY, Wu YX, Tong CS, et al. Cellular folding determinants and conformational plasticity of native C-reactive protein. *Front Immunol.* (2020) 31:538. doi: 10.3389/fimmu.2020.00583
66. Schamel WW, Alarcon B, Minguet S. The TCR is an allosterically regulated macromolecular machinery changing its conformation while working. *Immunol Rev.* (2019) 291:8–25. doi: 10.1111/imr.12788
67. Petrovic D, Rizzo VA, Kamerlin SCL, Sanchez-Ruiz JM. Conformational dynamics and enzyme evolution. *J R Soc Interface.* (2018) 15:20180330. doi: 10.1098/rsif.2018.0330
68. Dellisanti CD, Hanson SM, Chen L, Czajkowski C. Packing of the extracellular domain hydrophobic core has evolved to facilitate pentameric ligand-gated ion channel function. *J Biol Chem.* (2011) 286:3658–70. doi: 10.1074/jbc.M110.156851
69. Potempa LA, Yao ZY, Ji SR, Filep JG, Wu Y. Solubilization and purification of recombinant modified C-reactive protein from inclusion bodies using reversible anhydride modification. *Biophys Rep.* (2015) 1:18–33. doi: 10.1007/s41048-015-0003-2
70. Kinoshita CM, Ying S-C, Hugli TE, Siegel JN, Potempa LA, Jiang H, et al. Elucidation of a protease-sensitive site involved in the binding of calcium to C-reactive protein. *Biochemistry.* (1989) 28:9840–8. doi: 10.1021/bi00451a044
71. Kushner I, Somerville JA. Estimation of the molecular size of C-reactive protein and Cx-reactive protein in serum. *Biochim Biophys Acta.* (1970) 207:105–14. doi: 10.1016/0005-2795(70)90140-6
72. Swanson SJ, Mullenix MC, Mortensen RF. Monoclonal antibodies to the calcium-binding region peptide of human C-reactive protein alter its conformation. *J Immunol.* (1991) 147:2248–52.
73. Ji SR, Bai L, Shi JM, Li HY, Potempa LA, Filep JG, et al. Monomeric C-reactive protein activates endothelial cells via interaction with lipid raft membrane microdomains. *FASEB J.* (2009) 23:1806–16. doi: 10.1096/fj.08-116962
74. Trial J, Potempa LA, Entman M. The role of C-reactive protein in innate and acquired inflammation: new perspectives. *Inflam Cell Signal.* (2016) 3:e1409. doi: 10.14800/ics.1409
75. Crawford JR, Trial J, Nambi V, Hoogveen RC, Taffet GE, Entman ML. Plasma levels of endothelial microparticles bearing monomeric C-reactive protein are increased in peripheral artery disease. *J Cardiovasc Transl Res.* (2016) 9:184–93. doi: 10.1007/s12265-016-9678-0
76. Radosevich JA, Haines GK, Motie M, Schaul KW, Mehta N, Kolb K, et al. Immunohistochemical detection of epitopes expressed on CRP and modified CRP (i.e. neo-CRP) in human normal and diseased tissues. *FASEB J.* (1996) 10:1466a.
77. Diehl EE, Haines GK, Radosevich JA, Potempa LA. Immunohistological localization of modified C-reactive protein antigen in normal human vascular tissue. *Amer J Med Sci.* (2000) 319:79–83. doi: 10.1097/00000441-200002000-00002
78. Rees RF, Gewurz H, Siegel JN, Coon J, Potempa LA. Expression of a C-reactive protein neo-antigen (Neo-CRP) in inflamed rabbit liver and muscle. *Clin Immunol Immunopathol.* (1988) 48:95–107. doi: 10.1016/0090-1229(88)90160-2
79. Shephard EG, Anderson R, Rosen O, Myer MS, Fridkin M, Strachan AF, et al. Peptides generated from C-reactive protein by a neutrophil membrane protease. *J Immunol.* (1990) 145:1469–76.
80. Shephard EG, Anderson R, Rosen O, Fridkin M. C-reactive protein (CRP) peptides inactivate enolase in human neutrophils leading to depletion of intracellular ATP and inhibition of superoxide generation. *Immunology.* (1992) 76:79–85.
81. Shephard EG, Kelly SL, Anderson R, Fridkin M. Characterization of neutrophil-mediated degradation of human C-reactive protein and identification of the protease. *Clin Exp Immunol.* (1992) 87:509–13. doi: 10.1111/j.1365-2249.1992.tb03028.x
82. El Kebir D, Zhang Y, Wang L, Potempa LA, Wu Y, Fournier A, et al. C-reactive protein-derived peptide 201-206 inhibits neutrophil adhesion to

- endothelial cells and platelets through CD32. *J Leukocyte Biol.* (2011) 90:1167–75. doi: 10.1189/jlb.0111032
83. Rajab IM, Majerczyk D, Olson ME, Addams JMB, Choe ML, Nelson MS, et al. C-reactive protein in gallbladder diseases – diagnostic and therapeutic insights. *Biophys Rep.* (2020) 6:49–67. doi: 10.1007/s41048-020-00108-9
 84. Habersberger J, Strang F, Scheichl A, Htun N, Bassler N, Merivirta RM, et al. Circulating microparticles generate and transport monomeric C-reactive protein in patients with myocardial infarction. *Cardiovasc Res.* (2012) 96:64–72. doi: 10.1093/cvr/cvs237
 85. Słomka A, Urban SK, Lukacs-Kornek V, Zekanowska E, Kornek M. Large extracellular vesicles: have we found the holy grail of inflammation? *Front Immunol.* (2018) 9:2723–47. doi: 10.3389/fimmu.2018.02723
 86. Pepys MB, Hirschfield GM, Tennent GA, Gallimore JR, Kahan MC, Bellotti V, et al. Targeting C-reactive protein for the treatment of cardiovascular disease. *Nature.* (2006) 440:1217–21. doi: 10.1038/nature04672
 87. Slevin M, Matou S, Zeinolabediny Y, Corpas R, Weston R, Di Napoli M, et al. Monomeric C-reactive protein—a key molecule driving development of Alzheimer's disease associated with brain ischemia? *Sci Rep.* (2015) 5:13281–302. doi: 10.1038/srep13281
 88. Khreiss T, József L, Hossain S, Chan JSD, Potempa LA, Filep JG. Loss of pentameric symmetry of C-reactive protein is associated with delayed apoptosis of human neutrophils. *J Biol Chem.* (2002) 277:40775–81. doi: 10.1074/jbc.M205378200
 89. Khreiss T, József L, Potempa LA, Filep JG. Conformational rearrangement in C-reactive protein is required for proinflammatory actions on human endothelial cells. *Circulation.* (2004) 109:2016–22. doi: 10.1161/01.CIR.0000125527.41598.68
 90. Khreiss T, József L, Potempa LA, Filep JG. Opposing effects of C-reactive protein isoforms on shear-induced neutrophil-platelet adhesion and neutrophil aggregation in whole blood. *Circulation.* (2004) 110:2713–20. doi: 10.1161/01.CIR.0000146846.00816.DD
 91. Khreiss T, József L, Potempa LA, Filep JG. Loss of pentameric symmetry in C-reactive protein induces Interleukin-8 secretion through peroxynitrite signaling in human neutrophils. *Circul Res.* (2005) 97:690–7. doi: 10.1161/01.RES.0000183881.11739.CB

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer KP declared a past co-authorship with one of the authors LP to the handling editor.

Copyright © 2020 Rajab, Hart and Potempa. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Treatment of Pneumococcal Infection by Using Engineered Human C-Reactive Protein in a Mouse Model

Donald N. Ngwa, Sanjay K. Singh, Toh B. Gang and Alok Agrawal*

Department of Biomedical Sciences, James H. Quillen College of Medicine, East Tennessee State University, Johnson City, TN, United States

OPEN ACCESS

Edited by:

Liwu Li,
Virginia Tech, United States

Reviewed by:

Karlheinz Peter,
Baker Heart and Diabetes Institute,
Australia
Nisha Jain Garg,
University of Texas Medical Branch
at Galveston, United States

*Correspondence:

Alok Agrawal
agrawal@etsu.edu

Specialty section:

This article was submitted to
Molecular Innate Immunity,
a section of the journal
Frontiers in Immunology

Received: 23 July 2020

Accepted: 11 September 2020

Published: 07 October 2020

Citation:

Ngwa DN, Singh SK, Gang TB
and Agrawal A (2020) Treatment
of Pneumococcal Infection by Using
Engineered Human C-Reactive
Protein in a Mouse Model.
Front. Immunol. 11:586669.
doi: 10.3389/fimmu.2020.586669

C-reactive protein (CRP) binds to several species of bacterial pathogens including *Streptococcus pneumoniae*. Experiments in mice have revealed that one of the functions of CRP is to protect against pneumococcal infection by binding to pneumococci and activating the complement system. For protection, however, CRP must be injected into mice within a few hours of administering pneumococci, that is, CRP is protective against early-stage infection but not against late-stage infection. It is assumed that CRP cannot protect if pneumococci got time to recruit complement inhibitor factor H on their surface to become complement attack-resistant. Since the conformation of CRP is altered under inflammatory conditions and altered CRP binds to immobilized factor H also, we hypothesized that in order to protect against late-stage infection, CRP needed to change its structure and that was not happening in mice. Accordingly, we engineered CRP molecules (E-CRP) which bind to factor H on pneumococci but do not bind to factor H on any host cell in the blood. We found that E-CRP, in cooperation with wild-type CRP, was protective regardless of the timing of administering E-CRP into mice. We conclude that CRP acts via two different conformations to execute its anti-pneumococcal function and a model for the mechanism of action of CRP is proposed. These results suggest that pre-modified CRP, such as E-CRP, is therapeutically beneficial to decrease bacteremia in pneumococcal infection. Our findings may also have implications for infections with antibiotic-resistant pneumococcal strains and for infections with other bacterial species that use host proteins to evade complement-mediated killing.

Keywords: C-reactive protein, complement, factor H, pneumococcal infection, *Streptococcus pneumoniae*

INTRODUCTION

C-reactive protein (CRP), whose blood level increases in inflammatory states in humans, binds to several species of bacterial pathogens, including *Streptococcus pneumoniae* (1–9). CRP is also a component of the acute phase response and a critical host defense molecule of the innate immune system against pneumococcal infection (10, 11). CRP binds to pneumococci by recognizing the phosphocholine (PCh) molecules present on the pneumococcal cell wall C-polysaccharide (PnC)

(12). CRP is made of five identical subunits arranged as a cyclic pentamer (13). Each subunit has a PCh-binding site consisting of Phe⁶⁶, Thr⁷⁶ and Glu⁸¹ (13–18). Through the PCh-binding site, CRP can also interact with phosphoethanolamine (PEt) (17, 18). The binding of CRP to PCh and PEt requires two Ca²⁺ ions (13). Once complexed with a PCh-bearing ligand, human CRP activates the complement system in both human and murine sera (3, 19–21). The amino acid residues Tyr⁴⁰ and Glu⁴² investigated in the current study are part of the intersubunit contact region of the CRP pentamer (13–15).

In murine models of pneumococcal infection, human CRP has been shown to be protective against lethality; however, the molecular mechanism of anti-pneumococcal action of CRP remains undefined (18, 21–31). Interestingly, CRP is protective against pneumococcal infection only when injected 6 h before to 2 h after administering pneumococci into mice, that is, CRP is protective against early-stage infection but not against late-stage infection (24). This 36-year old observation provided us with an experimental strategy to define the mechanism of anti-pneumococcal functions of CRP in mice. CRP is protective against early-stage infection because of the ability of CRP-pneumococci complexes to activate the complement system (20, 31). The reason why CRP is not protective against late-stage pneumococcal infection is not known. It is assumed that after a few hours of initial infection, pneumococci recruit the complement inhibitory protein factor H on their surface to become complement attack-resistant (32–36).

It has been shown that the pentameric conformation of CRP is altered in a variety of experimental conditions mimicking an inflammatory milieu and, in its alternate pentameric conformation, CRP binds to immobilized complement inhibitor factor H also (37–47). We hypothesize that in order to protect mice against late-stage infection, a structural change in CRP is needed, followed by the interaction between structurally altered CRP and recruited factor H on the pneumococcal surface, and that was not happening in mice. We further hypothesize that an exogenously constructed, irreversible and stable CRP mutant capable of binding to factor H should be able to protect mice against late-stage infection; such a CRP molecule when administered into mice would bind to factor H on pneumococci *in vivo* and mask the complement inhibitory activity of factor H (48). Wild-type (WT) CRP should then be able to protect mice against otherwise complement-resistant pneumococci by activating the complement system if a CRP molecule which can cover factor H on pneumococci is present along with WT CRP.

In this study, to test our hypotheses, we engineered CRP (E-CRP) by site-directed mutagenesis and produced two types of E-CRP: One, E42Q/F66A/T76Y/E81A, that binds to immobilized factor H but does not bind to PCh (E-CRP-1) and another, Y40F/E42Q, that binds to both immobilized factor H and to PCh (E-CRP-2). We found that both E-CRP-1 and E-CRP-2 were protective against both early-stage and late-stage

infection in a murine model of pneumococcal infection. These findings indicate that CRP functions in two different structural conformations to fully protect against pneumococcal infection.

MATERIALS AND METHODS

Construction of Mutant CRP cDNAs

The template for construction of the CRP quadruple mutant E42Q/F66A/T76Y/E81A (E-CRP-1) was a cDNA for the CRP triple mutant F66A/T76Y/E81A cDNA (substitution of Phe⁶⁶ with Ala, Thr⁷⁶ with Tyr, and Glu⁸¹ with Ala). Mutagenic oligonucleotides, 5'-C CAC TTC TAC ACG **CAA** CTG TCC TCG ACC-3' and 5'-GGT CGA GGA CAG **TTG** CGT GTA GAA GTG G-3', to substitute Glu⁴² with Gln (codons shown in bold and italicized letters), were designed according to the sequence of the template cDNA and obtained from Integrated DNA Technologies. Mutagenesis was conducted using the QuickChange site-directed mutagenesis kit (Stratagene). Mutations were verified by nucleotide sequencing, utilizing the services of the Molecular Biology Core Facility of the university (**Supplementary Figure 1**). The construction of cDNAs for CRP mutants E42Q, F66A/T76Y/E81A and Y40F/E42Q (E-CRP-2) has been reported earlier (15–18).

Expression and Purification of CRP

CRP mutants were expressed in CHO cells using the ExpiCHO Expression System (Thermo Fisher Scientific) as described previously (31). Purification of E-CRP-1 from culture supernatants involved Ca²⁺-dependent affinity chromatography on a PEt-conjugated Sepharose column, followed by ion-exchange chromatography on a MonoQ column, and gel filtration on a Superose12 column, as reported previously for the CRP triple mutant F66A/T76Y/E81A (18). PEt-conjugated Sepharose was prepared as described previously (18). Briefly, CHO cell culture media was diluted (1:1) in 0.1 M borate buffer saline, pH 8.3, containing 3 mM CaCl₂, and passed through the PEt-sepharose column. After collecting the flow-through and washing the column with the same buffer, bound E-CRP-1 was eluted with 0.1 M borate buffer saline, pH 8.3, containing 5 mM EDTA. E-CRP-1 was then subjected to ion-exchange chromatography and bound E-CRP-1 was eluted with an NaCl gradient. E-CRP-1 containing fractions were pooled, concentrated, and further purified by gel filtration. The gel filtration column was equilibrated and eluted with TBS (10 mM Tris-HCl, 150 mM NaCl, pH 7.2) containing 5 mM EDTA. Eluted E-CRP-1 was immediately dialyzed against TBS containing 2 mM CaCl₂, stored at 4°C, and was used within a week. WT CRP and all other CRP mutants including E-CRP-2 were purified as described previously (49). The purity of CRP preparations was confirmed by denaturing 4–20% SDS-PAGE under reducing conditions.

For *in vivo* experiments, purified CRP was treated with Detoxi-Gel Endotoxin Removing Gel (Thermo Fisher Scientific) according to manufacturer's instructions. The concentration of endotoxin in CRP preparations was determined by using the Limulus Amebocyte Lysate kit QCL-1000 (Lonza).

Abbreviations: CRP, C-reactive protein; E-CRP-1, CRP mutant E42Q/F66A/T76Y/E81A; E-CRP-2, CRP mutant Y40F/E42Q; PCh, phosphocholine; PEt, phosphoethanolamine; PnC, pneumococcal C-polysaccharide; Pn-broth, pneumococci cultured in broth; Pn-mice, pneumococci isolated from infected mice; WT, wild-type.

Determination of Pentameric Structure of CRP

The pentameric structure of E-CRP was confirmed by employing gel filtration and denaturing SDS-PAGE. The gel filtration column was equilibrated with TBS containing 5 mM EDTA. E-CRP was injected into the column and eluted with TBS containing 5 mM EDTA at a flow rate of 0.3 ml/min. Fractions (60 fractions, 250 μ l each) were collected and absorbance at 280 nm measured to locate the elution volume of E-CRP. Gel filtration of WT CRP was carried out on the same column to determine the elution volume of pentameric CRP.

Pneumococci (Pn-Broth)

Streptococcus pneumoniae type 3, strain WU2, were made virulent by sequential i.v. passages in mice, and were stored in 1 ml aliquots at -80°C in Todd-Hewitt broth containing 0.5% yeast extract and 10% glycerol, as described previously (30). For each experiment, a separate 1 ml aliquot of pneumococci was thawed. Pneumococci were then grown in 50 ml Todd-Hewitt broth containing 0.5% yeast extract and incubated at 37°C with shaking at 125 rpm for 3 h (mid-log phase culture). The culture was centrifuged at 7,500 rpm for 15 min. The bacterial pellet was washed and resuspended in 10 ml normal saline and adjusted the volume until A_{600} was 0.29 to give a concentration of 3.5×10^8 cfu/ml ($A_{600} = 1.00 = 1.2 \times 10^9$ cfu/ml). This preparation of pneumococci cultured in broth was called as Pn-broth. The concentration, purity, and viability of pneumococci were confirmed by plating on sheep blood agar plates.

PCh-Binding, PEt-Binding, and Pneumococcus-Binding Assays

Binding activity of CRP for PCh was evaluated by using pneumococcal C-polysaccharide (PnC, from Statens Serum Institut) as the ligand, as described previously (29). Briefly, microtiter wells were coated with PnC in 100 μ l TBS, overnight at 4°C . The unreacted sites were blocked with TBS containing 0.5% gelatin for 1 h at room temperature. CRP, diluted in TBS containing 2 mM CaCl_2 , 0.1% gelatin and 0.02% Tween 20 (TBS-Ca), was then added in duplicate wells and incubated for 2 h at 37°C . After washing the wells with TBS-Ca, bound CRP was detected by using anti-CRP monoclonal antibody HD2.4 diluted in TBS-Ca. HRP-conjugated goat anti-mouse IgG diluted in TBS-Ca was used as the secondary antibody. Color was developed using ABTS substrate and the OD was read at 405 nm in a plate reader. Binding activity of CRP for PEt was evaluated by using biotinylated-PEt as the ligand, exactly as described previously (18).

Binding activity of CRP for whole pneumococci (broth-grown or isolated from infected mice) was evaluated as described previously (18). Briefly, microtiter wells were coated with 10^7 cfu of pneumococci overnight at 4°C . The unreacted sites in the wells were blocked with TBS containing 0.5% gelatin. CRP, diluted in TBS-Ca, was then added to the wells for 2 h at 37°C . After washing the wells with TBS-Ca, bound CRP was detected by using rabbit polyclonal anti-human CRP antibody. HRP-conjugated donkey anti-rabbit IgG was used as the secondary antibody. Color

was developed using ABTS substrate and the OD was read at 405 nm in a plate reader. Binding activity of CRP for whole pneumococci isolated from infected mice was evaluated both in the presence and absence of Ca^{2+} .

Isolation of Pneumococci (Pn-Mice) From Infected Mice

Mice were injected i.v. with 3.5×10^7 cfu of Pn-broth. After 40 h, blood was collected by cardiac puncture, in tubes containing 10% EDTA (1% v/v of blood). Blood was diluted with an equal volume of normal saline and centrifuged at 2,200 rpm for 2 min. The supernatant was recovered. The bacterial pellet was washed four times with normal saline, centrifuged at 2,200 rpm for 2 min after each wash, and continued to recover the supernatant. All recovered supernatants were then pooled and centrifuged at 11,000 rpm for 5 min. This time the supernatant was discarded, and the pellet was resuspended in normal saline for immediate use or resuspended in Todd-Hewitt broth containing 0.5% yeast extract and 10% glycerol for storage at -80°C . This preparation of pneumococci isolated from infected mice was called as Pn-mice. The concentration, purity, and viability of pneumococci were confirmed by plating on sheep blood agar plates.

Detection of Factor H on the Surface of Pn-Mice

Microtiter wells were coated with Pn-mice in TBS (10^7 cfu) overnight at 4°C . The unreacted sites in the wells were blocked with TBS containing 0.5% gelatin for 45 min at room temperature. Murine factor H present on the surface of Pn-mice was detected by using sheep polyclonal anti-mouse factor H antibody (R&D, AF4999) diluted in TBS-Ca. HRP-conjugated rabbit anti-sheep IgG (Thermo Fisher Scientific), in TBS-Ca, was used as the secondary antibody. Color was developed and the OD₄₀₅ read in a microtiter plate reader.

Factor H-Binding Assay

The binding activity of CRP for factor H was evaluated by using both human factor H (Complement Technology) and murine factor H (R&D), as described previously (46). Briefly, microtiter wells were coated with 2 μ g/ml of factor H in TBS, overnight at 4°C . The unreacted sites in the wells were blocked with TBS containing 0.5% gelatin for 45 min at room temperature. CRP diluted in TBS-Ca (TBS containing 2 mM CaCl_2 , 0.1% gelatin and 0.02% Tween 20) was added in duplicate wells. After incubating the plates for 2 h at 37°C , the wells were washed with TBS-Ca. Polyclonal rabbit anti-human CRP antibody (1 μ g/ml) (EMD Millipore Corp, 235752), diluted in TBS-Ca, was used to detect bound CRP. HRP-conjugated donkey anti-rabbit IgG (GE Healthcare), diluted in TBS-Ca, was used as the secondary antibody. Color was developed and the OD₄₀₅ read in a microtiter plate reader.

Clearance of E-CRP From Mouse Circulation

The clearance rate of E-CRP from the mouse blood was determined as described previously (18). Briefly, mice were

injected i.v. with 100 μ g of CRP in 100 μ l TBS containing 2 mM CaCl_2 through the tail vein. Four to five mice were used for each CRP species. After 8 h, blood was collected from the tip of the tail vein at four different time points up to 24 h. The concentration of CRP in the serum was measured by ELISA. The concentration of CRP in the serum at the first bleed was plotted as the 100% value.

Repurification of E-CRP From E-CRP-Spiked Mouse Serum

Purified E-CRP (400 μ g) was added to 2 ml C57BL/6 mouse serum (Innovative Research) and the final volume was made to 10 ml by adding 0.1 M borate buffered saline, pH 8.3, containing 3 mM CaCl_2 . The mixture was incubated for 30 min at 37°C. E-CRP was repurified by Ca^{2+} -dependent affinity chromatography on PET-Sepharose beads whose capacity to bind E-CRP was >400 μ g. After collecting the flow-through and washing the column with the same buffer, bound E-CRP was eluted with 0.1 M borate buffered saline, pH 8.3, containing 5 mM EDTA. To control the experiment, mouse serum alone (2 ml), without spiking with E-CRP, was used. The EDTA eluates were subjected to SDS-PAGE. The concentration of CRP in the EDTA eluates was measured by ELISA to calculate percent recovery.

Mice

Male C57BL/6J mice (Jackson Laboratories) were brought up and maintained according to protocols approved by the University Committee on Animal Care. Mice were 8–10 weeks old when used in experiments.

Mouse Protection Experiments

Separate mouse protection experiments were performed using two different preparations of purified WT CRP, E-CRP-1 and E-CRP-2. The endotoxin content in 25 μ g all CRP preparations was <1.5 endotoxin units. Mice were first injected i.v. with 3.5×10^7 cfu (based on A_{600}) of pneumococci in 100 μ l normal saline. The actual number of pneumococci injected, based on the plating results obtained on the next day, was $3.53 \pm 0.21 \times 10^7$ cfu. In the first set of experiments, mice were injected i.v. with either WT CRP, E-CRP-1 or E-CRP-2, 30 min after the administration of pneumococci. In the second set of experiments, mice were injected i.v. with either WT CRP, E-CRP-1, combination of WT CRP and E-CRP-1 (WT CRP first and, an hour later, E-CRP-1) or E-CRP-2, 12 h after the administration of pneumococci. In the third set of experiments, mice were injected i.v. with either WT CRP or E-CRP-1, four times (6, 12, 24 and 48 h) after the administration of pneumococci. CRP (25 μ g) was injected in 100 μ l TBS containing 2 mM CaCl_2 . The dose of 25 μ g of CRP with 3.5×10^7 cfu bacteria was chosen because, under these conditions, the protection of mice with WT CRP injected 30 min apart from the administration of pneumococci was same as reported previously (30). Survival of mice was recorded three times per day for 7 days. To determine bacteremia (cfu/ml) in the surviving mice, blood was collected daily for 5 days from the tip of the tail vein, diluted in normal saline, and plated on sheep blood agar for colony counting. The bacteremia value for dead mice was

recorded as 10^9 cfu/ml because mice died when the bacteremia exceeded 10^8 cfu/ml.

Statistical Analysis

All experiments were performed three times unless otherwise mentioned and comparable results were obtained each time. Results of a representative experiment are shown in the figures where the raw data (A_{280} or OD_{405}) were used to plot the curves. Survival curves were generated using the GraphPad Prism 4 software. To determine *p*-values for the differences in the survival curves among various groups, the survival curves were compared using the software's Logrank (Mantel-Cox) test. The scatter plots of the bacteremia data and the median bacteremia value for each group were generated using the GraphPad Prism 4 software. Bacteremia values of 0–100 were plotted as 100 and bacteremia values of $>10^8$ were plotted as 10^9 . To determine *p*-values for the differences in bacteremia among various groups at each time point, scatter plots were compared using the software's Mann-Whitney test. The software's Mann-Whitney test included all the dots in the scatter plots and not the median values for each time point.

RESULTS

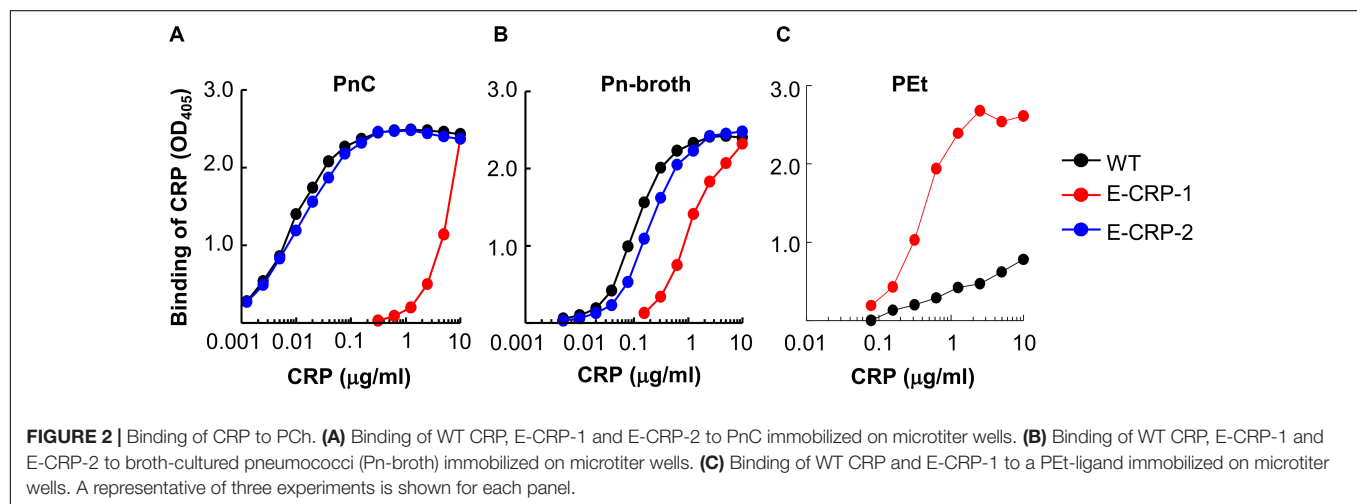
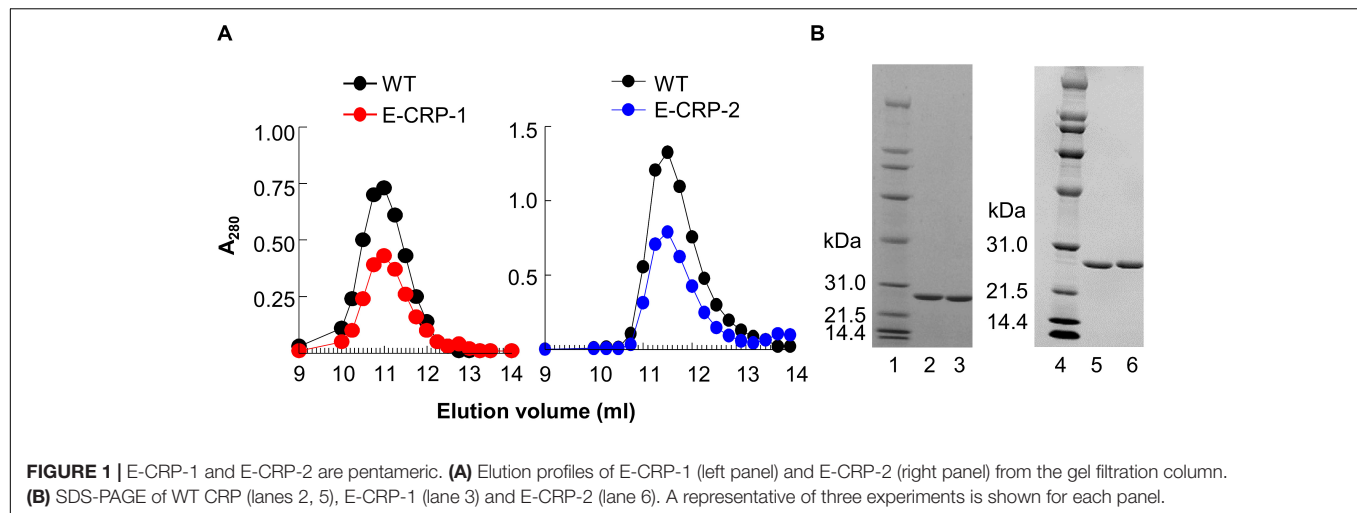
E-CRP-1 and E-CRP-2 Have the Desired Ligand-Binding Properties

The elution profiles of WT CRP, E-CRP-1 and E-CRP-2 from the gel filtration column were almost overlapping. Like WT CRP, both E-CRP-1 and E-CRP-2 eluted at 11 ml (Figure 1A). SDS-PAGE of purified proteins showed a single band and the molecular weight of the subunits of both E-CRP-1 and E-CRP-2 was same as WT CRP (Figure 1B). Thus, E-CRP-1 and E-CRP-2 were pentameric.

The PCh-binding ability of E-CRP-1 and E-CRP-2 was assessed by using two different PCh-containing ligands: PnC (Figure 2A) and Pn-broth (Figure 2B). Both, WT CRP and E-CRP-2 bound to both PCh-ligands in a CRP concentration-dependent manner. The binding of E-CRP-2 to PCh-ligands was comparable to that of WT CRP. However, as shown, for equivalent binding (OD_{405}) of WT CRP and E-CRP-1 to either PnC or Pn-broth, ~ 100 -times more of E-CRP-1 was required compared to WT CRP, indicating that the PCh-binding ability of E-CRP-1 was $\sim 99\%$ less than that of WT CRP. Thus, E-CRP-1 and E-CRP-2 had the desired PCh-binding activity.

Since E-CRP-1 lost its PCh-binding property, the PCh-affinity chromatography method could not be used to purify E-CRP-1. Therefore, the PET-binding activity of E-CRP-1 was tested (Figure 2C). E-CRP-1 bound to PET more efficiently than WT CRP. The avid binding of E-CRP-1 to PET facilitated the purification of E-CRP-1 by affinity chromatography using a PET-Sepharose column.

In factor H-binding assays, unlike WT CRP, both E-CRP-1 and E-CRP-2 bound readily to purified human and murine factor H immobilized on microtiter wells (Figure 3A). Thus, both E-CRP-1 and E-CRP-2 had the desired factor H-binding activity.



Surprisingly, triple mutant CRP, which was not investigated before for factor H binding (18), also bound to factor H (50). If it was known earlier that triple mutant CRP, without the addition of E42Q mutation, would bind to factor H, there would have been no need to generate quadruple mutant CRP. However, since the expression of quadruple mutant CRP was better than triple mutant CRP, we proceeded with quadruple mutant CRP (E-CRP-1) to test the hypothesis.

Next, we tested whether E-CRP-1 and E-CRP-2 bind to factor H on pneumococci which have recruited factor H on their surface. For this purpose, Pn-mice were isolated from infected mice. We first tested the presence of murine factor H on pneumococci. As shown (Figure 3B), factor H was present on Pn-mice but not on Pn-broth. Next, we determined binding of E-CRP-1 and E-CRP-2 to factor H-coated Pn-mice. As shown (Figure 3C), WT CRP bound to both Pn-broth and Pn-mice but only in the presence of Ca^{2+} , suggesting that the binding of WT CRP to Pn-mice was through PCh. In contrast, E-CRP-1 and E-CRP-2 bound to Pn-mice in the absence of Ca^{2+} also, suggesting that E-CRP-1 and E-CRP-2 bound to a molecule other than PCh, and that molecule could be factor H recruited

by pneumococci *in vivo*. However, interestingly, both E-CRP-1 and E-CRP-2 also bound to Pn-broth in EDTA, suggesting that E-CRP-1 and E-CRP-2 were capable of recognizing and binding to a pneumococcus' own surface protein. Thus, E-CRP-1 and E-CRP-2, but not WT CRP, interacted with pneumococci in a Ca^{2+} -independent and therefore PCh-independent manner.

Combined data indicated that both E-CRP-1 and E-CRP-2 were pentameric, their overall structure was similar to WT CRP, and both E-CRP-1 and E-CRP-2 had the desired ligand-binding properties to test our hypothesis.

Both E-CRP-1 and E-CRP-2 Are Suitable for *in vivo* Use

We determined the $T_{1/2}$ of CRP from mouse circulation. Based on the data obtained from four to five mice (Figure 4A), the average $T_{1/2}$ of WT CRP, E-CRP-1 and E-CRP-2 were 4.9, 8.0 and 7.5 h, respectively. Thus, the clearance of E-CRP-1 and E-CRP-2 was not markedly faster than that of WT CRP. In another approach to confirm that E-CRP-1 and E-CRP-2 were free in the mouse serum, we performed an experiment where E-CRP could

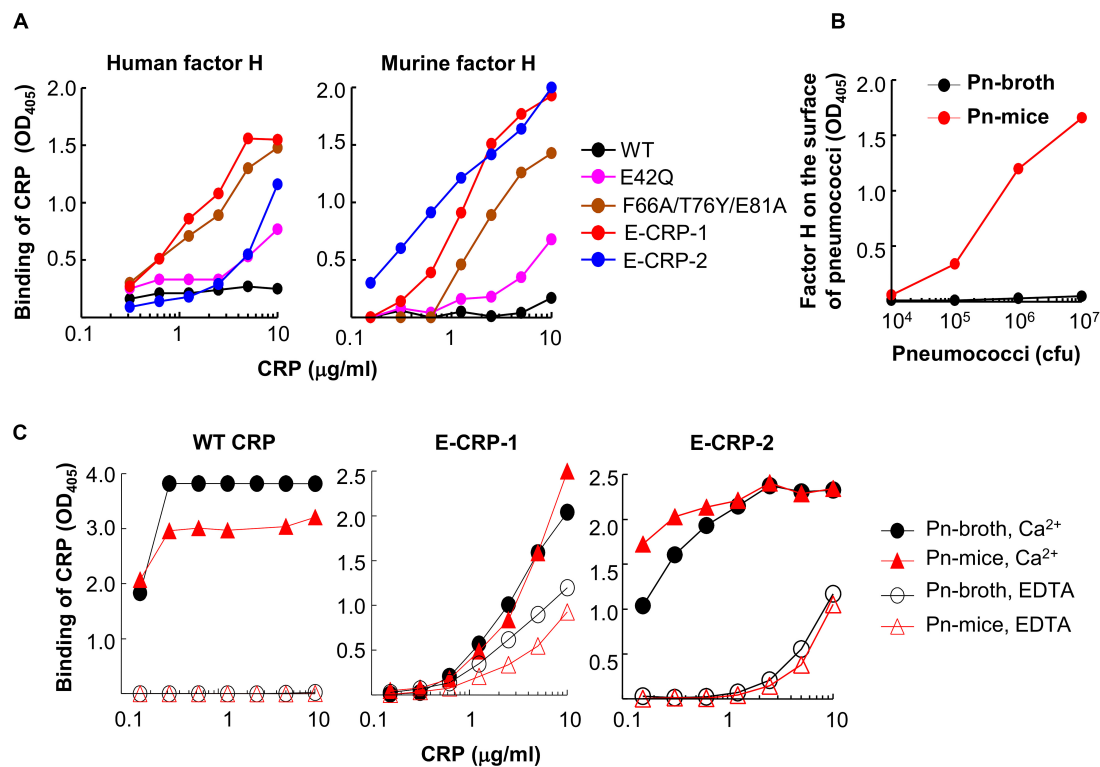


FIGURE 3 | Binding of CRP to factor H. **(A)** Binding of WT CRP, E-CRP-1, E-CRP-2 and other CRP mutants to purified human factor H (left panel) and murine factor H (right panel) immobilized on microtiter wells. **(B)** Presence of murine factor H on pneumococci isolated from the blood of infected mice (Pn-mice). **(C)** Binding of WT CRP, E-CRP-1 and E-CRP-2 to Pn-broth and Pn-mice in the presence and absence of Ca²⁺. A representative of three experiments is shown for each panel.

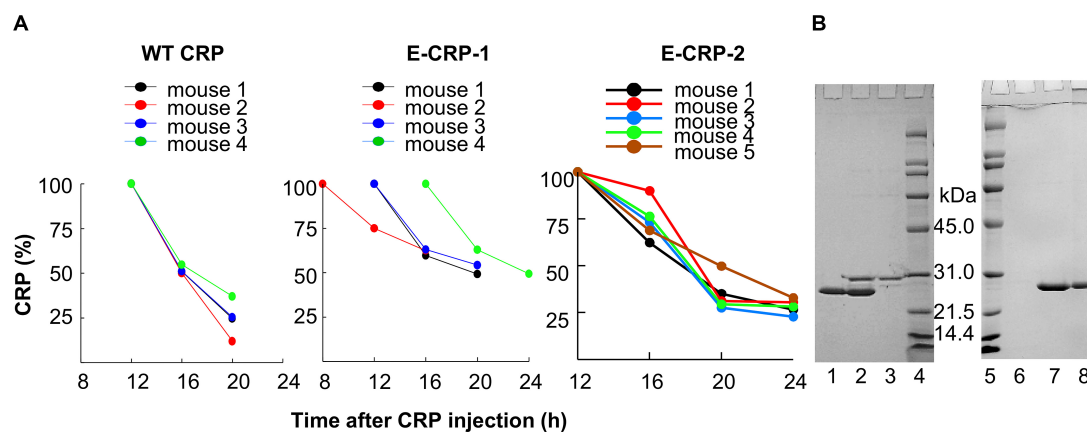


FIGURE 4 | E-CRP-1 and E-CRP-2 are suitable for *in vivo* use. **(A)** Clearance of WT CRP (left panel), E-CRP-1 (middle panel) and E-CRP-2 (right panel) from mouse circulation. **(B)** Repurification of E-CRP-1 and E-CRP-2 from purified E-CRP-1-spiked and E-CRP-2-spiked mouse sera, respectively. SDS-PAGE of repurified E-CRP-1 and E-CRP-2 is shown. Lane 1, purified E-CRP-1; Lane 2, EDTA eluate from the PET-affinity chromatography column through which mouse serum containing E-CRP-1 was passed in the presence of Ca²⁺; Lane 3, EDTA eluate from the PET-column through which mouse serum alone was passed. Lane 6, EDTA eluate from the PCh-affinity chromatography through which mouse serum alone was passed; Lane 7, EDTA eluate from the PCh-column through which mouse serum containing E-CRP-2 was passed in the presence of Ca²⁺; Lane 8, purified E-CRP-2.

be repurified from E-CRP-spiked mouse serum (**Figure 4B**). As shown (left panel), E-CRP-1 present in the mouse serum bound to PET in a Ca²⁺-dependent manner and could be eluted with EDTA (lane 2). The recovery of E-CRP-1 was 96%. Besides CRP,

no additional protein bands were found when compared with the non-specific bands seen with the serum alone control (compare lanes 2 and 3). Similar results were seen with E-CRP-2 (right panel). Thus, both E-CRP-1 and E-CRP-2 stayed free in the

mouse serum, were not sequestered by any other serum protein, and the mutations did not confer instability to E-CRP-1 and E-CRP-2 *in vivo*.

To test the possibility that E-CRP-1 and E-CRP-2 may be sequestered by cells in the mouse blood, E-CRP-spiked mouse blood was centrifuged at 8000 rpm for 5 min and the serum recovered. The amount of E-CRP in the recovered serum, as determined by ELISA, was the same as the amount of E-CRP mixed with the blood. These data showed that both E-CRP-1 and E-CRP-2 were suitable for use in mouse models of infection to test the hypothesis.

Role of Endogenous Murine CRP in the Animal Model

As shown in **Figure 5**, mice were protected, without administering human CRP, when up to 10^7 cfu of pneumococci were injected into mice, suggesting that endogenous murine WT CRP was sufficient to protect mice from lethality when bacteremia was relatively lower. Endogenous murine CRP was not enough to protect mice from lethality when $>10^7$ cfu of pneumococci were injected into mice. These data also suggest that if mice are administered with, for example, 3.5×10^7 cfu bacteria in the protection experiments, endogenous murine CRP can participate in protecting mice from lethality once bacteremia is lowered to $<10^7$ cfu of pneumococci in the animal model employed in this study.

E-CRP-1 Protects Mice Against Late-Stage Infection

All the data presented in this section show the combined results of two separate protection experiments using six to eight mice in each group in each experiment. Some protection experiments shown in **Figures 6–8** were performed together.

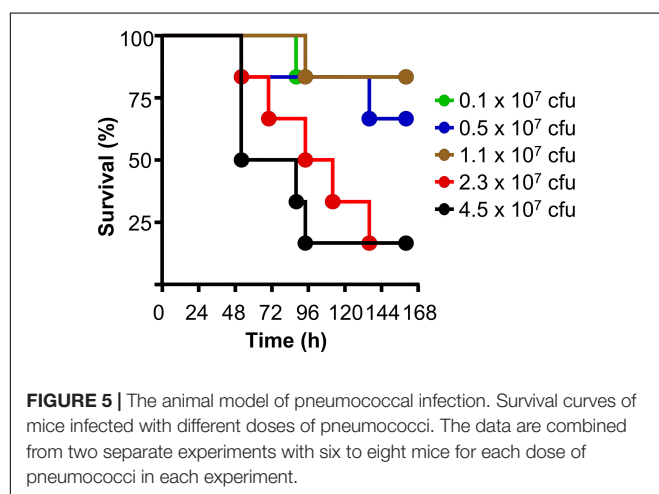
Figure 6A shows the results of experiments in which WT CRP and E-CRP-1 were injected into mice within 30 min of administering pneumococci. The median survival time (MST, the time taken for the death of 50% of mice) for mice injected with bacteria alone (group A) was 60 h. The MST for mice

injected with bacteria and either WT CRP (group B) or E-CRP-1 (group C) could not be calculated because $>50\%$ of mice survived. WT CRP and E-CRP-1 were not significantly different in protecting mice from lethality. Increase in survival was due to decrease in bacteremia (**Figures 6B,C**). By 44 h, in group A, median bacteremia increased dramatically, and mice died once bacteremia reached 10^9 cfu/ml; however, in groups B and C, median bacteremia reached only $\sim 10^5$ cfu/ml and then decreased dramatically afterward. There was $>99\%$ reduction in bacteremia in both WT CRP-treated and E-CRP-1-treated mice. Since E-CRP-1 does not bind to PCh, and hence cannot activate the complement system, these results indicated that the increased resistance to infection in E-CRP-1-treated mice was due to combined actions of E-CRP-1 and endogenous mouse WT CRP. Most likely, E-CRP-1 bound to a protein ligand present on the pneumococcal surface and, once bacteremia was already lower, endogenous mouse WT CRP bound to PCh to activate the complement system to reduce bacteremia further. Since the dose of injected E-CRP-1 was same as that of WT CRP, it is unlikely that the protection depended upon the residual PCh-binding activity of E-CRP-1.

Next, we injected E-CRP-1 into mice 12 h after administering pneumococci, a time point for CRP injection when WT CRP does not confer protection (**Figure 7A**). A gap of 12 h is clinically significant because all strategies for a sepsis drug have so far failed in human clinical trials (51, 52). We included WT CRP, 30 min regimen, in all experiments to ensure that the animal model was comparable from experiment to experiment. The MST for mice injected with either bacteria alone (group A) or with bacteria and WT CRP (group B) was 60 h. In contrast, the MST for mice injected with bacteria and E-CRP-1 (group C) was 90 h and the MST for mice injected with bacteria and both WT CRP and E-CRP-1 (group D) was 108 h. In the WT CRP-treated group, all mice died by 66 h. However, in the E-CRP-1-treated groups, it took 4 days until 60–70% mice died, and 30–40% mice survived up to 7 days. As reported previously (24, 29), WT CRP was not protective. These data again suggested that endogenous mouse WT CRP participated and that is why E-CRP-1 alone was not different from the combination of E-CRP-1 and WT CRP in protecting mice from lethality. In mice receiving E-CRP-1 (groups C and D), median bacteremia was reduced by $\sim 99\%$ as early as 44 h and lower bacteremia was maintained for up to 92 h (**Figures 7B,C**).

Next, we injected CRP into mice four times, at 6, 12, 24 and 48 h, after administering pneumococci, to determine whether multiple injections of E-CRP-1 were better than a single injection at 12 h (**Figure 8A**). The MST for mice injected with either bacteria alone (group A) or with bacteria and four doses of WT CRP (group C) was 60 h. Like a single dose of WT CRP at 12 h, multiple doses of WT CRP were also not protective. In contrast, the MST for mice injected with bacteria and multiple doses of E-CRP-1 (group D) was 108 h. In E-CRP-1-treated mice, median bacteremia was reduced by $\sim 99\%$ as early as 36 h and the reduction lasted for up to 72 h. There was ~ 48 h gain over WT CRP for bacteremia to reach the deadly levels (**Figures 8B,C**).

The protective ability of E-CRP-1 when injected at 30 min, 12 h or at multiple time points were compared



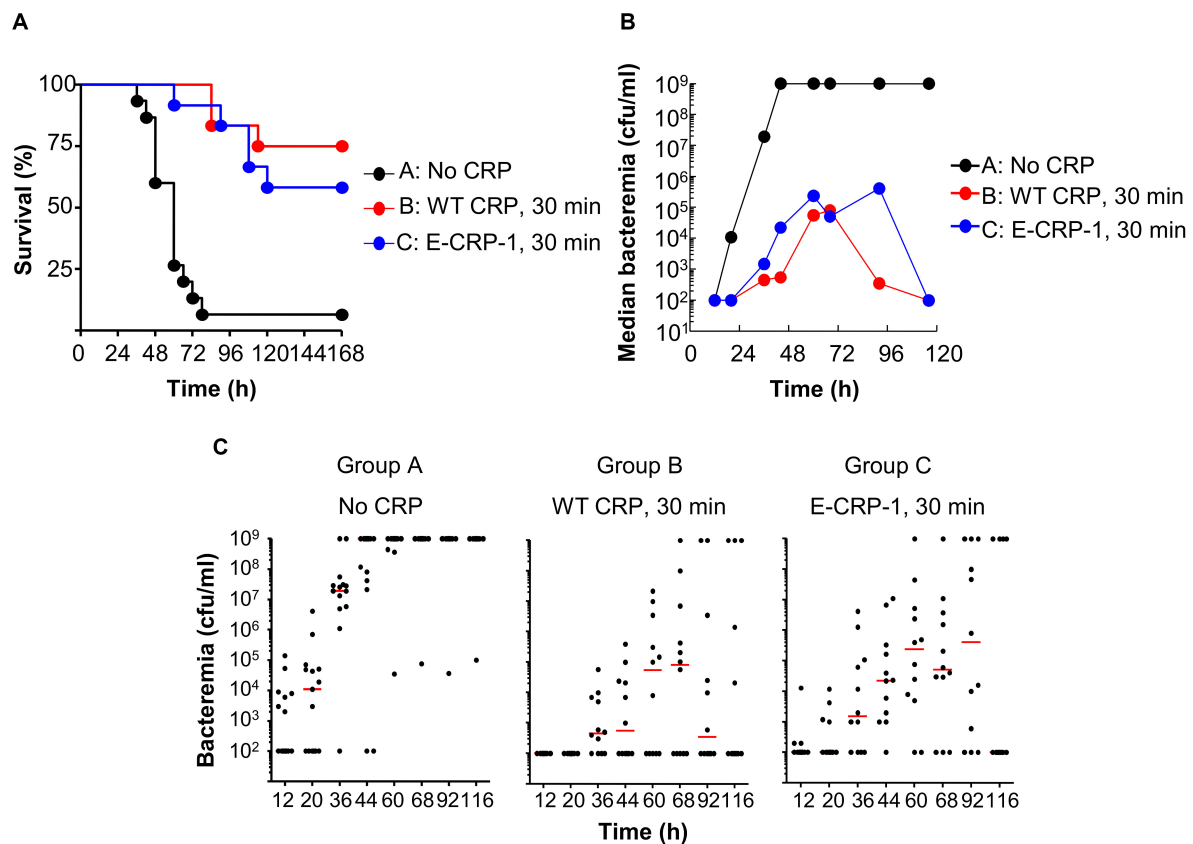


FIGURE 6 | Like WT CRP, E-CRP-1 is also protective against early-stage infection. The data are combined from two separate experiments with six to eight mice in each group in each experiment. **(A)** Survival curves. Pneumococci and CRP were injected 30 min apart. The *p*-values for the differences in the survival curves between groups A B, A C, and B C were <0.001, <0.001 and 0.43, respectively. **(B)** Bacteremia. Blood was collected from each surviving mouse shown in **A**. The median bacteremia values are plotted. For 36–116 h, the *p*-values for the differences between groups A B and A C were <0.001. The *p*-value for the difference between groups B C was >0.05 at all time points. **(C)** Scatter plots of the bacteremia data shown in **B**. The horizontal line in each group of mice represents median bacteremia.

(Supplementary Figure 2). The injection of E-CRP-1 at 12 h was found to be as effective as it was when administered within 30 min. Based on the statistical analyses of the survival curves and of the scatter plots for bacteremia (Figures 6–8), no significant difference was found between 30 min and 12 h regimens in either the survival of or bacteremia in both groups of mice (Supplementary Figures 2A,B). Likewise, four injections of E-CRP-1 and one injection of E-CRP-1 were equally effective in reducing bacteremia; there was no significant difference in either the survival of or bacteremia in these two groups of mice (Supplementary Figures 2C,D). In this study, mice were injected with E-CRP-1 four times within 48 h of administering pneumococci. It is possible that a different regimen for four injections of E-CRP-1 that improves its availability over the course of the infection, such as four injections spread over 4 days, would have shown results different from that used in this study and, therefore, be more protective than a single injection of E-CRP-1. Overall, the data indicate that E-CRP-1, unlike WT CRP, is protective against infection regardless of the time point of injecting E-CRP-1.

E-CRP-2 Also Protects Mice Against Late-Stage Infection

The results of protection experiments with E-CRP-2 are shown in Figure 9. The MST for mice injected with bacteria alone (group A) was 54 h. The MST for mice injected with E-CRP-2, 12 h after administering pneumococci (group C), was extended to 132 h. The MST for mice injected with E-CRP-2, 30 min after administering pneumococci (group B), could not be calculated because >50% of mice survived, as expected (Figure 9A). There was >99% reduction in bacteremia even when E-CRP-2 was given to mice 12 h after administering pneumococci and the lower bacteremia stayed as such for >96 h (Figures 9B,C). Since E-CRP-2 binds to PCh, like WT CRP does, we do not know the involvement of mouse endogenous WT CRP in this case.

DISCUSSION

In this study, two CRP mutants, E-CRP-1 and E-CRP-2, were employed to investigate the mechanisms of anti-pneumococcal function of CRP in a mouse model of pneumococcal infection.

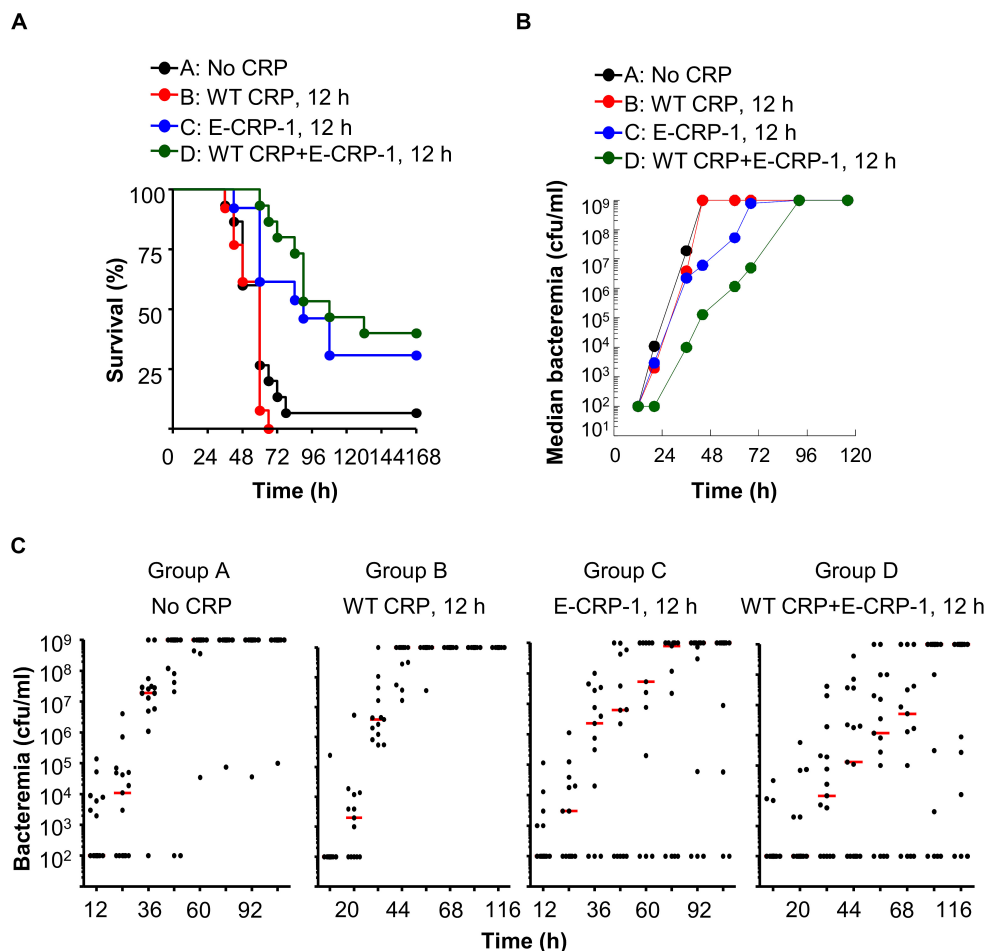


FIGURE 7 | Unlike WT CRP, E-CRP-1 is protective against late-stage infection. The data are combined from two separate experiments with six to eight mice in each group in each experiment. **(A)** Survival curves. Pneumococci were injected first; CRP was injected 12 h later. The *p*-values for the differences in the survival curves between groups A B, A C, A D, B C, B D and C D were 0.28, <0.01, <0.001, <0.001, <0.001 and 0.31, respectively. **(B)** Bacteremia. Blood was collected from each surviving mouse shown in **A**. The median bacteremia values are plotted. The *p*-values for the differences between groups A B and C D were >0.05 at all time points. For 44–92 h, the *p*-values for the differences between groups B C and B D were <0.01. **(C)** Scatter plots of the bacteremia data shown in **B**. The horizontal line in each group of mice represents median bacteremia.

E-CRP-1 binds to factor H but does not bind to PCh and E-CRP-2 binds to both factor H and PCh. Our major findings were: 1. CRP with mutations in either the intersubunit contact region or in the overlapping PCh-binding and Ca²⁺-binding sites binds to immobilized factor H. 2. Unlike WT CRP, both E-CRP-1 and E-CRP-2 were protective against late-stage infection. 3. Injecting WT CRP along with E-CRP-1 into mice did not enhance the protective ability of E-CRP-1. 4. Multiple injections of E-CRP-1 were not different from a single injection in protecting mice against infection. 5. Unlike WT CRP, both E-CRP-1 and E-CRP-2 bound to broth-grown pneumococci in the absence of Ca²⁺. Overall, these findings indicate that a conformationally altered form of CRP capable of binding to factor H is necessary for protection against late-stage pneumococcal infection.

We previously reported a triple mutant of CRP, F66A/T76Y/E81A, which does not bind to PCh and

a single mutant of CRP, E42Q, which binds to factor H (18, 46). A quadruple mutant of CRP (E-CRP-1), E42Q/F66A/T76Y/E81A, was constructed in which the F66A/T76Y/E81A mutations were introduced to abolish the PCh-binding and the E42Q mutation was added to confer the factor H-binding ability to mutated CRP. The ability to bind to PCh was abolished so that the observed effects could be attributed solely to the factor H-binding ability of E-CRP-1. While screening a library of CRP mutants for binding to factor H, we found that Y40F/E42Q CRP (E-CRP-2) bound to factor H more avidly than E42Q CRP did. That is why, E-CRP-2 was employed in this study, instead of E42Q CRP, as the molecule which binds to both PCh and factor H. The mechanism of interaction between various CRP mutants and immobilized factor H is currently being investigated in a separate project.

The data indicate that endogenous murine WT CRP also participated, along with E-CRP-1, in protecting mice against

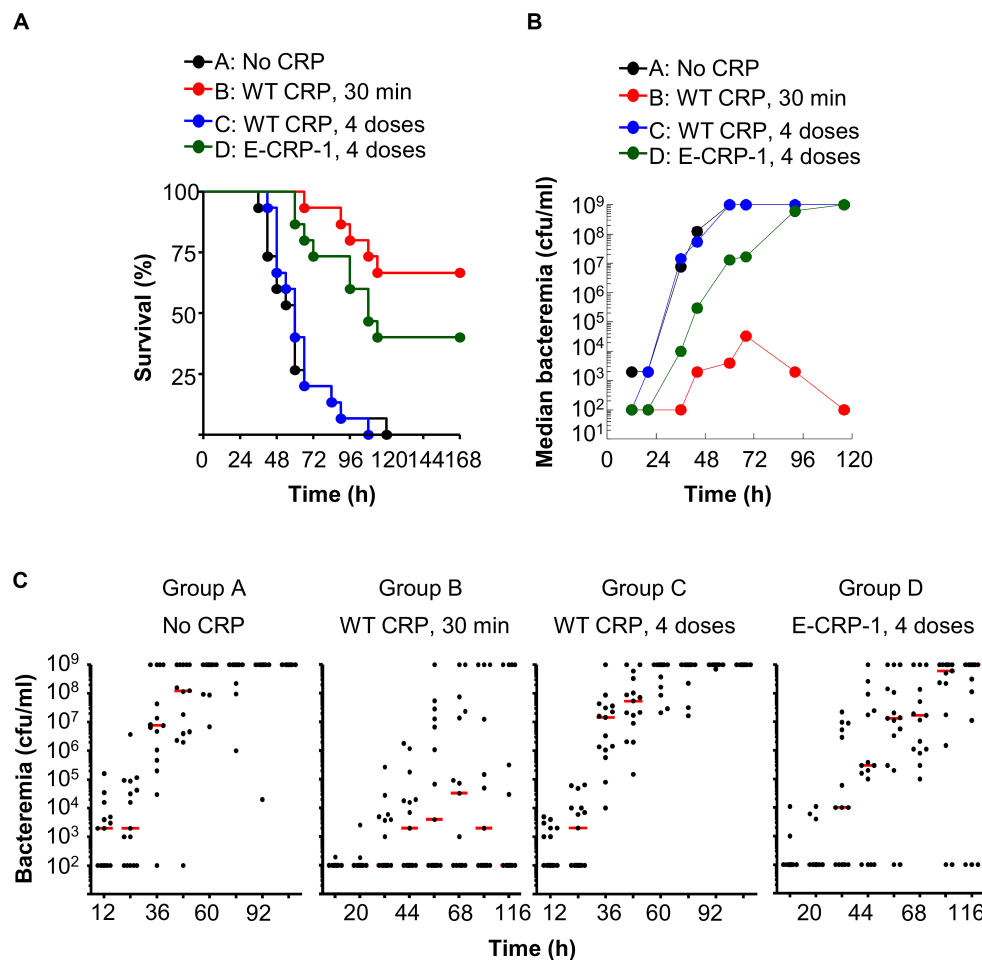


FIGURE 8 | One injection of E-CRP-1 was sufficient to protect against late-stage infection. The data are combined from two separate experiments with six to eight mice in each group in each experiment. **(A)** Survival curves. CRP was injected four times: 6, 12, 24 and 48 h after injecting pneumococci. The *p*-values for the differences in the survival curves between groups A B, A D, B C and C D were <0.001 . **(B)** Bacteremia. Blood was collected from each surviving mouse shown in **A**. The median bacteremia values are plotted. The *p*-value for the difference between groups A C was >0.05 at all-time points. For 36–116 h, the *p*-value for the difference between groups B C was <0.001 . For 44–116 h, the *p*-values for the differences between groups B D and C D were <0.05 . For 44–116 h, the *p*-value for the difference between groups A D was <0.01 . **(C)** Scatter plots of the bacteremia data shown in **B**. The horizontal line in each group of mice represents median bacteremia.

infection in our animal model. Both E-CRP-1 (cannot bind to PCh and hence unable to activate the complement system) and E-CRP-2 (can bind to PCh and hence able to activate the complement system) protected mice against late-stage infection. Since complement activation by CRP-complexes is necessary for protection (31), endogenous murine CRP must have participated along with E-CRP-1 in protecting mice against infection. Involvement of endogenous murine CRP in protection is also supported by the finding that the addition of human WT CRP to E-CRP-1 did not change the protective ability of E-CRP-1. The finding that the outcome of multiple injections of E-CRP-1 within 48 h of administering pneumococci was not different from a single injection at 12 h suggested that the effects of E-CRP-1 lasted for at least 48 h. Overall, the data indicate that CRP functions in two different conformations: in the WT conformation to bind to PCh and activate the

complement system and in the altered conformation to bind to factor H to remove the inhibitory effect of factor H on complement activation. Experiments employing CRP-deficient mice are in progress to confirm the participation of endogenous murine CRP in E-CRP-1-mediated protection against late-stage infection.

As expected, E-CRP-1 acquired the ability of E42Q CRP to bind to factor H. Unexpectedly, CRP triple mutant, F66A/T76Y/E81A, which was not investigated before for factor H-binding (18), also bound to factor H, without the E42Q mutation (50). These results were not available till we generated and tested E-CRP-1 for binding to factor H. Since the expression of E-CRP-1 cDNA was higher than the expression of the triple mutant cDNA, we used E-CRP-1 in this study. Previously, we reported that CRP triple mutant, which does not bind to PCh, protected mice against infection and we interpreted the

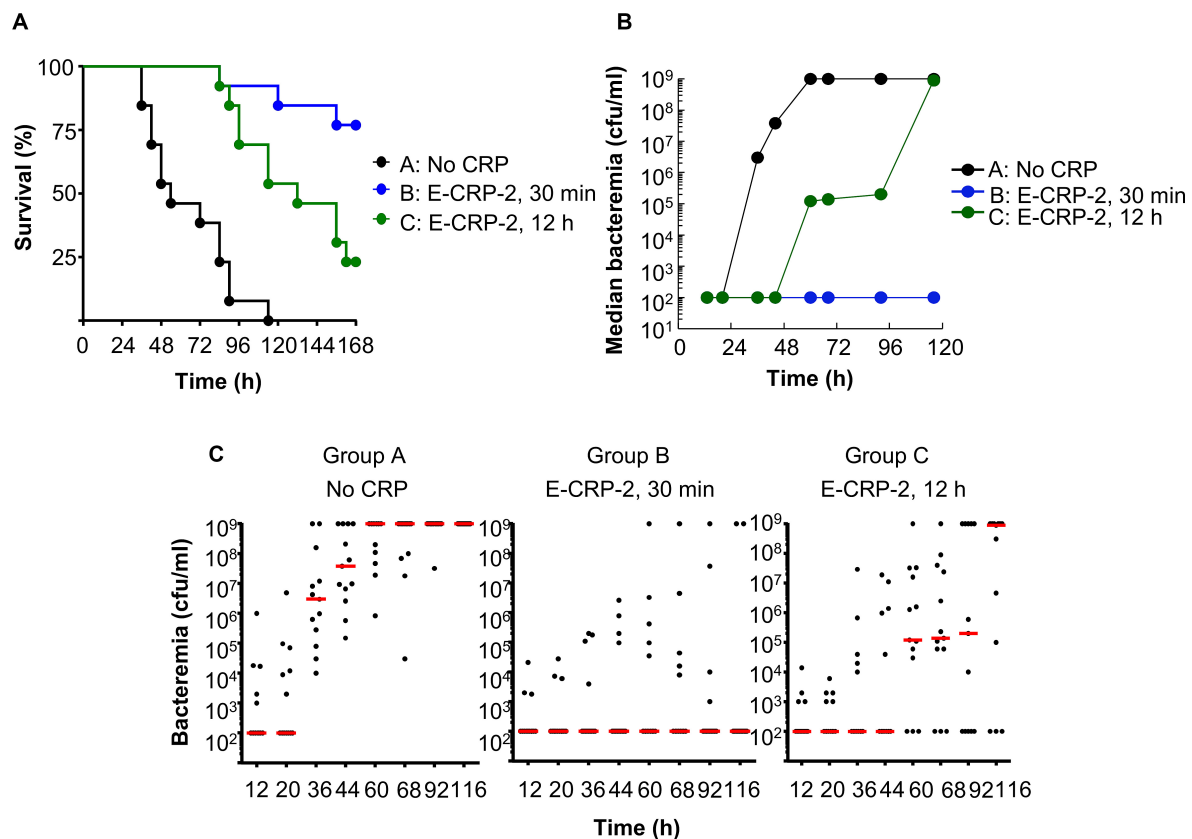


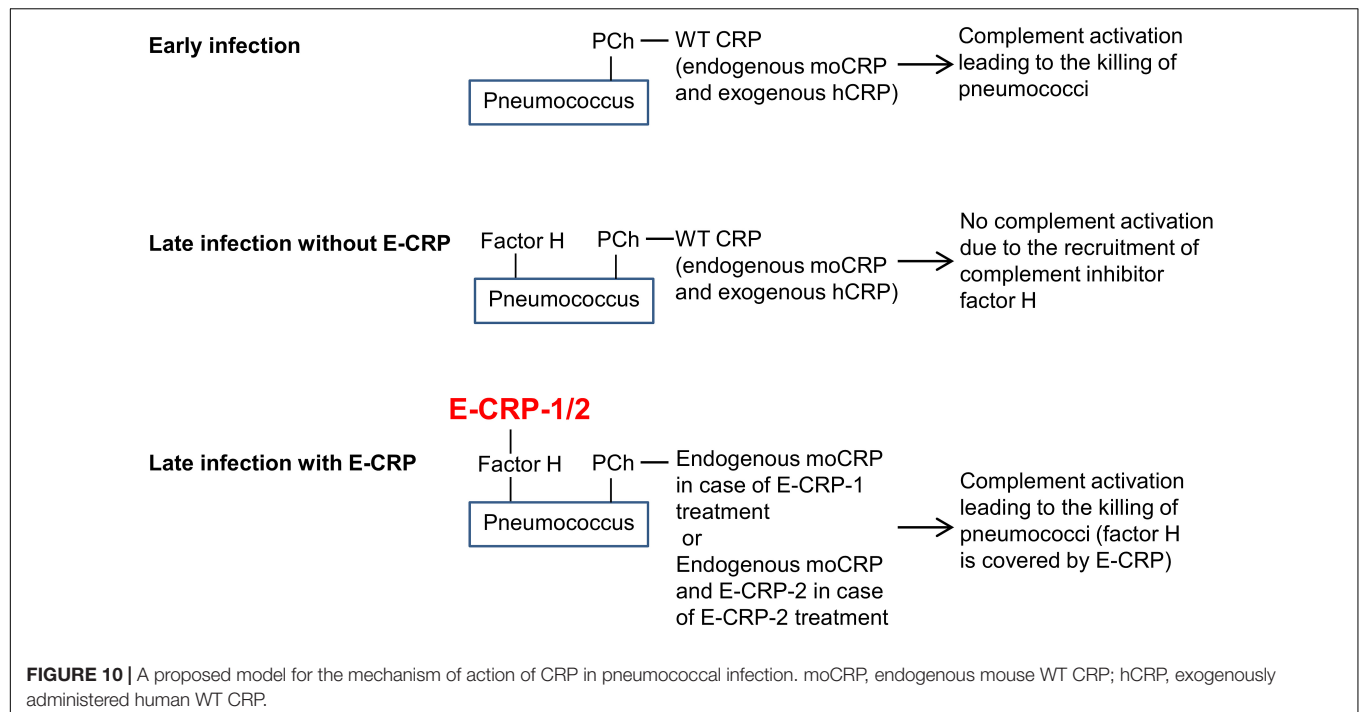
FIGURE 9 | Like E-CRP-1, E-CRP-2 is also protective against late-stage infection. The data are combined from two separate experiments with six to eight mice in each group in each experiment. **(A)** Survival curves. CRP was injected first; pneumococci were injected either 30 min or 12 h later. The *p*-values for the differences in the survival curves between groups A C and A B were <0.001 and between groups B C was 0.01. **(B)** Bacteremia in each surviving mouse shown in **A**. The median values of bacteremia are plotted. For 36–116 h, the *p*-values for the differences between groups A B and A C were <0.001 . The *p*-values for the differences between groups B C was >0.05 till 60 h and <0.05 after 60 h. **(C)** Scatter plots of the bacteremia data shown in **B**. The horizontal line in each group of mice represents median bacteremia.

data to suggest that CRP protects mice against pneumococcal infection without binding to pneumococci. Recent findings that triple mutant CRP can also bind to factor H (50) and that complement activation is critical for protection (31) indicate that in previously published experiments employing CRP triple mutant (18, 30), endogenous murine CRP had also participated, along with triple mutant CRP, in protecting mice against infection.

A model for the mechanism of action of E-CRP-1 and E-CRP-2 in protecting mice against infection is proposed (**Figure 10**). During early-stage infection, at a time when pneumococci have not recruited factor H yet on their surface to become complement-resistant, WT CRP is sufficient to decrease bacteremia. WT CRP (human or murine or both) would bind to PCh on pneumococci, activate the complement system, and reduce bacteremia. During late-stage infection, pneumococci recruit factor H and become resistant to WT CRP-activated complement-mediated killing. E-CRP-1 or E-CRP-2 would then bind to factor H on pneumococci, enabling complement activation by WT CRP/E-CRP-2-PCh complexes to proceed, resulting in the decrease in bacteremia. Thus, during late-stage

infection, two different structural conformations of CRP are required for protection.

Based on the model for E-CRP-1 and E-CRP-2, we propose a possible mechanism of action of endogenous CRP in humans in pneumococcal infection. Pneumococci are usually harbored in the nasopharynx but can spread to lungs and bloodstream. From the blood, after evading the attack by the complement system, pneumococci can spread to multiple other organs causing septicemia (53–55). Pneumococci release toxic substances including H_2O_2 , creating a localized inflammatory environment (56, 57). H_2O_2 has been found to modify the pentameric conformation of CRP, and H_2O_2 -treated CRP binds to factor H (47). The presence of conformationally altered pentameric CRP has been shown to be one of the features of inflamed sites (40, 42, 58). The binding of conformationally altered CRP to factor H on complement-resistant pneumococci would result in converting pneumococci back to being complement-sensitive. WT CRP can then bind to PCh on pneumococci and activate the complement system to attack pneumococci. In this regard, CRP is similar to serum amyloid A (SAA) which is another acute phase protein in humans (59). SAA



has recently been shown to exhibit pH-dependent antibacterial action against *Staphylococcus aureus*. Both CRP and SAA are produced not only by hepatocytes but also by a variety of cells in extrahepatic tissues (59–63). The expression of SAA is increased in abscesses of *S. aureus* cutaneous infected mice, and SAA then binds to bacterial cell surface and disrupts the cell membrane in acidic conditions (59). Similarly, it is possible that extrahepatically synthesized CRP may be responsible for the availability of conformationally altered CRP generated at sites of inflammation.

The finding that E-CRP-1 and E-CRP-2 bound to broth-grown pneumococci in the absence of Ca^{2+} suggests the involvement of a pneumococcal surface protein in the interaction between E-CRP-1/E-CRP-2 and pneumococci. Thus, the binding of E-CRP-1/E-CRP-2 to factor H may not be the only mechanism for protection against infection; as E-CRP-1/E-CRP-2 can interact directly with the surface virulence factors to eliminate their virulence. Since E-CRP-1 and E-CRP-2 bind to a variety of proteins immobilized on microtiter wells (data not shown), and not just to factor H, and because pneumococci also recruit other serum proteins to their surface, such as complement C1q, ficolins and complement inhibitor C4BP, our findings may be applicable to infections with a wide range of pneumococcal strains (64–71). The advantage of our strategy is that it is dependent on the recruited proteins and not on the serotype of pneumococci. We speculate that E-CRP could be therapeutically beneficial for infections with antibiotic-resistant pneumococcal strains, such as, strain 106 resistant to clindamycin, strain 109 resistant to clarithromycin, strain 999 resistant to penicillin, and others (72, 73). E-CRP could also be protective against infections with other bacterial species that use factor H to evade complement-mediated killing.

For example, *Bordetella pertussis*, *Borrelia burgdorferi*, *Borrelia hermsii*, *Haemophilus influenzae*, *Neisseria gonorrhoeae*, *Neisseria meningitis*, *Pseudomonas aeruginosa*, and *Streptococcus suis*, are all known to recruit factor H (74–79). It is possible that CRP plays a general antibacterial role, as exemplified in studies that show CRP protecting mice against infection with *Salmonella typhimurium* also (80).

We conclude that CRP functions in two different structural conformations. Our data provide a proof of concept that the structure of CRP is subtly modified *in vivo* to execute full anti-pneumococcal activities. We hypothesize that in individuals in whom the conformation of CRP remains unchanged, perhaps due to inappropriate inflammatory conditions around CRP, CRP is not fully functional during infection. If this hypothesis is correct, then our findings provide a new strategy to treat pneumococcal infection by injecting exogenously prepared pre-modified CRP, such as E-CRP-1 and E-CRP-2. Pre-modified CRP could also be therapeutically beneficial for infections with antibiotic-resistant pneumococcal strains and for infections with other bacterial species that use host proteins to evade complement-mediated killing.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/Supplementary Material.

ETHICS STATEMENT

The animal study was reviewed and approved by UCAC-ETSU.

AUTHOR CONTRIBUTIONS

DN, SS, and TG performed the experiments. AA conceived and designed the experiments. DN, SS, and AA analyzed the data. AA wrote the manuscript with input from DN. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by National Institutes of Health Grants AR068787, AI117730, and AI151561.

REFERENCES

1. Abernethy TJ, Avery OT. The occurrence during acute infections of a protein not normally present in the blood. I. Distribution of the reactive protein in patients' sera and the effect of calcium on the flocculation reaction with C polysaccharide of pneumococcus. *J Exp Med.* (1941) 73:173–82. doi: 10.1084/jem.73.2.173
2. Mold C, Rodgers CP, Kaplan RL, Gewurz H. Binding of human C-reactive protein to bacteria. *Infect Immun.* (1982) 38:392–5. doi: 10.1128/iai.38.1.392-395.1982
3. Agrawal A, Suresh MV, Singh SK, Ferguson DA Jr. The protective function of human C-reactive protein in mouse models of *Streptococcus pneumoniae* infection. *Endocr Metab Immune Disord Drug Targets.* (2008) 8:231–7. doi: 10.2174/187153008786848321
4. Ng PML, Le Saux A, Lee CM, Tan NS, Lu J, Thiel S, et al. C-reactive protein collaborates with plasma lectins to boost immune response against bacteria. *EMBO J.* (2007) 26:3431–40. doi: 10.1038/sj.emboj.7601762
5. Weiser JN, Pan N, McGowan KL, Musher D, Martin A, Richards J. Phosphorylcholine on the lipopolysaccharide of *Haemophilus influenzae* contributes to persistence in the respiratory tract and sensitivity to serum killing mediated by C-reactive protein. *J Exp Med.* (1998) 187:631–40. doi: 10.1084/jem.187.4.631
6. Langereis JD, van der Pasch ES, de Jonge MI. Serum IgM and C-reactive protein binding to phosphorylcholine of nontypeable *Haemophilus influenzae* increases complement-mediated killing. *Infect Immun.* (2019) 87:e299–19.
7. Serino L, Virji M. Genetic and functional analysis of the phosphorylcholine moiety of commensal *Neisseria* lipopolysaccharide. *Mol Microbiol.* (2002) 43:437–48. doi: 10.1046/j.1365-2958.2002.02755.x
8. Casey R, Newcombe J, McFadden J, Bodman-Smith KB. The acute-phase reactant C-reactive protein binds to phosphorylcholine-expressing *Neisseria meningitidis* and increases uptake by human phagocytes. *Infect Immun.* (2008) 76:1298–304. doi: 10.1128/iai.00741-07
9. Ng PML, Jin Z, Tan SSH, Ho B, Ding JL. C-reactive protein: a predominant LPS-binding acute phase protein responsive to *Pseudomonas* infection. *J Endotoxin Res.* (2004) 10:163–74. doi: 10.1177/09680519040100030301
10. Kushner I. The phenomenon of the acute phase response. *Ann N Y Acad Sci.* (1982) 389:39–48.
11. Simons JP, Loeffler JM, Al-Shawi R, Ellmerich S, Hutchinson WL, Tennent GA, et al. C-reactive protein is essential for innate resistance to pneumococcal infection. *Immunology.* (2014) 142:414–20. doi: 10.1111/imm.12266
12. Volanakis JE, Kaplan MH. Specificity of C-reactive protein for choline phosphate residues of pneumococcal C-polysaccharide. *Proc Soc Exp Biol Med.* (1971) 136:612–4. doi: 10.3181/00379727-136-35323
13. Shrive AK, Cheetham GMT, Holden D, Myles DAA, Turnell WG, Volanakis JE, et al. Three-dimensional structure of human C-reactive protein. *Nat Struct Biol.* (1996) 3:346–54.
14. Thompson D, Pepys MB, Wood SP. The physiological structure of human C-reactive protein and its complex with phosphocholine. *Structure.* (1999) 7:169–77. doi: 10.1016/s0969-2126(99)80023-9
15. Agrawal A, Xu Y, Ansardi D, Macon KJ, Volanakis JE. Probing the phosphocholine-binding site of human C-reactive protein by site-directed mutagenesis. *J Biol Chem.* (1992) 267:25352–8.

ACKNOWLEDGMENTS

We thank Dr. David E. Briles (University of Alabama at Birmingham, Birmingham, AL, United States) for the gift of *S. pneumoniae* type 3, strain WU2.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2020.586669/full#supplementary-material>

16. Agrawal A, Lee S, Carson M, Narayana SVL, Greenhough TJ, Volanakis JE. Site-directed mutagenesis of the phosphocholine-binding site of human C-reactive protein: role of Thr76 and Trp67. *J Immunol.* (1997) 158:345–50.
17. Agrawal A, Simpson MJ, Black S, Carey M-P, Samols DA. C-reactive protein mutant that does not bind to phosphocholine and pneumococcal C-polysaccharide. *J Immunol.* (2002) 169:3217–22. doi: 10.4049/jimmunol.169.6.3217
18. Gang TB, Hammond DJ Jr., Singh SK, Ferguson DA Jr., Mishra VK, Agrawal A, et al. The phosphocholine-binding pocket on C-reactive protein is necessary for initial protection of mice against pneumococcal infection. *J Biol Chem.* (2012) 287:43116–25. doi: 10.1074/jbc.m112.427310
19. Kaplan MH, Volanakis JE. Interaction of C-reactive protein complexes with the complement system. I. Consumption of human complement associated with the reaction of C-reactive protein with pneumococcal C-polysaccharide and with the choline phosphatides, lecithin, and sphingomyelin. *J Immunol.* (1974) 112:2135–47.
20. Volanakis JE. Complement activation by C-reactive protein complexes. *Ann N Y Acad Sci.* (1982) 389:235–50. doi: 10.1111/j.1749-6632.1982.tb22140.x
21. Suresh MV, Singh SK, Ferguson DA Jr., Agrawal A. Role of the property of C-reactive protein to activate the classical pathway of complement in protecting mice from pneumococcal infection. *J Immunol.* (2006) 176:4369–74. doi: 10.4049/jimmunol.176.7.4369
22. Mold C, Nakayama S, Holzer TJ, Gewurz H, Du Clos TW. C-reactive protein is protective against *Streptococcus pneumoniae* infection in mice. *J Exp Med.* (1981) 154:1703–8. doi: 10.1084/jem.154.5.1703
23. Yother J, Volanakis JE, Briles DE. Human C-reactive protein is protective against fatal *Streptococcus pneumoniae* infection in mice. *J Immunol.* (1982) 128:2374–6.
24. Nakayama S, Gewurz H, Holzer T, Du Clos TW, Mold C. The role of the spleen in the protective effect of C-reactive protein in *Streptococcus pneumoniae* infection. *Clin Exp Immunol.* (1983) 54:319–26.
25. Horowitz J, Volanakis JE, Briles DE. Blood clearance of *Streptococcus pneumoniae* by C-reactive protein. *J Immunol.* (1987) 138:2598–603.
26. Szalai AJ, Briles DE, Volanakis JE. Human C-reactive protein is protective against fatal *Streptococcus pneumoniae* infection in transgenic mice. *J Immunol.* (1995) 155:2557–63.
27. Szalai AJ, Briles DE, Volanakis JE. Role of complement in C-reactive protein-mediated protection of mice from *Streptococcus pneumoniae*. *Infect Immun.* (1996) 64:4850–3. doi: 10.1128/iai.64.11.4850-4853.1996
28. Mold C, Rodic-Polic B, Du Clos TW. Protection from *Streptococcus pneumoniae* infection by C-reactive protein and natural antibody requires complement but not Fcγ receptors. *J Immunol.* (2002) 168:6375–81. doi: 10.4049/jimmunol.168.12.6375
29. Suresh MV, Singh SK, Ferguson DA Jr., Agrawal A. Human C-reactive protein protects mice from *Streptococcus pneumoniae* infection without binding to pneumococcal C-polysaccharide. *J Immunol.* (2007) 178:1158–63. doi: 10.4049/jimmunol.178.2.1158
30. Gang TB, Hanley GA, Agrawal A. C-reactive protein protects mice against pneumococcal infection via both phosphocholine-dependent and phosphocholine-independent mechanisms. *Infect Immun.* (2015) 83:1845–52. doi: 10.1128/iai.03058-14

31. Singh SK, Ngwa DN, Agrawal A. Complement activation by C-reactive protein is critical for protection of mice against pneumococcal infection. *Front Immunol.* (2020) 11:1812. doi: 10.3389/fimmu.2020.01812
32. Kopp A, Hebecker M, Svobodová E, Józsi M, Factor H: a complement regulator in health and disease, and a mediator of cellular interactions. *Biomolecules.* (2012) 2:46–75. doi: 10.3390/biom2010046
33. Janulczyk R, Iannelli F, Sjöholm AG, Pozzi G, Björck L. Hic, a novel surface protein of *Streptococcus pneumoniae* that interferes with complement function. *J Biol Chem.* (2000) 275:37257–63. doi: 10.1074/jbc.m004572200
34. Jarva H, Janulczyk R, Hellwage J, Zipfel PF, Björck L, Meri S. *Streptococcus pneumoniae* evades complement attack and opsonophagocytosis by expressing the *pspC* locus encoded Hic protein that binds to short consensus repeats 8–11 of factor H. *J Immunol.* (2002) 168:1886–94. doi: 10.4049/jimmunol.168.4.1886
35. Lu L, Ma Z, Jokiranta TS, Whitney AR, DeLeo F, Zhang J-R. Species-specific interaction of *Streptococcus pneumoniae* with human complement factor H. *J Immunol.* (2008) 181:7138–46.
36. Andre GO, Converso TR, Politano WR, Ferraz LF, Ribeiro ML, Leite LC, et al. Role of *Streptococcus pneumoniae* proteins in evasion of complement-mediated immunity. *Front Microbiol.* (2017) 8:224. doi: 10.3389/fmicb.2017.00224
37. Wu Y, Potempa LA, El Kebir D, Filep JG. C-reactive protein and inflammation: conformational changes affect function. *Biol Chem.* (2015) 396:1181–97. doi: 10.1515/hsz-2015-0149
38. McFadyen JD, Zeller J, Potempa LA, Pietersz GA, Eisenhardt SU, Peter K. C-reactive protein and its structural isoforms: an evolutionary conserved marker and central player in inflammatory diseases and beyond. *Subcell Biochem.* (2020) 94:499–520. doi: 10.1007/978-3-030-41769-7_20
39. Suresh MV, Singh SK, Agrawal A. Interaction of calcium-bound C-reactive protein with fibronectin is controlled by pH: *In vivo* implications. *J Biol Chem.* (2004) 279:52552–7. doi: 10.1074/jbc.m409054200
40. Ji S-R, Wu Y, Zhu L, Potempa LA, Sheng F-L, Lu W, et al. Cell membranes and liposomes dissociate C-reactive protein (CRP) to form a new, biologically active structural intermediate: mCRPm. *FASEB J.* (2007) 21:284–94. doi: 10.1096/fj.06-6722com
41. Alnaas AA, Moon CL, Alton M, Reed SM, Knowles MK. Conformational changes in C-reactive protein affect binding to curved membranes in a lipid bilayer model of the apoptotic cell surface. *J Phys Chem B.* (2017) 121:2631–9. doi: 10.1021/acs.jpcc.6b11505
42. Braig D, Nero TL, Koch HG, Kaiser B, Wang X, Thiele JR, et al. Transitional changes in the CRP structure lead to the exposure of proinflammatory binding sites. *Nat Commun.* (2017) 8:14188.
43. Lv J-M, Wang M-Y. *In vitro* generation and bioactivity evaluation of C-reactive protein intermediate. *PLoS One.* (2018) 13:e0198375. doi: 10.1371/journal.pone.0198375
44. Agrawal A, Gang TB, Rusiñol AE. Recognition functions of pentameric C-reactive protein in cardiovascular disease. *Mediators Inflamm.* (2014) 2014:319215.
45. Hammond DJ Jr., Singh SK, Thompson JA, Beeler BW, Rusinol AE, Pangburn MK, et al. Identification of acidic pH-dependent ligands of pentameric C-reactive protein. *J Biol Chem.* (2010) 285:36235–44. doi: 10.1074/jbc.m110.142026
46. Singh SK, Thirumalai A, Hammond DJ Jr., Pangburn MK, Mishra VK, Johnson DA, et al. Exposing a hidden functional site of C-reactive protein by site-directed mutagenesis. *J Biol Chem.* (2012) 287:3550–8. doi: 10.1074/jbc.m111.310011
47. Singh SK, Thirumalai A, Pathak A, Ngwa DN, Agrawal A. Functional transformation of C-reactive protein by hydrogen peroxide. *J Biol Chem.* (2017) 292:3129–36. doi: 10.1074/jbc.m116.773176
48. Ngwa DN, Agrawal A. Structure-function relationships of C-reactive protein in bacterial infection. *Front Immunol.* (2019) 10:166. doi: 10.3389/fimmu.2019.00166
49. Thirumalai A, Singh SK, Hammond DJ Jr., Gang TB, Ngwa DN, Pathak A, et al. Purification of recombinant C-reactive protein mutants. *J Immunol Methods.* (2017) 443:26–32. doi: 10.1016/j.jim.2017.01.011
50. Pathak A, Singh SK, Thewke DP, Agrawal A. Conformationally altered C-reactive protein capable of binding to atherogenic lipoproteins reduces atherosclerosis. *Front Immunol.* (2020) 11:1780. doi: 10.3389/fimmu.2020.01780
51. Ulloa L, Brunner M, Ramos L, Deitch EA. Scientific and clinical challenges in sepsis. *Curr Pharm Des.* (2009) 15:1918–35. doi: 10.2174/138161209788453248
52. Marshall JC. Why have clinical trials in sepsis failed? *Trends Mol Med.* (2014) 20:195–203. doi: 10.1016/j.molmed.2014.01.007
53. Van der Poll T, Opal SM. Pathogenesis, treatment, and prevention of pneumococcal pneumonia. *Lancet.* (2009) 374:1543–56. doi: 10.1016/s0140-6736(09)61114-4
54. Henriques-Normark B, Tuomanen EI. The pneumococcus: epidemiology, microbiology, and pathogenesis. *Cold Spring Harb Perspect Med.* (2013) 3:a010215. doi: 10.1101/cshperspect.a010215
55. Ercoli G, Fernandes VE, Chung WY, Wanford JJ, Thomson S, Bayliss CD, et al. Intracellular replication of *Streptococcus pneumoniae* inside splenic macrophages serves as a reservoir for septicemia. *Nat Microbiol.* (2018) 3:600–10. doi: 10.1038/s41564-018-0147-1
56. Duane PG, Rubins JB, Weisel HR, Janoff EN. Identification of hydrogen peroxide as a *Streptococcus pneumoniae* toxin for rat alveolar epithelial cells. *Infect Immun.* (1993) 61:4392–7. doi: 10.1128/iai.61.10.4392-4397.1993
57. Rai P, Parrish M, Tay IJJ, Li N, Ackerman S, He F, et al. *Streptococcus pneumoniae* secretes hydrogen peroxide leading to DNA damage and apoptosis in lung cells. *Proc Natl Acad Sci USA.* (2015) 112:E3421–30. doi: 10.1128/iai.61.10.4392-4397.1993
58. Shi P, Li X-X, Zhu W, Yang H, Dong C, Li X-M. Immunohistochemical staining reveals C-reactive protein existing predominantly as altered conformation forms in inflammatory lesions. *Acta Biol Hung.* (2014) 65:265–73. doi: 10.1556/abiol.65.2014.3.3
59. Zheng H, Li H, Zhang J, Fan H, Jia L, Ma W, et al. Serum amyloid A exhibits pH dependent antibacterial action and contributes to host defense against *Staphylococcus aureus* cutaneous infection. *J Biol Chem.* (2020) 295:2570–81. doi: 10.1074/jbc.ra19.010626
60. Gould JM, Weiser JN. Expression of C-reactive protein in the human respiratory tract. *Infect Immun.* (2001) 69:1747–54. doi: 10.1128/iai.69.3.1747-1754.2001
61. Jabs WJ, Lögering BA, Gerke P, Kreft B, Wolber E-M, Klinger MHE, et al. The kidney as a second site of human C-reactive protein formation *in vivo*. *Eur J Immunol.* (2003) 33:152–61. doi: 10.1002/immu.20039.0018
62. Kearney JF, Gartland GL, Kilpatrick JM, Volanakis JE. CRP-associated determinant expressed by human monocytes. *Immunol Comm.* (1982) 11:275–82. doi: 10.3109/08820138209050727
63. Lütjhe FL, Blirup-Plum SA, Möller NS, Heegaard PMH, Jensen HE, Kirketerp-Møller K, et al. The host response to bacterial bone infection involves a local upregulation of several acute phase proteins. *Immunobiology.* (2020) 15:151914. doi: 10.1016/j.imbio.2020.151914
64. Frolet C, Beniazza M, Roux L, Gallet B, Noirclerc-Savoye M, Vernet T, et al. New adhesion functions of surface-exposed pneumococcal proteins. *BMC Microbiol.* (2010) 10:190. doi: 10.1186/1471-2180-10-190
65. Mukerji R, Mirza S, Roche AM, Widener RW, Crony CM, Rhee D-K, et al. Pneumococcal surface protein A inhibits complement deposition on the pneumococcal surface by competing with the binding of C-reactive protein to cell-surface phosphocholine. *J Immunol.* (2012) 189:5327–35. doi: 10.4049/jimmunol.1201967
66. Voss S, Hallström T, Saleh M, Burchhardt G, Pribyl T, Singh B, et al. The choline-binding protein PspC of *Streptococcus pneumoniae* interacts with the C-terminal heparin-binding domain of vitronectin. *J Biol Chem.* (2013) 288:15614–27. doi: 10.1074/jbc.m112.443507
67. Agarwal V, Hammerschmidt S, Malm S, Bergmann S, Riesbeck K, Blom AM. Enolase of *Streptococcus pneumoniae* binds human complement inhibitor C4b-binding protein and contributes to complement evasion. *J Immunol.* (2012) 189:3575–84. doi: 10.4049/jimmunol.1102934
68. Agarwal V, Sroka M, Fulde M, Bergmann S, Riesbeck K, Blom AM. Binding of *Streptococcus pneumoniae* endopeptidase O (PepO) to complement component C1q modulates the complement attack and promotes host cell adherence. *J Biol Chem.* (2014) 289:15833–44. doi: 10.1074/jbc.m113.530212
69. Zhang J, Yang L, Ang Z, Yoong SL, Tran TTT, Anand GS, et al. Secreted M-ficolin anchors onto monocyte transmembrane G protein-coupled receptor 43 and cross talks with plasma C-reactive protein to mediate immune signaling and regulate host defense. *J Immunol.* (2010) 185:6899–910. doi: 10.4049/jimmunol.1001225

70. Vassal-Stermann E, Lacroix M, Gout E, Laffly E, Pedersen CM, Martin L, et al. Human L-ficolin recognizes phosphocholine moieties of pneumococcal teichoic acid. *J Immunol.* (2014) 193:5699–708. doi: 10.4049/jimmunol.140.0127
71. Ma YJ, Lee BL, Garred P. An overview of the synergy and crosstalk between pentraxins and collectins/ficolins: their functional relevance in complement activation. *Exp Mol Med.* (2017) 49:e320. doi: 10.1038/emmm.2017.51
72. Tessier PR, Kim MK, Zhou W, Xuan D, Li C, Ye M, et al. Pharmacodynamic assessment of clarithromycin in a murine model of pneumococcal pneumonia. *Antimicrob Agents Chemother.* (2002) 46:1425–34. doi: 10.1128/aac.46.5.1425-1434.2002
73. Den Hollander JG, Knudsen JD, Mouton JW, Fuursted K, Frimodt-Møller N, Verbrugh HA, et al. Comparison of pharmacodynamics of azithromycin and erythromycin in vitro and in vivo. *Antimicrob Agents Chemother.* (1998) 42:377–82. doi: 10.1128/aac.42.2.377
74. Schneider MC, Prosser BE, Caesar JJE, Kugelberg E, Li S, Zhang Q, et al. *Neisseria meningitidis* recruits factor H using protein mimicry of host carbohydrates. *Nature.* (2009) 458:890–3. doi: 10.1038/nature07769
75. Beernink PT, Shaughnessy J, Braga EM, Liu Q, Rice PA, Ram S, et al. A meningococcal factor H binding protein mutant that eliminates factor H binding enhances protective antibody responses to vaccination. *J Immunol.* (2011) 186:3606–14. doi: 10.4049/jimmunol.1003470
76. Meri T, Amdahl H, Lehtinen MJ, Hyvärinen S, McDowell JV, Bhattacharjee A, et al. Microbes bind complement inhibitor factor H via a common site. *PLoS Pathog.* (2013) 9:e1003308. doi: 10.1371/journal.ppat.1003308
77. Li X, Liu P, Gan S, Zhang C, Zheng Y, Jiang Y, et al. Mechanisms of host-pathogen protein complex formation and bacterial immune evasion of *Streptococcus suis* protein Fhb. *J Biol Chem.* (2016) 291:17122–32. doi: 10.1074/jbc.m116.719443
78. Biagini M, Spinsanti M, De Angelis G, Tomei S, Ferlenghi I, Scarselli M, et al. Expression of factor H binding protein in meningococcal strains can vary at least 15-fold and is genetically determined. *Proc Natl Acad Sci USA.* (2016) 113:2714–9. doi: 10.1073/pnas.1521142113
79. Lewis LA, Rice PA, Ram S. Role of gonococcal neisserial surface protein A (NspA) in serum resistance and comparison of its factor H binding properties with those of its meningococcal counterpart. *Infect Immun.* (2019) 87:e658–18.
80. Szalai AJ, VanCott JL, McGhee JR, Volanakis JE, Benjamin WH Jr. Human C-reactive protein is protective against fatal *Salmonella enterica* serovar typhimurium infection in transgenic mice. *Infect Immun.* (2000) 68:5652–6. doi: 10.1128/iai.68.10.5652-5656.2000

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Ngwa, Singh, Gang and Agrawal. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



C-Reactive Protein and Cancer—Diagnostic and Therapeutic Insights

Peter C. Hart*, Ibraheem M. Rajab, May Alebraheem and Lawrence A. Potempa*

Roosevelt University, College of Science, Health and Pharmacy, Schaumburg, IL, United States

OPEN ACCESS

Edited by:

Kenji Daigo,
Nippon Medical School, Japan

Reviewed by:

Annette Karen Shrive,
Keele University, United Kingdom
Moises Armides Franco Molina,
Autonomous University of
Nuevo León, Mexico

*Correspondence:

Peter C. Hart
phart02@roosevelt.edu
Lawrence A. Potempa
lpotempa01@roosevelt.edu

Specialty section:

This article was submitted to
Molecular Innate Immunity,
a section of the journal
Frontiers in Immunology

Received: 17 August 2020

Accepted: 07 October 2020

Published: 19 November 2020

Citation:

Hart PC, Rajab IM, Alebraheem M and
Potempa LA (2020) C-Reactive
Protein and Cancer—Diagnostic and
Therapeutic Insights.
Front. Immunol. 11:595835.
doi: 10.3389/fimmu.2020.595835

Cancer disease describes any pathology involving uncontrolled cell growth. As cells duplicate, they can remain localized in defined tissues, forming tumor masses and altering their microenvironmental niche, or they can disseminate throughout the body in a metastatic process affecting multiple tissues and organs. As tumors grow and metastasize, they affect normal tissue integrity and homeostasis which signals the body to trigger the acute phase inflammatory response. C-reactive protein (CRP) is a predominant protein of the acute phase response; its blood levels have long been used as a minimally invasive index of any ongoing inflammatory response, including that occurring in cancer. Its diagnostic significance in assessing disease progression or remission, however, remains undefined. By considering the recent understanding that CRP exists in multiple isoforms with distinct biological activities, a unified model is advanced that describes the relevance of CRP as a mediator of host defense responses in cancer. CRP in its monomeric, modified isoform (mCRP) modulates inflammatory responses by inserting into activated cell membranes and stimulating platelet and leukocyte responses associated with acute phase responses to tumor growth. It also binds components of the extracellular matrix in involved tissues. Conversely, CRP in its pentameric isoform (pCRP), which is the form quantified in diagnostic measurements of CRP, is notably less bioactive with weak anti-inflammatory bioactivity. Its accumulation in blood is associated with a continuous, low-level inflammatory response and is indicative of unresolved and advancing disease, as occurs in cancer. Herein, a novel interpretation of the diagnostic utility of CRP is presented accounting for the unique properties of the CRP isoforms in the context of the developing pro-metastatic tumor microenvironment.

Keywords: C-reactive protein, monomeric C-reactive protein, inflammation, tumor microenvironment, acute phase response

INTRODUCTION

Cancer is a pervasive disease affecting many people across the globe. Worldwide, the prevalence of people living within 5 years of receiving a cancer diagnosis is estimated to be 43.8 million. In 2018, 18.1 million new cancer cases were diagnosed worldwide, and 9.6 million deaths were reported. The most reported new cases involve the lung and breast, with colorectal cancer third, prostate cancer

fourth, and stomach cancer fifth (1, 2). Each reported cancer is associated with variable overall life expectancies, years of life lost, and 5-year survival rate (3–6).

The term cancer describes a physiological condition in which body cells grow and replicate in an uncontrolled and unregulated fashion. While cancer is often categorized by the tissue in which the predominant uncontrolled growth originates, the disease is fundamentally defined by a loss of basic cellular processes that regulate proliferation. When cells proliferate, their mass and the mass of ancillary tissues in which the growth occurs increases, leading to localized areas of disrupted tissues which can overwhelm the natural protective immune responses that are designed to identify and destroy aberrantly growing cells, and to repair damaged tissues. Hence, in evaluating cancerous disease, consideration must be given not only aberrant cells, but the tissues, vasculature, and immune responses in which the malignant growth occurs. If cancerous growth is relatively slow and localized, treatment options include surgical resection and radiotherapy. If cancerous growths become unregulated and rapid, involved tissues are compromised and weakened, and tumor cells can break free to metastasize to other tissues. Treatment options for disseminated cancers become limited and prognosis for long term survival decreases. Strategies for treating cancers must therefore include an assessment of how tissue structures involved in cancerous growths may be compromised and how natural barriers might be strengthened to counteract the growth of tumor masses and to better coordinate the immune responses against tumor development.

Both innate and adaptive immune responses exist to recognize and remove diseased cells, primarily involving innate system humoral factors and natural killer cells. Innate responses involve neutrophils, M1 macrophages, natural killer cells, interferons, cytokines, and the acute phase response (7). Adaptive responses involve antibodies developed to specifically recognize malignant cells, and direct cytotoxic T-lymphocytes to specifically recognize intracellular abnormalities associated with uncontrolled cellular duplication that often display tumor specific antigens, leading to cell-mediated tumor cell apoptosis (8). However, a number of cancers present with “cold” tumors, showing limited or no infiltration of activated T cells within the tumor compartment (9). Many cancers are known to produce suppression factors that target effective immune system surveillance, thus giving cancer cells an advantage in the balance between continued proliferation and leukocyte-mediated apoptosis. The exciting development of biotherapeutic reagents known as checkpoint inhibitors, which function by neutralizing the immunosuppressive factors [i.e. by directly binding to Program Death Ligand-1 (PDL-1) or its receptor (PD-1)], shifts the balance back to natural immune function, slowing or stopping tumor cell growth in treated individuals (10). This is likely due to the prevention of PD-1-dependent T cell exhaustion; therefore, blockade increases the presence of cytotoxic T cells in the microenvironment capable of tumor cell lysis through perforin and granzyme secretions (11). While promoting the cytotoxic capacity of the local immune response can slow or prevent tumor growth in some cases (12), it is notable that certain distributions of leukocytes can also favor a

state of chronic inflammation that conversely would promote genomic instability, survival, and proliferation of tumor cells that can confer tumor development and progression [reviewed in (13)]. Thus, the dualistic nature of the immune system in cancer suggests that tight regulation of immune and inflammatory responses is necessary to prevent tumor growth and metastasis.

This report focuses on innate immunity and the acute phase response (APR) to cancerous growth. More specifically, it provides a new perspective on the diagnostic and therapeutic value of the prototypic acute phase reactant, C-reactive protein (CRP), and cancer. The concepts presented herein represent a unifying understanding of the value of CRP as a key protein of the natural inflammatory response and how both beneficial and pathological inflammation contribute to cancerous disease as well as the pathologies that involve any tissue damaged by trauma or disease.

USE OF CRP AS A DIAGNOSTIC MARKER IN CANCER

The US Food and Drug Administration (FDA) guidance for assessing the relevance of CRP as a diagnostic marker in any disease involving a host defense inflammatory response, describes CRP as a single protein that can be reliably measured from blood using various qualitative, semi-quantitative and quantitative measurements (14). Two concentration thresholds were established: 1.) conventional CRP levels (defined as CRP levels ≥ 10 $\mu\text{g/ml}$), and 2.) high sensitivity CRP levels (i.e. hsCRP; defined as CRP levels < 10 $\mu\text{g/ml}$). FDA guidance does not associate either CRP or hsCRP levels with specific diseases or risks for disease, cautioning that any interpretation of CRP levels must include the context of a specific clinical evaluation. However, high levels of CRP were found to be strongly associated with advanced disease severity in numerous cancer types (elaborated below). Hence, CRP measurements have potential utility as a diagnostic tool in assessing disease status and progression, including in cancer.

A comprehensive review of the literature on the diagnostic significance and therapeutic value of CRP blood levels in cancer proved to be problematic. Reported CRP levels varied from <1 $\mu\text{g/ml}$ to more than 175 $\mu\text{g/ml}$ [(15); see **Table 1** and **Figure 1**] and were most often reported with reference to the tissues affected with cancerous growths (e.g. lung, breast, gastrointestinal, esophageal, head and neck, sexual and reproductive organs, renal, pancreas, and blood). One complication to interpreting the value of CRP measurements in any such disease condition is that its relative blood level can change rapidly and in direct relationship to the stage and extent of progressive disease and/or associated complication (e.g. infections) that may accompany the disease progression. Another limitation is that many of these studies did not fully characterize the relationship between CRP and the discrete variables that define cancer staging (e.g., tumor size in TNM staging of breast cancer, etc.); however, the associations drawn between CRP with prognosis and disease severity are consistent among cancers and indicate the potential utility of

TABLE 1 | Overview of CRP values reported in various cancers distinguishing Conventional CRP levels (≥ 10 $\mu\text{g/ml}$) from High Sensitivity (hsCRP) levels (< 10 $\mu\text{g/ml}$).

Tissue affected by cancer	Notes and generalized conclusions	CRP levels reported*		References**
		Conventional CRP (≥ 10 $\mu\text{g/ml}$)	hsCRP (1–10 $\mu\text{g/ml}$)	
Lung Non-Small Cell	- Higher conventional levels correlate with tumor size and staging (2) - Higher levels (before or after surgery) are indicators of poor prognosis (1) - Smoking and hsCRP levels did not correlate with increased risk for lung adenocarcinoma (9) - Smoking and lung function with hsCRP can predict bronchial dysplasia than can progress to cancer (14) - Conventional CRP above 40 $\mu\text{g/ml}$ is predictive of metastasis (17) - CRP levels do correlate with level of inflammation which is more pronounced in cancer than COPD (18)	4–20 (1)	<3 (1)	(1) Alifano et al. (16)
		>20 (1)	5.4 ± 9 (3)	(2) Aref and Refaat (17)
		13.4 ± 8.6 (2)	6.4 ± 7.9 (3)	(3) Bittoni et al. (18)
		114.2 ± 60.1 (2)	<1 (4)	(4) Chaturvedi et al. (19)
		84 ± 88 (5)	≥ 5.6 (4)	(5) Hara et al. (20)
		≥ 10 (7)	<5 (5,6, 16)	(6) Hara et al. (21)
		7.1–100 (8)	≥ 5 (5,6)	(7) Jin et al. (22)
		≥ 10 (9)	0–0.7 (11)	(8) Jing et al. (23)
		1–30 (10)	0.7–1.76 (11)	(9) Koch et al. (24)
		4.14–87.9 (11)	1.77–4.13 (11)	(10) Liao et al. (25)
		143 max (12)	3 (12)	(11) Muller et al. (26)
		>101 –122.6 (15)	1–5 (13)	(12) Pastorino et al. (27)
		>40 (17)	>0.5 (14)	(13) Shinohara et al. (28)
		22.49 ± 2.31 (18)	$8.37 + 0.91$ (18)	(14) Sin et al. (29)
		20.42 ± 1.95 (18)		(15) Szturmowicz et al. (30)
		$10.27 + 2.12$ (19)		(16) Tomita et al. (31)
		66.35 (20)		(17) Torrecilla et al. (32)
Breast	- Elevated conventional CRP levels are associated with reduced overall and disease-free survival and increased risk of death (21) - hsCRP levels are not predictive in post-menopausal breast cancer incidence or in apparently healthy women (25)	≥ 16.4 (21)	1.5–10 (24,25)	(18) Vagueliené et al. (33)
		12 ± 8 (22)	4.93 ± 6.65 (26)	(19) Wei et al. (34)
		42 ± 12 (22)	5.26 ± 8.59 (26)	(20) Zhao et al. (35)
		>10 (23,24)	1.0 ± 1.3 (27)	(21) Allin et al. (36)
		0.1–39.5 (26)	1.5 ± 1.7 (27)	(22) Asegaonkar et al. (37)
		0.1–73 (26)	>5 (28)	(23) Gathirua-Mwangi et al. (38)
		0.6–33.6 (29)	$5.1 + 5.3$ (29)	(24) Guo et al. (39)
		>10 (30)	>3 (31)	(25) Nelson et al. (40)
			2.6 (32)	(26) Rodriguez-Gil et al. (41)
				(27) Sabiston et al. (42)
Colon and Rectum	- Higher hsCRP associates with higher risk for colon but not rectal cancer (33) - Conventional CRP levels associate with colorectal cancer mortality (36) - CRP gene rs1205 polymorphism was not associated with the risk of colorectal cancer (35)	>10 (36,38)	1.1–5.6 (33)	(28) Sickling et al. (43)
		30 (37)	1–4.7 (33)	(29) Thomson et al. (44)
		0–196 (37)	2.69 (34)	(30) Villaseñor et al. (45)
		0.01–22.8 (41)	1.97 (34)	(31) Wang et al. (46)
			<2.1 (36)	(32) Zhang et al. (47)
			2.2–5.0 (36)	(33) Aleksandrova et al. (48)
			5.1–10 (36)	(34) Erlinger et al. (49)
			3 (39)	(35) Fang and Ye (50)
			4.1 ± 3.2 (39)	(36) Goyal et al. (51)
			1–10 (39,42,43)	(37) Holm et al. (52)
Esophagus	Elevated serum CRP was associated with poor overall survival (44–47)		1.07–4.36 (40)	(38) Ishizuka et al. (53)
			2.86–5.20 (40)	(39) Lumachi et al. (54)
			0.6 (41)	(40) Nimptsch et al. (55)
			≤ 10 (44)	(41) Shibutani et al. (56)
Gastrointestinal Tract	- Elevated conventional CRP is associated with progressive disease and advanced stage disease and correlates with worse survival (49) - Higher conventional CRP levels correlate with advanced stage metastatic cancer (50) - Post-operative levels are useful to monitor infections (51)	17 (48)	<3 (49)	(42) Toyama et al. (57)
		0.08–231.7 (49)	1.6 (49)	(43) Zhou et al. (58)
		≥ 10 (50,52)		(44) Badakhshi et al. (59)
		177 (51)		(45) Huang et al. (60)
				(46) Katano et al. (61)
				(47) Zheng et al. (62)

(Continued)

TABLE 1 | Continued

Tissue affected by cancer	Notes and generalized conclusions	CRP levels reported*		References**
		Conventional CRP ($\geq 10 \mu\text{g/ml}$)	hsCRP (1–10 $\mu\text{g/ml}$)	
Head and Neck	- Pre-operative levels are prognostic of oral cancer (55) - Elevated levels associate with worse prognosis (53) - CRP may contribute to tongue squamous cell carcinoma (56) - CRP levels associate with perceived pain and inflammatory process (57)	<20 (57)	2–8 (53) <10 (54) ≥ 5 (55)	(53) Fang et al. (68) (54) He et al. (69) (55) Tai et al. (70) (56) Du et al. (71) (57) Oliveira et al. (72)
Liver	- CRP levels correlate with hepatocellular carcinoma aggressiveness (58) - Elevated CRP correlates with poor prognosis and is useful in staging (59)	10–50 (58) >50 (58) ≥ 10 (59)	<10 (58)	(58) Carr et al. (73) (59) Kinoshita et al. (74)
Reproductive Organs	Elevated CRP associates with increased risk and survival	36 $\pm$ 48 (60)	≤ 10 (61,68,69)	(60) Heffler et al. (75)
• Ovary	Higher Levels are measured in platinum-resistant ovarian tumors (68)	60 $\pm$ 66 (60)	1.24 $\pm$ 2.94 (65)	(61) Li et al. (76)
• Prostate	- CRP/Albumin ratios are prognostic (69) - Pre-diagnostic hsCRP levels are not predictive of risk (65) - CRP levels are not as predictive in clinically localized prostate cancer compared to advanced disease (66) - High levels associate with metastasis and worse survival (64)	28 $\pm$ 38 (60) >10 (61; 62) <12 (63) 0.02–29.9 (64)	<4 (66) ≥ 8.6 (67)	(62) Lundin et al. (77) (63) Graff et al. (78) (64) Liu et al. (79) (65) Platz et al. (80) (66) Schnoeller et al. (81) (67) Thurner et al. (82) (68) Xu et al. (83) (69) Liu et al. (84)
Pancreas	- Elevated levels associate with poor outcomes (70) - Neutrophil-lymphocyte ratios with CRP levels have prognostic value (71)	3–50 (70) 0.1–219 (71)	>4.5 (71) 3–10 (72)	(70) Chen et al. (85) (71) Inoue et al. (86) (72) Stevens et al. (87)
Kidney and Urinary tract	- CRP levels can predict mortality (73), treatment outcome and tumor recurrence in solid tumor renal cell carcinoma and digestive tumors [Shotriya et al. (15)] - Preoperative values are predictive of survival and end stage disease requiring hemodialysis (74) - Elevated preoperative levels correlate with aggressive tumor biology (76) - Elevated CRP/Alb ratio correlate with poor survival after or partial nephrectomy (78)	24.3 $\pm$ 50.1 (73) 1.5–15 (77)	<5 (74) <3 (75) <9 (76)	(73) Hsiao et al. (88) (74) Omae et al. (89) (75) Teishima et al. (90) (76) Aziz et al. (91) (77) Dai et al. (92) (78) Guo et al. (93)
Blood	- Higher CRP levels are a poor prognostic indicator in large B-cell lymphoma (79) - Measuring CRP has value in melanoma (80)	<15 (79)	<10 (80)	(79) Troppan et al. (94) (80) Fang et al. (95)
Sarcomas	Increased preoperative CRP is prognostic of poorer outcomes in bone cancer (82) and Soft tissue sarcoma (84)	43 (85) 0.1–342 (84) ≥ 8 (81, 82) 0.1–34.2 (83)		(81) Fang et al. (96) (82) Li et al. (97) (83) Nakamura et al. (98); Nakamura et al. (99) (84) Wang et al. (100) (85) Yanagisawa et al. (101)
Adult Solid Tumors: Renal Cell Solid Carcinoma & Digestive Solid Tumors	CRP as a predictor of prognosis, treatment outcome or tumor recurrence (86)	>10, >35, >50, >150 (86)	<1 – >9.8 (86)	(86) Shrotriya et al. (15)

*Conventional CRP levels ($\geq 10 \mu\text{g/ml}$) are summarized separately from high sensitivity levels (hsCRP: < 10 $\mu\text{g/ml}$) to help differentiate FDA defined diagnostic relevance from putative microinflammatory responses suggested by reported levels of hsCRP.

**The parenthetical number assigned to each reference is used to indicate where the CRP and hsCRP values, notes and general conclusions are derived within this table.

CRP as a prognostic index. Reports of CRP level in relationship to blood albumin level (i.e. the CRP/albumin ratio) as a novel prognostic index for survival for cervical cancer have appeared (64). In addition, the prognostic value of CRP in relationship to the neutrophil/lymphocyte ratio has also been proposed. Since CRP levels change rapidly during disease, some studies describe a “maximal CRP level” as a relevant diagnostic index. However, such values must be evaluated with reference to how prolonged cancer disease extends, the treatments used during disease, and the levels measured both before and after any surgical intervention.

Nonetheless, these maximal CRP values have been discussed as one criterion for assessing the extent of disease and the prognosis for recovery (27, 102).

Another complicating factor in interpreting the diagnostic and therapeutic value of CRP in cancer disease is the current focus on the value and significance of “high sensitivity CRP” levels (i.e. hsCRP). Baseline levels of CRP in purportedly healthy individuals are generally described as being <1–2 $\mu\text{g/ml}$. Some studies reviewed and included in reports summarized in **Table 1** describe and differently interpret cohort groups with hsCRP

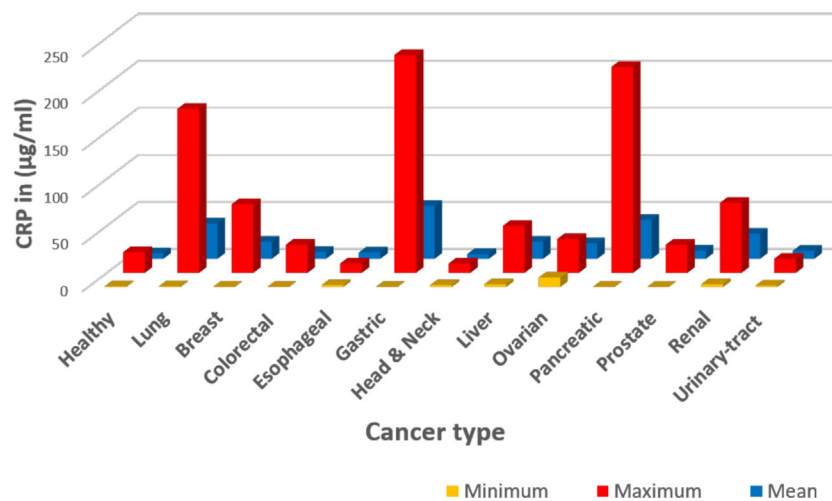


FIGURE 1 | Graphic representation of data summarized in **Table 1**. The CRP values were extracted from published references as detailed in **Table 1**. In this presentation, data were tabulated in Microsoft Excel based on cancer type, then the Excel functions were used in calculations: Minimum describes the lowest reported level; Maximum describes the highest reported level; and Mean describes the average of the reported values. One limitation of the reported data summarized here is the lack of specific clinical conditions ongoing when (or how frequent) CRP values were collected and measured.

levels between 1–3 and >3 µg/ml. While FDA offers no guidance on the diagnostic significance of such values in any disease, including cancer, it is important to note that numerous reports have appeared that associate baseline hsCRP levels more generally with populations of individuals grouped by gender, age, ethnicity, degree of fitness, and obesity. Indeed, with sensitive assays for CRP measurement now readily available, including point-of-care measurements where CRP values can be generated from a finger stick drop of blood in minutes, thousands of reports have appeared reporting that slightly elevated hsCRP levels are reflective of increased risk for developing and exacerbating cancerous growth (see **Table 1** for a list of references that discuss hsCRP in the defined cancers).

Table 1 offers one literature overview summarizing reported CRP blood levels as a function of cancer type, conventional CRP levels reported (i.e. CRP levels ≥ 10 µg/ml), high sensitivity CRP levels reported [hsCRP levels reported (< 10 µg/ml)] and stage of cancer disease and/or complications. A graphic presentation of reported CRP maximum, minimum and mean (average) values as a function of cancer type is presented in **Figure 1**. A simplified interpretation of all the data included in **Table 1** is presented in **Table 2**.

CRP—A MARKER OF TISSUE DAMAGE FIRST AND INFLAMMATION SECOND

Systemic CRP exists as a pentameric structure (pCRP) made up of five subunits, each containing calcium dependent bindings sites that interact with exposed phosphocholine ligand (PC) which can be expressed on activated plasma membrane (103). Exposure of PC groups requires phospholipid remodeling such

as occurs with Phospholipase A2 activity or oxidation of acyl chains (104). When pCRP binds to membrane anchored PC, juxtaposed apolar bonding energies contribute to the dissociation of the pentameric isoform into the modified, monomeric isoform (i.e. mCRP) which undergoes structural rearrangement to express a cholesterol binding site as is found in lipid rafts (**Figure 2**) (107, 110).

Since the appearance and concentration of pCRP in blood is not specific for cancer types, tissue locations, or stages of disease, and since its appearance correlates with an ongoing inflammatory response, what is the common denominator that triggers this protein to appear? One reflective focus involves evaluating how CRP may affect the fibrinolytic-like responses that are recognized as hallmarks of cancer growth. Indeed, cancer has been described as “a wound that never heals” (111, 112); any discussion of cancer growth and regulation must, therefore, include an understanding of the structure and function of the extracellular matrix and connective tissues within which the cancers are found. As tumors grow, endothelial cells become activated to allow platelets, neutrophils, and blood proteins (e.g. CRP) to enter tissues as part of normal protective inflammatory response. The goal of this early response is to help control the extent of disease growth and return the tissues to healthy homeostasis. Possible pathways by which CRP may participate in this host defense response include its binding reactivity with 1.) PC ligands which become accessible on stimulated endothelial cell membranes (113, 114), 2.) fibronectin (115–117), 3.) laminin (118), and 4.) collagen (107). CRP is also known to activate and regulate complement activity and bind immune complexes (119). Many reports also detail the interactions of CRP with endothelial cells (119), platelets (120), neutrophils (121), monocytes/macrophages (117, 122), epithelial cells (123), and fibroblasts (124).

TABLE 2 | Most consistent interpretations of the diagnostic significance of CRP in cancer.

1. Plasma CRP is not selective for any cancer type or tissue involving cancer.
2. Elevated CRP levels ($> 10 \mu\text{g/ml}$) are associated with active, advanced cancer disease.
3. Elevated CRP levels ($> 10 \mu\text{g/ml}$) can be diagnostic of complicating pathologies (e.g. infections).
4. Significantly, elevated CRP levels (above $50\text{--}100 \mu\text{g/ml}$) are associated with advanced disease, metastasis, and poor response prognosis.
5. The significance of hsCRP levels in cancer is not yet known and have no proven value.
6. Higher conventional CRP levels may be predictive of resistance to certain chemotherapeutic treatment (e.g. platinum resistance in ovarian cancer).
7. Elevated CRP is noted in and associated with aggressive hepatocellular carcinoma.
8. Any interpretation of the diagnostic significance of CRP requires consideration of when CRP levels are measured in relation to disease activity (i.e. quiescence or rapid growth phase) and in relation to therapeutic treatments and the response to such treatments.

For many decades, the exact role for CRP in the host defense APR remained undefined and controversial as different groups studying similar systems came to opposite conclusions. More recently, as CRP has been shown to exist in more than just a serum soluble cyclic pentamer disc configuration, it is now apparent that the effects of CRP on cell behavior and the tissue microenvironment are clearly dependent on its structural conformation. The highly soluble circulating pentameric CRP (pCRP) binds to PC on the cell surface (e.g., endothelial cells activated by inflammatory signals), which initiates the dissociation of pCRP into its distinct modified, monomeric

isoform (mCRP) which has notably reduced aqueous solubility. mCRP will self-aggregate and deposit in tissues or will internalize into plasma membranes and bind cholesterol. Cells activated by mCRP are known to stimulate intracellular signaling pathways, including activating the NF κ B transcription factor which propagates intracellular effects known as hallmarks of inflammation (125). Careful comparison studies have now established that mCRP (rather than pCRP) can interact with integral extracellular matrix (ECM) proteins (e.g., fibronectin, collagen) (98). With the awareness and understanding of both the pCRP and mCRP isoforms (including an understanding of how mCRP can be derived from pCRP), and the different roles each has on both cellular and tissue based components involved in acute inflammatory responses occurring in cancer, a consistent role for CRP as a diagnostic marker becomes apparent. A summary of the interactions of CRP with cell types and components of the extracellular environment is summarized in **Figure 3**.

The mCRP isoform can be formed from the pCRP isoform when membranes are activated and cellular responses are stimulated as occurs when tissue are damaged by any means (e.g. trauma, disease, cancerous growth). Once mCRP is formed, it will not reform pCRP; mCRP is readily degraded by proteolytic enzymes and peptides formed by its degradation feedback inhibit many of the acute phase responses stimulated by intact mCRP (126–129). In the earliest minutes of the APR, the rate of conversion of pCRP to mCRP is rapid. Over time, however (hours to days) the rate of conversion of pCRP to mCRP diminishes over time, resulting in quantifiable increases of

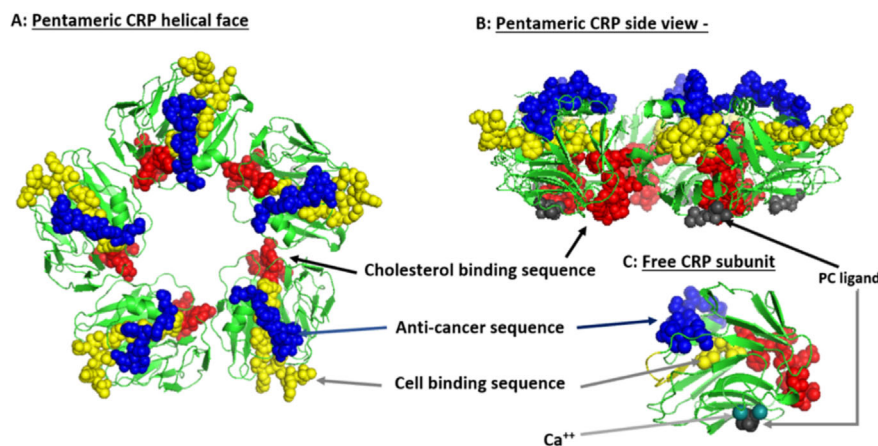


FIGURE 2 | Structural features of serum-soluble pentameric CRP. **(A)** illustrates the location and orientation peptide sequences in CRP reported to have cell-binding activity (shown in yellow and involving $_{27}\text{TKPLKAFVCLH}_{38}$) (105), anti-cancer activity (shown in blue and involving $_{176}\text{LGPFSPNVL}_{186}$) (106), cholesterol binding activity (shown in red and involving $_{35}\text{VCLHFYELSSSTR}_{47}$), and which also controls CRP binding to apolipoprotein B, C1q, fibronectin, and collagen (107), in relationship to the phosphocholine (PC) binding face (PC groups shown in gray and involving residues L_{64} , F_{66} , and T_{76}) and bound calcium ions (two per subunit, juxtaposed to each PC binding sites and involves residue E_{147}) (PDB code: 1B09; PC and calcium residues as defined by Thompson et al. (108) and Shrive et al. (109), respectively). **(B)** illustrates the orientation of these same residues when the discoid protein is laid flat (i.e. side view). **(C)** shows the orientation of same sequences on the isolated pCRP subunit (note: the exact orientation of these residues on the conformationally changed mCRP subunit has not been determined). The PC ligand binds in a shallow binding pocket controlled by calcium ions, with all PC sites on one face of the flattened discoid structure. The cholesterol binding sequences are near the PC binding sites so that when pCRP binds membrane associated PC, the cholesterol binding sequence is brought into proximity with intra-membraneous cholesterol (in lipid rafts) contributing to the conversion of pCRP into mCRP. The cell binding and anti-cancer peptides are oriented on the opposite face of the discoid protein where they can interact with effector leukocytes and activated inflammatory responses.

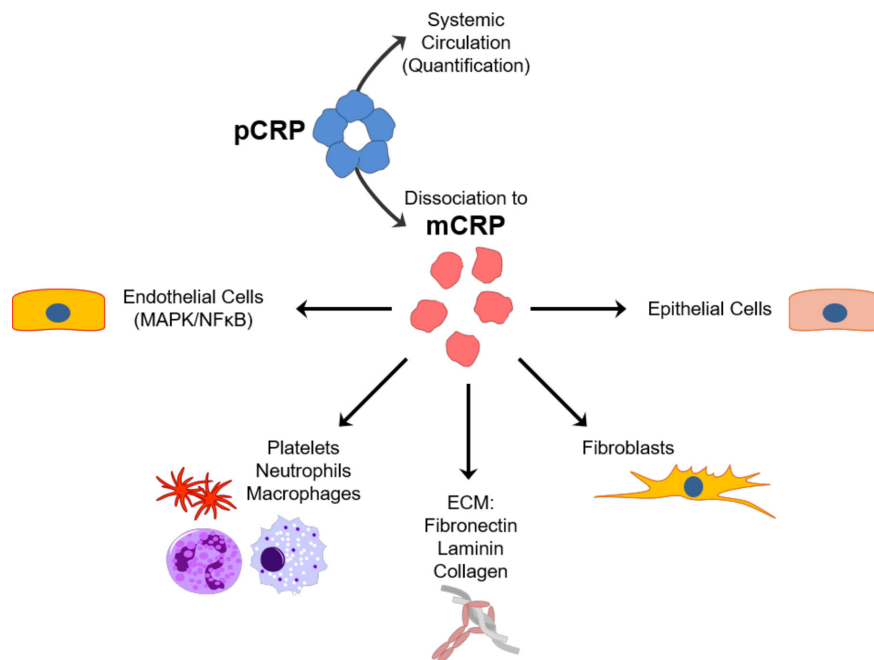


FIGURE 3 | Schematic representation of the predominant interactions of pCRP and mCRP isoforms. Pentameric CRP (pCRP) released from hepatocytes due to inflammation circulates through the systemic vasculature and serves as the pool of quantifiable CRP that is used in diagnostic testing. pCRP, however, once dissociated to monomeric CRP (mCRP) at lipid rafts of cells involved in inflammatory responses instead is highly biologically active. mCRP in turn interacts with a number of different cell types at the sites of inflammation, including endothelial cells, epithelial cells, fibroblasts and immune cells (platelets, neutrophils, macrophages) as well as components of the extracellular matrix (ECM) such as fibronectin, laminin and collagen.

pCRP in blood [recently reviewed in (130)]. If any injury persists and inflammatory mediators (such as IL-6 and IL-1 β) that signal hepatocytes to continue to synthesize acute phase proteins, quantifiable increases in plasma levels of pCRP will result. Increased levels of pCRP, therefore, suggest less pCRP is converted into mCRP. Since mCRP is a potent amplifier of the acute inflammatory response (110, 120, 131–133), any condition that limits its expression will cause a reduction in natural host defense responses. In the case of advancing tumor growth, this would lead to chronic inflammatory signaling that potentiates the “wound that never heals”. As affected tissues are still producing signals that direct the synthesis and release of pCRP, higher blood levels of pCRP are reflective of the persistence and severity of tissue damage associated with cancer growth and progression.

In line with this, CRP levels in blood do indeed correlate with the degree of inflammatory tissue involvement. In soft tissue sarcomas, CRP levels were associated with the degree of tumor infiltration determined by magnetic resonance imaging (MRI) as well as disease-specific survival (99). Similarly, tissue pathologies associated with COVID-19 disease complications identified by computerized tomography (CT) technology were also significantly associated with CRP levels and, importantly, CRP had high sensitivity and specificity to predict severity of the disease (134).

Using this tissue-based perspective as a common denominator, readers are encouraged to interpret CRP diagnostic levels in any clinical situation by first focusing on alterations in tissue structures

and second by assessing how it affects or regulates the inflammation that ensues. A brief overview of the extracellular matrix structures and ways that CRP may interact with such structures during inflammatory responses to cancer disease is included below.

THE EXTRACELLULAR TISSUE MICROENVIRONMENT, ACUTE PHASE RESPONSE, AND INFLAMMATION

In all animals, including humans, the first line of defense against disease is the structural connective tissue that not only presents a barrier to pathogens and toxins, but also contributes an appropriate macromolecular matrix for coordinated biochemical and immunological host defense reactions. Connective tissues include fibrous proteins, various cells, amorphous ground substance (e.g. proteoglycans and glycosaminoglycans), plasma constituents, ions, and water. Connective tissue can be loose or dense, regular or irregular, fibrous or elastic depending on the types of proteins and polysaccharides that are secreted locally or accumulate in the specific space filled by the connective tissue. The organization and interactions of the components, not only within the framework space but also at boundaries and surfaces, define the physical properties and function of each connective tissue. In tissues as wide ranging as skin and bone, connective tissues form an architectural framework (i.e. the extracellular matrix or ECM) that

serves not only as an inert space-filling scaffold, but as a physical structure that plays a dynamic role in organizing and regulating the physiological responses that occur within each tissue. While providing mechanical structure through the deposition of numerous molecules (e.g., collagens, laminins), the ECM also acts as an intermediary space in which growth factors, cytokines, metabolites, and other secreted factors can communicate between cells within tissue compartments (135–137).

When connective tissue is injured either by incision, accident, xenobiotic stress or disease, the APR is activated to stimulate innate host defenses to the injury and the wound healing process so to efficiently and effectively repair the injured tissue (138). These processes must work in concert with hematological and immunological mechanisms that are triggered to defend the body from anything that might threaten the homeostasis affecting not only localized tissues, but of the entire organism as well. The APR reaction involves the initial production of signals locally (e.g., IL-6, IL-1 β) at the site of injury, which, once secreted into the extracellular milieu, can have extensive physiological effects on cells within the local tissue or act systemically to promote production of acute phase proteins, such as hepatic CRP (138). This frequent early event during wound repair is characterized by such inflammatory responses that in turn facilitate the rapid recruitment and activation of immune cells, such as neutrophils and other leukocytes. Neutrophils migrate into the wound and scavenge for debris and foreign matter that must be removed. In uncomplicated repair, the movement of neutrophils into a wound is transient and resolved prior to the in-growth of vascular tissue (i.e. granulation tissue). If foreign materials are present, the neutrophil response may persist and thus complicate the wound healing process by having the conflicting processes of removal (scavenging) and repair occurring simultaneously. In such an event, the wound healing process is compromised and can lead to a greater amount of scar tissue and regenerated tissue that has only a percentage of the original tissue's functional activity (139, 140).

Depending on the extent of separation of the edges of the wound, healing can begin from the sides inward, or from the base upward. During the first few days of wound repair, epithelial cells and fibroblasts migrate across the wound surface into the regenerating tissue where they proliferate and differentiate. Such cells are specialized for the synthesis and secretion of the ECM substances and are fundamental to the architectural repair of the tissue framework. Fibroblasts are known to be very versatile cells that can reversibly transform into highly differentiated cells required for the connective tissue within which they are found. Differentiated cells secrete the types of collagen and ground substances needed for the repair and regeneration of that tissue.

INFLAMMATION CAN INDUCE CANCEROUS GROWTH

Physiological inflammation that occurs during wound healing involves many of the processes associated with *de novo* tumor development as well as mechanisms that endow cancers to metastasize (141–143). The exact directionality of whether

inflammation causes carcinogenic processes, or that tumor cells induce local inflammatory responses to facilitate their rapid growth and dissemination is unclear. Indeed, it is likely that there are reciprocal interactions between the host and tumor mediated through inflammatory processes that promote tumor initiation and progression. Overall, the current literature suggests that a lack of resolution to inflammation leads to chronic inflammatory signaling that is intimately linked to promoting the development and progression of cancer.

Tissues involved with cancerous growth have long been known to involve inflammation (111, 141). Mechanistic studies have demonstrated that components of inflammation, such as reactive oxygen species (ROS) and growth factors, can promote both the initiation of neoplastic cells and their proliferation (142, 144). While ROS and proteolytic enzymes produced by neutrophils and macrophages are key contributors to a favorable immune response to the stressed tissue, both superoxide anion and hydrogen peroxide have been shown to induce DNA damage that can contribute to mutagenesis, potentially giving rise to neoplastic cells (145). Unresolved (chronic) inflammation, therefore, would not only prolong immune infiltration at the site of injury, but could promote secondary genetic mutations that could exacerbate malignant conversion of otherwise benign neoplastic cells.

Chronic inflammation could also contribute to pro-tumorigenic processes by increased secretions of growth factors (e.g., PDGF, TGF β) that promote rapid cell proliferation, as well as cytokines that stimulate cell motility (146). Such inflammatory signals have been observed to induce tumor cell epithelial-to-mesenchymal transition (EMT), a process in which epithelial cancer cells dedifferentiate and adopt a fibroblast-like phenotype to promote rapid growth and enhance pro-metastatic signaling (147, 148), in multiple cancer types. Importantly, this mesenchymal phenotype promotes the production of metalloproteinases to breakdown collagen IV and other ECM proteins to facilitate tumor cell invasion through basement membrane (149) and trans-endothelial migration as cancer cells disseminate into tissues (150, 151). EMT is also known to be associated with upregulated secretion of cytokines and chemokines that allow cancer cells to reprogram surrounding stromal cells to provide a conducive environment for growth and metastasis (152). These data suggest that continuous (chronic) inflammatory responses in any tissue could promote the development of *de novo* malignancies and enhancement of the capacity for tumor cells to metastasize (153). Moreover, inflammatory signaling from the developing tumor could also act systemically to promote acute phase protein production (e.g., hepatic CRP) and provide a positive feedback loop to potentiate this pro-metastatic inflammatory environment (Figure 4).

CANCER CAN INDUCE AN INFLAMMATORY RESPONSE

Tumors that establish and grow in a tissue environment will induce an inflammatory response that will involve secretion of

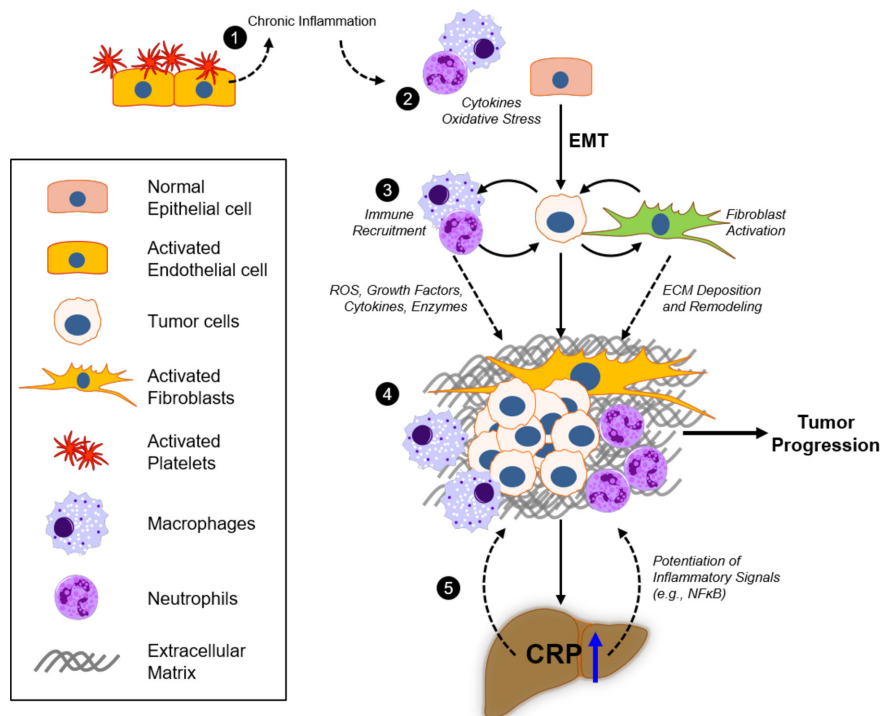


FIGURE 4 | Inflammatory responses of CRP in the extracellular matrix and tumor microenvironment. 1) Platelet recruitment to damaged tissue and fibrin accumulation represent acute phase inflammatory responses that, if injury remains unresolved, will contribute to excessive chronic immunoreactivity. 2) Continuous oxidative stress (reactive oxygen species; ROS) and cytokine production by activated macrophages and neutrophils promote tumorigenicity in epithelial cells, which can promote epithelial-to-mesenchymal transition (EMT) as a result. 3) Deposition of the extracellular matrix (ECM) components, including fibronectin, collagen, laminin, and fibrin, in the tumor microenvironment (TME) by fibroblasts and activated immune cells modulate tumor cell proliferation and invasion. 4) Bidirectional crosstalk in the TME promotes further proliferation of tumor and stromal cells, as well as deposition and remodeling of ECM to promote tumor growth and motility. 5) Excessive cytokine release (e.g., IL-6) from the TME increases systemic circulating levels that promote hepatocyte pCRP production. pCRP secretion and subsequent mCRP-dependent inflammatory signaling (e.g., in involved endothelial cells and neutrophils), as well as its direct action on the ECM, contribute to tumor progression through ROS and cytokine signaling in the TME.

chemokines that promote immune recruitment (154). Tumor cells will also exploit signaling pathways of localized cells that, under non-cancerous situations would promote fibrosis and tissue repair, in order to foster rapid growth and metastatic potential of the growing tumor mass (149, 155, 156). Under normal physiologic conditions, tissue repair occurs in several phases in response to mechanical injury, infection, or irritation from xenobiotics (156–158). Epithelial or endothelial injury stimulates platelet aggregation and subsequent recruitment of neutrophils and mononuclear cells to the site of injury is then followed by the activation and differentiation of monocytes to polarized macrophages, which secrete growth factors and cytokines to facilitate wound healing through stimulating migration and activation of fibroblasts (159). Activated fibroblasts (myofibroblasts) in turn deposit collagen and remodel the extracellular matrix (160), and in concert with immune cells, promote fibrosis and the resulting formation of granulation tissue to resolve tissue damage.

The tumor microenvironment (TME) has been shown to promote tumor cell proliferation, migration, invasion and intravasation (141, 161, 162) through metabolite and cytokine

secretion (163) and production of chemokines involved in immune recruitment (154, 164). Further, the TME favors differentiation of naïve monocytes to M2 macrophages which mediate fibrotic-like responses that facilitate tumor progression (165). Tumor-associated macrophages, in concert with activated fibroblasts and other stromal cell types in the TME, secrete laminin, and collagen to promote tumor cell motility as well as fibronectin, a key modulator of integrin-dependent adhesion and invasion (166–168). As observed in multiple cancers, activated stromal cells in the TME notably induce tumor cell EMT to promote not only proliferation, but also key metastatic processes, such as adhesion, migration, invasion, and colonization (163, 169, 170). Thus, the interplay between tissue structure/function and activated inflammatory responses may contribute to both protection from and exacerbation of disease.

Tumor cells that have undergone EMT secrete a number of growth factors observed during physiologic wound healing, including PDGF (171, 172), TGF β 1 (173), and fibrinogen (174), which are associated with immune cell granulation and enhanced tumor growth. Moreover, mesenchymal-like tumor cells and stromal cells common to the TME have been shown to

also secrete cytokines involved in the synthesis of CRP [IL-1 β (175)] as well as its secretion [IL-6 (176)], indicating a potential for systemic reaction to the progressing tumor. Locally, this crosstalk by these inflammatory mediators between tumor cells and their microenvironment promotes processes involved in metastasis *in vitro* (163, 169, 177), and markedly enhance the success of tumor growth and metastatic implantation *in vivo* (178, 179). Taken together, these studies indicate that progressing tumors enhance their development and metastasis in part through processes involved in wound healing and pro-inflammatory signaling, suggesting that cancer cell induced inflammation promotes tumor progression and thus disease severity.

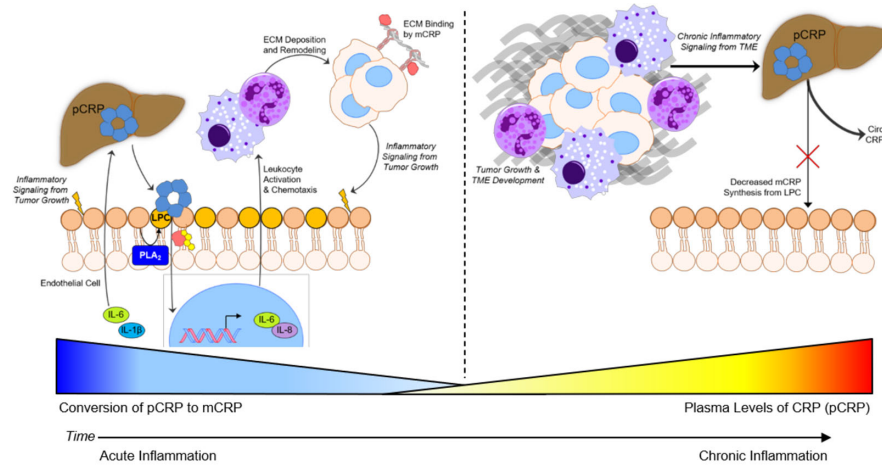
The structural support for the regenerating tissue (e.g. basement membranes, non-damaged adjacent tissue) and the connections to the supporting structure tissues are important factors in the effective repair of wounded tissue. Cells growing into the matrix both influence and are influenced by the macromolecules found in the tissue. This influence is mediated within a cell through the intracellular cytoskeleton, composed primarily of actin, intermediate filament, and microtubule proteins. The cytoskeleton helps orient and organize cells within the tissue matrix for optimized function. Connections between the intracellular spaces and the extracellular matrix provide for dynamic and active interactions. Such connections must be reestablished as part of the wound healing process as cells migrate into a wound to regenerate functional, healthy tissues (136, 180–185).

PROPOSED SIGNIFICANCE OF CRP AS A BIOMARKER AND AS A BIOLOGICAL RESPONSE MODIFIER IN CANCER DISEASE

The clinical studies assessing CRP levels in cancer reviewed above, in light of preclinical data regarding the molecular activity of CRP and its distinctive isoforms in the inflammatory microenvironment, may provide invaluable insight into the contribution of CRP to disease progression of cancers. Moreover, the unique biological activity of mCRP or pCRP could help elaborate the clinical interpretations of CRP levels in patients suffering from cancer, thereby presenting CRP as a potential non-invasive technique to assess the severity of tumor development or progression. In the TME the potential exists for CRP to resolve an inflammatory environment through stimulating retention of monocytes by direct binding to fibronectin (117), which could aid in resolution of a pro-inflammatory (pro-tumorigenic) signaling milieu. Similarly, its ability to limit neutrophil chemotaxis through inhibition of IL-8-dependent migration (121) may also prevent an exacerbation of an inflammatory TME conducive for tumor growth. Conversely, a number of studies suggest the possibility for CRP to positively stimulate leukocyte production of cytokines such as IL-8 and MCP-1 [reviewed in (119)]. Depending on the

broader context of stromal cells within the TME, this could indicate that CRP is either 1) enhancing cytotoxic T lymphocyte recruitment and subsequent tumor cell lysis, or 2) prolonging immune recruitment which could potentially lead to a sustained pro-inflammatory, and thus pro-tumorigenic, microenvironment. Therefore, while CRP may facilitate leukocyte retention and subsequent tumor cell lysis early in tumor development, an unresolved neoplastic growth may result in persistent signaling that potentiates both hepatic CRP production and excessive local inflammation, similar to what is observed in other pathologies that fail to resolve following injury and in line with the putative role of CRP in inflammation. While there is tremendous overlap in the matrix characteristics and signaling processes observed in both inflamed tissues and developing tumors, more direct measurement of the physiologic activity of CRP in the TME is both warranted and necessary. Assessing the capacity for CRP to regulate immune cell phenotypes, as well as its ability to modulate behavior of other stromal cells and tumor cells in the TME, can only be accomplished using *in vivo* studies or sophisticated 3D organotypic models (e.g., organoid cultures) to recapitulate the specific TME. Through these methods it may be possible to fully dissect the impact of CRP on the interactions between tumor and stromal compartments in order to assess its role in tumor development and metastasis.

The emerging relevance of the functional states of CRP isoforms suggest a complex relationship between its response in early inflammation related to *de novo* tumorigenesis and more advanced disease. The activity of mCRP in acute phase response illustrates its tremendous overlap in requisite components and signaling mechanisms of an actively developing tumor milieu. Further, given the observations that systemic levels of both IL-6 and IL-1 β are elevated in multiple advanced cancers (186, 187), it is conceivable that the evolving tumor and its microenvironment may contribute to an exacerbation of CRP *de novo* synthesis and continuous secretion, potentially in excess of a slowing rate of conversion to mCRP that results in a demonstrable (and quantifiably significant) increase in systemic pCRP levels (Figure 5). This rise in pCRP, and indeed what has been measured in traditional clinical assessments, is thus more likely representative of continued tissue damage resulting from persistent development of neoplasms *in situ*. However, whether the rate of conversion of pCRP to mCRP during tumorigenesis or metastatic progression is like that observed in other inflammatory diseases remains unknown and requires exhaustive investigation. These potential relationships merit further preclinical assessment of the activity of mCRP in the growing tumor microenvironment and early metastatic niches of cancers *in vivo* to inform the exact nature of this inflammatory mediator and the significance of pCRP plasma levels throughout disease progression. Moreover, the recent advances in quantifying mCRP through enzyme-linked assays present a potential way forward for not only identifying the significance of mCRP as a diagnostic marker during disease progression *per se* (188), but could also be adapted to evaluate the molecular role of mCRP in cell-cell communication in the TME. Importantly,



- Step 1:** Blood vessels are perturbed by the growth of cancer cells – endothelial cells (ECs) get activated and form lyso-phosphocholine (LPC) via phospholipase A2 (PLA₂) intramembrane activity
- Step 2:** EC's secrete IL-6 and IL-1 β to send a signal to hepatocytes to release pCRP and to start translating and releasing more
- Step 3:** pCRP binds to activated ECs on PC and pCRP enters into tissues where it interacts with ECM components
- Step 4:** On activated ECs, pCRP undergoes conformational change to produce mCRP which enters into cholesterol-rich lipid rafts
- Step 5:** mCRP coordinates and amplifies platelet, neutrophil and monocyte/macrophage activities
- Step 6:** In the ECM – mCRP can bind to fibronectin, laminin and collagen affecting how the ECM and the host defense response reacts to the growing cancer cells
- Step 7:** As the ECs deactivate, LPCs are no longer produced and thus pCRP binding to membranes is inhibited, resulting in decreased conversion to mCRP; the level of pCRP in blood then rises
- Step 8:** As pCRP levels increase, the APR subsides; however, chronic underlying inflammatory signaling can lead to continued tissue damage, cancer cell propagation, and unresolved disease

FIGURE 5 | Schematic relationship between pCRP and mCRP as a function of inflammation in cancer.

TABLE 3 | Proposed diagnostic significance of CRP as a marker of inflammation associated with tissue damage.

- CRP blood levels (which only measure the soluble pentameric isoform) should be interpreted as a diagnostic index of tissue health and homeostasis rather than inflammation.
- Baseline levels of CRP in health, in controlled disease or in disease remission will be < 10 $\mu\text{g/ml}$. Levels closer to 1–3 $\mu\text{g/ml}$ are better indicators of good health and control of disease.
- As tissues become involved with rapidly growing cancers, hepatic production and release of pCRP increase proportionate to the level by which tissues are affected/damaged by the growing tumors.
- Even though stores of CRP are immediately released, there is a lag in quantifying pCRP in blood as it undergoes conformational rearrangement and enters membrane lipid rafts and activates potent pro-inflammatory signaling pathways.
- Conformationally-altered CRP is rapidly consumed (proteolyzed); peptides released regulate biofeedback to down-regulate the acute inflammatory response.
- When the rate of conformational rearrangement slows, pCRP levels measured in blood increase.
- If tissues remain damaged by unresolved disease, blood levels of pCRP will remain elevated.
- Elevated pCRP blood levels indicate that a weakened inflammatory response persists which may be insufficient to remove cancerous cells and restore tissue homeostasis.
- pCRP levels above 10 $\mu\text{g/ml}$ climbing above 50–100 $\mu\text{g/ml}$ are an index of the degree of ongoing tissue damage.
- pCRP levels may also indicate complicating pathologies (e.g. infections).
- pCRP values above 100 $\mu\text{g/ml}$ indicate extensive ongoing tissue damage and are consistent with poor prognosis for treatment response and survival.
- pCRP levels should be drawn at various times (weekly to monthly) to initially diagnose the presence and severity of primary disease, to assess response to treatment (over time), and to evaluate disease recurrence and prognosis.
- pCRP levels taken before and after surgical intervention may help diagnose the response to surgery and the reestablishment of tissue homeostasis.
- The significance of hsCRP levels (i.e. CRP levels < 10 $\mu\text{g/ml}$) is currently unknown.

further development of such assays that can reliably distinguish between mCRP, pCRP, or possibly membrane-bound CRP will be essential in addressing the limitation of the reviewed clinical studies in that they only measure conventional or hsCRP (**Table 1**), which does not allow for a deeper appreciation of the contextual biological activity of CRP. While the predominant utility of CRP as a biomarker has traditionally been as a non-invasive diagnostic, it may also be useful to measure CRP by immunohistochemical methods or in tumor explant lysates, especially in the evaluation of CRP under controlled conditions in *in vivo* xenograft experiments. In combination with molecular techniques to directly identify interactions of mCRP with tumor and stromal cells, as well as other components of the TME (e.g., ECM proteins), these methods provide approaches that may elucidate the exact impact of mCRP on tumor cell proliferation, migration, invasion, and chemoresistance.

Taking this diversity in the physiologic activity of CRP isoforms into account in the context of cancer may give further insight into the relationship between inflammation and cancer and, moreover, improve the clinical evaluation of cancer progression using this biomarker in patient assessment. Regardless, there are already several clinically important interpretations from the current preclinical and clinical data that may help refine assessment of CRP as a diagnostic tool in cancer, which are presented in **Table 3**. Importantly, the data suggest that pCRP levels exceeding 50–100 µg/ml indicates pervasive tissue damage and is associated with poor survival. In general, this may relate to the correlation of high CRP levels

and metastatic potential of many tumor types, as outlined in **Table 1**, and is in line with the concept that unresolved inflammation may drive tumor development as well as enhance dissemination and metastasis. This proposed utility of CRP levels to estimate cancer progression are in line with what has been described in assessing disease severity in a number of inflammatory diseases, including analysis of the recent SARS-CoV-2 viral infection (COVID-19) (189). Further evaluation of the role of CRP in cancer will undoubtedly improve its ability as a biomarker to indicate disease severity and progression more precisely, and thus may reveal it as an indispensable asset in clinical decision making.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version. IR and LP were responsible for the curation of the meta-analysis performed in the review of published works on CRP levels among different cancers.

REFERENCES

- Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* (2018) 68 (6):394–424. doi: 10.3322/caac.21492
- WHO: World Health Organization. *International Agency for Research on Cancer. Press release N° 263*. Geneva, Switzerland (2018). Available at: <https://www.who.int/cancer/PRGlobocanFinal.pdf> (Accessed on January 2nd, 2020). 12 September.
- Botta L, Dal Maso L, Guzzinati S, Panato C, Gatta G, Trama A, et al. Changes in life expectancy for cancer patients over time since diagnosis. *J Adv Res* (2019) 20:153–9. doi: 10.1016/j.jare.2019.07.002
- Gu X, Zheng R, Xia C, Zeng H, Zhang S, Zou X, et al. Interactions between life expectancy and the incidence and mortality rates of cancer in China: a population-based cluster analysis. *Cancer Commun* (2018) 38:44. doi: 10.1186/s40880-018-0308-x
- Cao B, Bray F, Beltrán-Sánchez H, Ginsburg O, Soneji S, Soerjomataram I. Benchmarking life expectancy and cancer mortality: global comparison with cardiovascular disease 1981–2010. *BMJ* (2017) 357:j2765. doi: 10.1136/bmj.j2765
- Allemani C, Weir HK, Carreira H, Harewood R, Spika D, Wang X-S, et al. Global surveillance of cancer survival 1995–2009: analysis of individual data for 25 676 887 patients from 279 population-based registries in 67 countries (CONCORD-2). *Lancet* (2015) 385:977–1010. doi: 10.1016/S0140-6736(14)62038-9
- Goldberg JL, Sondel PM. Enhancing Cancer Immunotherapy Via Activation of Innate Immunity. *Semin Oncol* (2015) 42(4):562–72. doi: 10.1053/j.seminoncol.2015.05.012
- Yarchoan M, Hopkins A, Jaffee E. Tumor mutational burden and response rate to PD-1 inhibition. *N Eng J Med* (2017) 377(25):2500–1. doi: 10.1056/NEJMc1713444
- Bonaventura P, Shekarian T, Alcazer V, Valladeau-Guilemond J, Valsesia-Wittmann S, Amigorena S, et al. Cold tumors: a therapeutic challenge for immunotherapy. *Front Immunol* (2019) 10:16. doi: 10.3389/fimmu.2019.0016
- Sharma P, Allison JP. Dissecting the mechanisms of immune checkpoint therapy. *Nat Rev Immunol* (2020) 20(2):75–6. doi: 10.1038/s41577-020-0275-8
- Intlekofer AM, Thompson CB. At the bench: preclinical rationale for CTLA-4 and PD-1 blockade as cancer immunotherapy. *J Leukoc Biol* (2013) 94 (1):25–39. doi: 10.1189/jlb.1212621
- Martinez-Lostao L, Anel A, Pardo J. How do cytotoxic lymphocytes kill cancer cells? *Clin Cancer Res* (2015) 21(22):5047–56. doi: 10.1158/1078-0432.CCR-15-0685
- Gonzalez H, Hagerline C, Werb Z. Roles of the immune system in cancer: from tumor initiation to metastatic progression. *Genes Dev* (2018) 32(19–20):1267–84. doi: 10.1101/gad.314617.118
- FDA. US. Department of Health and Human Services. Guidance for Industry and FDA Staff. Review Criteria for Assessment of C-reactive protein (CRP), High Sensitivity C-Reactive Protein (hsCRP) and Cardiac C-Reactive Protein (cCRP) Assays. Document issued on September 22, 2005 Content current as of: 03/13/2018.
- Shrotriya S, Walsh D, Bennani-Baiti N, Thomas S, Lorton C. C-Reactive Protein Is an Important Biomarker for Prognosis Tumor Recurrence and Treatment Response in Adult Solid Tumors: A Systematic Review. *PloS One* (2015) 10(12):e0143080. doi: 10.1371/journal.pone.0143080
- Alifano M, Falcoz PE, Seegers V, Roche N, Schussler O, Younes M, et al. Preresection serum C-reactive protein measurement and survival among patients with resectable non-small cell lung cancer. *J Thorac Cardiovasc Surg* (2011) 142:1161–7. doi: 10.1016/j.jtcvs.2011.07.021
- Aref H, Refaat S. CRP evaluation in non-small cell lung cancer. *Egyptian J Chest Dis Tuberculosis* (2014) 63:717–22. doi: 10.1016/j.ejcdt.2014.02.003

18. Bittoni MA, Focht BC, Clinton SK, Buckworth J, Harris RE. Prospective evaluation of C-reactive protein, smoking and lung cancer death in the Third National Health and Nutrition Examination Survey. *Int J Oncol* (2015) 47:1537–44. doi: 10.3892/ijo.2015.3141
19. Chaturvedi AK, Caporaso NE, Katki HA, Wong H-L, Chatterjee N, Pine SR, et al. C-Reactive Protein and Risk of Lung Cancer. *J Clin Oncol* (2010) 28 (16):2719–26. doi: 10.1200/JCO.2009.27.0454
20. Hara M, Matsuzaki Y, Shimizu T, Tomita M, Ayabe T, Enomoto Y, et al. Preoperative Serum C-reactive Protein Level in Non-small Cell Lung Cancer. *Anticancer Res* (2007) 27:3001–4.
21. Hara M, Yonei A, Ayabe T, Tomita M, Nakamura K, Onitsuka T. Postoperative serum C-Reactive Protein levels in non-small cell lung cancer patients. *Ann Thorac Cardiovasc Surg* (2010) 16:85–90.
22. Jin Y, Sun Y, Shi X, Zhao J, Shi L, Yu X. Prognostic value of circulating C-reactive protein levels in patients with non-small cell lung cancer: A systematic review with meta-analysis. *J Cancer Res Ther* (2014) 10(Special Issue 2):Suppl:C160–6. doi: 10.4103/0973-1482.145854
23. Jing X, Huang C, Zhou H, Li C, Fan L, Chen J, et al. Association between serum C-reactive protein value and prognosis of patients with non-small cell lung cancer: a meta-analysis. *Int J Clin Exp Med* (2015) 8(7):10633–9.
24. Koch A, Fohlin H, Sörenson S. Prognostic Significance of C-reactive protein and Smoking in Patients with Advanced Non-small Cell Lung Cancer Treated with First-Line Palliative Chemotherapy. *J Thoracic Oncol* (2009) 4(3):326–32. doi: 10.1097/JTO.0b013e31819578c8
25. Liao C, Yu Z, Guo W, Liu Q, Wu Y, Li Y, et al. Prognostic value of circulating inflammatory factors in non-small cell lung cancer: a systematic review and meta-analysis. *Cancer Biomark* (2014) 14(6):469–81. doi: 10.3233/CBM-140423
26. Muller DC, Larose TL, Hodge A, Guida F, Langhammer A, Grankvist K, et al. Circulating high sensitivity C reactive protein concentrations and risk of lung cancer: nested case-control study within Lung Cancer Cohort Consortium. *BMJ* (2018) 364:k4981. doi: 10.1136/bmj.k4981
27. Pastorino U, Morelli D, Leuzzi G, Gisabella M, Suatoni P, Taverna F, et al. Baseline and postoperative C-reactive protein levels predict mortality in operable lung cancer. *Eur J Cancer* (2017) 79:90–7. doi: 10.1016/j.jca.2017.03.020
28. Shinohara S, Otsuki R, Onitsuka T, Machida K, Matsuo M, Nakagawa M, et al. Postoperative C-reactive Protein Is a Predictive Biomarker for Survival After Non-small Cell Lung Cancer Resection. *Anticancer Res* (2019) 39:2193–8. doi: 10.21873/anticancer.13334
29. Sin DD, Man SFP, McWilliams A, Lam S. Progression of airway dysplasia and C - reactive protein in smokers at high risk of lung cancer. *Am J Respir Crit Care Med* (2006) 173:535–9. doi: 10.1164/rccm.200508-1305OC
30. Szturmowicz M, Rudziński P, Kacprzak A, Langfort R, Bestry I, Broniarek-Samson B, et al. Prognostic value of serum C-reactive protein (CRP) and cytokeratin 19 fragments (Cyfra 21-1) but not carcinoembryonic antigen (CEA) in surgically treated patients with non-small cell lung cancer. *Pneumonol Alergol Pol* (2014) 82:422–9. doi: 10.5603/PiAP.2014.0055
31. Tomita M, Shimizu T, Ayabe T, Onitsuka T. Elevated Preoperative Inflammatory Markers Based on Neutrophil-to-Lymphocyte Ratio and C-Reactive Protein Predict Poor Survival in Resected Non-small Cell Lung Cancer. *Anticancer Res* (2012) 32:3535–8.
32. Torrecilla JA, Scrimini S, Saulea J, García-Cosío FB, Noguera A, Iglesias A, et al. Role of C reactive protein in non-small cell lung cancer staging. *Eur Respir J* (2011) 38(Suppl 55):2806.
33. Vagulienė N, Žemaitis M, Miliuskas S, Urbonienė D, Šitkauskienė B, Sakalauskas R. Comparison of C-reactive Protein Levels in Patients with Lung Cancer and Chronic Obstructive Pulmonary Disease. *Medicina (Kaunas)* (2011) 47(8):421–7. doi: 10.3390/medicina47080059
34. Wei L, Du Y, Wu W, Li L. Changes of tumor markers and C reactive protein in different status of lung cancer. *Int J Clin Exp Pathol* (2016) 9 (11):11984–8.
35. Zhao Z, Li X, Zhao Y, Wang D, Li Y, Liu L, et al. Role of C-reactive protein and procalcitonin in discriminating between infectious fever and tumor fever in non-neutropenic lung cancer patients. *Medicine* (2018) 97:33 (e11930). doi: 10.1097/MD.00000000000011930
36. Allin KH, Nordestgaard BG, Flyger H, Bojesen SE. Elevated pre-treatment levels of plasma C-reactive protein are associated with poor prognosis after breast cancer: a cohort study. *Breast Cancer Res* (2011) 13:R55. doi: 10.1186/bcr2891
37. Asegaonkar SB, Takalkar UV, Kodlikeri P, Pagdhune A, Bonduliya V, Thorat AP. Serum high sensitivity C-reactive protein in breast cancer patients. *Int J Res Med Sci* (2014) 2(4):1408–11. doi: 10.5455/2320-6012.ijrms20141131
38. Gathirua-Mwangi WG, Song Y, Monahan P, Champion VL, Zollinger T. Associations of metabolic syndrome and C-reactive protein with mortality from total cancer, obesity-linked cancers and breast cancer among women in NHANES III. *Int J Cancer* (2018) 143(3):535–42. doi: 10.1002/ijc.31344
39. Guo L, Liu S, Zhang S, Chen Q, Zhang M, Quan P, et al. C-reactive protein and risk of breast cancer: A systematic review and meta-analysis. *Sci Rep* (2015) 5:10508. doi: 10.1038/srep10508
40. Nelson SH, Brasky TM, Patterson RE, Laughlin GA, Kritz-Silverstein D, Edwards BJ, et al. The association of the C-reactive protein inflammatory biomarker with breast cancer incidence and mortality in the Women's Health Initiative. *Cancer Epidemiol Biomarkers Prev* (2017) 26(7):1100–6. doi: 10.1158/1055-9965.EPI-16-1005
41. Rodriguez-Gil JL, Takita C, Wright J, Reis IM, Zhao W, Brian EL, et al. Inflammatory biomarker C-Reactive Protein and radiotherapy-induced early adverse skin reactions in breast cancer patients. *Cancer Epidemiol Biomarkers Prev* (2014) 23(9):1873–83. doi: 10.1158/1055-9965.EPI-14-0263
42. Sabiston CM, Wrosch C, Castonguay AL, Sylvester BD. Changes in physical activity behavior and C-reactive protein in breast cancer patients. *Ann Behav Med* (2018) 52:545–51. doi: 10.1093/abm/kax010
43. Sicking I, Edlund K, Wesbuer E, Weyer V, Battista MJ, Lebrecht A, et al. Prognostic Influence of pre-operative C-Reactive Protein in node-negative breast cancer patients. *PloS One* (2014) 9(10):e111306. doi: 10.1371/journal.pone.0111306
44. Thomson CA, Thompson PA, Wright-Bea J, Nardi E, Frey GR, Stopeck A. Metabolic Syndrome and Elevated C-Reactive Protein in Breast Cancer Survivors on Adjuvant Hormone Therapy. *J Women's Health* (2009) 18 (12):2041–7. doi: 10.1089/jwh.2009.1365
45. Villaseñor A, Flatt SW, Marinac C, Natarajan L, Pierce JP, Patterson RE. Postdiagnosis C - reactive protein and Breast Cancer Survivorship: Findings from the WHEL Study. *Cancer Epidemiol Biomarkers Prev* (2013) 23(1):189–99. doi: 10.1158/1055-9965.EPI-13-0852
46. Wang J, Lee IM, Tworoger SS, Buring JE, Ridker PM, Rosner B, et al. Plasma C-reactive protein and risk of breast cancer in two prospective studies and a meta-analysis. *Cancer Epidemiol Biomarkers Prev* (2015) 24(8):1199–206. doi: 10.1158/1055-9965.EPI-15-0187
47. Zhang SM, Lin J, Cook NR, Lee I-M, Manson JE, Buring JE, et al. C - reactive protein and risk of breast cancer. *J Natl Cancer Inst* (2007) 99:890–94. doi: 10.1093/jnci/djk202
48. Aleksandrova K, Jenab M, Boeing H, Jansen E, Bueno-de-Mesquita HB, Rinaldi S, et al. Circulating C-Reactive Protein Concentrations and Risks of Colon and Rectal Cancer: A Nested Case-Control Study Within the European Prospective Investigation into Cancer and Nutrition. *Am J Epidemiol* (2010) 172(4):407–18. doi: 10.1093/aje/kwq135
49. Erlinger TP, Platz EA, Rifai N, Helzlsouer KJ. C - reactive protein and the Risk of Incident Colorectal Cancer. *JAMA* (2004) 291(5):585–90. doi: 10.1001/jama.291.5.585
50. Fang D, Ye Y. C-reactive protein gene rs1205 polymorphism is not associated with the risk of colorectal cancer. *Biosci Rep* (2017) 37: BSR20170872. doi: 10.1042/BSR20170872
51. Goyal A, Terry MB, Jin Z, Siegel AB. C-Reactive Protein and Colorectal Cancer Mortality in U.S. Adults. *Cancer Epidemiol Biomarkers Prev* (2014) 23 (8):1609–18. doi: 10.1158/1055-9965.EPI-13-0577
52. Holm M, Saraswat M, Joenväärä S, Ristimäki A, Haglund C, Renkonen R. Colorectal cancer patients with different C-reactive protein levels and 5-year survival times can be differentiated with quantitative serum proteomics. *PloS One* (2018) 13(4):e0195354. doi: 10.1371/journal.pone.0195354
53. Ishizuka M, Nagata H, Takagi K, Kubota K. C-Reactive Protein is Associated with Distant Metastasis of T3 Colorectal Cancer. *Anticancer Res* (2012) 32:1409–16.
54. Lumachi F, Basso SMM, Santeufemia DA, Ermani M, Lo Re G, Chiara GB. Preoperative Serum C - reactive protein and its Prognostic Significance in Patients with Stage III-IV Colorectal Cancer. *Anticancer Res* (2014) 34:7263–6.

55. Nimptsch K, Aleksandrova K, Boeing H, Janke J, Lee Y-A, Jenab M, et al. Association of CRP genetic variants with blood concentrations of C-reactive protein and colorectal cancer risk. *Int J Cancer* (2015) 136:1181–92. doi: 10.1002/ijc.29086
56. Shibutani M, Maeda K, Nagahara H, Ohtani H, Sugano K, Ikeya T, et al. Elevated preoperative serum C-reactive protein levels are associated with poor survival in patients with colorectal cancer. *Hepatogastroenterology* (2014) 61(136):2236–40.
57. Toiyama Y, Fujikawa H, Koike Y, Saigusa S, Inoue Y, Tanaka K, et al. Evaluation of preoperative C-reactive protein aids in predicting poor survival in patients with curative colorectal cancer with poor lymph node assessment. *Oncol Lett* (2012) 5:1881–8. doi: 10.3892/ol.2013.1308
58. Zhou B, Shu B, Yang J, Liu J, Xi T, Xing Y. C-reactive protein, interleukin-6 and the risk of colorectal cancer: a meta-analysis. *Cancer Causes Control* (2014) 25:1397–405. doi: 10.1007/s10552-014-0445-8
59. Badakhshi H, Kaul D, Zhao KL. Association between the inflammatory biomarker, C-reactive protein, and the response to radiochemotherapy in patients with esophageal cancer. *Mol Clin Oncol* (2016) 4(4):643–7. doi: 10.3892/mco.2016.753
60. Huang Y, Feng JF, Liu JS, Chen QX. Prognostic role of serum C-reactive protein in esophageal cancer: a systematic review and meta-analysis. *Ther Clin Risk Manag* (2015) 11:89–94. doi: 10.2147/TCRM.S70954
61. Katano A, Takahashi W, Yamashita H, Yamamoto K, Ando M, Yoshida M, et al. The impact of elevated C-reactive protein level on the prognosis for oro-hypopharynx cancer patients treated with radiotherapy. *Sci Rep* (2017) 7(1):17805. doi: 10.1038/s41598-017-18233-w
62. Zheng TL, Cao K, Liang C, Zhang K, Guo HZ, Li DP, et al. Prognostic value of C-reactive protein in esophageal cancer: a meta-analysis. *Asian Pac J Cancer Prev* (2014) 15(19):8075–81. doi: 10.7314/apjcp.2014.15.19.8075
63. Baba H, Kuwabara K, Ishiguro T, Hatano S, Matsuzawa T, Fukuchi M, et al. C-reactive Protein as a Significant Prognostic Factor for Stage IV Gastric Cancer Patients. *Anticancer Res* (2013) 33:5591–6.
64. Chang C-C, Sun C-F, Pai H-J, Wang W-K, Hsieh C-C, Kuo L-M, et al. Preoperative Serum C -reactive protein and Gastric Cancer; Clinical-pathological Correlation and Prognostic Significance. *Med J* (2010) 33:301–12.
65. Shimura T, Kitagawa M, Yamada T, Ebi M, Mizoshita T, Tanida S, et al. C-reactive Protein is a Potential Prognostic Factor for Metastatic Gastric Cancer. *Anticancer Res* (2012) 32:491–6.
66. Shishido Y, Fujitani K, Yamamoto K, Hirao M, Tsujinaka T, Sekimoto M. C-reactive protein on postoperative day 3 as a predictor of infectious complications following gastric cancer resection. *Gastric Cancer* (2016) 19:293–301. doi: 10.1007/s10120-014-0455-y
67. Yu Q, Yu X-F, Zhang S-D, Wang H-H, Wang H-Y, Teng L-S. Prognostic Role of C-reactive protein in Gastric Cancer: A Meta-analysis. *Asian Pacific J Cancer Prev* (2013) 14(10):5735–40. doi: 10.7314/APJCP.2013.14.10.5735
68. Fang Y, Xu C, Wu P, Zhang L-H, Li D-W, Sun J-H, et al. Prognostic role of C-reactive protein in patients with nasopharyngeal carcinoma A meta-analysis and literature review. *Medicine* (2017) 96:45(e8463). doi: 10.1097/MD.00000000000008463
69. He X, Li J-P, Liu X-H, Zhang J-P, Zeng Q-Y, Chen H, et al. Prognostic value of C-reactive protein/albumin ratio in predicting overall survival of Chinese cervical cancer patient's overall survival: comparison among various inflammation-based factors. *J Cancer* (2018) 9(10):1877–84. doi: 10.7150/jca.23320
70. Tai SF, Chien H-T, Young C-K, Tsao C-K, de Pablo A, Fan K-H, et al. Roles of preoperative C-reactive protein are more relevant in buccal cancer than other subsites. *World J Surg Oncol* (2017) 15:47. doi: 10.1186/s12957-017-1116-5
71. Du J, Hu W, Yang C, Wang Y, Wang X, Yang P. C-reactive protein is associated with the development of tongue squamous cell carcinoma. *Acta Biochim Biophys Sin* (2018) 50(3):238–45. doi: 10.1093/abbs/gmy004
72. Oliveira KG, von Zeidler SV, Lamas AZ, de Podesta JRV, Sena A, Souza ED, et al. Relationship of inflammatory markers and pain in patients with head and neck cancer prior to anticancer therapy. *Braz J Med Biol Res* (2014) 47(7):600–4. doi: 10.1590/1414-431X20143599
73. Carr BI, Akkiz H, Guerra V, Üsküdar O, Kuran S, Karaogullarından Ü, et al. C-reactive protein and hepatocellular carcinoma: analysis of its relationship to tumor factors. *Clin Pract (Lond)* (2018) 15(Spec Issue):625–34. doi: 10.4172/clinical-practice.1000409
74. Kinoshita A, Onoda H, Imai N, Iwaku A, Oishi M, Tanaka K, et al. The addition of C-reactive protein to validated staging systems improves their prognostic ability in patients with hepatocellular carcinoma. *Oncology* (2014) 86:308–17. doi: 10.1159/000360704
75. Hefler L, Concin N, Hofstetter G, Marth C, Mustea A, Sehoul J, et al. Serum C-Reactive Protein as independent prognostic variable in patients with ovarian cancer. *Clin Cancer Res* (2008) 14(3):710–4. doi: 10.1158/1078-0432.CCR-07-1044
76. Li J, Jiao X, Yuan Z, Qiu H, Guo R. C-reactive protein and risk of ovarian cancer: A systematic review and meta-analysis. *Medicine* (2017) 96:34(e7822). doi: 10.1097/MD.00000000000007822
77. Lundin E, Dossus L, Clendenen T, Krogh V, Grankvist K, Wulff M, et al. C-reactive protein and ovarian cancer: a prospective study nested in three cohorts (Sweden, USA, Italy). *Cancer Causes Control* (2009) 20(7):1151–9. doi: 10.1007/s10552-009-9330-2
78. Graff JN, Beer TM, Liu B, Sonpavde G, Taioli E. Pooled analysis of C-Reactive Protein levels and mortality in prostate cancer patients. *Clin Genitourin Cancer* (2015) 13(4):e217–21. doi: 10.1016/j.clgc.2015.01.011
79. Liu Z-Q, Chu L, Fang J-M, Zhang X, Zhao H-X, Chen Y-J, et al. Prognostic role of C-reactive protein in prostate cancer: a systematic review and meta-analysis. *Asian J Androl* (2014) 16:467–71. doi: 10.4103/1008-682X.123686
80. Platz EA, De Marzo AM, Erlinger TP, Rifai N, Visvanathan K, Hoffman SC, et al. No association between pre-diagnostic plasma C-reactive protein concentration and subsequent prostate cancer. *Prostate* (2004) 59(4):393–400. doi: 10.1002/pros.10368
81. Schnoeller TJ, Steinestel J, Jentzmik F, Schrader AJ. Do preoperative serum C-reactive protein levels predict the definitive pathological stage in patients with clinically localized prostate cancer? *Int Urol Nephrol* (2015) 47:765–70. doi: 10.1007/s11255-015-0952-x
82. Thurner EM, Krenn-Pilko S, Langsenlehner U, Stojakovic T, Pichler M, Gerger A, et al. The elevated C-reactive protein level is associated with poor prognosis in prostate cancer patients treated with radiotherapy. *Eur J Cancer* (2015) 51(5):610–9. doi: 10.1016/j.ejca.2015.01.002
83. Xu L, Zhao Q, Huang S, Li S, Wang J, Li Q. Serum C-reactive protein acted as a prognostic biomarker for overall survival in metastatic prostate cancer patients. *Tumour Biol* (2015) 36(2):669–73. doi: 10.1007/s13277-014-2670-x
84. Liu Y, Chen S, Zheng C, Ding M, Zhang L, Wang L, et al. The prognostic value of the preoperative c-reactive protein/albumin ratio in ovarian cancer. *BMC Cancer* (2017) 17:285. doi: 10.1186/s12885-017-3220-x
85. Chen J, Jing X, Deng X, Gao F, Wang X, Han D, et al. Prognostic value of serum C-reactive protein in pancreatic cancer: a meta-analysis. *Int J Clin Exp Med* (2018) 11(11):11789–96.
86. Inoue D, Ozaka M, Matsuyama M, Yamada I, Takano K, Saiura A, et al. Prognostic value of neutrophil-lymphocyte ratio and level of C-reactive protein in a large cohort of pancreatic cancer patients: a retrospective study in a single institute in Japan. *Japanese J Clin Oncol* (2015) 45(1):61–6. doi: 10.1093/jco/hyu159
87. Stevens L, Pathak S, Nunes QM, Pandanaboyana S, Macutkiewicz C, Smart N, et al. Prognostic significance of pre-operative C-reactive protein and the neutrophil-lymphocyte ratio in resectable pancreatic cancer: a systematic review. *HPB* (2015) 17:285–91. doi: 10.1111/hpb.12355
88. Hsiao W, Herrel LA, Yu C, Kattan MW, Canter DJ, Carthon BC, et al. Nomograms incorporating serum C-reactive protein effectively predict mortality before and after surgical treatment of renal cell carcinoma. *Int J Urol* (2015) 22(3):264–70. doi: 10.1111/iju.12672
89. Omae K, Kondo T, Tanabe K. High preoperative C-reactive protein values predict poor survival in patients on chronic hemodialysis undergoing nephrectomy for renal cancer. *Urol Oncol* (2015) 33(2):e9–13. doi: 10.1016/j.urolonc.2014.07.004
90. Teishima J, Kobatake K, Hayashi T, Seno Y, Ikeda K, Nagamatsu H, et al. Prognostic significance of C-reactive protein in patients with intermediate-risk metastatic renal cell carcinoma treated with molecular targeted therapy. *Oncol Lett* (2014) 8(2):881–5. doi: 10.3892/ol.2014.2207
91. Aziz A, Rink M, Gakis G, Kluth LA, Dechet C, Miller F, et al. Preoperative C-reactive protein in the serum: a prognostic biomarker for upper urinary tract

- urothelial carcinoma treated with radical nephroureterectomy. *Urol Int* (2014) 93(3):352–60. doi: 10.1159/000362248
92. Dai J, Tang K, Xiao W, Yu G, Zeng J, Li W, et al. Prognostic Significance of C-reactive protein in Urological Cancers: A Systematic Review and Meta-analysis. *Asian Pac J Cancer Prev* (2014) 15(8):3369–75. doi: 10.7314/apjcp.2014.15.8.3369
 93. Guo S, He X, Chen Q, Yang G, Yao K, Dong P, et al. The C-reactive protein/albumin ratio, a validated prognostic score, predicts outcome of surgical renal cell carcinoma patients. *BMC Cancer* (2017) 17:171. doi: 10.1186/s12885-017-3119-6
 94. Troppan KT, Schlick K, Deutsch A, Melchardt T, Egle A, Stojakovic T, et al. C-reactive protein level is a prognostic indicator for survival and improves the predictive ability of the R-IPi score in diffuse large B-cell lymphoma patients. *Br J Cancer* (2014) 111:55–60. doi: 10.1038/bjc.2014.277
 95. Fang S, Wang Y, Sui D, Liu H, Ross MI, Gershenwald JE, et al. C-Reactive Protein as a marker of melanoma progression. *J Clin Oncol* (2015) 33:1389–96. doi: 10.1200/JCO.2014.58.0209
 96. Fang E, Wang X, Feng J, Zhao X. The Prognostic Role of Glasgow Prognostic Score and C - reactive protein to Albumin Ratio for Sarcoma: A System Review and Meta-Analysis. *Dis Markers* (2020) 2020:14 pages. doi: 10.1155/2020/8736509. Article ID 8736509.
 97. Li W, Luo X, Liu Z, Chen Y, Li Z. Prognostic value of C-reactive protein levels in patients with bone neoplasms: A meta-analysis. *PloS One* (2018) 13(4):e0195769. doi: 10.1371/journal.pone.0195769
 98. Nakamura T, Grimer R, Gaston C, Francis M, Charman J, Graunt P, et al. The value of C-reactive protein and comorbidity in predicting survival of patients with high grade soft tissue sarcoma. *Eur J Cancer* (2013) 49(2):377–85. doi: 10.1016/j.ejca.2012.09.004
 99. Nakamura T, Matsumine A, Matsubara T, Asanuma K, Yada Y, Hagi T, et al. Infiltrative tumor growth patterns on magnetic resonance imaging associated with systemic inflammation and oncological outcome in patients with high-grade soft-tissue sarcoma. *PloS One* (2017) 12(7):e0181787. doi: 10.1371/journal.pone.0181787
 100. Wang X, Liu S, Zhao X, Fang E, Zhao X. The value of C-reactive protein as an independent prognostic indicator for disease specific survival in patients with soft tissue sarcoma: A meta-analysis. *PloS One* (2019) 14(7):e0219215. doi: 10.1371/journal.pone.0219215
 101. Yanagisawa M, Gingrich AA, Judge S, Li C-S, Wang N, Thorpe SW, et al. Serum C-reactive protein and neutrophil/lymphocyte ratio after neoadjuvant radiotherapy in soft tissue sarcoma. *Anticancer Res* (2018) 38(3):1491–7. doi: 10.21873/anticancer.12376
 102. Shrotriya S, Walsh D, Nowacki AS, Lorton C, Aktas A, Hüllihen B, et al. cSerum C-reactive protein is an important and powerful prognostic biomarker in most adult solid tumors. *PloS One* (2018) 13(8):e0202555. doi: 10.1371/journal.pone.0202555
 103. Rajab IM, Majerczyk D, Olson ME, Addams JMB, Choe ML, Nelson MS, et al. C-reactive protein in gallbladder diseases: diagnostic and therapeutic insights. *Biophysics Rep* (2020). doi: 10.1007/s41048-020-00108-9
 104. Tyurina YY, St. Croix CM, Watkins SC, Watson AM, Epperly MW, Tamil S, et al. Redox (phospho)lipidomics of signaling in inflammation and programmed cell death. *J Leukoc Biol* (2019) 106(1):57–81. doi: 10.1002/JLB.3MIR0119-004RR
 105. Zen Q, Zhong W, Mortensen RF. Binding site on human C-reactive Protein (CRP) recognized by the Leukocyte CRP-receptor. *J Cell Biochem* (1997) 64:140–51. doi: 10.1002/(SICI)1097-4644(199701)64:1<140::AID-JBC16>3.0.CO;2-p
 106. Thomassen MJ, Meeker DP, Deodhar SD, Wiedemann HP, Barna BP. Activation of human monocytes and alveolar macrophages by a synthetic peptide of C-reactive protein. *J Immunother Emphasis Tumor Immunol* (1993) 13(1):1–6. doi: 10.1097/00002371-1993010000-00001
 107. Li H-Y, Wang J, Meng F, Jia Z-K, Su Y, Bai Q-F, et al. An intrinsically disordered motif mediates diverse actions of monomeric C-reactive protein. *J Biol Chem* (2016) 291(16):8795–804. doi: 10.1074/jbc.M115.695023
 108. Thompson D, Pepys MB, Wood SP. The physiological structure of human C-reactive protein and its complex with phosphocholine. *Structure* (1999) 7(2):169–77. doi: 10.1016/S0969-2126(99)80023-9
 109. Shrive AK, Cheetham GMT, Holden D, Myles DAA, Turnell WG, Volanakis JE, et al. Three-dimensional structure of human C-reactive protein. *Nat Struct Biol* (1996) 3:346–54. doi: 10.1038/nsb0496-346
 110. Wu Y, Potempa LA, Kebir DE, Filep JG. C-reactive protein and inflammation: conformational changes affect function. *Biol Chem* (2015) 396(11):1181–97. doi: 10.1515/hsz-2015-0149
 111. Dvorak HF. Tumors: wounds that do not heal. *New Engl J Med* (1986) 315:1650–9. doi: 10.1056/NEJM198612253152606
 112. Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? *Lancet* (2001) 357(9255):539–45. doi: 10.1016/S0140-6736(00)04046-0
 113. Agrawal A, Xu Y, Ansardi D, Macon KJ, Volanakis JE. Probing the phosphocholine-binding site of human C-reactive protein by site-directed mutagenesis. *J Biol Chem* (1992) 267:25352–8.
 114. Volanakis JE. Human C-reactive protein: expression, structure, and function. *Mol Immunol* (2001) 38(2-3):189–97. doi: 10.1016/s0161-5890(01)00042-6
 115. Salonen E-M, Vartio T, Hedman K, Vaheri A. Binding of fibronectin by the acute phase reactant C-reactive protein. *J Biol Chem* (1984) 259:1496–501.
 116. Tseng J, Mortensen RF. Binding of human C-reactive protein (CRP) to plasma fibronectin occurs via the phosphorylcholine-binding site. *Mol Immunol* (1988) 25:679–86. doi: 10.1016/0161-5890(88)90103-4
 117. Ullah N, Ma F-R, Jin Han J, Liu X-L, Fu Y, Liu Y-T, et al. Monomeric C-reactive Protein Regulate Mediated Monocyte Adhesion. *Mol Immunol* (2020) 117:122–30. doi: 10.1016/j.molimm.2019.10.013
 118. Swanson SJ, McPeck MM, Mortensen RF. Characteristics of the binding of human C-reactive protein (CRP) to laminin. *J Cell Biochem* (1989) 40:121–32. doi: 10.1002/jcb.240400112
 119. Sproston NR, Ashworth JJ. Role of C-Reactive Protein at Sites of Inflammation and Infection. *Front Immunol* (2018) 9:754. doi: 10.3389/fimmu.2018.00754
 120. McFadyen J, Kiefer J, Loseff-Silver J, Braig D, Potempa LA, Eisenhardt SU, et al. Dissociation of C-reactive protein localizes and amplifies inflammation: Evidence for a direct biological role of CRP and its conformational changes. *Front Immunol* (2018) 9:1351. doi: 10.3389/fimmu.2018.01351.ecollection. Article 1351.
 121. Zhong W, Zen Q, Tebo J, Schlottmann K, Coggeshall M, Mortensen RF, et al. Effect of human C-reactive protein on chemokine and chemotactic factor-induced neutrophil chemotaxis and signaling. *J Immunol* (1998) 161(5):2533–40.
 122. Trial J, Potempa LA, Entman ML. The Role of C-reactive Protein in Innate and Acquired Inflammation: New Perspectives. *Inflammation Cell Signaling* (2016) 3:e1409. doi: 10.14800/ics.1498
 123. Zhang L, Shen ZY, Wang K, Li W, Shi J-M, Kombo Orsoro E, et al. C-reactive protein exacerbates epithelial-mesenchymal transition through Wnt/ β -catenin and ERK signaling in streptozocin-induced diabetic nephropathy. *FASEB J* (2019) 33(5):6551–63. doi: 10.1096/fj.201801865RR
 124. Nakai K, Tanaka H, Yamanaka K, takahashi Y, Murakami F, Matsuike R, et al. Effects of C-reactive protein on the expression of matrix metalloproteinases and their inhibitors via Fc γ receptors on 3T3-L1 adipocytes. *Int J Med Sci* (2017) 14(5):484–93. doi: 10.7150/ijms.18059
 125. Li H-Y, Wang J, Wu Y-X, Zhang L, Liu Z-P, Filep J, et al. Topological localization of monomeric C-reactive protein determines proinflammatory endothelial cell responses. *J Biol Chem* (2014) 289(20):14283–90. doi: 10.1074/jbc.M114.555318
 126. Shephard EG, Anderson R, Rosen O, Myer MS, Fridkin M, Strachan AF, et al. Peptides generated from C-reactive protein by a neutrophil membrane protease. *J Immunol* (1990) 145:1469–76.
 127. Shephard EG, Anderson R, Rosen O, Fridkin M. C-reactive protein (CRP) peptides inactivate enolase in human neutrophils leading to depletion of intracellular ATP and inhibition of superoxide generation. *Immunology* (1992a) 76(1):79–85.
 128. Shephard EG, Kelly SL, Anderson R, Fridkin M. Characterization of neutrophil-mediated degradation of human C-reactive protein and identification of the protease. *Clin Exp Immunol* (1992b) 87(3):509–13. doi: 10.1111/j.1365-2249.1992.tb0328.x
 129. El Kebir D, Zhang Y, Wang L, Potempa LA, Wu Y, Fournier A, et al. C-reactive protein-derived peptide 201-206 inhibits neutrophil adhesion to endothelial cells and platelets through CD32. *J Leukocyte Biol* (2011) 90(6):1167–75. doi: 10.1074/jlb.0111032
 130. Rajab IM, Hart PC, Potempa LA. How C-reactive protein structural isoforms with distinctive bioactivities affect disease progression. *Front Immunol* (2020) 11:2126. doi: 10.3389/fimmu.2020.02126

131. Theile JR, Zeller J, Kiefer J, Braig D, Kreuzaler S, Lenz Y, et al. A conformational change in C-reactive protein enhances leukocyte recruitment and reactive oxygen species generation in ischemia/reperfusion injury. *Front Immunol* (2018) 6:675. doi: 10.3389/fimmu.2018.00675
132. Thiele JR, Zeller J, Bannasch H, Stark GB, Peter K, Eisenhardt SU. Targeting C-Reactive Protein in Inflammatory Disease by Preventing Conformational Changes. *Mediators Inflammation* (2015) 2015:372432. doi: 10.1155/2015/372432
133. Braig D, Nero TL, Koch H-G, Kaiser B, Wang X, Thiele JR, et al. Characterization of transitional changes in the CRP structure leading to the exposure of pro-inflammatory binding sites. *Nat Commun* (2017) 23:14188. doi: 10.1038/ncomms14188
134. Tan C, Huang Y, Shi F, Tan K, Ma Q, Chen Y, et al. C-reactive protein correlates with CT findings and predicts severe COVID-19 early. *J Med Virol* (2020) 92(7):856–62. doi: 10.1002/jmv.25871
135. Birk DE, Silver FH, Trelstad RL. Matrix assembly. In: ED Hay, editor. *Cell Biology of Extracellular Matrix, 2nd edition*. New York: Plenum Press (1991). p. 221–54.
136. Aumailley M, Gayraud B. Structure and biological activity of the extracellular matrix. *J Mol Med* (1998) 76:253–65. doi: 10.1007/s001090050215
137. Yue B. Biology of the extracellular matrix: an overview. *J Glaucoma* (2014) 23(8):S20–3. doi: 10.1097/IJG.0000000000000108
138. Cray C. Acute phase proteins in animals. *2012 Prog Mol Biol Transl Sci* (2012) 105:113–50. doi: 10.1016/B978-0-12-394596-9.00005-6
139. Hunt TK. Basic principles of wound healing. *J Trauma* (1990) 30:S122–128. doi: 10.1097/00005373-199012001-00025
140. Kim WJ, Gittes GK, Longaker MT. Signal transduction in wound pharmacology. *Arch Pharm Res* (1998) 21:487–95. doi: 10.1007/BF02975363
141. Balkwill FR, Mantovani A. Cancer-related inflammation: common themes and therapeutic opportunities. *Semin Cancer Biol* (2012) 22(1):33–40. doi: 10.1016/j.semcancer.2011.12.005
142. Coussens LM, Werb Z. Inflammation and cancer. *Nature* (2002) 420(6917):860–7. doi: 10.1038/nature01322
143. Murata M. Inflammation and cancer. *Environ Health Prev Med* (2018) 23(1):50. doi: 10.1186/s12199-018-0740-1
144. Landskron G, De la Fuente M, Thuwajit P, Thuwajit C, Hermoso MA. Chronic inflammation and cytokines in the tumor microenvironment. *J Immunol Res* (2014) 2014:149185. doi: 10.1155/2014/149185
145. Colotta F, Allavena P, Sica A, Garlanda C, Mantovani A. Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. *Carcinogenesis* (2009) 30(7):1073–81. doi: 10.1093/carcin/bgp127
146. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. *Cell* (2010) 140(6):883–99. doi: 10.1016/j.cell.2010.01.025
147. Giannoni E, Parri M, Chiarugi P. EMT and oxidative stress: a bidirectional interplay affecting tumor malignancy. *Antioxid Redox Signal* (2012) 16(11):1248–63. doi: 10.1089/ars.2011.4280
148. Xu J, Lamouille S, Derynck R. TGF-beta-induced epithelial to mesenchymal transition. *Cell Res* (2009) 19(2):156–72. doi: 10.1038/cr.2009.5
149. Gilles C, Newgreen D, Sato H, Thompson E. Matrix Metalloproteases and Epithelial-to-Mesenchymal Transition: Implications for Carcinoma Metastasis. In: *Madame Curie Bioscience Database*. (Austin, TX: Landes Bioscience 2000-2013 (2013). Available at: <https://www.ncbi.nlm.nih.gov/books/NBK6387>.
150. Drake JM, Strohschein G, Bair TB, Moreland JG, Henry MD. ZEB1 enhances transendothelial migration and represses the epithelial phenotype of prostate cancer cells. *Mol Biol Cell* (2009) 20(8):2207–17. doi: 10.1091/mbc.e08-10-1076
151. Bergeman J, Caillier A, Houle F, Gagne LM, Huot ME. Localized translation regulates cell adhesion and transendothelial migration. *J Cell Sci* (2016) 129(21):4105–17. doi: 10.1242/jcs.191320
152. Dominguez C, David JM, Palena C. Epithelial-mesenchymal transition and inflammation at the site of the primary tumor. *Semin Cancer Biol* (2017) 47:177–84. doi: 10.1016/j.semcancer.2017.08.002
153. Todoric J, Antonucci L, Karin M. Targeting Inflammation in Cancer Prevention and Therapy. *Cancer Prev Res (Phila)* (2016) 9(12):895–905. doi: 10.1158/1940-6207.CAPR-16-0209
154. Lim SY, Yuzhalin AE, Gordon-Weeks AN, Muschel RJ. Targeting the CCL2-CCR2 signaling axis in cancer metastasis. *Oncotarget* (2016) 7(19):28697–710. doi: 10.18632/oncotarget.7376
155. Stone RC, Pastar I, Ojeh N, Chen V, Liu S, Garzon KI, et al. Epithelial-mesenchymal transition in tissue repair and fibrosis. *Cell Tissue Res* (2016) 365(3):495–506. doi: 10.1007/s00441-016-2464-0
156. Shaw TJ, Martin P. Wound repair at a glance. *J Cell Sci* (2009) 122(Pt 18):3209–13. doi: 10.1242/jcs.031187
157. Gonzalez AC, Costa TF, Andrade ZA, Medrado AR. Wound healing - A literature review. *Bras Dermatol* (2016) 91(5):614–20. doi: 10.1590/abd1806-4841.20164741
158. Guo S, Di Pietro LA. Factors affecting wound healing. *J Dent Res* (2010) 89(3):219–29. doi: 10.1177/0022034509359125
159. Mack M. Inflammation and fibrosis. *Matrix Biol* (2018) 68-69:106–21. doi: 10.1016/j.matbio.2017.11.010
160. Darby IA, Laverdet B, Bonte F, Desmouliere A. Fibroblasts and myofibroblasts in wound healing. *Clin Cosmet Invest Dermatol* (2014) 7:301–11. doi: 10.2147/CCID.S50046
161. Balkwill F, Capasso M, Hagemann T. The tumor microenvironment at a glance. *J Cell Sci* (2012) 125(Pt 23):5591–6. doi: 10.1242/jcs.116392
162. Tao L, Huang G, Song H, Chen Y, Chen L. Cancer associated fibroblasts: An essential role in the tumor microenvironment. *Oncol Lett* (2017) 14(3):2611–20. doi: 10.3892/ol.2017.6497
163. Fiori ME, Di Franco S, Villanova L, Bianca P, Stassi G, De Maria R. Cancer-associated fibroblasts as abettors of tumor progression at the crossroads of EMT and therapy resistance. *Mol Cancer* (2019) 18(1):70. doi: 10.1186/s12943-019-0994-2
164. Tokunaga R, Zhang W, Naseem M, Puccini A, Berger MD, Soni S, et al. CXCL9, CXCL10, CXCL11/CXCR3 axis for immune activation - A target for novel cancer therapy. *Cancer Treat Rev* (2018) 63:40–7. doi: 10.1016/j.ctrv.2017.11.007
165. Weigel E, Smith C, Liu PG, Robison R, O'Neill K. Macrophage polarization and its role in cancer. *J Clin Cell Immunol* (2015) 6(4):1–8. doi: 10.4172/2155-9899.1000338
166. Giannelli G, Antonaci S. Biological and clinical relevance of laminin-5 in cancer. *Clin Exp Metastasis* (2000) 18(6):439–43.
167. Clark A, Vignjevic DM. Modes of cancer cell invasion and the role of the microenvironment. *Curr Opin Cell Biol* (2015) 36:13–22.
168. Wang JP, Hielscher A. Fibronectin: how its aberrant expression in tumors may improve therapeutic targeting. *J Cancer* (2017) 8(4):674–82.
169. Lin Y, Xu J, Lan H. Tumor-associated macrophages in tumor metastasis: biological roles and clinical therapeutic applications. *J Hematol Oncol* (2019) 12(1):76. doi: 10.1186/s13045-019-0760-3
170. Jung HY, Fattet L, Yang J. Molecular pathways: linking tumor microenvironment to epithelial-mesenchymal transition in metastasis. *Clin Cancer Res* (2015) 21(5):962–8. doi: 10.1158/1078-0432.CCR-13-3173
171. Jechlinger M, Sommer A, Moriggl R, Seither P, Kraut N, Capodieci P. Autocrine PDGFR signaling promotes mammary cancer metastasis. *J Clin Invest* (2006) 116(6):1561–70. doi: 10.1172/JCI24652
172. Neri S, Miyashita T, Hashimoto H, Suda Y, Ishibashi M, Kii H, et al. Fibroblast-led cancer cell invasion is activated by epithelial-mesenchymal transition through platelet-derived growth factor BB secretion of lung adenocarcinoma. *Cancer Lett* (2017) 395:20–30. doi: 10.1016/j.canlet.2017.02.026
173. Lebrun JJ. The Dual Role of TGF beta in Human Cancer: From Tumor Suppression to Cancer Metastasis. *ISRN Mol Biol* (2012) 2012:381428. doi: 10.5402/2012/381428
174. Simpson-Haidaris PJ, Rybarczyk B. Tumors and fibrinogen. The role of fibrinogen as an extracellular matrix protein. *Ann N Y Acad Sci* (2001) 936:406–25. doi: 10.1111/j.1749-6632.2001.tb03525.x
175. Tulotta C, Ottewill P. The role of IL-1B in breast cancer bone metastasis. *Endocr Relat Cancer* (2018) 25(7):R421–34. doi: 10.1530/ERC-17-0309
176. Masjedi A, Hashemi V, Hojjat-Farsangi M, Ghalamfarsa G, Azizi G, Yousefi M, et al. The significant roles of interleukin-6 and its signaling pathway in the immunopathogenesis and treatment of breast cancer. *BioMed Pharmacother* (2018) 108:1415–24. doi: 10.1016/j.biopha.2018.09.177

177. Suarez-Carmona M, Lesage J, Cataldo D, Gilles C. EMT and inflammation: inseparable actors of cancer progression. *Mol Oncol* (2017) 11(7):805–23. doi: 10.1002/1878-0261.12095
178. Liu T, Zhou L, Li D, Andl T, Zhang Y. Cancer-Associated Fibroblasts Build and Secure the Tumor Microenvironment. *Front Cell Dev Biol* (2019) 7:60. doi: 10.3389/fcell.2019.00060
179. Song W, Mazziere R, Yang T, Gobe GC. Translational Significance for Tumor Metastasis of Tumor-Associated Macrophages and Epithelial-Mesenchymal Transition. *Front Immunol* (2017) 8:1106. doi: 10.3389/fimmu.2017.01106
180. Turner CE, Burridge K. Transmembrane molecular assemblies in cell-extracellular matrix interactions. *Curr Opin Cell Biol* (1991) 5:849–53. doi: 10.1016/0955-0674(91)90059-8
181. Damsky CH, Werb Z. Signal transduction by integrin receptors for extracellular matrix: cooperative processing of extracellular information. *Curr Opin Cell Biol* (1992) 5:772–81. doi: 10.1016/0955-0674(92)90100-q
182. Roy F, DeBlois C, Doillon CJ. Extracellular matrix analogs as carriers for growth factors: *in vitro* fibroblast behavior. *J BioMed Mat Res* (1993) 27:389–97. doi: 10.1002/jbm.820270312
183. Scott JE. Extracellular matrix, supramolecular organization and shape. *J Anat* (1995) 187:259–69.
184. Yanagishita M. Function of proteoglycans in the extracellular matrix. *Acta Pathol Jpn* (1993) 43:283–93. doi: 10.1111/j.1440-1827.1993.tb02569.x
185. McCarthy J, Turley EA. Effects of extracellular matrix components on cell locomotion. *Crit Rev Oral Biol Med* (1993) 4:619–37. doi: 10.1177/10454411930040050101
186. Lippitz BE, Harris RA. Cytokine patterns in cancer patients: A review of the correlation between interleukin 6 and prognosis. *Oncoimmunology* (2016) 5(5):e1093722. doi: 10.1080/2162402X.2015.1093722
187. Xu J, Yin Z, Cao S, Gao W, Liu L, Yin Y, et al. Systematic review and meta-analysis on the association between IL-1B polymorphisms and cancer risk. *PLoS One* (2013) 8(5):e63654. doi: 10.1371/journal.pone.0063654
188. Zhang L, Li HY, Li W, Shen ZY, Wang YD, Ji SR, et al. An ELISA assay for quantifying monomeric C-reactive protein in plasma. *Front Immunol* (2018) 9:511. doi: 10.3389/fimmu.2018.00511
189. Potempa LA, Rajab IM, Hart PC, Bordon J, Fernandez-Botran R. Insights into the use of C-reactive protein as a diagnostic index of disease severity in COVID-19 infections. *Am J Trop Med Hyg* (2020) 103(2):561–63. doi: 10.4269/ajtmh.20-0473

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Hart, Rajab, Alebraheem and Potempa. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



OPEN ACCESS

Edited by:

Kenji Daigo,
Nippon Medical School, Japan

Reviewed by:

Jukka Meurman,
University of Helsinki, Finland
Peter Celec,
Comenius University, Slovakia
Ana María Gutiérrez,
University of Murcia, Spain

*Correspondence:

Christopher Sjöwall
christopher.sjowall@liu.se

*ORCID:

Jonas Wetterö
orcid.org/0000-0002-6916-5490
Sarah von Löhneysen
orcid.org/0000-0003-4691-6447
Flordelyn Cobar
orcid.org/0000-0002-2580-8669
Margareta Kristenson
orcid.org/0000-0001-8141-2581
Peter Garvin
orcid.org/0000-0001-7429-0889
Christopher Sjöwall
orcid.org/0000-0003-0900-2048

[†]These authors have contributed
equally to this work

Specialty section:

This article was submitted to
Molecular Innate Immunity,
a section of the journal
Frontiers in Immunology

Received: 16 September 2020

Accepted: 27 November 2020

Published: 08 January 2021

Citation:

Wetterö J, von Löhneysen S, Cobar F,
Kristenson M, Garvin P and Sjöwall C
(2021) Pronounced Diurnal Pattern of
Salivary C-Reactive Protein
(CRP) With Modest Associations to
Circulating CRP Levels.
Front. Immunol. 11:607166.
doi: 10.3389/fimmu.2020.607166

Pronounced Diurnal Pattern of Salivary C-Reactive Protein (CRP) With Modest Associations to Circulating CRP Levels

Jonas Wetterö^{1†}, Sarah von Löhneysen^{2†}, Flordelyn Cobar^{3†}, Margareta Kristenson^{4†}, Peter Garvin^{5†} and Christopher Sjöwall^{1*†}

¹ Division of Inflammation and Infection, Department of Biomedical and Clinical Sciences, Linköping University, Linköping, Sweden, ² Faculty of Mathematics and Computer Science, University of Leipzig, Leipzig, Germany, ³ Division of Rheumatology, Department of Medicine Solna, Karolinska Institutet and Rheumatology, Karolinska University Hospital, Stockholm, Sweden, ⁴ Division of Society and Health, Department of Health, Medicine and Caring Sciences, Linköping University, Linköping, Sweden, ⁵ Research and Development Unit in Region Östergötland, Department of Health, Medicine and Caring Sciences, Linköping University, Linköping, Sweden

C-reactive protein (CRP), a humoral component of the innate immune system with important functions in host-defense, is extensively used as a sensitive biomarker of systemic inflammation. During inflammation, hepatocyte-derived CRP rises dramatically in the blood due to increased interleukin-6 (IL-6) levels. Reliable detection of CRP in saliva, instead of blood, would offer advantages regarding sampling procedure and availability but using saliva as a diagnostic body fluid comes with challenges. The aims of this study were to evaluate associations between salivary CRP, total protein levels in saliva and serum CRP. Furthermore, we examined associations with plasma IL-6, body mass index (BMI), tobacco smoking and age. Salivary CRP was investigated by ELISA in 107 middle-aged participants from the general population. We employed spectrophotometric determination of total protein levels. Correlation analyses were used for associations of salivary CRP with serum CRP (turbidimetry), plasma IL-6 (Luminex®), BMI and smoking habits. Salivary median CRP was 68% higher ($p=0.009$), and total protein levels were 167% higher ($p<0.0001$), in morning compared to evening saliva. The correlation coefficients between serum and salivary CRP were low to moderate, but stronger for evening than morning saliva. Plasma IL-6 correlated significantly with serum CRP ($r_s=0.41$, $p<0.01$), but not with morning or evening salivary CRP. Non-smokers showed 103% higher salivary CRP levels ($p=0.015$), whereas serum CRP was independent of smoking status. As opposed to CRP in serum, salivary CRP was not associated with BMI. Salivary CRP was 90% higher among the age interval 60–69 years compared to subjects aged 45–59 ($p=0.02$) while serum CRP levels did not differ between the age groups. In conclusion, CRP in saliva did not straightforwardly reflect serum concentrations. This raises questions regarding adequate reflection of biological events. The pronounced diurnal salivary CRP pattern accentuates the importance of standardizing the time-point of sampling.

Keywords: saliva, C-reactive protein, pentraxins, inflammation, interleukin 6, biomarker

INTRODUCTION

The classical acute phase reactant C-reactive protein (CRP), originally described by Tillett & Francis in 1930, belongs to the pentraxin family and consists of five identical 23-kDa globular subunits (1, 2). CRP plays multiple important roles in innate immunity and host-defense. It binds to specific ligands like phosphorylcholine and activates the classical complement pathway through complement protein (C) 1q binding (3, 4).

Similarly to immunoglobulin G (IgG), CRP also has a binding site for Fc- γ receptors of phagocytic cells. However, in contrast to antibodies, ligand binding is less specific and does not require affinity maturation. Furthermore, the capacity of CRP to recognize nuclear components on the surface of apoptotic cells, and thereby to facilitate their clearance, could potentially act as a protector against autoimmunity (5). The pentameric structure of CRP is dependent upon the presence of calcium, although it can also be disrupted irreversibly into monomers under denaturing conditions, such as an acidic local microenvironment. Monomeric/modified CRP can be deposited in tissues, but the biological relevance of this remains unclear and warrants further investigation (5–8).

As a marker of acute inflammation, CRP has a short half-life of about 19 h in the circulation and declines rapidly after resolution of the acute phase response and it is widely used clinically to monitor infections, particularly those caused by bacteria, and inflammatory diseases, both acute infections and chronic conditions (5). In systemic lupus erythematosus, however, the circulating levels of CRP are generally low, despite inflammatory activity. Although some investigators have reported extrahepatic CRP production, human CRP is mainly synthesized by primary hepatocytes after stimulation through its main inducer interleukin (IL) 6, but impeded by interferon- α (9, 10). CRP has been thoroughly studied as a biomarker of future cardiovascular events (11, 12). Whether or not CRP plays a causal role in the development of atherosclerosis is debated (13).

The interest of mucosal immunity in general and the use of human *salivary* CRP as a diagnostic body fluid is increasing (14). Saliva contains a unique mixture of proteins, nucleic acids, electrolytes and hormones derived from systemic as well as local sources (15). Advantages of using saliva as a diagnostic tool are that the collection is non-invasive, relatively easy and rapid, safe, stress- and pain-free and cheap because sampling is possible without professionals (15–17). Yet there are also challenges in handling saliva. Biomarkers are often less concentrated in saliva than in serum, and therefore harder to detect by traditional analytic approaches, and their concentrations can be influenced by circadian cycles or especially sampling/processing methods (15). In addition, saliva collection can be performed either by a “passive” approach, meaning that the saliva is gathered in the mouth and then transferred into a sampling tube, or by an “active” mechanically stimulated approach. The latter stimulation of salivary flow is achieved by chewing on a solid object. It is also possible to use cotton swabs, which absorb the saliva, and then release saliva by centrifugation. Unstimulated saliva originates mainly from the submandibular gland whereas parotid saliva production increases intensively with stimulation (18). All of these sources of variation

must be considered to evaluate the usefulness of saliva as a diagnostic fluid. Regarding CRP, an essential question is if salivary levels reflect the same biological events as the serum levels do. Different studies investigated the association between salivary and serum concentrations with inconsistent results (19–22). Some indications of a diurnal rhythm of salivary CRP with elevated levels in the morning have been reported (20, 23).

In the present study, we asked whether CRP levels vary similarly to the total protein levels in saliva, and if any substantial diurnal variation occurs. The general aim was thus to evaluate associations between salivary CRP, total protein levels in saliva and serum CRP in collected samples from middle-aged individuals of the general population. In addition, we examined associations between salivary CRP and body mass index (BMI), tobacco smoking and age.

MATERIALS AND METHODS

Participants

In the present study, samples were obtained from 107 participants (61 men, 46 women; median age of 57, range 45–69 years) randomly selected from the *Life conditions, Stress and Health Study* (LSH) cohort at Linköping University, aimed at investigating the pathways that link psychosocial factors to cardiovascular diseases (24). The LSH cohort is almost ten times larger ($n=1007$) and includes individuals who were randomly chosen from the general population in the Region of Östergötland, Sweden. Participants in the LSH cohort provided saliva and blood samples and answered questionnaires regarding their socioeconomic, psychological and health status. Exclusion criteria were self-reported severe disease that hindered the possibility to participate, e.g. terminal cancer, severe dementia and psychiatric disorders. Participants with symptoms of infection were instructed to return for sampling after recovery (24).

Collection of Saliva

The saliva was sampled at home at three time-points over three consecutive days (d_1 , d_2 , d_3) using Salivette[®] cotton swabs (Sarstedt AG & Co., Nümbrecht, Germany). For saliva collection, the swab was placed in the mouth and only chewed on if the salivation was low and participants felt discomfort or mouth dryness. The participants were instructed to take samples immediately after awakening (t_1), 30 min after awakening (t_2), and just before going to bed (t_3) following their normal sleeping habits. The reported time for t_1 was 6:30 (mean and median), range 04:23 to 11:00, for d_1 , d_2 and d_3 (standard deviation 57 min). For >75% of the study participants, samples were collected between 5:30 and 7:30. The reported time for t_3 was 22:15 (mean and median), range 19:00 to 0:35 (standard deviation 65 min). For >75% of the study participants, samples were collected between 21:15 and 23:15. Participants were also told to avoid physical exercise, food intake and smoking 1 h prior to sampling. After collection, the samples were stored in a refrigerator until centrifugation. Thereafter, samples were frozen at -70°C until analysis (25). To standardize for any potential differences in viscosity of the saliva, the samples used

herein were centrifuged at 1500g for 15 min after thawing, and aliquots were taken from the supernatants for upcoming analyses.

Serum C-Reactive Protein and Plasma IL-6

All blood samples were obtained at primary health care centers between 6.30 and 9.00 AM (typically between 7.30 and 8.00 AM) in a fasting state of the first day of the week following the saliva-sampling period (*nota bene* salivary CRP was measured in the samples from d_3). Aliquots of sera were stored at -70°C until analysis (24, 25). CRP levels were detected in serum utilizing a highly sensitive latex-enhanced turbidimetric immunoassay (Roche Diagnostics GmbH, Vienna, Austria) with a lower detection limit of 0.03 mg/L, the coefficient of variance (CV) was 1.7% and the detection rate of 100% (26).

Levels of IL-6 were determined in ethylenediaminetetraacetic acid (EDTA)-plasma with an ultra-sensitive bead kit technology (Invitrogen Co., Carlsbad, CA, USA) on a Luminex[®] 100TM system (Austin, TX, USA). The lower detection limits were set at 1.68 pg/ml for IL-6. The proportion of samples with levels above this limit was 40% for IL-6. The CV was 7.0% for IL-6.

Body Mass Index and Tobacco Smoking

The participants' BMI were calculated based on weight in kilograms divided by the square of their height in meters (median BMI 23.3, range 18.7–39.5). The division of tobacco smokers (17.3%) and non-smokers (82.7%) was based on self-reported data from the questionnaires. Participants smoking ≥ 1 cigarette per day were considered as “current smoker”. All participants reporting “never smoked” or “quit smoking” were categorized as “non-smokers”.

Salivary C-Reactive Protein Assay

CRP in saliva was measured in morning (t_1) and evening (t_3) samples of the third sampling day (d_3) using Salimetrics' salivary CRP ELISA kit (State College, PA, USA) according to the manufacturer's instruction. The optical density was read on a Sunrise[™] microplate reader (Tecan, Männedorf, Switzerland) at 450 nm (reference wavelength 630 nm) and measurement data were processed using Magellan[™] software V.7.1 (Tecan). All samples were analyzed in duplicates as a pilot study in samples from healthy donors that had been spiked with CRP isolated from human plasma (Sigma-Aldrich Co., St. Louis, MO, USA) showed this to be sufficiently reliable when we tested for intra-assay precision. Further, the impact of thawing-freezing cycles of the samples was investigated and CRP levels were observed to be largely stable for at least four times of thawing. For the measurements, the saliva samples were diluted 1:10 in dilution buffer (supplied with the kit). If CRP levels above the higher detection limit (≈ 3 ng/ml) occurred, higher dilution factors were assessed. The within assay CV was 5.4% and the between assay CV 11.4%.

Total Protein Measurements

Total protein was quantified in morning (t_1) and evening (t_3) saliva of two sampling days (d_1 and d_3), giving four values for each participant. Concentrations were obtained by measuring the absorbance at 280 nm with a Nanodrop[®] ND-1000 spectrophotometer (Thermo

Fisher Scientific, Wilmington, DE, USA) using the Nanodrop[®] ND-1000 software V.3.8.1. A general reference setting was utilized based on the assumption that a 0.1% (1 mg/ml) protein solution produces an absorbance of 1.0 AU (Absorbance Unit) at 280 nm. Amino acids with aromatic rings are the primary reason for the absorbance peak at 280 nm. All samples were analyzed undiluted in duplicates (CV=1.2%).

Statistics

Analyses were performed using SPSS[®] v.20 (IBM Co., Armonk, NY, USA) and figures made in GraphPad Prism 5.03 (GraphPad Software, La Jolla, CA, USA) or in R statistical software, version 3.5.1 (Vienna, Austria). P-values < 0.05 were considered statistically significant. IL-6 and salivary CRP levels below the lower detection limit were given half the limit's value. To investigate the influence of sex and age on CRP, Mann-Whitney U ranking analysis was employed. Regarding age, participants < 60 years of age ($n=65$) were compared to those ≥ 60 years ($n=42$). Correlations between salivary morning/evening CRP, total protein levels, serum CRP, BMI and plasma IL-6 were investigated by Spearman (r_s). Bland-Altman graphs were used to illustrate and analyze the level of agreement between CRP in saliva and serum. Diurnal variations of CRP, total protein and CRP-percentages of total protein, respectively, were investigated by Wilcoxon. To evaluate if the differences between morning and evening values were the same for high and low CRP levels and ratios, the participants were grouped into two classes by morning salivary CRP (≤ 1.3 ng/ml; > 1.3 ng/ml). To examine if high CRP levels ($>$ median) coincide with high levels of BMI and IL-6 and if smokers had elevated CRP levels, we used Mann-Whitney U test. For this analysis, the daily mean salivary CRP concentration was employed. To evaluate the performance of salivary CRP to identify (biomarker-defined) “systemic inflammation”, the 90th percentile of serum CRP values among the 107 participants was used to define a cut-off. Sensitivity (proportion of samples correctly identified with systemic inflammation), specificity (proportion of samples correctly identified without systemic inflammation) and accuracy (proportion of correctly classified samples) were calculated, including 95% confidence intervals (CI) using the Wilson score method.

RESULTS

C-Reactive Protein Concentrations in Saliva and Serum

Salivary morning CRP levels ranged from 0.97 to 1674.20 ng/ml with median and interquartile range (IQR) 2.44 (0.50–10.80) ng/ml. Salivary evening CRP ranged < 0.97 –223.9, median (IQR) 1.45 (1.05–2.72) ng/ml (**Table 1**). The detectability rate for 1:10 diluted saliva samples was 77%. Twelve outliers of morning and eight of evening CRP samples were identified exceeding four times the respective IQR. Five participants were outliers regarding both morning, evening and serum CRP. The high levels of CRP in saliva were not due to visible blood contamination in these individuals. To avoid confounding contributions from blood in saliva, visibly blood contaminated samples were excluded from further statistical analyses, although their CRP values were within the normal range

(0.5–9.42 ng/ml). Serum CRP concentrations ranged from 0.03 to 30.43 mg/L with median IQR 0.90 (0.34–2.43 mg/L) (Table 1). No statistically significant differences were seen between men and women for any of the measures; salivary morning, evening or serum CRP concentrations. Participants aged 60–69 years had significantly higher salivary CRP levels than those aged 45–59 years ($p=0.02$, $n=102$; IQR=3.28 ng/ml (1.33–13.49) and 1.73 ng/ml (0.79–5.46), Figure 1A), but no significant difference in serum CRP was observed (Figure 1B) and neither between men and women.

Diurnal Variation of Salivary C-Reactive Protein and Total Protein

Total protein levels ranged from 1.46 to 37.76 mg/ml with a mean [standard deviation (SD)] of 6.92 (5.17) mg/ml. Levels at all four sampling occasions (d_1 : morning and evening, d_3 : morning and evening) correlated significantly ($r_s=0.37$ – 0.78 ; $p<0.01$). Strongest correlations were found for the two morning ($r_s=0.61$, $p<0.001$) and the two evening measurements ($r_s=0.78$, $p<0.001$) (Table 2). A significant decrease of the total protein levels toward the evening was observed for both days of sampling (d_1 : $p<0.001$, $n=87$; d_3 : $p<0.001$, $n=101$) (Figures 1C, D). IQRs were 6.20 (4.20–9.55) mg/ml (morning) and 2.97 (2.42–3.89) mg/ml (evening) for d_1 and 8.10 (5.42–12.25) mg/ml (morning) and 3.10 (2.53–9.55) mg/ml (evening) for d_3 . An additional test performed on morning and evening samples from 5 individuals (laboratory personnel) confirmed these findings (data not shown). The correlation between salivary morning and evening CRP levels was strong ($r_s=0.64$, $p<0.001$) and, as illustrated in Figure 2, the Bland–Altman graph revealed a fair level of agreement ($p=0.48$). Higher CRP levels in mornings compared to evenings were found ($p=0.009$, $n=100$) (Figures 3A, C), but this difference disappeared after adjustment for total protein ($p=0.59$, $n=100$) (Figures 3B, D).

To specifically investigate the diurnal patterns, the morning samples were stratified into two groups: low (≤ 1.3 ng/ml) and high (> 1.3 ng/ml) CRP levels. In the CRP^{high} subgroup, morning levels were significantly higher regardless when absolute CRP or CRP/total protein percentage were compared with the corresponding evening samples (both $p<0.01$, $n=63$). IQRs were 7.67 (2.62–28.19) ng/ml (morning) and 1.90 (1.34–4.27) ng/ml (evening) or 8.89×10^{-5} (3.56×10^{-5} – 19.80×10^{-5}) % (morning) and 6.95×10^{-5} (4.49×10^{-5} – 10.64×10^{-5}) % (evening). On the contrary, the CRP^{low} subgroup showed significantly lower absolute CRP levels and CRP percentages in the morning, than in the evening ($p<0.01$, $n=37$) (Figures 3E, F). The IQRs were 0.50

(0.50–0.74) ng/ml (morning) and 1.05 (0.50–1.37) ng/ml (evening) or 0.68×10^{-5} (0.57×10^{-5} – 1.50×10^{-5}) % (morning) and 3.07×10^{-5} (1.60×10^{-5} – 4.74×10^{-5}) % (evening).

To further evaluate the impact of time in relation to saliva collection, a *post hoc* analysis was performed. We assessed any potential correlation between actual time-point of sampling and CRP in morning as well as evening saliva. None of these analyses reached statistical significance.

Associations Between Salivary C-Reactive Protein With Serum C-Reactive Protein

Based on mean values of morning and evening saliva divided by serum CRP, the achieved saliva-to-serum concentration ratio reached 1:1084. We found low correlation between salivary evening CRP and serum CRP ($r_s=0.27$, $p<0.01$), but the level of agreement was poor ($p=0.0045$). As opposed to evening salivary CRP, morning CRP levels were not significantly correlated with serum CRP and the level of agreement was mediocre (Figure 4A). In the 60–69 year age interval, the level of agreement was best and both morning ($p=0.26$) and evening ($p=0.94$) salivary CRP could be used interchangeably with serum CRP (Figures 4B–F).

We further explored the strength of correlation below and above a clinically relevant serum CRP cut-off at ≥ 3 mg/L (11). Salivary evening CRP showed stronger correlation with serum CRP for high serum values ($r_s=0.54$, $p<0.05$) than for low ($r_s=0.28$, $p<0.01$), whereas this classification did not affect correlations between salivary morning CRP and serum CRP (Table 3). When high salivary CRP values (> 10 ng/ml) were excluded, the correlation between salivary morning CRP and serum CRP reached statistical significance ($r_s=0.24$, $p<0.05$).

Associations Between Salivary/Serum C-Reactive Protein and Plasma IL-6

Detectable IL-6 values ranged from 1.77 to 22.93 pg/ml and median IQR for all samples was 0.56 (0.56–2.85). As expected, participants with high serum CRP ($>$ median) had significantly higher IL-6 levels ($p=0.03$) than those with lower serum CRP concentrations ($\leq$ median). For all available samples ($n=97$), IL-6 levels correlated with serum CRP ($r_s=0.41$, $p<0.01$); however, when samples with IL-6 below detection limit were excluded the significance did not remain (Table 4). Using median split of daily mean salivary CRP values, we found no difference in IL-6 levels ($p=0.30$, $n=98$). These results remained essentially constant when testing separately for morning and evening saliva values (data not shown). In concordance, neither morning nor evening salivary CRP did correlate significantly with IL-6 levels (Table 4).

TABLE 1 | Descriptive statistics for C-reactive protein (CRP) levels in saliva and serum (levels below the detection limit were given the value 0.5 ng/mL; morning, $n=27$, and evening, $n=19$).

	Salivary CRP; morning [ng/ml]	Salivary CRP; evening [ng/ml]	Serum CRP [mg/L]
Range	<0.97–1674.2	<0.97–223.9	0.03–30.4
Mean (SD)	57.6 (222.75)	6.0 (24.07)	2.4 (4.68)
Median (IQR)	2.44 (0.50–10.80)	1.45 (1.05–2.72)	0.90 (0.34–2.43)
<i>n</i>	102	100	99

SD, standard deviation; IQR, interquartile range (25th to 75th percentiles).

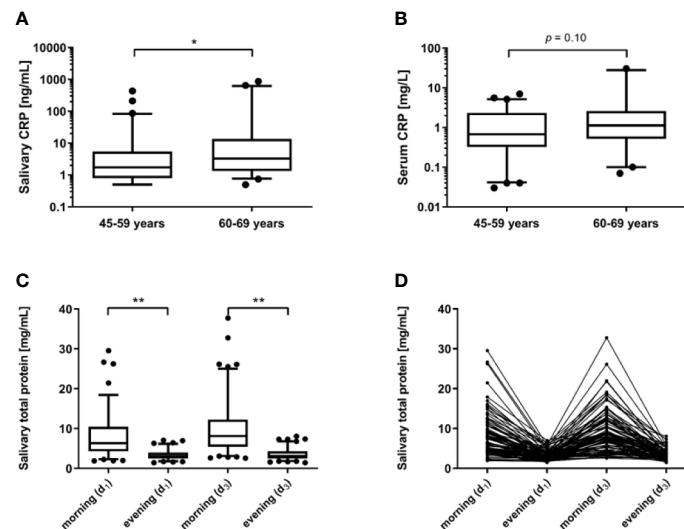


FIGURE 1 | C-reactive protein (CRP) in **(A)** saliva samples ($n=102$, shown are mean values of measurements in morning and evening saliva), and **(B)** sera ($n=96$). Participants were grouped into two age classes (45–59 years and 60–69 years, respectively) and compared using Mann-Whitney U test. Box plots reflect the interquartile range (IQR) with the median shown as a line and the 5th–95th percentiles as bars (* $p<0.05$). Spectrophotometric measurement of salivary total protein levels in morning and evening samples of the first (d_1) and third day (d_3) of sampling. **(C)** Differences in morning and evening levels of the same day were identified by Wilcoxon tests. Box plots reflect the IQR with the median shown as a line and the 5th–95th percentiles as bars (** $p<0.01$). **(D)** The four related total protein levels shown for each participant.

TABLE 2 | Spearman (r_s) correlations between total protein measures originated from morning and evening saliva collected at day 1 (d_1) and day 3 (d_3) of the sampling period.

	Salivary total protein; evening (d_1)	Salivary total protein; morning (d_3)	Salivary total protein; evening (d_3)
Salivary total protein; morning (d_1)	$r_s = 0.45^{**}$ $n = 93$	$r_s = 0.61^{***}$ $n = 94$	$r_s = 0.39^{**}$ $n = 94$
Salivary total protein; evening (d_1)		$r_s = 0.38^{**}$ $n = 93$	$r_s = 0.78^{***}$ $n = 93$
Salivary total protein; morning (d_3)			$r_s = 0.37^{**}$ $n = 101$

** $p < 0.01$.

*** $p < 0.001$.

Associations Between C-Reactive Protein, Body Mass Index and Tobacco Smoking

We found no significant difference in BMI levels between participants with low salivary and high salivary CRP ($p=0.59$, $n=99$). Higher BMI levels were found among participants with high serum CRP ($>$ median), whereas those with low serum CRP concentrations ($\leq$ median) had lower BMI levels ($p=0.01$, $n=96$). CRP in serum correlated with BMI ($r_s=0.43$, $p<0.01$), whereas morning and evening salivary CRP values showed no significant correlations with BMI (Table 4). Regarding tobacco smoking, non-smokers had significantly higher daily mean salivary CRP levels than smokers did ($p=0.015$, $n=98$; IQR=2.30 (1.11–7.74) and 1.13 (0.5–3.71) ng/ml, respectively). As compared to smokers, non-smokers showed borderline significant higher CRP using morning ($p=0.08$) and evening ($p=0.05$) saliva. In contrast, serum CRP levels were found to be independent of smoking ($p=0.27$).

Specificity and Sensitivity of Salivary C-Reactive Protein to Identify Elevated Serum C-Reactive Protein

By using the 90th percentile of serum CRP values among the 107 participants, a cut-off of 5.0 mg/L was achieved and used to define “systemic inflammation” based on laboratory findings. As demonstrated in Table 5, the specificity of both morning and evening saliva to detect individuals with biomarker-defined systemic inflammation was decent. However, the sensitivity was poor, particularly regarding morning saliva.

DISCUSSION

In the present study, we analyzed CRP and total protein in saliva from middle-aged individuals of the general population. A striking finding was the noticeable diurnal variation in salivary

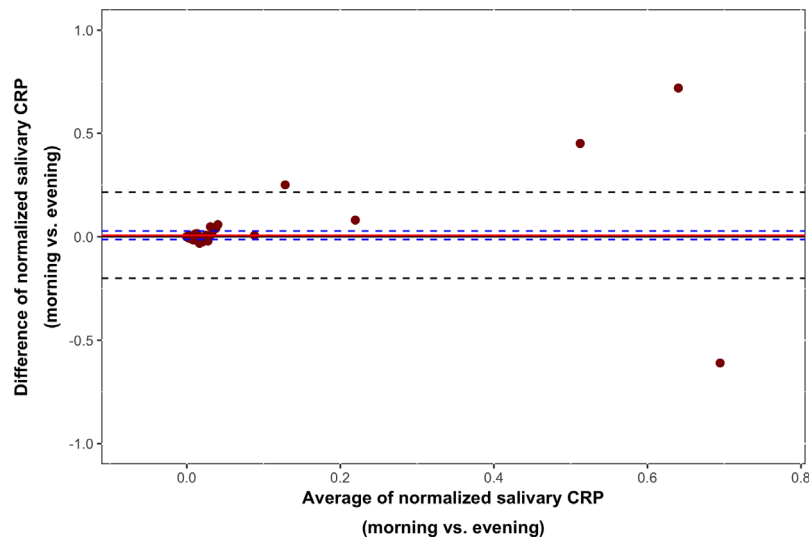


FIGURE 2 | A Bland-Altman graph illustrating the level of agreement between salivary morning and evening C-reactive protein (CRP) levels based on analysis of 100 participants. The level of agreement suggested interchangeability ($p=0.48$).

CRP and total protein with higher levels in mornings compared to evenings. Another observation was the considerable range of CRP in saliva and dilution factors had to be applied for some samples. These high levels of CRP were not be explained by visible blood contaminations, but might be due to acute local or systemic inflammatory processes. The observed similarity of CRP levels in saliva of men and women is in accordance with previous observations in serum (27) and saliva (23). Further, associations between aging and elevated CRP levels in serum have been reported in several studies (28) whereas such

connection was not found in saliva (19, 21). In contrast, however, we found a dependency of age also in saliva as the 60–69 year old participants had significantly higher salivary CRP levels as compared to the 45–59 year old group.

The finding of diurnal variation in salivary CRP is in line with earlier studies, which suggested a similar pattern and, importantly, used comparable sampling times at awaking and bedtime (20, 23). It has been reported that serum levels of CRP are also affected by diurnal variations with a peak at 3pm and slightly higher levels in the morning than in the evening (27). In

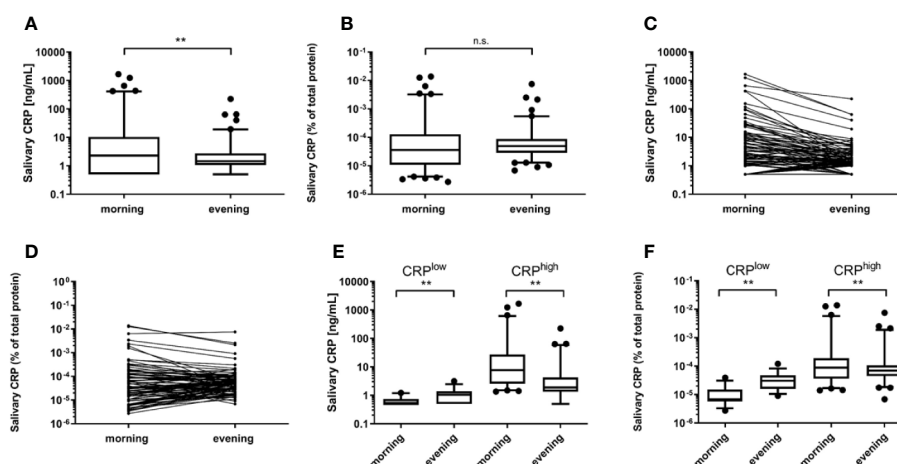
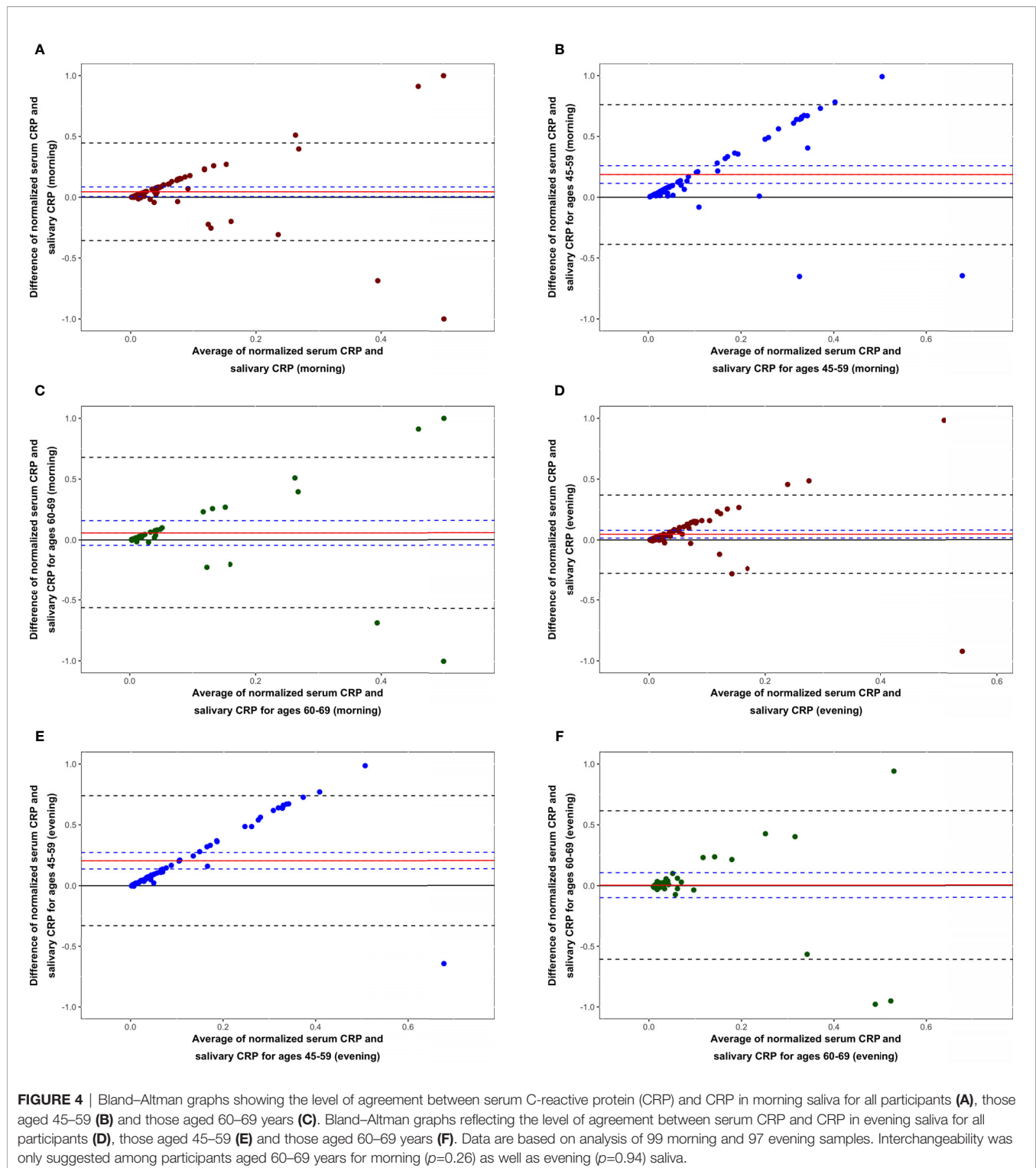


FIGURE 3 | C-reactive protein (CRP) levels in morning and evening saliva samples. Displayed are absolute CRP levels (**A**) and CRP levels relative to the total protein concentrations (**B**). Related morning and evening absolute CRP concentrations (**C**) and percentages (**D**) are shown for each participant. Morning CRP levels were classified as low (≤ 1.3 ng/ml) and high (> 1.3 ng/ml) concentrations, respectively, and each group was compared to the related evening values. Given are the results for absolute CRP (**E**) and CRP percentages of total protein (**F**). Differences in morning and evening levels of the same day were identified by Wilcoxon tests. Box plots reflect the IQR with the median shown as a line and the 5th–95th percentiles as bars. Significant differences are highlighted by ** $p < 0.01$ (n.s., not significant).



contrast, some smaller studies indicate that serum levels do not vary during the day (29, 30). We found a similar diurnal pattern for the salivary total protein concentrations, with a clear decrease toward the evening. Again, this is concordant to another study with similar sampling time points (23) whereas studies with later

morning sampling hours and fewer participants reported stable, or even rising, total protein concentrations over the day (31, 32).

Adjustment for total protein concentrations affected the diurnal pattern of salivary CRP. As both variables decreased toward the evening, the ratio was not significantly different over

TABLE 3 | Correlations between serum C-reactive protein (CRP), salivary CRP, and salivary CRP/total protein ratio calculated by Spearman's (r_s).

	Salivary CRP; morning	Salivary CRP; morning (% of total protein)	Salivary CRP; evening	Salivary CRP; evening (% of total protein)
Serum CRP	$r_s = 0.19$ $n = 99$	$r_s = 0.20$ $n = 99$	$r_s = 0.27^{**}$ $n = 97$	$r_s = 0.20^*$ $n = 97$
Serum CRP (≤ 3 mg/mL)	$r_s = 0.19$ $n = 80$	$r_s = 0.20$ $n = 80$	$r_s = 0.28^{**}$ $n = 79$	$r_s = 0.20^*$ $n = 79$
Serum CRP (> 3 mg/mL)	$r_s = 0.25$ $n = 19$	$r_s = 0.14$ $n = 19$	$r_s = 0.54^*$ $n = 18$	$r_s = 0.36$ $n = 18$

Values are shown for all samples as well as for the low and high serum CRP levels (≤ 3 mg/mL, > 3 mg/mL).

* $p < 0.05$.

** $p < 0.01$.

TABLE 4 | Spearman (r_s) correlations between serum C-reactive protein (CRP), salivary CRP, BMI and plasma IL-6.

	Serum CRP	Salivary CRP; morning	Salivary CRP; evening
BMI	$r_s = 0.43^{**}$ $n = 96$	$r_s = 0.06$ $n = 99$	$r_s = 0.15$ $n = 97$
IL-6	$r_s = 0.41^{**}$ $n = 97$	$r_s = -0.04$ $n = 98$	$r_s = 0.06$ $n = 96$
IL-6 [#]	$r_s = 0.10$ $n = 35$	$r_s = 0.23$ $n = 35$	$r_s = 0.22$ $n = 35$

Subjects with undetectable IL-6 levels were given half the detection limit's value. Additionally, correlations were calculated only for participants with detectable IL-6.

[#]IL-6 above detection limit.

** $p < 0.01$.

TABLE 5 | Performance of salivary morning and evening C-reactive protein (CRP) to identify "systemic inflammation", defined by the 90th percentile of serum CRP values.

	Sensitivity	Specificity	Accuracy
Salivary CRP; morning ($n=105$)	0.10 (0.11–0.31)	0.89 (0.85–0.91)	0.82 (0.77–0.85)
Salivary CRP; evening ($n=103$)	0.22 (0.18–0.43)	0.91 (0.87–0.93)	0.85 (0.81–0.88)

Sensitivity, specificity and accuracy, including 95% confidence intervals (in parentheses) are given.

the day when all samples were considered. However, the cut-off at 1.3 ng/mL of morning CRP, the ratio was decreasing toward the evening and contrariwise rising below this cut-off. As the low salivary CRP levels could be considered as baseline levels present in all healthy subjects, the differences in the low CRP subgroup could probably be regarded as a normal fluctuation. The low CRP subgroup contained mainly subjects with levels below the detection limit in the morning where a decrease toward the evening was not observed. It seems unlikely that the fall within the high CRP subgroup is only generated by higher dilution of saliva in the evening as there was still a decrease after correction for total protein. Furthermore, contrasting daily patterns for other salivary proteins such as α -amylase have been reported (33). Interestingly, the same authors showed that α -amylase also decreased strongly in the first 30 min after awaking and then started to rise over the day with the highest values in the evening.

To settle a standardized protocol of saliva sampling, the collection method is of utmost importance. Mechanical stimulation achieves higher flow rates than the passive drooling (34) and can be more comfortable for the donor. It seems that CRP and total protein levels do not differ between these two different collection methods or the use of salivettes (34, 35). It

should be noticed that the sampling method used in this study is somewhat in between passive collection and mechanical stimulation as the sampling was performed using cotton swabs. Only in cases of too low flow rate, the participants were chewing on the swab and thus stimulating salivation. Taken together it seems beneficial to use salivettes, but not entirely necessary. Our results accentuate the need to standardize a sampling protocol and especially the sampling time point.

The CRP saliva-to-serum concentration ratio of the present study is in fairly agreement with that of a previous study, 1:1,084 versus 1:1,663 (19). Positive correlation between salivary and serum CRP have initially been reported in animal studies, such as in pigs (36). Since then several studies in healthy and diseased individuals investigated this association in humans with diverging results. One group reported no significant correlation between CRP in serum and saliva in healthy donors, although there was a trend toward it among a subgroup with high CRP levels (22). Opposed to them, other studies indicated moderate to strong correlations in healthy donors and in women exposed to intimate partner violence (19, 20), and further correlation was found in patients with ischemic heart disease (21). Our results present a low to moderate correlation between serum and salivary evening CRP,

but stronger when stratifying for high serum CRP. Morning CRP did only show significant correlation when individuals with high salivary CRP levels (>10 ng/ml) were excluded. Very high salivary CRP morning levels could be due to such an acute phase reaction, already having started to decrease at the time of serum collection, and vice versa. Low levels of salivary CRP has been shown to predict low plasma levels more accurately than high salivary levels predicted high serum concentrations (20). Based on our data, we conclude that serum and salivary CRP levels do correlate but to a lower extent than previously reported. Only in subjects of the 60–69 year age interval, the statistical analyses suggested interchangeability between serum and saliva CRP.

Smoking, BMI and plasma IL-6 are known to be associated with systemic inflammation. Earlier, such correlations were reported also for salivary CRP and those variables (19). A study of women exposed to intimate partner violence could confirm the results for BMI, but not for smoking (21), contrasting our results without significant association between salivary CRP and BMI. The higher salivary CRP levels among non-smokers was unexpected and warrants further investigation. Nevertheless, anti-inflammatory properties of nicotine have been documented in obesity and nicotine-mediated modulation of hypothalamic-pituitary-adrenal axis activity was suggested as a potential mechanism (37, 38).

An essential issue when considering salivary CRP as a biomarker is to understand where it's produced and/or the route of CRP entry to saliva – a question that has not been fully elucidated. Because of its high molecular weight and relative insolubility in lipids, it seems unlikely that CRP enters by diffusion or ultrafiltration through the tight junctions of the cells from the circulation (15, 16). The entry *via* the gingival crevicular fluid (GCF), a liquid found in the gingival sulcus between free gingival and the tooth is one possibility. CRP in GCF has been reported to be of systemic origin (39). The presence of CRP in GCF could be a result of systemic inflammation induced by periodontitis or disease elsewhere in the body. Salivary CRP levels may be elevated in patients with periodontal diseases (40), which emphasizes the need to consider oral health in following studies. Although the main origin of CRP is the liver, there have also been signs of CRP production by the salivary glands as indicated by elevated mRNA levels (41). The correlation of salivary CRP with detectable circulating IL-6, however, is another indication for derivation from the liver. It could be that by some unknown mechanism CRP is attracted to local oral sites of inflammation without affecting the systemic CRP concentrations.

The study has some limitations that should be mentioned. We acknowledge the lack of data on oral health status and salivation rate, which both might have biased the results and should be considered as limitations. Furthermore, sera were generally collected 3 to 4 days after the saliva used for CRP determination (d_3). It cannot be excluded that this procedure could have affected the results. Finally, the definition of “systemic inflammation” was based on serum CRP levels only (no physical examination of the participants was performed). However, all individuals with symptoms of infection or severe disease had already been excluded (24).

To summarize, we observed evident diurnal variation of salivary CRP and total protein with higher levels at awaking time in the mornings compared to evenings. This highlights the need to standardize collecting times before introduction in clinical routine. The correlation between saliva and serum was low to moderate; stronger in the subgroup of serum CRP >3 mg/L, but interchangeability was suggested only among older participants. We found no clear impact of sex, but higher age and non-smoking were associated with increased levels of salivary CRP. The sensitivity of salivary CRP to detect individuals with elevated serum CRP was not impressive. In contrast to serum CRP, salivary CRP did not correlate with BMI or plasma IL-6. Further investigations are needed to clarify the relevance and suitability of CRP (and possibly other pentraxins) as salivary biomarkers before reliable introduction in clinical routine is possible.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The LSH study protocol was approved by the regional ethics review board in Linköping (No. 02–324). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. JW and CS had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. JW, SL, MK, PG, and CS conceptualized and designed the study. SL, MK, and PG acquired the data. JW, SL, FC, MK, PG, and CS analyzed and interpreted the data. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by grants from the Swedish Rheumatism Association (R-844801), the Region Östergötland (ALF Grants) (LiO-932055), the Swedish Society of Medicine (SLS-590231), the King Gustaf V's 80-year Anniversary foundation (FAI-2018-0504) and the King Gustaf V and Queen Victoria's Freemasons' foundation.

REFERENCES

- Bottazzi B, Garlanda C, Teixeira MM. Editorial: The Role of Pentraxins: From Inflammation, Tissue Repair and Immunity to Biomarkers. *Front Immunol* (2019) 10:2817. doi: 10.3389/fimmu.2019.02817
- Lu J, Mold C, Du Clos TW, Sun PD. Pentraxins and Fc Receptor-Mediated Immune Responses. *Front Immunol* (2018) 9:2607. doi: 10.3389/fimmu.2018.02607
- Sjöwall C, Wetterö J, Bengtsson T, Askendal A, Almroth G, Skogh T, et al. Solid-phase classical complement activation by C-reactive protein (CRP) is inhibited by fluid-phase CRP-C1q interaction. *Biochem Biophys Res Commun* (2007) 352:251–58. doi: 10.1016/j.bbrc.2006.11.013
- Ma YJ, Garred P. Pentraxins in Complement Activation and Regulation. *Front Immunol* (2018) 9:3046. doi: 10.3389/fimmu.2018.03046
- Sjöwall C, Wetterö J. Pathogenic implications for autoantibodies against C-reactive protein and other acute phase proteins. *Clin Chim Acta* (2007) 378:13–23. doi: 10.1016/j.cca.2006.12.002
- Sjöwall C, Olin AI, Skogh T, Wetterö J, Mörgelin M, Nived O, et al. Bengtsson AA. C-reactive protein, immunoglobulin G and complement co-localize in renal immune deposits of proliferative lupus nephritis. *Autoimmunity* (2013) 46:205–14. doi: 10.3109/08916934.2013.764992
- Yao Z, Zhang Y, Potempa LA, Rajab I, Ji L, Lv L, et al. A redox sensitivity-based method to quantify both pentameric and monomeric C-reactive protein in a single assay. *J Immunol Methods* (2019) 470:40–5. doi: 10.1016/j.jim.2019.04.009
- Rajab IM, Hart PC, Potempa LA. How C-Reactive Protein Structural Isoforms With Distinctive Bioactivities Affect Disease Progression. *Front Immunol* (2020) 11:2126. doi: 10.3389/fimmu.2020.02126
- Enocsson H, Sjöwall C, Skogh T, Eloranta ML, Rönnblom L, Wetterö J. Interferon-alpha mediates suppression of C-reactive protein: explanation for muted C-reactive protein response in lupus flares? *Arthritis Rheumatol* (2009) 60:3755–60. doi: 10.1002/art.25042
- Enocsson H, Sjöwall C, Kastbom A, Skogh T, Eloranta ML, Rönnblom L, et al. Association of serum C-reactive protein levels with lupus disease activity in the absence of measurable interferon- α and a C-reactive protein gene variant. *Arthritis Rheumatol* (2014) 66:1568–73. doi: 10.1002/art.38408
- Pearson TA, Bazzarre TL, Daniels SR, Fair JM, Fortmann SP, Franklin BG, et al. American Heart Association Expert Panel on Population and Prevention Science, American Heart Association guide for improving cardiovascular health at the community level: a statement for public health practitioners, healthcare providers, and health policy makers from the American Heart Association Expert Panel on Population and Prevention Science. *Circulation* (2003) 107:499–511. doi: 10.1161/01.cir.0000054482.38437.13
- Ridker PM. C-reactive protein: Eighty years from discovery to emergence as a major risk marker for cardiovascular disease. *Clin Chem* (2009) 55:209–15. doi: 10.1373/clinchem.2008.119214
- Grad E, Danenberg HD. C-reactive protein and atherothrombosis: Cause or effect? *Blood Rev* (2013) 27:23–9. doi: 10.1016/j.blre.2012.12.001
- Engeland CG, Bosch JA, Rohleder N. Salivary Biomarkers in Psychoneuroimmunology. *Curr Opin Behav Sci* (2019) 28:58–65. doi: 10.1016/j.cobeha.2019.01.007
- Pfaffe T, Cooper-White J, Beyerlein P, Kostner K, Punyadeera C. Diagnostic potential of saliva: current state and future applications. *Clin Chem* (2011) 57:675–87. doi: 10.1373/clinchem.2010.153767
- Kaufman E, Lamster IB. The diagnostic applications of saliva – a review. *Crit Rev Oral Biol Med* (2002) 13:197–212. doi: 10.1177/154411130201300209
- Szabo YZ, Slavish DC, Graham-Engeland JE. The effect of acute stress on salivary markers of inflammation: A systematic review and meta-analysis. *Brain Behav Immun* (2020) 88:887–900. doi: 10.1016/j.bbi.2020.04.078
- Dodds MW, Johnson DA, Yeh CK. Health benefits of saliva: a review. *J Dent* (2005) 33:223–33. doi: 10.1016/j.jdent.2004.10.009
- Ouellet-Morin I, Danese A, Williams B, Arseneault L. Validation of a high-sensitivity assay for C-reactive protein in human saliva. *Brain Behav Immun* (2011) 25:640–6. doi: 10.1016/j.bbi.2010.12.020
- Out D, Hall RJ, Granger DA, Page GG, Woods SJ. Assessing salivary C-reactive protein: longitudinal associations with systemic inflammation and cardiovascular disease risk in women exposed to intimate partner violence. *Brain Behav Immun* (2012) 26:543–51. doi: 10.1016/j.bbi.2012.01.019
- Punyadeera C, Dimeski G, Kostner K, Beyerlein P, Cooper-White J. One-step homogeneous C-reactive protein assay for saliva. *J Immunol Methods* (2011) 373:19–25. doi: 10.1016/j.jim.2011.07.013
- Dillon MC, Opris DC, Kopanczyk R, Lickliter J, Cornwell HN, Bridges EG, et al. Detection of homocysteine and C-reactive protein in the saliva of healthy adults: comparison with blood levels. *Biomark Insights* (2010) 5:57–61. doi: 10.4137/bmi.s5305
- Izawa S, Miki K, Liu X, Ogawa N. The diurnal patterns of salivary interleukin-6 and C-reactive protein in healthy young adults. *Brain Behav Immun* (2013) 27:38–41. doi: 10.1016/j.bbi.2012.07.001
- Garvin P, Nilsson L, Carstensen J, Jonasson L, Kristenson M. Circulating matrix metalloproteinase-9 is associated with cardiovascular risk factors in a middle-aged normal population. *PLoS One* (2008) 3(3):e1774. doi: 10.1371/journal.pone.0001774
- Garvin P, Carstensen J, Kristenson M. Pooling ambulatory saliva cortisol samples over consecutive days – as reliable as arithmetic means. *Scand J Clin Lab Invest* (2008) 68:508–12. doi: 10.1080/00365510701832229
- Sjöwall C, Ernerudh J, Bengtsson AA, Sturfelt G, Skogh T. Reduced anti-TNF α autoantibody levels coincide with flare in systemic lupus erythematosus. *J Autoimmun* (2004) 22:315–23. doi: 10.1016/j.jaut.2004.02.003
- Rudnicka AR, Rumley A, Lowe GD, Strachan DP. Diurnal, seasonal, and blood-processing patterns in levels of circulating fibrinogen, fibrin D-dimer, C-reactive protein, tissue plasminogen activator, and von Willebrand factor in a 45-year-old population. *Circulation* (2007) 115:996–1003. doi: 10.1161/CIRCULATIONAHA.106.635169
- Ballou SP, Lozanski FB, Hodder S, Rzewnicki DL, Mion LC, Sipe JD, et al. Quantitative and qualitative alterations of acute-phase proteins in healthy elderly persons. *Age Ageing* (1996) 25:224–30. doi: 10.1093/ageing/25.3.224
- Meier-Ewert HK, Ridker PM, Rifai N, Price N, Dinges DF, Mullington JM. Absence of diurnal variation of C-reactive protein concentrations in healthy human subjects. *Clin Chem* (2001) 47:426–30. doi: 10.1093/clinchem/47.3.426
- Mills PJ, Natarajan L, von Känel R, Ancoli-Israel S, Dimsdale JE. Diurnal variability of C-reactive protein in obstructive sleep apnea. *Sleep Breath* (2009) 13:415–20. doi: 10.1007/s11325-009-0268-0
- Harden CJ, Perez-Carrion K, Babakordi Z, Plummer SF, Hepburn N, Barker ME, et al. Evaluation of the salivary proteome as a surrogate tissue for systems biology approaches to understanding appetite. *J Proteomics* (2012) 75:2916–23. doi: 10.1016/j.jprot.2011.12.010
- Rantonen PJ, Meurman JH. Correlations between total protein, lysozyme, immunoglobulins, amylase, and albumin in stimulated whole saliva during daytime. *Acta Odontol Scand* (2000) 58:160–65. doi: 10.1080/000163500429154
- Nater UM, Rohleder N, Schlotz W, Ehler U, Kirschbaum C. Determinants of the diurnal course of salivary α -amylase. *Psychoneuroendocrinology* (2007) 32:392–401. doi: 10.1016/j.psyneuen.2007.02.007
- Mohamed R, Campbell JL, Cooper-White J, Dimeski G, Punyadeera C. The impact of saliva collection and processing methods on CRP, IgE, and Myoglobin immunoassays. *Clin Transl Med* (2012) 1(1):19. doi: 10.1186/2001-1326-1-19
- Golatoski C, Salazar MG, Dhople VH, Hammer E, Kocher T, Jehmlich N, et al. Comparative evaluation of saliva collection methods for proteome analysis. *Clin Chim Acta* (2013) 419:42–6. doi: 10.1016/j.cca.2013.01.013
- Gutiérrez AM, Martínez-Subiela S, Eckersall PD, Cerón JJ. C-reactive protein quantification in porcine saliva: a minimally invasive test for pig health monitoring. *Vet J* (2009) 181:261–5. doi: 10.1016/j.tvjl.2008.03.021
- Lakhan SE, Kirchgessner A. Anti-inflammatory effects of nicotine in obesity and ulcerative colitis. *J Transl Med* (2011) 9:129. doi: 10.1186/1479-5876-9-129
- Wong JA, Pickworth WB, Waters AJ, al'Absi M, Leventhal AM. Cortisol levels decrease after acute tobacco abstinence in regular smokers. *Hum Psychopharmacol* (2014) 29:152–62. doi: 10.1002/hup.2382

39. Megson E, Fitzsimmons T, Dharmapathi K, Bartold PM. C-reactive protein in gingival crevicular fluid may be indicative of systemic inflammation. *J Clin Periodontol* (2010) 37:797–804. doi: 10.1111/j.1600-051X.2010.01603.x
40. Christodoulides N, Mohanty S, Miller CS, Langub MC, Floriano PN, Dharshan P, et al. Application of microchip assay system for the measurement of C-reactive protein in human saliva. *Lab Chip* (2005) 5:261–9. doi: 10.1039/b414194f
41. Wei W, Parvin N, Tsumura K, Akamatsu T, Tada J, Kanamori N, et al. Induction of C-reactive protein, serum amyloid P component, and kininogens in the submandibular and lacrimal glands of rats with experimentally induced inflammation. *Life Sci* (2001) 69:359–68. doi: 10.1016/s0024-3205(01)01129-8

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Wetterö, von Löhneysen, Cobar, Kristenson, Garvin and Sjöwall. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



C-Reactive Protein-Based Strategy to Reduce Antibiotic Dosing for the Treatment of Pneumococcal Infection

Donald N. Ngwa[†], Sanjay K. Singh[†] and Alok Agrawal^{*}

Department of Biomedical Sciences, James H. Quillen College of Medicine, East Tennessee State University, Johnson City, TN, United States

OPEN ACCESS

Edited by:

Richard H. Gomer,
Texas A&M University, United States

Reviewed by:

Alexander J. Szalai,
University of Alabama at Birmingham,
United States

Rachel Victoria Jimenez,
Moffitt Cancer Center, United States

*Correspondence:

Alok Agrawal
agrawal@etsu.edu

[†]These authors have contributed
equally to this work

Specialty section:

This article was submitted to
Molecular Innate Immunity,
a section of the journal
Frontiers in Immunology

Received: 23 October 2020

Accepted: 03 December 2020

Published: 20 January 2021

Citation:

Ngwa DN, Singh SK and Agrawal A
(2021) C-Reactive Protein-Based
Strategy to Reduce Antibiotic
Dosing for the Treatment of
Pneumococcal Infection.
Front. Immunol. 11:620784.
doi: 10.3389/fimmu.2020.620784

C-reactive protein (CRP) is a component of innate immunity. The concentration of CRP in serum increases in microbial infections including *Streptococcus pneumoniae* infection. Employing a mouse model of pneumococcal infection, it has been shown that passively administered human wild-type CRP protects mice against infection, provided that CRP is injected into mice within two hours of administering pneumococci. Engineered CRP (E-CRP) molecules have been reported recently; unlike wild-type CRP, passively administered E-CRP protected mice against infection even when E-CRP was injected into mice after twelve hours of administering pneumococci. The current study was aimed at comparing the protective capacity of E-CRP with that of an antibiotic clarithromycin. We established a mouse model of pneumococcal infection in which both E-CRP and clarithromycin, when used alone, provided minimal but equal protection against infection. In this model, the combination of E-CRP and clarithromycin drastically reduced bacteremia and increased survival of mice when compared to the protective effects of either E-CRP or clarithromycin alone. E-CRP was more effective in reducing bacteremia in mice treated with clarithromycin than in untreated mice. Also, there was 90% reduction in antibiotic dosing by including E-CRP in the antibiotic-treatment for maximal protection of infected mice. These findings provide an example of cooperation between the innate immune system and molecules that prevent multiplication of bacteria, and that should be exploited to develop novel combination therapies for infections against multidrug-resistant pneumococci. The reduction in antibiotic dosing by including E-CRP in the combination therapy might also resolve the problem of developing antibiotic resistance.

Keywords: C-reactive protein, clarithromycin, pneumococcal infection, *Streptococcus pneumoniae*, combination therapy

INTRODUCTION

C-reactive protein (CRP) is a critical host defense molecule of the innate immune system (1, 2). CRP binds to cells and molecules, host or foreign, which have accessible phosphocholine (PCh) moieties (3, 4). One example of CRP-ligands is pneumococcal C-polysaccharide found on the surface of *Streptococcus pneumoniae* (5). Once bound to a PCh-bearing ligand, CRP activates the complement

system in both human and murine sera to damage and, if possible, eliminate the ligand (6–9). Experiments employing human CRP transgenic mice, CRP-deficient mice, and normal mice in which human wild-type CRP (WT CRP) was passively administered have all revealed that CRP is protective against pneumococcal infection (2, 10–15). It has also been shown that the anti-pneumococcal activity of CRP *in vivo* is due to the ability of ligand-complexed CRP to activate the complement system (9, 16–18). However, WT CRP was found to be protective only when given to mice within 2 h of administering pneumococci in the mouse model of infection in which WT CRP is passively administered (16).

CRP is a pentameric molecule comprised of five identical subunits (19, 20). Recently, two types of engineered pentameric CRP (E-CRP), E-CRP-1, and E-CRP-2, generated by oligonucleotide-directed site-specific mutagenesis of WT CRP cDNA, have been reported (21). In E-CRP-1, four amino acid residues were mutated (E42Q/F66A/T76Y/E81A). In E-CRP-2, two amino acid residues were mutated (Y40F/E42Q). Tyr⁴⁰ and Glu⁴² are present in the intrinsically disordered region of CRP. Glu⁴² is also a part of the inter-subunit contact region. Phe⁶⁶, Thr⁷⁶ and Glu⁸¹ form the PCh-binding site of CRP (19, 20). E-CRP-1 does not bind to PCh while E-CRP-2, like WT CRP, binds to PCh. Both E-CRP-1 and E-CRP-2 bind to complement inhibitor factor H recruited by pneumococci on their surface in mouse circulation; WT CRP does not bind to immobilized factor H (22–25). Unlike passively administered WT CRP, both E-CRP-1 and E-CRP-2 protected mice against infection even when E-CRP was administered 12 h after administering pneumococci, indicating that a conformationally altered form of CRP that can bind to immobilized factor H is required for CRP-mediated protection of mice against late-stage pneumococcal infection (1, 21). Accordingly, it was hypothesized that in individuals in whom the conformation of CRP remains unchanged, perhaps due to inappropriate inflammatory conditions around CRP, CRP is not fully functional during infection (21). It was proposed that the use of E-CRP might be beneficial for treatment of infections against antibiotic-resistant pneumococci (21).

Antibiotics are commonly used to treat pneumococcal infection in humans (26). The aim of this study was to directly compare the protective effects (increase in survival and decrease in bacteremia) of E-CRP and antibiotics. Since the antibiotic clarithromycin has been used previously in mouse models of pneumococcal infection and was found to be protective when administered into mice later during the infection (27), clarithromycin was selected for the current study. Both E-CRP-1 and E-CRP-2 were included in the study to compare the protective effects with that of clarithromycin. A mouse model of pneumococcal infection was employed in which E-CRP-1, E-CRP-2, and clarithromycin, when used singly, provided minimal but equal protection against infection. This model was suitable to investigate the protection against infection when E-CRP-1 or E-

CRP-2 and clarithromycin were combined for the treatment of mice. The results of the experiments indicate that E-CRP is more effective in reducing bacteremia in mice when used in the presence of clarithromycin.

MATERIALS AND METHODS

Preparation of CRP

The cDNAs for E-CRP-1 (E42Q/F66A/T76Y/E81A mutant CRP) and E-CRP-2 (Y40F/E42Q mutant CRP) were constructed and expressed in CHO cells using the ExpiCHO Expression System (Thermo Fisher Scientific), as described earlier (21). Purification of E-CRP-1 from cell culture supernatants involved Ca²⁺-dependent affinity chromatography on a phosphoethanolamine-conjugated Sepharose column, followed by ion-exchange chromatography on a MonoQ column and gel filtration on a Superose12 column, as described earlier (14). E-CRP-2 was purified by Ca²⁺-dependent affinity chromatography on a PCh-conjugated Sepharose column, followed by ion-exchange chromatography on a MonoQ column and gel filtration on a Superose12 column, as described earlier (8). Native WT CRP was purified from discarded human pleural fluid by Ca²⁺-dependent affinity chromatography on a PCh-conjugated Sepharose column, followed by ion-exchange chromatography on a MonoQ column and gel filtration on a Superose12 column, as described earlier (8), and was used throughout this study. Purified CRP was dialyzed against 10 mM Tris-HCl, pH 7.2, containing 150 mM NaCl and 2 mM CaCl₂, and was subsequently treated with Detoxi-Gel Endotoxin Removing Gel (Thermo Fisher Scientific) according to manufacturer's instructions. The concentration of endotoxin in CRP preparations was determined by using the Limulus Amebocyte Lysate kit QCL-1000 (Lonza). Purified CRP was stored at 4°C and used within a week for mouse protection experiments.

Pneumococci

Streptococcus pneumoniae type 3, strain WU2, was obtained as a gift from Dr. David Briles (University of Alabama at Birmingham, Birmingham, AL, USA). Pneumococci were made virulent by sequential i.v. passages in mice and were stored in aliquots at –80°C, as described previously (14, 15). For each experiment, a separate aliquot of pneumococci was thawed and cultured, as described previously (14, 15). Cultured pneumococci were resuspended in normal saline at a concentration of 3.5×10^8 cfu/ml based on the absorbance of the resuspension at 600 nm ($A_{600} = 1.00 = 1.2 \times 10^9$ cfu/ml). Within 2 h, 100 μ l (3.5×10^7 cfu) of pneumococci suspension was injected into each mouse, as reported previously (14, 15, 21). The concentration of pneumococci was confirmed next day by plating on sheep blood agar plates.

Mice

Male C57BL/6J mice (Jackson Laboratories) were brought up and maintained according to protocols approved by the

Abbreviations: CRP, C-reactive protein; E-CRP-1, engineered CRP mutant E42Q/F66A/T76Y/E81A; E-CRP-2, engineered CRP mutant Y40F/E42Q; moCRP, endogenous mouse CRP; PCh, phosphocholine; WT CRP, native wild-type CRP purified from discarded human body fluids.

University Committee on Animal Care. Mice were 8–10 weeks old when used in experiments.

Determination of the Experimental Dose of Clarithromycin

The antibiotic clarithromycin (Santa Cruz Biotechnology, sc-205634) was reconstituted in acetone at a concentration of 50 mg/ml and stored at 4°C for a maximum of 5 days. To determine the experimental dose for *in vivo* experiments, clarithromycin (50 mg/ml) was diluted in acetone at 40, 4.0, 0.4 and 0.04 mg/ml, and 50 µl of each concentration was injected i.v. into mouse, four times, at 12, 36, 60 and 84 h after the administration of pneumococci. Thus, the final amount of clarithromycin in each group of mice was 2, 0.2, 0.02 and 0.002 mg per mouse per injection. As shown in **Figure 1**, it was clear that a dose of 0.02 mg clarithromycin per mouse per injection was most suitable to evaluate the effects of the combination of clarithromycin and E-CRP on the protection of mice against infection. The dose of 0.02 mg of clarithromycin resulted in a survival curve which fell in the middle so that a shift of the curve, either above or below, could be observed when E-CRP is added.

Mouse Protection Experiments

Two separate protection experiments were performed using 25 µg of purified WT CRP, E-CRP-1 and E-CRP-2, and 3.5×10^7 cfu of pneumococci, as described earlier (14, 15, 21). The average amount of endotoxin in 25 µg of all CRP preparations was 1.2 ± 1.1 endotoxin units. Mice were first injected i.v. with 3.5×10^7 cfu of pneumococci. The actual number of pneumococci injected, based on the plating results obtained on the next day, was $3.55 \pm 0.44 \times 10^7$ cfu. Mice were injected i.v. with either WT CRP, E-CRP-1 or E-CRP-2, 12 h after the administration of pneumococci. Since clarithromycin is soluble in normal saline at 0.2 mg/ml, stock clarithromycin (50 mg/ml in acetone) was diluted in normal saline to a final concentration of 0.2 mg/ml, and 100 µl was injected i.v. per mouse at 12, 36, 60 and 84 h after the administration of pneumococci, to achieve a final dose of 0.02 mg of clarithromycin per mouse per injection. Survival of mice was recorded three times per day for 7 days. To determine bacteremia (cfu/ml) in the surviving mice, blood was collected daily for 5 days from the tip of the tail vein, diluted in normal saline, and plated on sheep blood agar for colony counting. The bacteremia value for dead mice was recorded as 10^9 cfu/ml because mice died when the bacteremia exceeded 10^8 cfu/ml.

Statistical Analysis

Survival curves were generated using the GraphPad Prism 4 software. To determine *p*-values for the differences in the survival curves among various groups, the survival curves were compared using the software's Logrank (Mantel-Cox) test. The scatter plots of the bacteremia data and the median bacteremia value for each group were generated using the GraphPad Prism 4 software. Bacteremia values of 0–100 were plotted as 100 and bacteremia values of $>10^8$ were plotted as 10^9 . To determine *p*-values for the differences in bacteremia among various groups at each time point, scatter plots were compared using the software's Mann-Whitney test. The software's Mann-Whitney test included all the

dots in the scatter plots and not just the median values for each time point.

RESULTS

Anti-Pneumococcal Effects of E-CRP-1, Clarithromycin, and Their Combination

As shown in **Figure 2**, and as reported previously (21), E-CRP-1 increased significantly the median survival time (MST, the time taken for the death of 50% of mice) of mice infected with pneumococci. The MST for mice injected with bacteria alone (group A) was 60 h while the MST for mice injected with E-CRP-1 (group B) was 84 h. Similarly, clarithromycin also significantly increased the survival of mice infected with pneumococci; the MST for mice treated with clarithromycin (group C) was 96 h. The increase in the MST for mice treated with either E-CRP-1 or clarithromycin were not significantly different from each other. In this mouse model of pneumococcal infection, it has been reported previously that WT CRP does not increase the MST if mice received WT CRP 12 h after receiving pneumococci (21). The protection in response to the combination of WT CRP and

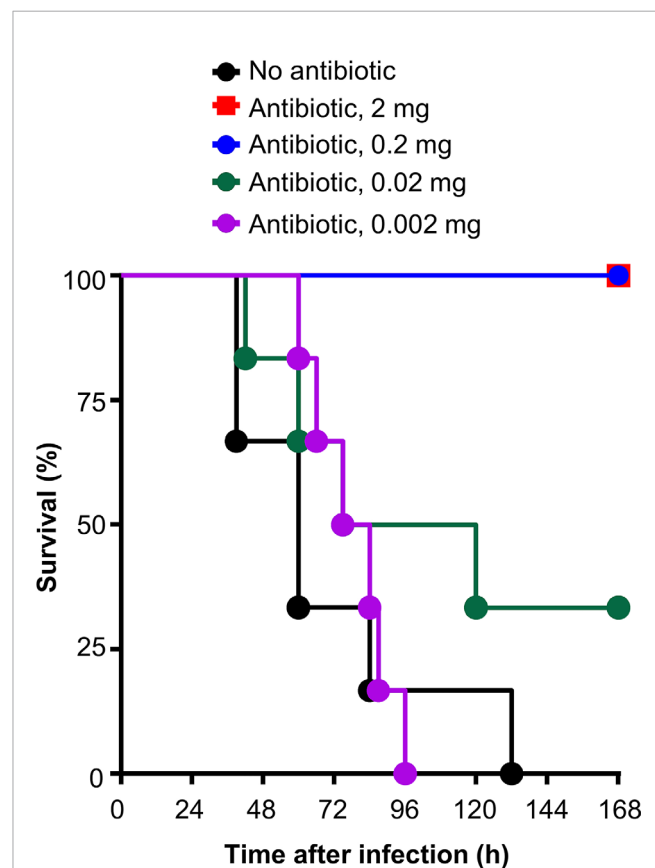


FIGURE 1 | Survival of mice infected with pneumococci and treated with different doses of clarithromycin. Clarithromycin was injected four times, at 12, 36, 60 and 84 h, after the administration of pneumococci. Six mice were used for each dose of clarithromycin.

clarithromycin (group D) was similar to that of clarithromycin alone. The MST for mice treated with the combination of WT CRP and clarithromycin was 84 h similar to the MST for mice treated with clarithromycin alone (group C). In contrast to WT CRP, the combination of E-CRP-1 and clarithromycin significantly increased the survival of infected mice (group E). The MST for mice treated with both E-CRP-1 and clarithromycin was significantly different than the MST for mice treated with either agent alone. The MST for mice treated with E-CRP-1 and clarithromycin could not be calculated since ~90% of mice survived till the end of the experiment. In all other groups, >75% mice died within 5 days. The combination of E-CRP-1 and 0.02 mg clarithromycin provided the same protection (Figure 2) in terms of survival of mice as was seen with 0.2 mg of clarithromycin when used alone (Figure 1). Thus, there was 90%

reduction in the dose of clarithromycin when used in combination with E-CRP-1.

The protective effects of E-CRP-1 (group B) and clarithromycin (group C) on the survival of mice, when used alone, were due to significant decrease in bacteremia (Figures 3A, B). E-CRP-1, as reported previously (21), and clarithromycin, both significantly decreased bacteremia. The decrease in bacteremia by E-CRP-1 and by clarithromycin were not significantly different from each other. Consistent with the survival data, the combination of WT CRP and clarithromycin (group D) did not significantly affect the protective ability of clarithromycin in terms of decreasing bacteremia. The dramatic increase in the survival of infected mice by the combination of E-CRP-1 and clarithromycin (group E) was due to the drastic decrease in bacteremia. The decrease in bacteremia in mice treated with both agents was significantly different from the decrease in bacteremia when mice were treated with either of the two agents alone. Bacteremia did not rise beyond 10^4 cfu/ml in mice treated with both E-CRP-1 and clarithromycin. The median bacteremia was maintained at the reduced level from the beginning to the end of the experiment.

Overall, based on the statistical analyses of the survival curves (Figure 2) and of the scatter plots for bacteremia (Figure 3A), highly significant differences were found between the groups of mice treated with both E-CRP-1 and clarithromycin and the groups of mice treated with either agent alone. The anti-pneumococcal effects of E-CRP-1 were enhanced in the presence of low-dose clarithromycin. Combining E-CRP-1 with clarithromycin in the treatment of pneumococcal infection in this mouse model reduced the dose of clarithromycin by 90%.

Anti-Pneumococcal Effects of E-CRP-2, Clarithromycin, and Their Combination

As shown in Figure 4, and as reported previously (21), E-CRP-2 increased significantly the MST of mice infected with pneumococci. The MST for mice injected with bacteria alone (group A) was 54 h while the MST for mice injected with E-CRP-2 (group B) was 132 h. Clarithromycin also significantly increased the survival of mice infected with pneumococci; the MST for mice treated with clarithromycin (group C) was 108 h. The increase in the MST for mice treated with either E-CRP-2 or clarithromycin were not significantly different from each other. The combination of E-CRP-2 and clarithromycin significantly increased the survival of infected mice (group D). The MST for mice treated with both E-CRP-2 and clarithromycin was significantly different than the MST for mice treated with either agent alone. The MST for mice treated with the combination of E-CRP-2 and clarithromycin could not be calculated since ~80% of mice survived till the end of the experiment. In all other groups, >50% mice died in 5 days. The combination of E-CRP-2 and 0.02 mg clarithromycin provided the same protection (Figure 4) in terms of survival of mice as was seen with 0.2 mg of clarithromycin when used alone (Figure 1). Thus, like E-CRP-1, there was 90% reduction in the dose of clarithromycin when combined with E-CRP-2.

The protective effects of E-CRP-2 (group B) and clarithromycin (group C) on the survival of mice, when used

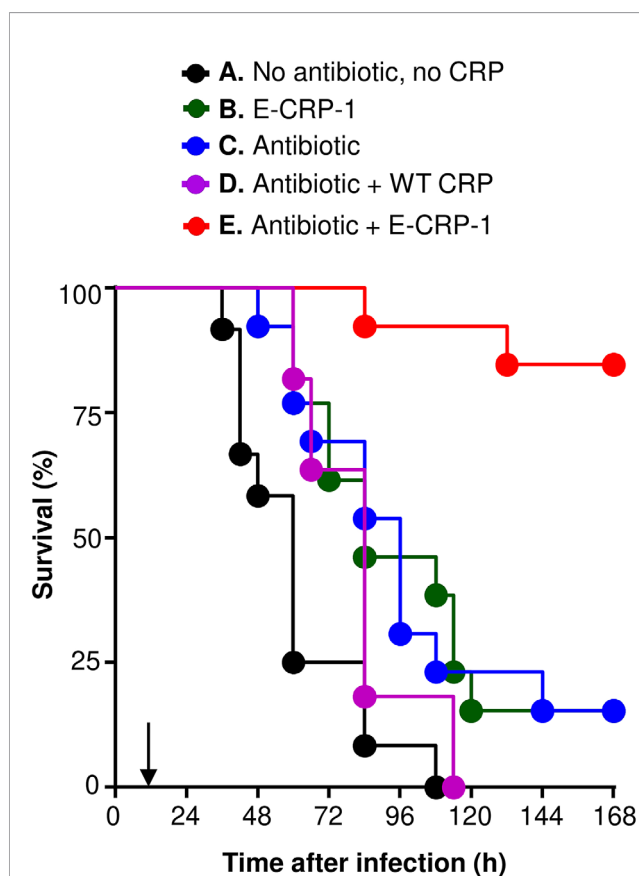


FIGURE 2 | Survival of mice infected with pneumococci and treated with E-CRP-1 and clarithromycin. E-CRP-1 or WT CRP was injected 12 h after administering pneumococci and is indicated by an arrow on the x-axis. Clarithromycin (0.02 mg) was injected four times, at 13, 36, 60 and 84 h, after the administration of pneumococci. The data are combined from two separate experiments with six to eight mice in each group in each experiment. The *p*-values for the differences in the survival curves between groups A B and A C were 0.004 and 0.006, respectively. The *p*-value for the difference in the survival curves between groups B and C was 0.94. The *p*-values for the differences in the survival curves between groups C D and C E were 0.23 and <0.001, respectively. The *p*-values for the differences in the survival curves between groups B E and D E were <0.001.

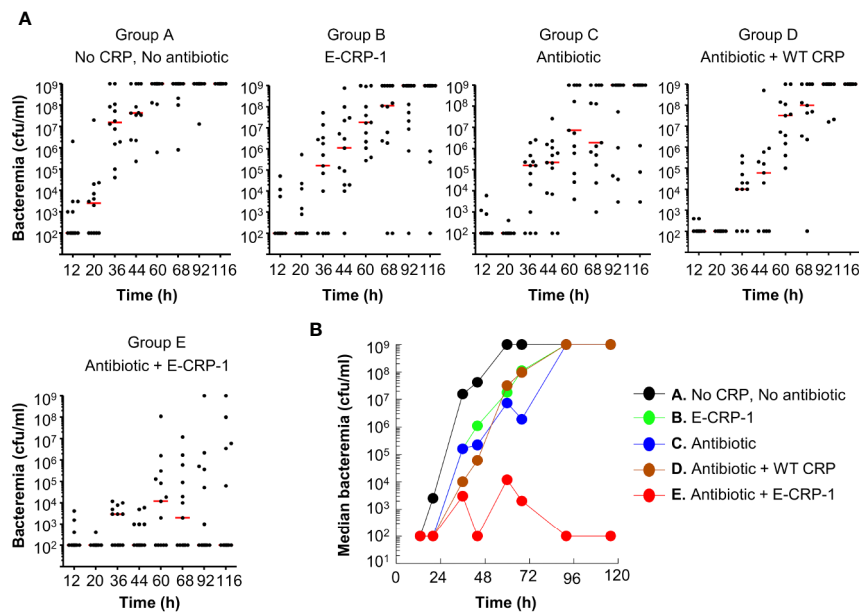


FIGURE 3 | Bacteremia in mice infected with pneumococci and treated with E-CRP-1 and clarithromycin. Blood was collected from each surviving mouse shown in **Figure 2**. **(A)** Scatter plots of the bacteremia data. The horizontal red line in each group of mice represents median bacteremia. **(B)** The median bacteremia values, derived from **(A)** For 36–92 h, the p -value for the difference between groups A and B was <0.05 . For 20–68 h, the p -value for the difference between groups A and C was <0.05 . For all time points, the p -values for the differences between groups B C and C D were >0.05 . For 36–116 h, the p -values for the differences between groups C E, B E, and D E were <0.05 , with most significant difference ($p < 0.005$) between 44–92 h.

alone, were due to significant decrease in bacteremia (**Figures 5A, B**). E-CRP-2, as reported previously (21), and clarithromycin, both significantly decreased bacteremia. The decrease in bacteremia by E-CRP-2 and by clarithromycin were not significantly different from each other. The dramatic increase in the survival of infected mice by the combination of E-CRP-2 and clarithromycin (group D) was due to the drastic decrease in bacteremia. The decrease in bacteremia in mice treated with both agents was significantly different from the decrease in bacteremia when mice were treated with either of the two agents alone. Bacteremia did not rise beyond 10^5 cfu/ml in mice treated with both E-CRP-2 and clarithromycin. The median bacteremia was maintained at the reduced level from the beginning to the end of the experiment.

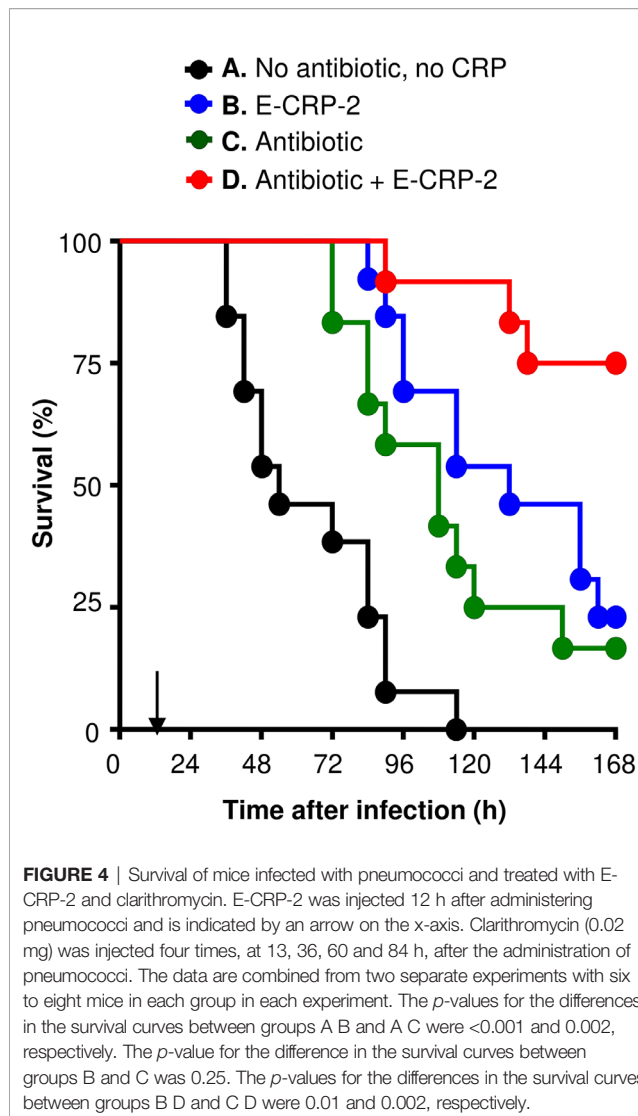
Overall, based on the statistical analyses of the survival curves (**Figure 4**) and of the scatter plots for bacteremia (**Figure 5A**), highly significant differences were found between the groups of mice treated with the combination of E-CRP-2 and clarithromycin and the groups of mice treated with either agent alone. The anti-pneumococcal effects of E-CRP-2 were enhanced in the presence of low-dose clarithromycin. Combining E-CRP-2 with clarithromycin in the treatment of pneumococcal infection in this mouse model reduced the dose of clarithromycin by 90%.

DISCUSSION

E-CRP molecules capable of binding to factor H recruited on the surface of pneumococci have been shown to protect mice against

late-stage pneumococcal infection (21). Antibiotics protect against pneumococcal infection too (27). The aim of this study was to compare the protective effects of E-CRP with that of an antibiotic clarithromycin employing the same animal model. Our major findings were: 1. Both E-CRP-1 and E-CRP-2, two different molecules capable of binding to factor H recruited on the surface of pneumococci, protected and acted synergistically with clarithromycin to drastically reduce bacteremia and enhance the survival of mice with late-stage pneumococcal infection. 2. The combination of either E-CRP-1 or E-CRP-2 and 0.02 mg clarithromycin provided the same protection as was seen with 0.2 mg of clarithromycin when used alone. There was 90% reduction in the dose of clarithromycin when E-CRP was combined with clarithromycin for the treatment of infected mice. WT CRP did not do so.

Both E-CRP and clarithromycin decrease bacteremia, but via different mechanisms. E-CRP-mediated decrease in bacteremia is due to the activation of the complement system component of innate immunity. Clarithromycin is a macrolide antibiotic that has bacteriostatic action against gram-positive bacteria and some gram-negative bacteria including anaerobes. Clarithromycin is believed to function by binding to the ribosome within the microorganism and inhibiting protein synthesis, and thus inhibiting bacterial growth (26–29). A possible explanation for the synergy between E-CRP and clarithromycin is that the binding of E-CRP to factor H, and perhaps also to other recruited complement inhibitor proteins on pneumococci (21), and subsequent attack by the complement system are more efficient when pneumococci are static; and a low-dose



clarithromycin treatment is sufficient to do that. In addition to the bacteriostatic action of clarithromycin, an increasing body of evidence suggests that clarithromycin possesses considerable anti-inflammatory and immunomodulatory properties, such as macrophage activation and inhibition of neutrophilic inflammation (26, 30–32). However, it is not clear whether the anti-inflammatory and immunomodulatory properties of clarithromycin participated in the synergy between clarithromycin and E-CRP in protection against infection. Nevertheless, our data provide a proof of concept that there is cooperation between E-CRP and clarithromycin in reducing bacteremia.

A single dose of E-CRP (25 μ g) combined with a tiny amount of clarithromycin (0.02 mg) was the best prescription among all others in this study and in a previously published study (21) for nearly complete protection of our experimental mice. Neither E-CRP (25 μ g) nor clarithromycin (0.02 mg) could do it singly, indicating a previously unknown pathway through which the

innate immune system responds to antibiotic treatment. The synergy between various CRP species and clarithromycin in reducing bacteremia is just another example of cooperation between a molecule of the innate immune system and antibiotics. It has been reported previously that the classical complement pathway-mediated immunity against antibiotic-resistant pneumococci was enhanced in the presence of sub-inhibitory concentrations of antibiotics cefditoren and ceftriaxone. The binding of CRP to pneumococci was also enhanced in the presence of serum plus either cefditoren or ceftriaxone. Complement activation was also enhanced in the presence of specific anti-pneumococcal antibodies and sub-inhibitory concentrations of antibiotics such as cefditoren, ceftriaxone and amoxicillin (33, 34). It has been suggested that using antibiotics to enhance complement activation might help reduce the impact of antibiotic resistance in pneumococcal infection (33, 34). The cooperation mechanisms between the molecules of the innate immune system and antibiotics should be exploited to develop novel combination therapies to treat infections with antibiotic-resistant pneumococci.

Combination therapies using antimicrobials with different mechanisms of action are used to treat infections against antibiotic-resistant pneumococci and, at the same time, are formulated to prevent the spread of the resistance (35–41). Combination therapies have been shown not only to be more effective against antibiotic-resistant bacteria but also significantly reduce any risk of bacteria developing resistance as seen in monotherapy. The power of E-CRP to reduce antibiotic dosing could be significant and might further assist in these goals to prevent emergence of antibiotic-resistant pneumococci. Usually, combination therapies involve low doses of two antibiotics from different classes. It has been suggested that antibiotic-antibiotic combination therapy may reduce and slow the development of antibiotic resistance. Eliminating one antibiotic from the combination altogether and substituting it with E-CRP should then be, in principle, more effective in reducing and slowing the development of antibiotic resistance. This strategy is similar to antibiotic-non-antibiotic combination therapies, such as adjuvant-antibiotic therapy, where one of the two antibiotics is substituted with an adjuvant with the goal to prevent the development of antibiotic resistance (35–41). We propose that E-CRP should be considered for inclusion in combination therapies. The ability of E-CRP to drastically reduce bacteremia even with a fraction of the normal dose of clarithromycin might contribute further to prevent the development and spread of antibiotic resistance. Our data suggest that, by adding E-CRP to E-CRP-antibiotic combination therapy, the dose of the remaining antibiotic can be kept low.

We conclude that the efficiency of the innate immune system is enhanced in the presence of antibiotics. Our findings provide another example of cooperation between the innate immune system and molecules that prevent multiplication of bacteria, and that should be exploited to develop novel combination therapies for infections against antibiotic-resistant pneumococci. The reduction in antibiotic

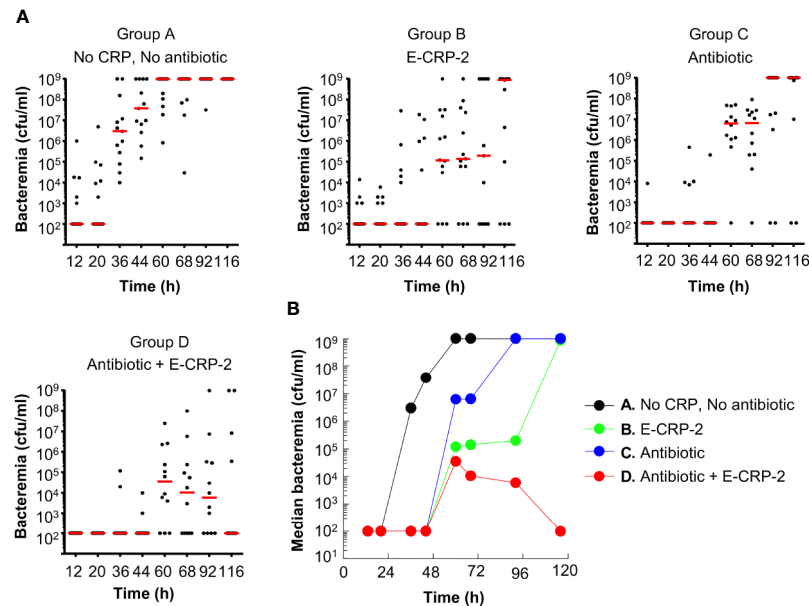


FIGURE 5 | Bacteremia in mice infected with pneumococci and treated with E-CRP-2 and clarithromycin. Blood was collected from each surviving mouse shown in **Figure 4**. **(A)** Scatter plots of the bacteremia data. The horizontal red line in each group of mice represents median bacteremia. **(B)** The median bacteremia values, derived from **(A)** For 36–116 h, the p -value for the difference between groups A and B was <0.005 . The p -values for the difference between groups A and C was <0.005 and <0.05 for 44–68 h and 92–116 h, respectively. For all time points, the p -values for the differences between groups B C and B D were >0.05 . For 60 to 116 h, the p -value for the difference between groups C and D was <0.05 .

dosing by the strategy to include E-CRP in the combination therapy using antibiotics might also resolve the problem of developing and spreading antibiotic resistance.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by University Committee on Animal Care, ETSU.

REFERENCES

- Ngwa DN, Agrawal A. Structure-function relationships of C-reactive protein in bacterial infection. *Front Immunol* (2019) 10:166. doi: 10.3389/fimmu.2019.00166
- Simons JP, Loeffler JM, Al-Shawi R, Ellmerich S, Hutchinson WL, Tennent GA, et al. C-reactive protein is essential for innate resistance to pneumococcal infection. *Immunology* (2014) 142:414–20. doi: 10.1111/imm.12266
- Volanakis JE, Kaplan MH. Specificity of C-reactive protein for choline phosphate residues of pneumococcal C-polysaccharide. *Proc Soc Exp Biol Med* (1971) 136:612–4. doi: 10.3181/00379727-136-35323
- Agrawal A. CRP after 2004. *Mol Immunol* (2005) 42:927–30. doi: 10.1016/j.molimm.2004.09.028

AUTHOR CONTRIBUTIONS

DN and SS performed the experiments. AA conceived and designed the experiments. DN, SS, and AA analyzed the data. AA wrote the manuscript with input from DN. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the National Institutes of Health grants AR068787, AI117730, and AI151561.

- Liu T-Y, Gotschlich EC. The chemical composition of pneumococcal C-polysaccharide. *J Biol Chem* (1963) 238:1928–34.
- Kaplan MH, Volanakis JE. Interaction of C-reactive protein complexes with the complement system. I. Consumption of human complement associated with the reaction of C-reactive protein with pneumococcal C-polysaccharide and with the choline phosphatides, lecithin, and sphingomyelin. *J Immunol* (1974) 112:2135–47.
- Volanakis JE. Complement activation by C-reactive protein complexes. *Ann N Y Acad Sci* (1982) 389:235–50. doi: 10.1111/j.1749-6632.1982.tb22140.x
- Suresh MV, Singh SK, Ferguson DA Jr, Agrawal A. Role of the property of C-reactive protein to activate the classical pathway of complement in protecting mice from pneumococcal infection. *J Immunol* (2006) 176:4369–74. doi: 10.4049/jimmunol.176.7.4369

9. Singh SK, Ngwa DN, Agrawal A. Complement activation by C-reactive protein is critical for protection of mice against pneumococcal infection. *Front Immunol* (2020) 11:1812. doi: 10.3389/fimmu.2020.01812
10. Szalai AJ, Briles DE, Volanakis JE. Human C-reactive protein is protective against fatal *Streptococcus pneumoniae* infection in transgenic mice. *J Immunol* (1995) 155:2557–63.
11. Yother J, Volanakis JE, Briles DE. Human C-reactive protein is protective against fatal *Streptococcus pneumoniae* infection in mice. *J Immunol* (1982) 128:2374–6.
12. Mold C, Nakayama S, Holzer TJ, Gewurz H, Du Clos TW. C-reactive protein is protective against *Streptococcus pneumoniae* infection in mice. *J Exp Med* (1981) 154:1703–8. doi: 10.1084/jem.154.5.1703
13. Suresh MV, Singh SK, Ferguson DAJr, Agrawal A. Human C-reactive protein protects mice from *Streptococcus pneumoniae* infection without binding to pneumococcal C-polysaccharide. *J Immunol* (2007) 178:1158–63. doi: 10.4049/jimmunol.178.2.1158
14. Gang TB, Hammond DJJr, Singh SK, Ferguson DAJr, Mishra VK, Agrawal A, et al. The phosphocholine-binding pocket on C-reactive protein is necessary for initial protection of mice against pneumococcal infection. *J Biol Chem* (2012) 287:43116–25. doi: 10.1074/jbc.M112.427310
15. Gang TB, Hanley GA, Agrawal A. C-reactive protein protects mice against pneumococcal infection via both phosphocholine-dependent and phosphocholine-independent mechanisms. *Infect Immun* (2015) 83:1845–52. doi: 10.1128/IAI.03058-14
16. Nakayama S, Gewurz H, Holzer T, Du Clos TW, Mold C. The role of the spleen in the protective effect of C-reactive protein in *Streptococcus pneumoniae* infection. *Clin Exp Immunol* (1983) 54:319–26.
17. Szalai AJ, Briles DE, Volanakis JE. Role of complement in C-reactive protein-mediated protection of mice from *Streptococcus pneumoniae*. *Infect Immun* (1996) 64:4850–3. doi: 10.1128/IAI.64.11.4850-4853.1996
18. Mold C, Rodic-Polic B, Du Clos TW. Protection from *Streptococcus pneumoniae* infection by C-reactive protein and natural antibody requires complement but not Fcγ receptors. *J Immunol* (2002) 168:6375–81. doi: 10.4049/jimmunol.168.12.6375
19. Shrive AK, Cheetham GMT, Holden D, Myles DAA, Turnell WG, Volanakis JE, et al. Three-dimensional structure of human C-reactive protein. *Nat Struct Biol* (1996) 3:346–54. doi: 10.1038/nsb0496-346
20. Thompson D, Pepys MB, Wood SP. The physiological structure of human C-reactive protein and its complex with phosphocholine. *Structure* (1999) 7:169–77. doi: 10.1016/S0969-2126(99)80023-9
21. Ngwa DN, Singh SK, Gang TB, Agrawal A. Treatment of pneumococcal infection by using engineered human C-reactive protein in a mouse model. *Front Immunol* (2020) 11:586669. doi: 10.3389/fimmu.2020.586669
22. Hammond DJJr, Singh SK, Thompson JA, Beeler BW, Rusinol AE, Pangburn MK, et al. Identification of acidic pH-dependent ligands of pentameric C-reactive protein. *J Biol Chem* (2010) 285:36235–44. doi: 10.1074/jbc.M110.142026
23. Singh SK, Thirumalai A, Hammond DJJr, Pangburn MK, Mishra VK, Johnson DA, et al. Exposing a hidden functional site of C-reactive protein by site-directed mutagenesis. *J Biol Chem* (2012) 287:3550–8. doi: 10.1074/jbc.M111.310011
24. Singh SK, Thirumalai A, Pathak A, Ngwa DN, Agrawal A. Functional transformation of C-reactive protein by hydrogen peroxide. *J Biol Chem* (2017) 292:3129–36. doi: 10.1074/jbc.M116.773176
25. Pathak A, Singh SK, Thewke DP, Agrawal A. Conformationally altered C-reactive protein capable of binding to atherogenic lipoproteins reduces atherosclerosis. *Front Immunol* (2020) 11:1780. doi: 10.3389/fimmu.2020.01780
26. Zuckerman JM. The newer macrolides: Azithromycin and clarithromycin. *Infect Dis Clin North Am* (2000) 14:449–62. doi: 10.1016/S0891-5520(05)70257-9
27. Tessier PR, Kim M-K, Zhou W, Xuan D, Li C, Ye M, et al. Pharmacodynamic assessment of clarithromycin in a murine model of pneumococcal pneumonia. *Antimicrob Agents Chemother* (2002) 46:1425–34. doi: 10.1128/AAC.46.5.1425-1434.2002
28. Alvarez-Elcoro S, Enzler MJ. The macrolides: erythromycin, clarithromycin, and azithromycin. *Mayo Clin Proc* (1999) 74:613–34. doi: 10.4065/74.6.613
29. Kakegawa T, Hirose S. Mode of inhibition of protein synthesis by metabolites of clarithromycin. *Chemotherapy* (1990) 38:317–23. doi: 10.11250/chemotherapy1953.38.317
30. Yanagihara K, Tomono K, Kuroki M, Kaneko Y, Sawai T, Ohno H, et al. Intrapulmonary concentrations of inflammatory cytokines in a mouse model of chronic respiratory infection caused by *Pseudomonas aeruginosa*. *Clin Exp Immunol* (2000) 122:67–71. doi: 10.1046/j.1365-2249.2000.01343.x
31. Ianaro A, Ialenti A, Maffia P, Sautebin L, Rombolà L, Carnuccio R, et al. Anti-inflammatory activity of macrolide antibiotics. *J Pharmacol Exp Ther* (2000) 292:156–63.
32. Giamarellos-Bourboulis EJ, Adamis T, Laoutaris G, Sabracos L, Koussoulas V, Mouktaroudi M, et al. Immunomodulatory clarithromycin treatment of experimental sepsis and acute pyelonephritis caused by multidrug-resistant *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* (2004) 48:93–9. doi: 10.1128/AAC.48.1.93-99.2004
33. Ramirez-Sevillano E, Rodriguez-Sosa C, Cafini F, Giménez MJ, Navarro A, Sevillano D, et al. Cefditoren and ceftriaxone enhance complement-mediated immunity in the presence of specific antibodies against antibiotic-resistant pneumococcal strains. *PLoS One* (2012) 7:e44135. doi: 10.1371/journal.pone.0044135
34. Yuste J, Giménez MJ, Jado I, Fenoll A, Aguilar L, Casal J. Enhanced decrease of blood colony counts by specific anti-pneumococcal antibodies in the presence of sub-inhibitory concentrations of amoxicillin. *J Antimicrob Chemother* (2001) 48:594–5. doi: 10.1093/jac/48.4.594
35. Majhi A, Adhikary R, Bhattacharyya A, Mahanti S, Bishayi B. Levofloxacin-ceftriaxone combination attenuates lung inflammation in a mouse model of bacteremic pneumonia caused by multidrug-resistant *Streptococcus pneumoniae* via inhibition of cytolytic activities of pneumolysin and autolysin. *Antimicrob Agents Chemother* (2014) 58:5164–80. doi: 10.1128/AAC.03245-14
36. Martínez JA, Horcajada JP, Almela M, Marco F, Soriano A, García E, et al. Addition of a macrolide to a beta-lactam-based empirical antibiotic regimen is associated with lower in-hospital mortality for patients with bacteremic pneumococcal pneumonia. *Clin Infect Dis* (2003) 36:389–95. doi: 10.1086/367541
37. Waterer GW. Optimal antibiotic treatment in severe pneumococcal pneumonia—time for real answers. *Eur J Clin Microbiol Infect Dis* (2005) 24:691–2. doi: 10.1007/s10096-005-0019-5
38. Caballero J, Rello J. Combination antibiotic therapy for community-acquired pneumonia. *Ann Intensive Care* (2011) 1:48. doi: 10.1186/2110-5820-1-48
39. Worthington RJ, Melander C. Combination approaches to combat multidrug-resistant bacteria. *Trends Biotechnol* (2013) 31:177–84. doi: 10.1016/j.tibtech.2012.12.006
40. Schneider EK, Reyes-Ortega F, Velkov T, Li J. Antibiotic-non-antibiotic combinations for combating extremely drug-resistant Gram-negative ‘superbugs’. *Essays Biochem* (2017) 61:115–25. doi: 10.1042/EBC20160058
41. Ejim L, Farha M, Falconer S, Wildenhain J, Coombes B, Tyers M, et al. Combinations of antibiotics and nonantibiotic drugs enhance antimicrobial efficacy. *Nat Chem Biol* (2011) 7:348–50. doi: 10.1038/nchembio.559

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Ngwa, Singh and Agrawal. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



C-Reactive Protein Levels in Systemic Lupus Erythematosus Are Modulated by the Interferon Gene Signature and CRP Gene Polymorphism rs1205

Helena Enocsson^{1*}, Birgitta Gullstrand², Maija-Leena Eloranta³, Jonas Wetterö¹, Dag Leonard³, Lars Rönnblom³, Anders A. Bengtsson² and Christopher Sjöwall¹

¹ Department of Biomedical and Clinical Sciences, Division of Inflammation and Infection, Linköping University, Linköping, Sweden, ² Department of Clinical Sciences Lund, Division of Rheumatology, Lund University, Lund, Sweden, ³ Department of Medical Sciences, Rheumatology, Uppsala University, Uppsala, Sweden

OPEN ACCESS

Edited by:

Kenji Daigo,
Nippon Medical School, Japan

Reviewed by:

Marta E. Alarcon-Riquelme,
Junta de Andalucía de Genómica e
Investigación Oncológica (GENYO),
Spain

Angelo A. Manfredi,
Vita-Salute San Raffaele University,
Italy

*Correspondence:

Helena Enocsson
Helena.enocsson@liu.se

Specialty section:

This article was submitted to
Molecular Innate Immunity,
a section of the journal
Frontiers in Immunology

Received: 28 October 2020

Accepted: 17 December 2020

Published: 28 January 2021

Citation:

Enocsson H, Gullstrand B,
Eloranta M-L, Wetterö J, Leonard D,
Rönnblom L, Bengtsson AA and
Sjöwall C (2021) C-Reactive Protein
Levels in Systemic Lupus
Erythematosus Are Modulated by the
Interferon Gene Signature and CRP
Gene Polymorphism rs1205.
Front. Immunol. 11:622326.
doi: 10.3389/fimmu.2020.622326

Objectives: Patients with systemic lupus erythematosus (SLE) often display modest elevations of C-reactive protein (CRP) despite raised disease activity and increased interleukin (IL-) 6. We asked to what extent IL-6 levels, the CRP polymorphism rs1205, and the type I interferon (IFN) gene signature affects the basal CRP levels in patients with SLE during a quiescent phase of the disease.

Methods: CRP and IL-6 were analyzed in plasma from 57 patients meeting established classification criteria for SLE. The CRP polymorphism rs1205 was assessed and gene expression analyzed including four type I IFN-regulated genes (IGS).

Results: CRP was increased in patients with detectable IL-6 levels ($p=0.001$) and decreased among IGS-positive subjects ($p=0.033$). A multiple linear regression model revealed IL-6 to have a positive association with CRP levels, whereas both IGS-positivity and CRP genotype (rs1205) AA/GA were negatively associated with CRP-levels.

Conclusion: Our data offer an explanation to the modest CRP levels seen in viral infections and IFN- α driven autoimmunity and corroborate prior observations showing an IFN- α dependent downregulation of CRP. The latter observation, together with the fact that the CRP-lowering polymorphism rs1205 is overrepresented in human SLE, could explain low basal CRP and inadequate CRP-responses among patients with active SLE.

Keywords: C-reactive protein, type I interferons, systemic lupus erythematosus, inflammation, biomarker, pentraxins, interferon, gene variants

INTRODUCTION

The acute-phase protein C-reactive protein (CRP) is a key actor in the clearance of bacteria and dying cells. Its pentameric structure encompasses an effector face with affinity for C1q and Fc γ -receptors and a recognition face with the ability to bind phosphocholine on e.g., dying cells and pathogens, but also nuclear constituents exposed during apoptosis (*i.e.*, snRNP and histones) (1, 2).

These qualities enable CRP to contribute to efficient clearance of cell remnants and immune complexes by complement activation/modulation, opsonization, and phagocytosis, biological processes considered dysfunctional in systemic lupus erythematosus (SLE) (3, 4). Indeed, protective effects of CRP in the disease process have been demonstrated in animal models of lupus (5–7).

Due to increased CRP production from hepatocytes upon interleukin (IL-) 6 stimulation, CRP is widely used to monitor infectious and inflammatory conditions (8). With few exceptions, CRP reflects ongoing inflammation/tissue damage, seemingly without discrimination of the underlying trigger. However, whereas bacterial infections generally result in impressive CRP levels, the response in many viral infections is typically less pronounced (9). Similarly, modest CRP-responses are recorded in autoimmune diseases characterized by increased type I interferon (IFN) activity, e.g., SLE (8), and lack of correlation between IL-6 and CRP has been demonstrated in patients with SLE (10). Type I IFNs are strong activators of the anti-viral immune response, but may also contribute to autoantibody production in several autoimmune conditions (11).

IFN- α , the most studied type I IFN in autoimmunity, has previously been evaluated in relation to regulation of pentraxins (12–15). We have previously demonstrated an inhibitory effect of IFN- α in IL-6/IL-1 β -induced CRP gene transcription and protein production from hepatocytes (12). Thus, the presence of IFN- α *per se* could contribute to modest CRP levels during IFN- α associated disease flares of type I IFN-driven autoimmunity as well as in viral infections. Furthermore, a combined effect of the CRP-lowering polymorphism of the CRP gene (rs1205) (16) and detectable IFN- α levels resulted in inability of CRP to reflect inflammatory activity among SLE patients (13). However, circulating IFN- α is difficult to detect with standard methods despite evidence of an increased gene transcription of type I IFN-regulated genes. Thus, a selection of type I IFN-regulated genes is typically assessed as a marker of an increased type I IFN activity, *i.e.*, the type I IFN gene signature (IGS).

The aim of this study was to confirm and extend the knowledge from previous *in vivo* and *in vitro* studies showing an impact of type I IFNs (12, 13, 15) and rs1205 (13) on CRP levels among patients with SLE. Herein, the objective was to evaluate the potential impact of the IGS and rs1205 in patients unbiased from high disease activity.

MATERIALS AND METHODS

Subjects

All patients (Table 1) were followed within the frame of an observational research program at the University Hospital in Linköping (17). Each participant ($n=57$) was classified with SLE according to the 1982 American College of Rheumatology and/or the 2012 Systemic Lupus International Collaborating Clinics criteria (18, 19).

The patients donated peripheral blood and the disease activity, defined by SLE disease activity index 2000 (SLEDAI-2K),

TABLE 1 | Clinical characteristics of the 57 included patients with systemic lupus erythematosus (SLE).

Variable	Median (range) or frequency (%)
Age (years)	43 (23–63)
Females	50 (88)
Disease duration (years)	8 (1–35)
SLEDAI-2K	2 (0–8)
1982 ACR criteria fulfilled (number)	5 (3–9)
Ever (current or prior) tobacco smoker	14 (25)
1982 ACR criteria fulfilled:	
ACR 1: malar rash	23 (40)
ACR 2: discoid lupus	2 (3.5)
ACR 3: photosensitivity	26 (46)
ACR 4: oral ulcers	13 (23)
ACR 5: arthritis	45 (79)
ACR 6: serositis	22 (39)
ACR 7: renal disorder	19 (33)
ACR 8: neurologic disorder	6 (11)
ACR 9: hematologic disorder	38 (67)
ACR 10: immunologic disorder	35 (61)
ACR 11: antinuclear antibodies	57 (100)
Ongoing immunomodulation:	
Antimalarials	51 (90)
Prednisolone	30 (53)
Prednisolone dose (mg/day)	2.5 (0–15)
Mycophenolate mofetil	15 (26)
Methotrexate	5 (8.8)
Azathioprine	3 (5.3)
Other DMARDs/biologics	8 (14)
^a CRP genotype (rs1205)	
Two major alleles (GG)	24 (44)
One minor allele (AG)	23 (42)
Two minor alleles (AA)	8 (15)

^aGenotype analysis available for 55 patients.

ACR, American College of Rheumatology; DMARDs, disease-modifying anti-rheumatic drugs; SLEDAI-2K, SLE disease activity index 2000.

was recorded from their closest regular visit to rheumatologist. All patients were considered to have low disease activity but could be serologically active and clinically quiescent at sampling (20, 21). Each participant gave written informed consent, and the study protocol was approved by the regional ethics board in Linköping.

Clinical Laboratory Analyses

Venous blood was drawn, and plasma was prepared and blood lipids were analyzed at the local Clinical Chemistry unit, including triglycerides, total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), non-HDL, IL-6, and CRP. We utilized a high sensitivity method for CRP with 0.15 mg/L as limit of quantification (LOQ). For IL-6, LOQ was 1.5 ng/L.

Gene Expression

The type I IFN gene signature (IGS) was based on gene expression of IFI27, IFI44, IFI44L, and RSAD2 (22). Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized whole blood by density gradient centrifugation and lysed by RLT-buffer. Total RNA was extracted using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). Quantification and purity of the RNA was assessed on a DS-11

spectrophotometer (DeNovix Inc. Wilmington, DE, USA). Total RNA was reverse transcribed using iScriptTM cDNA synthesis kit (Bio Rad, Hercules, California, USA) to obtain cDNA for RT-qPCR. The RT-qPCR was performed by preheating for 10 min at 95°C, followed by 40 cycles of 95°C 15 s and 60°C for 60 s using StepOnePlus (Applied Biosystems, CA, USA). TaqManTM Gene Expression assay (FAM) (Applied Biosystems, CA, USA) were used with the following primers from Thermo Fisher Scientific, IFI44L: Hs00199115_m1, RSAD2: Hs00369813_m1, IFI27: Hs00271467_m1, IFI44: Hs00197427_m1, GAPDH: Hs03929097_g1.

Fold-change of gene expression was determined by the relative quantification method ($\Delta\Delta Ct$) after normalization to the housekeeping gene (GAPDH). A subsequent log-transformation was performed to achieve comparability between genes. IGS-score was expressed as mean of log-transformed fold change in relation to a control sample (consisting of a pooled sample from healthy control donors) of the four genes (detailed in **Supplementary Data 1**). The patient distribution revealed two groups regarding the IGS; a cut-off of 0.5 was applied to separate IGS-positive patients from IGS-negative.

CRP Genotyping

DNA was extracted from peripheral blood using Qiagen Blood Midi Kit (Qiagen) and genotyping was performed using Illumina Infinium Global Screening Array-Multi Disease version3 (Illumina Inc. California, USA) at the SNP&SEQ Technology Platform at Uppsala University. The genotyping is detailed in **Supplementary Data 2**.

Statistics

CRP levels were not normally distributed why Mann-Whitney U test, Kruskal-Wallis test, or Spearman's correlation analysis were applied. CRP and IL-6 were log-transformed (10log) prior to linear regression analysis with CRP or IL-6 as dependent variables. A two-sided p-value of <0.05 was considered statistically significant.

RESULTS

CRP Is Associated With IL-6 and IGS-Status

Levels of CRP correlated significantly with IL-6 ($\rho=0.415$, $p=0.001$; **Figure 1A**) and with age ($\rho=0.292$, $p=0.027$). No correlation was observed between CRP levels and IGS-score (**Figure 1B**). Since 28 patients had non-detectable IL-6, and 21 were judged IGS-negative, IL-6 and IGS were further tested as binary variables. CRP levels were significantly higher among those with detectable IL-6 ($p=0.001$; **Figure 1C**) and lower among IGS-positive subjects ($p=0.033$; **Figure 1D**). The CRP genotype (rs1205) was not significantly associated with CRP, neither based on number of rare alleles, nor based on presence or absence of one or two rare alleles (binary variables; **Figure 1E**).

No significant correlations between CRP and corticosteroid dose, SLEDAI-2K or blood lipids were observed. Neither did we

observe any differences in CRP related to sex, tobacco smoking, or use of antimalarials, corticosteroids, mycophenolate mofetil, methotrexate or use of any immunosuppressive agent (disease-modifying anti-rheumatic drugs except antimalarials, $n=28$). Finally, CRP levels did not associate with disease phenotypes (*i.e.*, fulfilled classification criteria).

IL-6, IGS, and CRP Genotype Versus CRP Levels

To evaluate impact of CRP genotype rs1205, IL-6, and IGS-positivity, these variables were included in a linear regression model with 10log CRP as the dependent variable. Age was further added as an independent variable due to its correlation with CRP.

A stepwise analysis revealed IGS-positivity ($p=0.017$) and alleles AA/AG of rs1205 ($p=0.041$) to be associated with low levels of CRP. Age and IL-6 levels did not associate with CRP in the regression analysis (**Table 2**). However, a regression analysis with IL-6 as binary variable resulted in a significant association of both IL-6 ($p=0.012$), CRP genotype (AA/AG) ($p=0.030$), and IGS-positivity ($p=0.030$) with CRP levels (**Table 2** and visualized in **Figure 2**).

We observed no associations between IGS-status and CRP genotype, IL-6 (binary), or CRP genotype as determined by χ^2 (Fisher's exact test). A regression analysis with 10log IL-6 as the dependent variable and CRP genotype and IGS-status as dependent variables did not reach statistical significance, indicating that the effect of IGS and CRP genotype on CRP levels were independent of IL-6.

DISCUSSION

The present study demonstrates that low CRP levels coincide with the CRP polymorphism rs1205 and the presence of an activated type I IFN system, as determined by the IGS-score. Our observations therefore add further support to an important role of type I IFNs in the regulation of circulating CRP *via* an inhibitory effect by IFN- α on CRP production (12, 15). Furthermore, we previously demonstrated an association between CRP and SLE disease activity when patients with the rs1205 minor allele and detectable serum IFN- α were excluded (13). Thus, it is plausible that our findings together with the fact that the CRP-lowering polymorphism rs1205 is overrepresented among SLE patients (16), explain the low basal CRP and inadequate CRP-responses in those with active SLE.

The extensive use of CRP as a biomarker motivate research aiming to unravel its regulation in different inflammatory conditions. Indeed, viral infections generally result in lower CRP-responses compared with bacterial infections (9), and SLE patients rarely mount a CRP-response that corresponds to their inflammatory activity (10, 23) or circulating IL-6 levels (10).

IL-6 is the main inducer of CRP-production, and it is therefore not surprising with a positive impact of IL-6 on circulating CRP (24). Herein, it was however evident that the CRP polymorphism, as well as type I IFN activity, coincides with

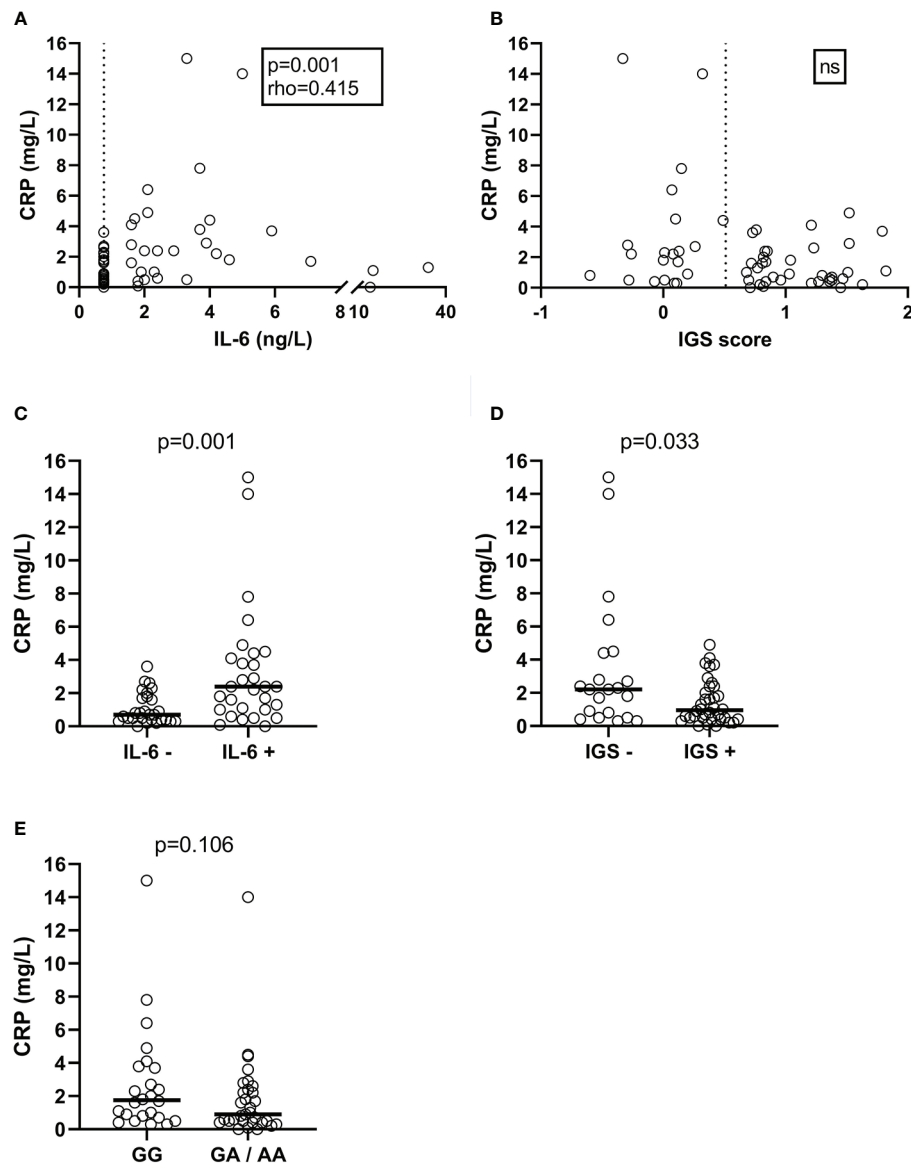


FIGURE 1 | Plasma CRP levels in SLE patients, and the correlation and association with plasma IL-6 concentrations **(A)**, the IGS score measured in peripheral blood mononuclear cells **(B)**, detectable plasma IL-6 levels **(C)**, IGS positivity **(D)** and CRP rs1205 genotype **(E)**. Dashed lines represent cut-off levels for IL-6 detection and IGS positivity, respectively.

a limited ability of IL-6 to induce a CRP-response. In fact, none of the IL-6-positive patients with the rs1205 minor allele and IGS-positivity reached a higher CRP level than 3.6 mg/L, *i.e.*, the highest CRP found among IL-6-negative subjects regardless of rs1205 genotype or IGS-status.

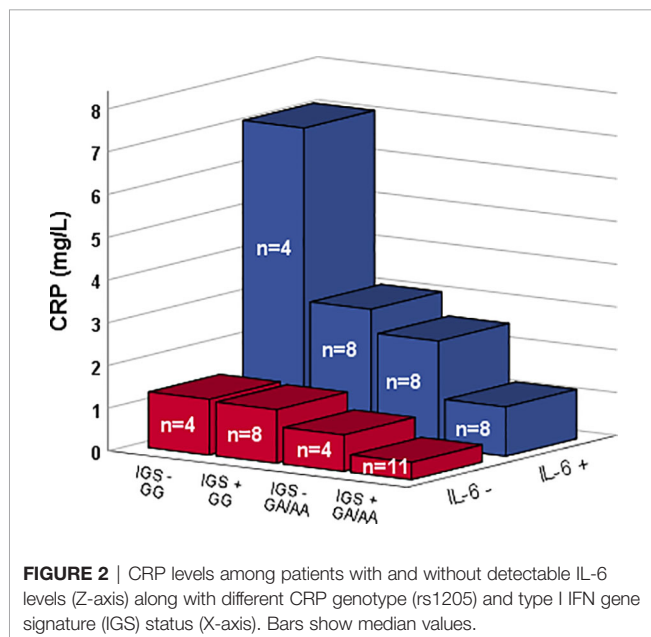
Hypothetically, other polymorphisms or presence of soluble receptors for *e.g.*, IL-6 and IFN- α could also impact CRP levels. As alternative explanations to the muted CRP response in SLE, increased consumption of CRP has been put forward. Increased complement consumption, due to inflammation and increased cell death, is a well-known feature of SLE flares (25). CRP has similar functions in the clearance of debris and could potentially

be utilized and eliminated at a higher rate in patients with SLE. However, the elimination rate of CRP in SLE patients seems unaltered compared with healthy controls (26). Furthermore, anti-CRP autoantibodies, which are more frequently found in SLE, are not directed toward native circulating CRP, but toward epitopes that are exposed upon CRP dissociation on surfaces (27).

Limitations of the present study encompassed a relatively small number of patients and the lack of a replication cohort as well as a control group with unrelated inflammatory disease. The patients included had no known concomitant infections and low/stable disease activity, which reduce the potential impact of

TABLE 2 | Variables associated with CRP levels (10log CRP) in stepwise linear regression analyses.

	p-value	Standardized beta	Model R ²
Model 1			0.16
IGS positivity	0.017	−0.32	
rs1205 AA or AG	0.041	−0.27	
<i>Excluded in the model:</i>			
Age			
IL-6 levels			
Model 2			0.26
Detectable IL-6	0.012	0.32	
rs1205 AA or AG	0.030	−0.27	
IGS positivity	0.030	−0.27	
<i>Excluded in the model:</i>			
Age			

**FIGURE 2 |** CRP levels among patients with and without detectable IL-6 levels (Z-axis) along with different CRP genotype (rs1205) and type I IFN gene signature (IGS) status (X-axis). Bars show median values.

specific SLE-manifestations and comorbidities affecting the CRP-response. During bacterial infections, SLE patients usually present with an adequate CRP-response (28) which may be due to the massive increase of IL-6 that overrides the inhibitory effect of type I IFNs and/or genetic variants of CRP. In addition, bacterial infections could result in a shift of the immune activation with reduced type I IFN production. CRP itself has furthermore been shown to inhibit type I IFN production (29, 30) and it is tempting to speculate in a reciprocal regulation between pentraxins and type I IFNs. Finally, also leukocyte secretion/release of the closely related protein pentraxin-3 (PTX3) appear to be regulated by IFN- α *in vitro* (14).

Given the essential biological functions of pentraxins, such as CRP, in facilitating silent removal of cellular debris *via* Fc-receptors and by interacting with the complement system (1, 4) it is perhaps not surprising that administration of CRP to a murine lupus model prevent and reverse ongoing nephritis (5, 7). The crucial role of type I IFNs in SLE was once again

demonstrated by the encouraging results of the phase 3 trial investigating the anti-IFN α/β receptor anifrolumab in patients with active SLE (31). These observations, together with our results, suggests that the consequences of CRP gene polymorphisms and IL-6/type I IFN interactions in SLE deserves further attention.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Regional Ethics Review Board in Linköping, Sweden. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

HE and CS conceived the original idea and project planning. HE, BG, JW, AAB, and CS contributed to the study design. CS collected clinical patient data. HE, BG, and M-LE carried out the laboratory work. M-LE, DL, and LR planned and supervised the genotyping. HE, BG, and CS analyzed the data. HE, LR, and CS drafted the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was funded by grants from the Swedish Research Council for Medicine and Health, the Swedish Society of Medicine and the Ingegerd Johansson donation, the Swedish Rheumatism Association, the Region Östergötland (ALF grants), the King Gustaf V's 80-year Anniversary Foundation, the King Gustaf V and Queen Victoria's Freemasons' Foundation, the Gustafsson, Selander, Alfred Österlund, Anna-Greta Crafoord and the Greta and Johan Kock's Foundations.

ACKNOWLEDGMENTS

We are grateful to participating patients and to study nurse Marianne Petersson, Linköping. Pascal Pucholt and Johanna Sandling, Uppsala University, are acknowledged for genotype data extraction. Genotyping was performed by the SNP&SEQ Technology Platform in Uppsala (www.genotyping.se).

The facility is part of the National Genomics Infrastructure supported by the Swedish Research Council for Infrastructures and Science for Life Laboratory, Sweden. The SNP&SEQ Technology Platform is supported by the Knut and Alice Wallenberg Foundation.

REFERENCES

- Lu J, Mold C, Du Clos TW, Sun PD. Pentraxins and Fc Receptor-Mediated Immune Responses. *Front Immunol* (2018) 9:2607. doi: 10.3389/fimmu.2018.02607
- Pathak A, Agrawal A. Evolution of C-Reactive Protein. *Front Immunol* (2019) 10:943. doi: 10.3389/fimmu.2019.00943
- Munoz LE, Lauber K, Schiller M, Manfredi AA, Herrmann M. The role of defective clearance of apoptotic cells in systemic autoimmunity. *Nat Rev* (2010) 6(5):280–9. doi: 10.1038/nrrheum.2010.46
- Ma YJ, Garred P. Pentraxins in Complement Activation and Regulation. *Front Immunol* (2018) 9:3046. doi: 10.3389/fimmu.2018.03046
- Du Clos TW, Zlock LT, Hicks PS, Mold C. Decreased autoantibody levels and enhanced survival of (NZB x NZW) F1 mice treated with C-reactive protein. *Clin Immunol Immunopathol* (1994) 70(1):22–7. doi: 10.1006/clin.1994.1005
- Szalai AJ, Weaver CT, McCrory MA, van Ginkel FW, Reiman RM, Kearney JF, et al. Delayed lupus onset in (NZB x NZW)F1 mice expressing a human C-reactive protein transgene. *Arthritis Rheum* (2003) 48(6):1602–11. doi: 10.1002/art.11026
- Rodriguez W, Mold C, Marnell LL, Hutt J, Silverman GJ, Tran D, et al. Prevention and reversal of nephritis in MRL/lpr mice with a single injection of C-reactive protein. *Arthritis Rheum* (2006) 54(1):325–35. doi: 10.1002/art.21556
- Gabay C, Kushner I. Acute-phase proteins and other systemic responses to inflammation. *N Engl J Med* (1999) 340(6):448–54. doi: 10.1056/NEJM199902113400607
- Nakayama T, Sonoda S, Urano T, Yamada T, Okada M. Monitoring both serum amyloid protein A and C-reactive protein as inflammatory markers in infectious diseases. *Clin Chem* (1993) 39(2):293–7. doi: 10.1093/clinchem/39.2.293
- Gabay C, Roux-Lombard P, de Moerloose P, Dayer JM, Vischer T, Guerne PA. Absence of correlation between interleukin 6 and C-reactive protein blood levels in systemic lupus erythematosus compared with rheumatoid arthritis. *J Rheumatol* (1993) 20(5):815–21.
- Crow MK, Ronnblom L. Type I interferons in host defence and inflammatory diseases. *Lupus Sci Med* (2019) 6(1):e000336. doi: 10.1136/lupus-2019-000336
- Enocsson H, Sjöwall C, Skogh T, Eloranta ML, Rönnblom L, Wetterö J. Interferon-alpha mediates suppression of C-reactive protein: explanation for muted C-reactive protein response in lupus flares? *Arthritis Rheumatol* (2009) 60(12):3755–60. doi: 10.1002/art.25042
- Enocsson H, Sjöwall C, Kastbom A, Skogh T, Eloranta ML, Rönnblom L, et al. Association of serum C-reactive protein levels with lupus disease activity in the absence of measurable interferon-alpha and a C-reactive protein gene variant. *Arthritis Rheumatol* (2014) 66(6):1568–73. doi: 10.1002/art.38408
- Wirestam L, Enocsson H, Skogh T, Eloranta ML, Ronnblom L, Sjöwall C, et al. Interferon-alpha coincides with suppressed levels of pentraxin-3 (PTX3) in systemic lupus erythematosus and regulates leucocyte PTX3 in vitro. *Clin Exp Immunol* (2017) 189(1):83–91. doi: 10.1111/cei.12957
- Vaez A, Jansen R, Prins BP, Hottenga JJ, de Geus EJ, Boomsma DI, et al. In Silico Post Genome-Wide Association Studies Analysis of C-Reactive Protein Loci Suggests an Important Role for Interferons. *Circ Cardiovasc Genet* (2015) 8(3):487–97. doi: 10.1161/CIRCGENETICS.114.000714
- Russell AI, Cunningham Graham DS, Shepherd C, Robertson CA, Whittaker J, Meeks J, et al. Polymorphism at the C-reactive protein locus influences gene expression and predisposes to systemic lupus erythematosus. *Hum Mol Genet* (2004) 13(1):137–47. doi: 10.1093/hmg/ddh021
- Ighe A, Dahlstrom O, Skogh T, Sjöwall C. Application of the 2012 Systemic Lupus International Collaborating Clinics classification criteria to patients in a regional Swedish systemic lupus erythematosus register. *Arthritis Res Ther* (2015) 17:3. doi: 10.1186/s13075-015-0521-9
- Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, Rothfield NF, et al. The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* (1982) 25(11):1271–7. doi: 10.1002/art.1780251101
- Petri M, Orbai AM, Alarcon GS, Gordon C, Merrill JT, Fortin PR, et al. Derivation and validation of the Systemic Lupus International Collaborating Clinics classification criteria for systemic lupus erythematosus. *Arthritis Rheum* (2012) 64(8):2677–86. doi: 10.1002/art.34473
- Steiman AJ, Gladman DD, Ibanez D, Urowitz MB. Prolonged serologically active clinically quiescent systemic lupus erythematosus: frequency and outcome. *J Rheumatol* (2010) 37(9):1822–7. doi: 10.3899/jrheum.100007
- Svensson C, Eriksson P, Zachrisson H, Sjöwall C. High-frequency ultrasound of multiple arterial areas reveals increased intima media thickness, vessel wall appearance and atherosclerotic plaques in systemic lupus erythematosus. *Front Med* (2020) 7:581336. doi: 10.3389/fmed.2020.581336
- Strauss R, Rose T, Flint SM, Klotsche J, Haupl T, Peck-Radosavljevic M, et al. Type I interferon as a biomarker in autoimmunity and viral infection: a leukocyte subset-specific analysis unveils hidden diagnostic options. *J Mol Med (Berl)* (2017) 95(7):753–65. doi: 10.1007/s00109-017-1515-7
- Rezaieyazdi Z, Sahebari M, Hatef MR, Abbasi B, Rafatpanah H, Afshari JT, et al. Is there any correlation between high sensitive CRP and disease activity in systemic lupus erythematosus? *Lupus* (2011) 20(14):1494–500. doi: 10.1177/0961203311418706
- Agrawal A, Cha-Molstad H, Samols D, Kushner I. Transactivation of C-reactive protein by IL-6 requires synergistic interaction of CCAAT/enhancer binding protein beta (C/EBP beta) and Rel p50. *J Immunol* (2001) 166(4):2378–84. doi: 10.4049/jimmunol.166.4.2378
- Leffler J, Bengtsson AA, Blom AM. The complement system in systemic lupus erythematosus: an update. *Ann Rheumatic Dis* (2014) 73(9):1601–6. doi: 10.1136/annrheumdis-2014-205287
- Vigushin DM, Pepys MB, Hawkins PN. Metabolic and scintigraphic studies of radioiodinated human C-reactive protein in health and disease. *J Clin Invest* (1993) 91(4):1351–7. doi: 10.1172/JCI116336
- Sjöwall C, Wetterö J. Pathogenic implications for autoantibodies against C-reactive protein and other acute phase proteins. *Clin Chim Acta* (2007) 378(1–2):13–23. doi: 10.1016/j.cca.2006.12.002
- Becker GJ, Waldburger M, Hughes GR, Pepys MB. Value of serum C-reactive protein measurement in the investigation of fever in systemic lupus erythematosus. *Ann Rheum Dis* (1980) 39(1):50–2. doi: 10.1136/ard.39.1.50
- Mold C, Du Clos TW. C-reactive protein inhibits plasmacytoid dendritic cell interferon responses to autoantibody immune complexes. *Arthritis Rheum* (2013) 65(7):1891–901. doi: 10.1002/art.37968
- C Svanberg, H Enocsson, K Martinsson, LA Potempa, I Rajab, J Wetterö, et al editors. Pentameric, but Not Monomeric C-reactive Protein, Limits the SnRNP-immune Complex Triggered Type I Interferon Response: Implications for Lupus Pathogenesis. *Arthritis Rheumatol* (2019) 71 Supplement:10.
- Morand EF, Furie R, Tanaka Y, Bruce IN, Askanase AD, Richez C, et al. Trial of Anifrolumab in Active Systemic Lupus Erythematosus. *N Engl J Med* (2020) 382(3):211–21. doi: 10.1056/NEJMoa1912196

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Enocsson, Gullstrand, Eloranta, Wetterö, Leonard, Rönnblom, Bengtsson and Sjöwall. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



The Role of PTX3 in Mineralization Processes and Aging-Related Bone Diseases

Umberto Tarantino^{1,2*}, Chiara Greggì^{2,3,4}, Ida Cariati^{2,3,4}, Virginia Veronica Visconti^{2,3,4}, Monica Gasparini^{1,2}, Marco Cateni^{1,2}, Elena Gasbarra^{1,2}, Annalisa Botta³, Antonietta Salustri³ and Manuel Scimeca³

¹ Department of Clinical Science and Translational Medicine, University of Rome "Tor Vergata", Rome, Italy, ² Department of Orthopedics and Traumatology, Policlinico Tor Vergata (PTV) Foundation, Rome, Italy, ³ Department of Biomedicine and Prevention, University of Rome "Tor Vergata", Rome, Italy, ⁴ PhD students' Program in Medical-Surgical and Biotechnologies and Translational Medicine, Faculty of Medicine and Surgery, University of Rome "Tor Vergata", Rome, Italy

OPEN ACCESS

Edited by:

Kenji Daigo,
Nippon Medical School, Japan

Reviewed by:

Ronca Roberto,
University of Brescia, Italy
Yves Delneste,
Institut National de la Santé et de la
Recherche Médicale (INSERM),
France

*Correspondence:

Umberto Tarantino
umberto.tarantino@uniroma2.it

Specialty section:

This article was submitted to
Molecular Innate Immunity,
a section of the journal
Frontiers in Immunology

Received: 29 October 2020

Accepted: 16 December 2020

Published: 29 January 2021

Citation:

Tarantino U, Greggì C, Cariati I,
Visconti VV, Gasparini M, Cateni M,
Gasbarra E, Botta A, Salustri A and
Scimeca M (2021) The Role of PTX3 in
Mineralization Processes and Aging-
Related Bone Diseases.
Front. Immunol. 11:622772.
doi: 10.3389/fimmu.2020.622772

The Long Pentraxin 3 (PTX3) is a multifunctional glycoprotein released by peripheral blood leukocytes and myeloid dendritic cells in response to primary pro-inflammatory stimuli, that acts as a non-redundant component of the humoral arm of innate immunity. In addition to the primary role in the acute inflammatory response, PTX3 seems to be involved in other physiological and pathological processes. Indeed, PTX3 seems to play a pivotal role in the deposition and remodeling of bone matrix during the mineralization process, promoting osteoblasts differentiation and activity. Recently, PTX3 was seen to be involved in the ectopic calcifications' formation in breast cancer disease. In this regard, it has been observed that breast cancer tumors characterized by high expression of PTX3 and high amount of Breast Osteoblast Like Cells (BOLCs) showed several Hydroxyapatite (HA) microcalcifications, suggesting a likely role for PTX3 in differentiation and osteoblastic activity in both bone and extra-bone sites. Furthermore, given its involvement in bone metabolism, several studies agree with the definition of PTX3 as a molecule significantly involved in the pathogenesis of age-related bone diseases, such as osteoporosis, both in mice and humans. Recent results suggest that genetic and epigenetic mechanisms acting on *PTX3* gene are also involved in the progression of these diseases. Based on these evidences, the aim of our systemic review was to offer an overview of the variety of biological processes in which PTX3 is involved, focusing on bone mineralization, both in a physiological and pathological context.

Keywords: PTX3, inflammation, mineralization, bone diseases, ectopic calcification, fracture healing, osteoporosis, aging

INTRODUCTION

The Long Pentraxin 3 (PTX3) is the prototypic long pentraxin that was first identified in the early 1990s as a cytokine-inducible gene in endothelial cells and fibroblasts (1). Long pentraxins have an unrelated amino-terminal region coupled to a carboxy-terminal pentraxin-like domain. Experimental evidences indicated that PTX3, originally identified as an early induced protein in

response to pro-inflammatory stimuli, is a soluble Pattern Recognition Molecule (PRM) involved in the humoral arm of the innate immune response (2, 3). However, in recent years several studies have shown that PTX3 is involved in other physiological and pathological processes such as tissue injury response, ectopic calcifications formation and in bone homeostasis (**Figure 1**). The human *PTX3* gene (MIM# 602492) is located on chromosome 3q25 and contains three exons and two introns. Mature PTX3 is characterized by a unique long N-terminal Domain (NTD - amino acids 18-179), and a pentraxin-like C-terminal domain (PTX - amino acids 179-381), homologous to the short pentraxins (4, 5). The molecule has a complex octameric structure composed of two covalently linked tetramers (6) and characterized by a peculiar asymmetric and elongated shape in which two domains are interconnected by a linear region (7). Hemostasis-related function of PTX3 in tissue repair and matrix formation has recently been described (8, 9). In several mouse models of tissue damage has been demonstrated that PTX3 promoted pericellular fibrinolysis in damaged site binding fibrin and plasminogen (Plg), thus ensuring appropriate tissue repair (10). Thus, in line with these studies, PTX3 plays an essential role in the orchestration of the tissue injury response. Furthermore, several studies demonstrate the important role played by PTX3 in ectopic calcification processes in extraosseous sites. Indeed, it

has been seen to be involved in HA crystals formation and breast cancer cells differentiation into Breast Osteoblast Like Cells (BOLCs). Finally, recent investigations highlighted a possible role of PTX3 in osteoblast activity and bone matrix formation in both human and mouse tissues (11-14). Noteworthy, in these studies the impairment of PTX3 expression was correlated to the occurrence of osteoporosis and more in general with a significant decrease of osteoblast differentiation. Starting from these considerations, this narrative review aims to report the most recent data concerning the role of PTX3 in bone diseases.

ROLE OF PTX3 IN ECTOPIC CALCIFICATIONS

In recent years, several investigations highlighted the role of PTX3 in ectopic calcifications (**Table 1**). Specifically, Scimeca et al. demonstrated that the impairment of PTX3 expression inhibited the synthesis of HA (13). This result was also confirmed by *ex vivo* investigations that aimed to describe the molecular mechanisms involved in the ectopic calcifications' formation. These elemental signs of human diseases are frequently detected in pathological tissues as consequence of cell homeostasis deregulation. The most common calcifications found in non-bone tissues are breast microcalcifications (21).

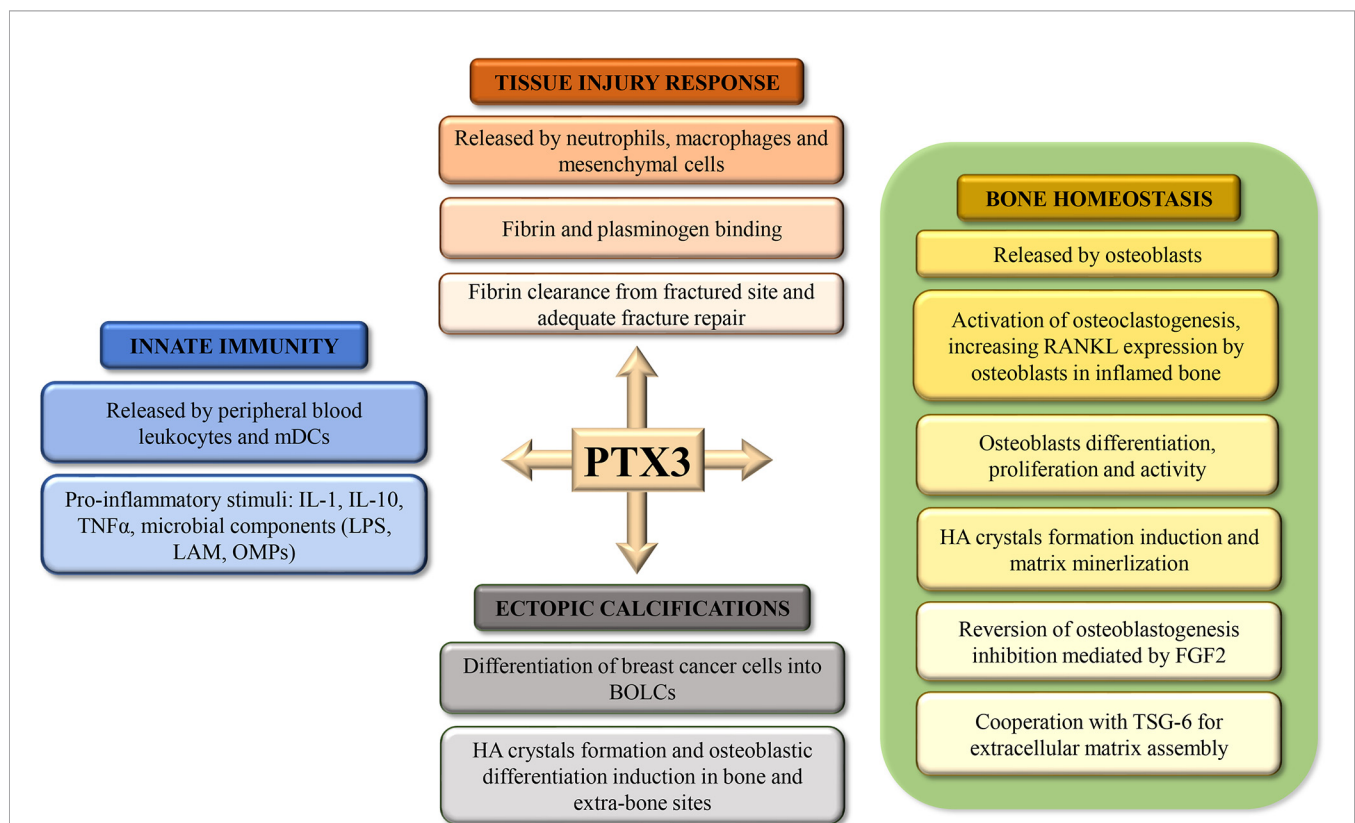


FIGURE 1 | Schematic representation of the roles of Pentraxin 3 (PTX3) in tissue injury response, bone homeostasis, ectopic calcification, and innate immunity.

TABLE 1 | Pentraxin 3 (PTX3) involvement in pathophysiological state of bone tissue's metabolism.

Pathophysiological state	Species	System	Biological process	Reference
ECTOPIC CALCIFICATION	Human	BOLCs	Differentiation of breast cancer cells	(15)
	Human	BOLCs	Differentiation and microcalcifications formation	(16)
OSTEOLYTIC CONDITION OF INFLAMED BONE	Human	pOBs	Osteoclastogenesis induction by increasing RANKL production	(17)
FRACTURE HEALING	Mouse	Non-hematopoietic periosteal cells	Periosteal reaction induction during fracture repair	(18)
OSTEOPOROSIS	Human	OBs	Differentiation, proliferation and function	(13)
BONE METABOLISM	Human	Plasma	Inverse correlation between plasma level and BMD	(19)
FRACTURE REPAIR	Mouse	OBs, OCs	Matrix mineralization sequestering FGF2	(12)
BONE METABOLISM	Mouse	MC3T3-E1 cells	Signature genes (RUNX, ALP, OSC, OSX) overexpression	(20)

BOLCs, breast osteoblast like cells; pOBs, osteoblast's precursor; OBs, osteoblasts; OCs, osteoclasts.

Deposits of calcium salts in breast are associated to the presence of lesions, both benign and malignant. Thus, detection of microcalcifications in breast tissues by imaging analysis, such as echography, Magnetic Resonance Imaging (MRI) or most commonly by mammography, currently represents the main breast cancer screening investigation (21). After the pioneering studies of Morgan et al. (22–24), numerous investigations have been performed about the origin of breast microcalcifications. Our group proposed an innovative idea in which BOLCs developed into breast cancer mass are responsible of the production of HA crystals (15, 16, 25, 26). The progressive accumulation of these calcium salts induces the formation of calcified nodules detectable by mammography. One of the mechanisms that drive the differentiation of breast cancer cells into the BOLCs and also the formation of HA crystals is mediated by PTX3 (16). Specifically, we found that breast cancer tumors with a high BOLCs amount and high PTX3 expression were characterized by several HA microcalcifications (16). In addition, microcalcifications produced by BOLCs showed a histological aspect like an osteon. Noteworthy, we also found that the presence of PTX3-positive BOLCs significantly increases the risk of developing breast cancer metastasis to bone within 5 years from first histological diagnosis (27). Therefore, it is possible to hypothesize that PTX3 can induce osteoblastic differentiation and activity in both bone and extra-bone sites such as the breast. In this respect, we described the same mechanisms for the calcifications' formation and bone metastasis in prostate cancer (28). A role of PTX3 was described also for the atheromatic lesions formation (29–31). However, to the best of our knowledge, no association has been identified between PTX3 expression and the formation of calcified nodules into the atheroma. Nevertheless, in our experience, the macrophages responsible for the atheromatic calcifications formation are frequently positive for PTX3 expression (32). Thus, it might be reasonable that osteoblastic differentiation process of atheromatic macrophages could in turn be driven by PTX3. It is important to remember that PTX3 is only one of the numerous possible mediators involved in the osteoblastic differentiation both in bone and ectopic sites, even though its activity seem to be fundamental for the mineralization process.

PTX3, OSTEOPOROSIS, AND FRACTURE HEALING

Recent studies have demonstrated the importance of the role played by PTX3 in bone tissue metabolism (Table 1). Particularly, the involvement of this molecule in aging-related bone diseases, such as osteoporosis, and fracture healing process has been studied. Given its recently emerging role, attention has been focused on PTX3 involvement in bone homeostasis. Particularly, great interest has been shown for its involvement in the pathogenesis of chronic bone diseases such as osteoporosis and in the fracture healing process, both in mice and humans (13, 14, 17, 18, 20, 33). It has been shown that PTX3 is able to induce Receptor Activator of Nuclear factor Kappa-B Ligand (RANKL) expression by osteoblasts that promote the activation of osteoclasts in an *in vitro* culture system (17). Lee and colleagues speculated that PTX3 could perform a pathological function: its expression in bone can be elevated by TNF- α , which increases RANKL expression by osteoblast's precursors, that, in turn, induces an excessive formation of osteoclasts. These results suggest that PTX3 can act as an inflammatory mediator that contributes to the deterioration of the osteolytic conditions of the inflamed bone. On the other hand, it has been proposed that PTX3 is involved in bone formation rather than resorption, as demonstrated by Kelava et al. in 2014, who studying the relationship between PTX3 expression and bone quality in mice deficient for the *PTX3* gene, demonstrated that PTX3 null mice had lower bone mass than wild-type mice (18). To establish the effective involvement of PTX3 in bone pathophysiology, its expression and function in human osteoblasts of osteoporotic, osteoarthritic patients and young subjects not affected by bone pathologies were studied (14). Immunohistochemical analysis performed on bone head biopsies showed a close association between bone health and the number of osteoblasts expressing PTX3. It was observed that the percentage of PTX3 positive osteoblasts was significantly lower in osteoporotic patients than in young and osteoarthritic patients of the same age (13). Furthermore, the treatment with an anti-PTX3 antibody of human osteoblast primary cultures derived from young patients has greatly influenced the behavior of osteoblasts: they lost the morphological and molecular characteristics typical of

mature osteoblasts, acquiring fibroblast-like shape and strongly decreasing RANKL and RUNX2 expression. Finally, PTX3 inhibition negatively influenced osteoblasts proliferation and their ability to form cell groups and HA microcrystals. Overall, it has been hypothesized that bone regeneration requires PTX3 both for the induction of the fracture healing process and for the recruitment of differentiated osteoblasts (14). Some researchers are testing the possibility to use PTX3 plasma levels as a biomarker for the bone quality and to stratify the population based on the risk to develop an osteoporotic condition. In this context, a paradox effect has been described by Lee and colleagues (19). In particular, authors evaluated PTX3 plasma levels on a sub-cohort of 1440 subjects (757 men and 683 women) founding that PTX3 levels were inversely associated with Bone Mineral Density (BMD) of the lumbar spine and femoral neck in men but not in women (19). This data could be explained as a systemic reaction of the body to offset the impairment of PTX3 in specific tissue as the bone. In agreement with these evidences, Grčević and colleagues performed an *in vivo* study in which PTX3 KO mice (*Ptx3*^{-/-}) was used to test the role of PTX3 in bone turnover and repair (12). By using the closed transversal tibial fracture model, authors found that *Ptx3*^{-/-} female mice formed significantly less mineralized callus during the anabolic phase following fracture injury compared to wild-type mice. These results indicate that PTX3 plays an important role in bone homeostasis and in proper matrix mineralization during fracture repair, a reflection of this molecule function in tissue homeostasis and repair (12). Moreover, PTX3 can interact with Fibroblast Growth Factor 2 (FGF2), which is highly expressed in the bone matrix, exerting a negative effect on osteoblastogenesis and bone remodeling. It has been demonstrated *in vitro* that PTX3 produced by osteoblasts is able to bind FGF2 and inhibit the negative effects exerted on the differentiation of bone marrow stromal cells into osteoblasts, acting as a protective factor for bone (33). Finally, a study performed by Liu et al. investigated the role played by PTX3 in promoting osteoblastic differentiation in MC3T3-E1 cells, confirming the essential role of PTX3 in the bone metabolism (20). Authors showed that PTX3 is abundantly expressed in MC3T3-E1 cells and that its expression is inducible by the introduction of osteogenic induction medium. In addition, the overexpression of PTX3 was able to significantly increase the expression of some of the most important genes related to bone metabolism such as Runt-related Transcription Factor 2 (RUNX2), ALP, Osteocalcin (OCN) and Osterix (OSX), suggesting that the PTX3 overexpression promotes osteoblastic differentiation and activity (20). As concern the possible molecular pathways involved in the bone metabolism regulation by PTX3, it is demonstrated that the effects of PTX3 on osteoblasts are mediated by its induction of the PI3K/Akt signaling pathway. From mechanistic point of view, Liu et al. displayed that the action of PTX3 requires the activation of PI3K and Akt, and deactivation of PI3K by its inhibitor LY294002 weakens the PTX3-mediated induction of osteoblast signature genes, ALP and matrix mineralization (20). These data provide the rationale to develop new strategies for bone metabolism

regulation in human subjects. Indeed, the possibility to modulate the molecular signaling involved in the PTX3-related osteoblast differentiation could represents an opportunity for future innovative therapies for osteoporotic patients.

PTX3 GENETIC VARIABILITY AND AGING-RELATED BONE DISEASES

In recent years, increasing research has focused on the association between the Single-Nucleotide Polymorphisms (SNPs) of *PTX3* gene and human diseases. However, there are still few reports about the effect of PTX3 genetic variability and aging-related bone diseases. Previous studies have described that rs2305619 and rs1840680 SNPs have functional significance on the relative PTX3 protein amount. Results indicated that the A allele of both SNPs is associated with higher levels of circulating PTX3 (34, 35). The mechanism by which these SNPs affect PTX3 plasma levels has still to be clarified but possibly rs2305619 and rs1840680 are in linkage disequilibrium with a genetic variant located in a regulatory region of the gene. Variable levels of PTX3, depending on different genetic variants, may therefore have a direct role in the pathogenesis of inflammation and ossification. On this basis, Zhang and Ding (36) analysed the effects of three PTX3 polymorphisms in genetic susceptibility to Ankylosing Spondylitis (AS). AS is a complex inflammatory disease characterized by a chronic long-term inflammation of the joints of the spine over time. As an autoimmune disease, AS develops through complex interactions between the individual genetic background and environmental factors (37). Genotyping of rs23056219, rs3816527, and rs3845978 SNPs in *PTX3* gene has been conducted by allelic discrimination assay in 101 AS patients and 93 controls. Results showed that the C allele and the CC genotype of rs3816527 have a positive effect on AS occurrence. Similarly, in rs3845978, individuals carrying the T allele and the CT genotype developed AS earlier. The three SNPs belong to a haplotype block and their combined analysis revealed that G-C-T and A-C-C haplotypes may increase the genetic susceptibility to AS (36). Another genetic aspect linked to the increased risk to develop bone disease is the so called “inflamm-aging”, the age-related elevations in proinflammatory cytokines associated with shorter immune cell telomere lengths. Telomeres maintain the “youthful” status of immune cells, and shortened telomere lengths are considered a main biological hallmark of cellular aging (38) leading to an irreversible state of replication-induced cellular senescence (39). Although leukocyte telomere lengths shorten as a natural consequence of aging, the continuous exposure to increased proinflammatory agents may accelerate this process (40). It has been demonstrated that increased PTX3 production is required to prevent overactivation of the inflammatory signalling pathway, and elevated concentrations of circulating PTX3 are considered an indicator of appropriate immune function in healthy young adults (41). This observation has been further confirmed in the study by Pavanello et al (42), who recently demonstrated that the upregulation of PTX3 plasma levels is associated with longer telomere lengths in

healthy middle aged adults (41). Last but not least, epigenetic mechanisms have been also implicated in the regulation of human PTX3 expression, since methylation of 5' UTR regions (enhancers and promoter) has been deemed responsible of PTX3 gene silencing in several disease conditions including cancer, inflammation and atherosclerosis (43, 44). Chronic inflammation induces a novel epigenetic program that is conserved in intestinal adenomas and in colorectal cancer (45). It is therefore possible to speculate that the persistent inflammatory stimuli from an aged immune system may be also involved in the progression of age-related bone diseases through either genetic (telomere shortening) and epigenetic (DNA hypermethylation) mechanisms acting on the *PTX3* gene.

DISCUSSION

In this systematic review, we discussed and summarised the effects of PTX3 on various biological mechanisms, focusing our attention on the role played by this protein in the physiology and pathophysiology of mineralization process, in bone formation and ectopic calcifications, as well as its involvement in the onset of age-related bone diseases. In fact, PTX3 seems to be involved in tissue remodelling and repair processes, both under physiological and pathological conditions (1, 33). In this regard, our group demonstrated for the first time the ability of PTX3 to induce an increase of both cell proliferation and HA microcrystal formation in osteoblasts culture characterized by an impairment of PTX3 expression. These results allowed us to speculate the existence of a close correlation between PTX3 expression by osteoblasts and bone quality, suggesting a central role for PTX3 in proliferation, differentiation, and function. The ability of PTX3 to participate in HA microcrystals' formation has also been confirmed by *ex vivo* studies to describe the molecular mechanisms involved in ectopic calcifications' formation, which are often detected in pathological tissues, resulting in an altered cellular homeostasis. PTX3 is known to mediate the differentiation of breast cancer cells into BOLCs cells and the formation of HA crystals (26). Based on these evidences, PTX3 can be considered a new possible marker for poor differentiated breast cancer (46). Furthermore, since a significantly greater difference in PTX3 expression between malignant and benign lesions in the presence of microcalcifications was found, it can be assumed that PTX3 plays an essential role in the formation of ectopic calcifications

in breast. Besides being considered one of the many possible mediators involved in osteoblastic differentiation both in bone and ectopic sites, several studies agree with the definition of PTX3 also as a molecule significantly involved in the pathogenesis of age-related bone diseases, such as osteoporosis, and in the fracture healing process. In this regard, to establish the effective involvement of PTX3 in bone pathophysiology, Scimeca and colleagues studied the expression and function of this protein in osteoblasts from osteoporotic and osteoarthritic patients and young subjects not affected by bone diseases (13). It is important to highlight the role of PTX3 in bone regeneration, since its presence would appear to be crucial both for inducing the fracture healing process and recruitment of differentiated osteoblasts. In recent years, it has been assumed that the onset and progression of age-related bone diseases are also influenced by genetic and epigenetic mechanisms acting on the *PTX3* gene. Indeed, it is possible to speculate that genetic variability, together with epigenetic modifications, in *PTX3* regulatory regions could lead to an alteration of PTX3 levels and may be directly involved in the pathogenesis of inflammation and ossification. Based on these scientific evidences, we can certainly conclude that PTX3 may represent a new regulator of bone metabolism. Given its evident involvement in the physiological and pathophysiological processes that occur in bone tissue, PTX3 could represent a new potential diagnostic marker and therapeutic target for the treatment of age-related bone diseases, representing an important goal in the era of personalized medicine. Considering the multiple roles played by PTX3 in both physiological and pathological contexts, we believe it is necessary to address future studies towards a better and deeper understanding of the underlying mechanisms, in order to make possible the development of targeted therapeutic approaches. Finally, we believe that the use of PTX3 as a marker can contribute significantly to a reduction in costs associated with diagnostic procedures and treatment of bone metabolism disorders.

AUTHOR CONTRIBUTIONS

CG, VV, IC, AB, MC, MG, and MS wrote the initial draft of the manuscript. UT, EG, MS, and AS contributed to the editing and revising of this work. All authors contributed to the article and approved the submitted version.

REFERENCES

1. Bottazzi B, Inforzato A, Messa M, Barbagallo M, Magrini E, Garlanda C, et al. The pentraxins PTX3 and SAP in innate immunity, regulation of inflammation and tissue remodelling. *J Hepatol* (2016) 64:1416–27. doi: 10.1016/j.jhep.2016.02.029
2. Bottazzi B, Doni A, Garlanda C, Mantovani A. An integrated view of humoral innate immunity: Pentraxins as a paradigm. *Annu Rev Immunol* (2010) 28:157–83. doi: 10.1146/annurev-immunol-030409-101305
3. Fornari R, Marocco C, Francomano D, Fittipaldi S, Lubrano C, Bimonte VM, et al. Insulin growth factor-1 correlates with higher bone mineral density and lower inflammation status in obese adult subjects. *Eat Weight Disord* (2018) 23:375–81. doi: 10.1007/s40519-017-0362-4
4. Garlanda C, Bottazzi B, Bastone A, Mantovani A. Pentraxins at the crossroads between innate immunity, inflammation, matrix deposition, and female fertility. *Annu Rev Immunol* (2005) 23:337–66. doi: 10.1146/annurev-immunol.23.021704.115756
5. Breviaro F, D'Aniello EM, Golay J, Peri G, Bottazzi B, Bairoch A, et al. Interleukin-1-inducible genes in endothelial cells. Cloning of a new gene related to C-reactive protein and serum amyloid P component. *J Biol Chem* (1992) 267:22190–7.
6. Inforzato A, Rivieccio V, Morreale AP, Bastone A, Salustri A, Scarchilli L, et al. Structural characterization of PTX3 disulfide bond network and its multimeric status in cumulus matrix organization. *J Biol Chem* (2008) 283:10147–61. doi: 10.1074/jbc.M708535200
7. Inforzato A, Baldock C, Jowitt TA, Holmes DF, Lindstedt R, Marcellini M, et al. The angiogenic inhibitor long pentraxin PTX3 forms an asymmetric octamer with two binding sites for FGF2. *J Biol Chem* (2010) 285:17681–92. doi: 10.1074/jbc.M109.085639

8. Doni A, Musso T, Morone D, Bastone A, Zambelli V, Sironi M, et al. An acidic microenvironment sets the humoral pattern recognition molecule PTX3 in a tissue repair mode. *J Exp Med* (2015) 212:905–25. doi: 10.1084/jem.20141268
9. Cappuzzello C, Doni A, Dander E, Pasqualini F, Nebuloni M, Bottazzi B, et al. Mesenchymal stromal cell-derived PTX3 promotes wound healing via fibrin remodeling. *J Invest Dermatol* (2016) 136:293–300. doi: 10.1038/JID.2015.346
10. Bonacina F, Barbieri SS, Cutuli L, Amadio P, Doni A, Sironi M, et al. Vascular pentraxin 3 controls arterial thrombosis by targeting collagen and fibrinogen induced platelets aggregation. *Biochim Biophys Acta - Mol Basis Dis* (2016) 1862:1182–90. doi: 10.1016/j.bbdis.2016.03.007
11. Parente R, Sobacchi C, Bottazzi B, Mantovani A, Grčević D, Inforzato A. The Long Pentraxin PTX3 in Bone Homeostasis and Pathology. *Front Immunol* (2019) 10:2628. doi: 10.3389/fimmu.2019.02628
12. Grčević D, Sironi M, Valentino S, Deban L, Cvija H, Inforzato A, et al. The long pentraxin 3 plays a role in bone turnover and repair. *Front Immunol* (2018) 9:417. doi: 10.3389/fimmu.2018.00417
13. Scimeca M, Salustri A, Bonanno E, Nardozi D, Rao C, Piccirilli E, et al. Impairment of PTX3 expression in osteoblasts: A key element for osteoporosis. *Cell Death Dis* (2017) 8:3125. doi: 10.1038/cddis.2017.514
14. Tarantino U, Feola M, Celi M, Scimeca M. PTX3: A new mediator of bone metabolism and osteoporosis. *Muscles Ligaments Tendons J* (2017) 7:200–1. doi: 10.11138/mltj/2017.7.1.200
15. Bonfiglio R, Scimeca M, Urbano N, Bonanno E, Schillaci O. Breast microcalcifications: Biological and diagnostic perspectives. *Future Oncol* (2018) 14:3097–9. doi: 10.2217/fon-2018-0624
16. Scimeca M, Bonfiglio R, Menichini E, Albonici L, Urbano N, De Caro MT, et al. Microcalcifications drive breast cancer occurrence and development by macrophage-mediated epithelial to mesenchymal transition. *Int J Mol Sci* (2019) 20:5633. doi: 10.3390/ijms20225633
17. Lee E-J, Song D-H, Kim Y-J, Choi B, Chung Y-H, Kim S-M, et al. PTX3 Stimulates Osteoclastogenesis by Increasing Osteoblast RANKL Production. *J Cell Physiol* (2014) 229:1744–52. doi: 10.1002/jcp.24626
18. Kelava T, Ivcevic S, Katavic V, Kovacic N, Cvija H, Petrovic KZ, et al. Increased expression of PTX3 in non-hematopoietic periosteal cells during fracture healing. *Bone Abstr* (2014). doi: 10.1530/boneabs.3.pp68
19. Lee R, Ahn HR, Shin MH, Kim HN, Lee YH, Choi SW, et al. Association between plasma pentraxin 3 levels and Bone mineral density in elderly Koreans: The Dong-gu Study. *J Korean Med Sci* (2018) 33. doi: 10.3346/jkms.2018.33.e165
20. Liu Y, Wang H, Zhou XZ, Li N, Guo YC, Chen TP. Pentraxin 3 promotes the osteoblastic differentiation of MC3T3-E1 cells through the PI3K/Akt signaling pathway. *Biosci Rep* (2020) 40:20201165. doi: 10.1042/BSR20201165
21. Tan PS, Alsheh Ali M, Eriksson M, Hall P, Humphreys K, Czene K. Mammography features for early markers of aggressive breast cancer subtypes and tumor characteristics - a population-based cohort study. *Int J Cancer* (2020). doi: 10.1002/ijc.33309
22. Cox RF, Hernandez-Santana A, Ramdass S, McMahon G, Harmey JH, Morgan MP. Microcalcifications in breast cancer: Novel insights into the molecular mechanism and functional consequence of mammary mineralisation. *Br J Cancer* (2012) 106:525–37. doi: 10.1038/bjc.2011.583
23. Cox RF, Morgan MP. Microcalcifications in breast cancer: Lessons from physiological mineralization. *Bone* (2013) 53:437–50. doi: 10.1016/j.bone.2013.01.013
24. Morgan MP, Cooke MM, McCarthy GM. Microcalcifications associated with breast cancer: An epiphenomenon or biologically significant feature of selected tumors? *J Mammary Gland Biol Neoplasia* (2005) 10:181–7. doi: 10.1007/s10911-005-5400-6
25. Scimeca M, Bonfiglio R, Montanaro M, Bonanno E. Osteoblast-like cells in human cancers: New cell type and reliable markers for bone metastasis. *Future Oncol* (2018) 14:9–11. doi: 10.2217/fon-2017-0472
26. Bonfiglio R, Scimeca M, Toschi N, Pistolesi CA, Giannini E, Antonacci C, et al. Radiological, Histological and Chemical Analysis of Breast Microcalcifications: Diagnostic Value and Biological Significance. *J Mammary Gland Biol Neoplasia* (2018) 23:89–99. doi: 10.1007/s10911-018-9396-0
27. Scimeca M, Antonacci C, Toschi N, Giannini E, Bonfiglio R, Buonomo CO, et al. Breast Osteoblast-like Cells: A Reliable Early Marker for Bone Metastases From Breast Cancer. *Clin Breast Cancer* (2018) 18:e659–69. doi: 10.1016/j.clbc.2017.11.020
28. Scimeca M, Urbano N, Rita B, Mapelli SN, Catapano CV, Carbone GM, et al. Prostate Osteoblast-Like Cells: A Reliable Prognostic Marker of Bone Metastasis in Prostate Cancer Patients. *Contrast Media Mol Imaging* (2018) 2018. doi: 10.1155/2018/9840962
29. Ninomiya I, Kanazawa M, Uemura M, Onodera O. Elevated serum pentraxin 3 levels might predict the diagnosis of branch atheromatous disease at a very early stage. *Eur J Neurol* (2020) 27:1279–84. doi: 10.1111/ene.14249
30. Zlibut A, Bocsan IC, Pop RM, Vesa SC, Bheecarry K, Revnic R, et al. Role of pentraxin-3 in risk assessment of patients with metabolic syndrome. *Physiol Int* (2019) 106:283–93. doi: 10.1556/2060.106.2019.18
31. Garlanda C, Bottazzi B, Moalli F, Deban L, Molla F, Latini R, et al. Pentraxins and Atherosclerosis: The Role of PTX3. *Curr Pharm Des* (2011) 17:38–46. doi: 10.2174/138161211795049750
32. Mantovani A, Garlanda C, Locati M. Macrophage diversity and polarization in atherosclerosis: A question of balance. *Arterioscler Thromb Vasc Biol* (2009) 29:1419–23. doi: 10.1161/ATVBAHA.108.180497
33. Doni A, Stravalaci M, Inforzato A, Magrini E, Mantovani A, Garlanda C, et al. The long pentraxin PTX3 as a link between innate immunity, tissue remodeling, and cancer. *Front Immunol* (2019) 10:712. doi: 10.3389/fimmu.2019.00712
34. Barbati E, Specchia C, Vilella M, Rossi ML, Barlera S, Bottazzi B, et al. Influence of Pentraxin 3 (PTX3) Genetic Variants on Myocardial Infarction Risk and PTX3 Plasma Levels. *PLoS One* (2012) 7. doi: 10.1371/journal.pone.0053030
35. Diamond JM, Meyer NJ, Feng R, Rushefski M, Lederer DJ, Kawut SM, et al. Variation in PTX3 is associated with primary graft dysfunction after lung transplantation. *Am J Respir Crit Care Med* (2012) 186:546–52. doi: 10.1164/rccm.201204-0692OC
36. Zhang X, Ding W. Association of genetic variants in pentraxin 3 gene with ankylosing spondylitis. *Med Sci Monit* (2016) 22:2911–6. doi: 10.12659/MSM.896562
37. Zhu W, He X, Cheng K, Zhang L, Chen D, Wang X, et al. Ankylosing spondylitis: etiology, pathogenesis, and treatments. *Bone Res* (2019) 7:1–16. doi: 10.1038/s41413-019-0057-8
38. Fasching CL. Telomere length measurement as a clinical biomarker of aging and disease. *Crit Rev Clin Lab Sci* (2018) 55:443–65. doi: 10.1080/10408363.2018.1504274
39. Zou Y, Sfeir A, Gryaznov SM, Shay JW, Wright WE. Does a sentinel or a subset of short telomeres determine replicative senescence? *Mol Biol Cell* (2004) 15:3709–18. doi: 10.1091/mbc.E04-03-0207
40. Jurk D, Wilson C, Passos JF, Oakley F, Correia-Melo C, Greaves L, et al. Chronic inflammation induces telomere dysfunction and accelerates ageing in mice. *Nat Commun* (2014) 2:1–14. doi: 10.1038/ncomms5172
41. Yamasaki K, Kurimura M, Kasai T, Sagara M, Kodama T, Inoue K. Determination of physiological plasma pentraxin 3 (PTX3) levels in healthy populations. *Clin Chem Lab Med* (2009) 47:471–7. doi: 10.1515/CCLM.2009.110
42. Pavanetto S, Stendardo M, Mastrangelo G, Bonci M, Bottazzi B, Campisi M, et al. Inflammatory long pentraxin 3 is associated with leukocyte telomere length in night-shift workers. *Front Immunol* (2017) 8:516. doi: 10.3389/fimmu.2017.00516
43. Guo TM, Huang LL, Liu K, Ke L, Luo ZJ, Li YQ, et al. Pentraxin 3 (PTX3) promoter methylation associated with PTX3 plasma levels and neutrophil to lymphocyte ratio in coronary artery disease. *J Geriatr Cardiol* (2016) 13:712–7. doi: 10.11909/j.issn.1671-5411.2016.08.010
44. Abu-Remaih M, Bender S, Raddatz G, Ansari I, Cohen D, Gutekunst J, et al. Chronic inflammation induces a novel epigenetic program that is conserved in intestinal adenomas and in colorectal cancer. *Cancer Res* (2015) 75:2120–30. doi: 10.1158/0008-5472.CAN-14-3295
45. Shiraki A, Kotooka N, Komoda H, Hirase T, Oyama Ji, Node K. Pentraxin-3 regulates the inflammatory activity of macrophages. *Biochem Biophys Res* (2016) 5:290–5. doi: 10.1016/j.bbrep.2016.01.009
46. Scimeca M, Antonacci C, Colombo D, Bonfiglio R, Buonomo OC, Bonanno E. Emerging prognostic markers related to mesenchymal characteristics of poorly differentiated breast cancers. *Tumor Biol* (2016) 37:5427–35. doi: 10.1007/s13277-015-4361-7

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Tarantino, Greggi, Cariati, Visconti, Gasparini, Cateni, Gasbarra, Botta, Salustri and Scimeca. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Therapeutic Lowering of C-Reactive Protein

Rachel V. Jimenez^{1*} and Alexander J. Szalai²

¹ Department of Immunology, H. Lee Moffitt Cancer Center & Research Institute, Tampa, FL, United States, ² Division of Clinical Immunology & Rheumatology, Department of Medicine, The University of Alabama at Birmingham, Birmingham, AL, United States

OPEN ACCESS

Edited by:

Richard H. Gomer,
Texas A&M University, United States

Reviewed by:

Mark B. Pepys,
University College London, United Kingdom
Ahmed Sheriff,
Charité – Universitätsmedizin Berlin, Germany

*Correspondence:

Rachel V. Jimenez
rachel.jimenez@moffitt.org

Specialty section:

This article was submitted to
Molecular Innate Immunity,
a section of the journal
Frontiers in Immunology

Received: 20 October 2020

Accepted: 15 December 2020

Published: 29 January 2021

Citation:

Jimenez RV and Szalai AJ (2021)
Therapeutic Lowering of
C-Reactive Protein.
Front. Immunol. 11:619564.
doi: 10.3389/fimmu.2020.619564

In the blood of healthy individuals C-reactive protein (CRP) is typically quite scarce, whereas its blood concentration can rise robustly and rapidly in response to tissue damage and inflammation associated with trauma and infectious and non-infectious diseases. Consequently, CRP plasma or serum levels are routinely monitored in inpatients to gauge the severity of their initial illness and injury and their subsequent response to therapy and return to health. Its clinical utility as a faithful barometer of inflammation notwithstanding, it is often wrongly concluded that the biological actions of CRP (whatever they may be) are manifested only when blood CRP is elevated. In fact over the last decades, studies done in humans and animals (e.g. human CRP transgenic and CRP knockout mice) have shown that CRP is an important mediator of biological activities even in the absence of significant blood elevation, i.e. even at baseline levels. In this review we briefly recap the history of CRP, including a description of its discovery, early clinical use, and biosynthesis at baseline and during the acute phase response. Next we overview evidence that we and others have generated using animal models of arthritis, neointimal hyperplasia, and acute kidney injury that baseline CRP exerts important biological effects. In closing we discuss the possibility that therapeutic lowering of baseline CRP might be a useful way to treat certain diseases, including cancer.

Keywords: pentraxin, antisense oligonucleotide, autoimmunity, cancer, cardiovascular

PREFACE

This article was written for *Frontiers in Immunology's* research topic *Diagnostic and Therapeutic Applications of Pentraxin and Pentraxin-Associated Proteins*. Our goal was to review the available evidence - generated by us and by others - that supports targeting of CRP as a therapeutic approach for the treatment of human diseases including cancer. To provide what we think is necessary context we include a brief historical overview of CRP and an update on our understanding of CRP's general biology and its mode of regulation, and we briefly summarize the use of CRP measurements in clinical practice (although in full disclosure neither of the authors is a clinician with direct experience in that realm). We have done our best to be non-partisan, fair, complete, and balanced, and to give credit to researchers in the field where we think credit is due. Finally, since this is a review paper of limited length we were necessarily judicious and have not referenced the entirety of the excellent work done by all the experts in the field. Those caveats notwithstanding, we sincerely hope that what we have written here stimulates in the reader an appetite for more

information about CRP. To feed that appetite we encourage the readers to devour one or more of the many excellent reviews already written by other experts (1–12).

HISTORICAL OVERVIEW OF C-REACTIVE PROTEIN

C-Reactive Protein Gained Acceptance as a Non-Specific Biomarker of Disease Soon After Its Initial Discovery

C-reactive protein (CRP) was discovered during the seminal studies of pneumococcal pneumonia being performed in the laboratory of Oswald T. Avery (1877–1955). There in 1930 in the blood of pneumonia patients, William S. Tillett (1892–1974) and Thomas Francis, Jr. (1900–1969) identified high titers of a substance that was reactive with (and could precipitate from solution) the carbohydrate-rich “C fraction” of *S. pneumonia* (i.e. the fraction containing pneumococcal C-polysaccharide) (13). Today we call this precipitating substance C-reactive protein or CRP. Tillett and Francis also observed the drastic decline and eventual disappearance of the precipitin from patient’s sera coincident with resolution of their febrile period – the first report of CRP’s characteristic rise and fall during what later became known as the “acute phase response” (APR). At the time, to detect CRP Tillett and Francis and their contemporaries relied on a flocculation reaction, wherein CRP in the serum reacted with C-polysaccharide in solution to form a visible precipitate. This assay requires high amounts of CRP and consequently, CRP could only be detected in the serum of patients experiencing severe disease; thus at the time CRP was thought to be absent from the serum of healthy people. Soon thereafter it was recognized that monitoring CRP blood levels might have clinical utility, and this was solidly established by a trio of papers published by Avery’s group in 1941 (14–16). Still today elevation of blood CRP is recognized as a useful “biomarker of inflammation”, and so CRP blood levels are incorporated into numerous disease assessment guidelines. With the eventual development of more accurate and more sensitive techniques for its measurement, CRP was found to be present in normal serum too (17–19) and the first rigorous study of normal CRP values soon followed (20). These findings challenged the earlier *status quo* that CRP was a precipitin present only during infection and raised the possibility that CRP plays a role in normal homeostasis. Recognizing that assays of CRP were of increasing clinical diagnostic value, in 1987 the World Health Organization spearheaded the development of an International Standard for Human CRP. That standard (coded 85/506), comprised of an extract of freeze-dried pooled human serum to which a quantity of human CRP has been added, is stable and is available in ampoules and was shown to be suitable for use as a standard in seven different immunoassays [(21), pp. 21–22]. Preparation 85/506 was meant to be the gold standard against which all national and international secondary CRP standards are meant to be calibrated.

Blood CRP levels in populations of ostensibly healthy people have a positively skewed log-normal distribution, and baseline blood CRP levels in individuals can vary with age, sex, race, genetics, and obesity (22–25). Consequently, even in people whose CRP levels fall within the reference ranges first established by Shine et al. (20) (median 0.8 mg/L with 90% under 3.0 mg/L and 99% under 10 mg/L), CRP values can still vary widely from one healthy person to another. The range of values deemed normal for a physiologic measurement in healthy persons is further complicated by the fact that blood CRP concentrations between 3 and 10 mg/L are considered by some to be indicative of ‘low-grade’ inflammation, i.e. mild inflammation resulting from a variety of persistent metabolic stresses (e.g. atherosclerosis, obesity, obstructive sleep apnea, insulin resistance, hypertension, type 2 diabetes, etc. (4). There is similar wide variability in CRP levels among those whose values are above the normal range; with levels greater than 10 mg/L generally considered to indicate clinically significant inflammation (i.e. the type accompanied by *rubor, calor, dolor*, and *tumor*) and levels above 100 mg/L considered indicative of infection. As mentioned above baseline CRP values are now included in a variety of clinical guidelines for disease assessment. Perhaps the best known example of this is the reference ranges for serum CRP now articulated by the Centers for Disease Control and Prevention and the American Heart Association to estimate cardiovascular risk in otherwise healthy individuals: low-, average-, and high-risk values defined as < 10, 10 – 30, and > 30 mg/L (26). Indeed according to some reports, high baseline CRP is a more robust predictor of future adverse coronary events than is dyslipidemia (27, 28). From cardiovascular disease to cancer to acute kidney injury high CRP levels have been shown to correlate with worse prognosis, and recent evidence indicates CRP is a very good predictor of adverse outcomes for COVID-19 patients (29). Interestingly, animal studies indicate that CRP’s association with autoimmune diseases might be reversed, i.e. the onset of multiple sclerosis, arthritis, and lupus, is delayed in CRP transgenic mice (3, 30, 31). Whether higher baseline CRP predicts later onset of autoimmunity in patients remains to be ascertained. CRP thus has a long history and its elevation in the blood above the normal range has well-established utility as a biomarker of inflammation and disease. As will be discussed later, emerging new evidence indicates that blood CRP can also actively participate in physiological processes when it is in the range deemed normal.

The Advent of Molecular Biology Ushered in a Deeper Understanding of C-Reactive Protein Structure, Regulation, and Biological Activity

Not only is CRP the prototypical human acute phase protein (32, 33) it is also the prototypical pentraxin (34), an evolutionarily highly conserved class of pattern recognition molecules with the same or highly similar primary, secondary, tertiary, and quaternary structures; human CRP being composed of five globular monomers of 206 amino acids each, non-covalently bound together into a planar ring with a central pore (**Figure 1**)

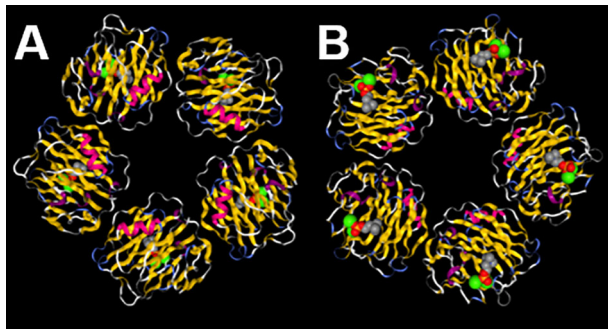


FIGURE 1 | C-reactive protein (CRP) is a pentamer with two distinct faces. **(A)** On the A face each CRP monomer displays an α -helical stretch of residues (fuchsia) adjacent to the C1q and FcR binding sites and on the opposite B face **(B)** each monomer displays residues that help coordinate two Ca^{2+} ions (green spheres) that assist with binding to ligands (phosphocholine shown in gray, red, and orange spheres). The image is a reproduction of structure 1B09 as reported by (35, 36) and was created using NGL (37) accessed on PDB (38–40).

(34, 35, 41). As mentioned earlier the first known biological activity of CRP identified was its ability to bind to and precipitate with the pneumococcal C-polysaccharide (13). This action was later found to rely on CRP binding to phosphocholine (a major constituent of C-polysaccharide) in a Ca^{2+} -dependent manner (42–44). While C-polysaccharide/phosphocholine is the canonical and most well-studied ligand for CRP, it is likely not the only ligand to which CRP can bind. For example numerous different groups have reported that CRP also binds to apoptotic cell membranes (45), to various nuclear antigens (46–48), to the oxidized LDL receptor (49), and to many other ligands (9, 12, 50, 51), although some of these reactivity's have been disputed (52). Additionally, CRP is known to activate the classical pathway of complement by binding and thereby activating C1q (53–55), and to activate numerous myeloid, lymphoid, and endothelial cell responses by binding to a variety of Fc receptors (10, 56). In general it is most likely that by binding one or more of these ligands, by activating complement, and by engaging Fc receptors that CRP mediates its well-described protective influences against bacterial infection (57–61). As will be detailed later, these varied ligand and receptor interactions also support CRP's participation in various non-infectious diseases. Insofar as is known, the CRP's of different species share the same or similar ligand binding profiles as human CRP (12).

Seminal studies conducted using both rabbit and human cells showed that the hepatocyte is the main site of CRP synthesis and assembly (62, 63). That finding and the subsequent mapping of the human *CRP* gene to chromosome 1 (64) led to more detailed studies of the control of *CRP* expression. While a few studies have reported that some pre-assembled CRP is kept in intracellular pools – from which it is expeditiously released during the APR (65, 66) – the overwhelming evidence from human cells and transgenic mice (67) has solidly established that *CRP* expression – and thus CRP blood levels – is controlled mainly at the transcriptional level. Today, transcriptional

regulation of *CRP* is known to be coordinated by a host of cytokines and hepatic transcription factors, with IL-6 and IL-1 β being the main cytokines regulating CRP blood levels during inflammatory episodes (68–70). The latter have been shown to bind to various overlapping transcription factor binding elements in the *CRP* promoter, in a region proximal to its coding sequence (**Figure 2**). It is now certain that the levels of blood CRP in healthy individuals is influenced by genetic variation in this promoter region (6, 24), but it is still uncertain whether genetic variation in these or other regulatory elements contributes to differences in the ability to upregulate *CRP* during inflammation. Notwithstanding the latter gap in knowledge, the available evidence indicates that in the absence of inflammation baseline *CRP* expression can be maintained by the transcription factors hepatic nuclear factor (HNF) 1 alpha (HNF1 α) and 3 (HNF3) (69). In contrast during inflammation, the increased availability of IL-6 and/or IL-1 β supports increased production of the transcription factors C/EBP, STAT3, cFos, and NF- κ B, which act synergistically with the *CRP* promoter-bound HNFs to drive high levels of *CRP* transcription (68, 69, 71–73). There is also some evidence that the proximal promoter of *CRP*, perhaps in conjunction with the *CRP* 3'UTR and the downstream pseudogene *CRPP1* can assemble into an enhanceosome (**Figure 2**) that favors more prolonged transcription of *CRP* (67, 74–76). Thus it is most likely that during an APR, the increased quantity and variety of transcription factors available (coupled with their increased nuclear residency?) supports more efficient transcription of *CRP*, thus accounting for the dramatic increase in circulating CRP levels (from ~1 mg/L to upwards of ~200 mg/L) that can be seen within hours during the acute phase response. Presumably when inflammation is resolved and the availability of cytokines and transcription factors is lowered, transcriptional control of *CRP* is handed back to HNFs (69).

TARGETED LOWERING OF C-REACTIVE PROTEIN

Blood C-Reactive Protein Lowering as a Therapeutic for Cardiovascular Disease?

The association of CRP blood levels with cardiovascular disease (CVD) has been of keen interest for many decades, and today baseline CRP is recognized both as an independent marker and predictor of myocardial infarction (MI), stroke, and death from coronary heart disease in ostensibly healthy people. Conclusive evidence for CRP causality in human CVD is still lacking [for more insight see the comprehensive critical reviews by (5, 7)] and the entire issue remains hotly debated, but the results of at least four different clinical trials suggest a role for CRP in the atherogenic process (27, 77–79). Also there is indirect evidence from many different groups suggesting that CRP is causally related to CVD. For example CRP is detected in human atherosclerotic lesions (80–84) and it can activate human endothelial cells (82, 85, 86). The evidence from transgenic animals is equally confusing, with some studies (including

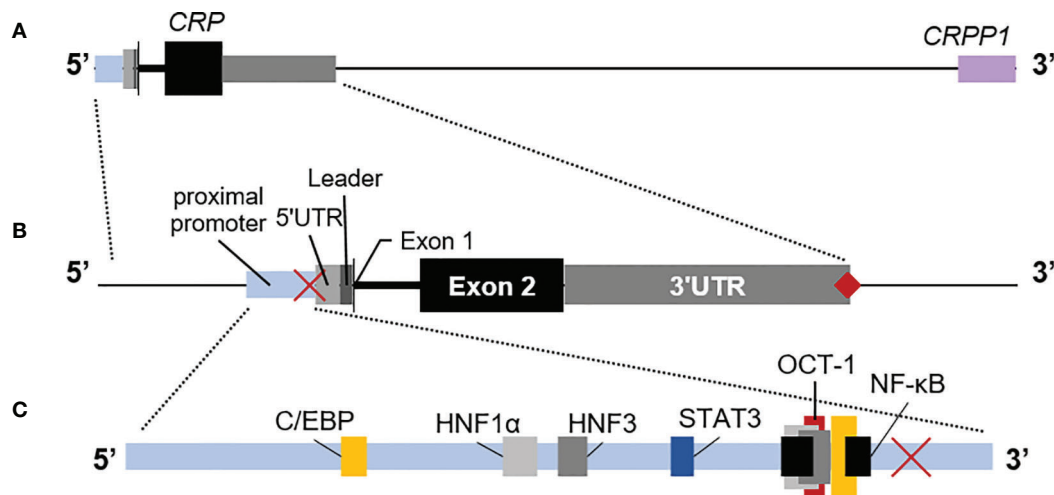


FIGURE 2 | The human C-reactive protein (*CRP*) gene. **(A)** Downstream of *CRP* is the *CRP* pseudo-gene (*CRPP1*, lilac box), that is likely involved in *cis*-acting regulation of basal *CRP* expression. **(B)** The proximal promoter of *CRP* (light blue box) is typically mapped as the region ~300 nucleotides upstream of the transcription start site, including the TATA binding site at -29 to -26 (red x). The short 5' untranslated region (5'UTR; light gray box) precedes the coding sequence for the 18 amino acid-long leader peptide (dark gray box). Exon 1 encodes the first three amino acids of mature *CRP* (thin black vertical line) and is immediately followed by the intron. The remainder of *CRP* is encoded by exon 2 (black box). *CRP* has a long 3'UTR sequence (light gray box) with a poly(A) signal (♦). Each region is drawn approximately to scale. **(C)** The relative locations in the *CRP* promoter of binding elements for hepatic nuclear factor (HNF) 1α and HNF3 that contribute to regulation of constitutive expression of *CRP* (gray boxes) and for C/EBP (yellow boxes), NF-κB (black boxes), and STAT3 (blue box) that contribute to regulation of expression of *CRP* during the acute phase response. Note that C/EBP and NF-κB and the repressor OCT-1 (red box) utilize overlapping elements.

some of our own) indicating human CRP might be pathogenic in CVD (87–90) whereas others indicating it is not (91, 92). While the exact contribution of CRP to human CVD remains equivocal and the debate still rages (93), the current authors will not be surprised if future placebo-controlled double-blind studies showed that CRP contributes to the pathophysiology of human CVD.

Causal or not, it has long been accepted that elevated CRP marks the presence of disease. If in certain cases elevated CRP worsens disease, then lowering CRP levels might lead to improvement. CRP lowering can be achieved by lifestyle changes (94) or by targeting inflammatory cytokines [e.g. see (28, 95)], but until recently there was no way to directly and selectively target CRP. This was the major impediment to clinical trials designed to directly test the possible benefits of CRP lowering. Over the last decades several groups have developed approaches to overcome this nagging problem. For example Pepys' group developed a small molecule inhibitor of CRP (1,6-bis(phosphocholine)-hexane) that they tested in a preclinical rat model (96). The compound works by crosslinking two CRP molecules, thereby blocking its ability to bind endogenous ligands while increasing its clearance from the blood. In rats that had received injections of human CRP prior to ligation of their coronary arteries, the signs of subsequent MI were worsened, indicating that human CRP exacerbates MI. Co-administration of human CRP plus 1,6-bis(phosphocholine)-hexane ablated the exacerbating influence of human CRP (96). This study underscored for the first time the promise of inhibiting CRP as a new approach for cardio-protection in acute MI. Our own group also recently tested a different

method of CRP lowering that relies on an antisense oligonucleotide (ASO) (3). ASOs have been shown to be highly effective at promoting the selective degradation of their target mRNAs and to have minimal off-target effects, and several ASOs have already been approved by the United States Food and Drug Administration for use in a variety of disease settings (97, 98). Since CRP is synthesized in the liver and ASOs have a propensity to accumulate in the liver (99), we designed ASOs to selectively target *CRP* mRNA and thereby efficiently reduce blood CRP. We showed that a human *CRP*-specific ASO was effective at lowering baseline CRP and was well-tolerated in healthy volunteers (100), and subsequently others showed that a human *CRP*-specific ASO attenuated CRP elevation (up to 69% compared to placebo) in humans challenged with endotoxin (101). Using species-specific *CRP*-targeting ASOs we showed the approach was also efficacious in rats and mice subjected to experimentally induced MI (89, 90, 102). Specifically, the rat *CRP*-specific ASO achieved a >60% reduction of rat blood CRP levels and improved their heart function and pathology following MI, and treating human CRP transgenic mice with a human *CRP*-specific ASO reduced blood human CRP by >70%. A third approach to CRP lowering is selective apheresis, which has recently been tested with some success in a small number of patients suffering from MI (103, 104) and with mixed results in patients with COVID-19 (105).

Blood C-Reactive Protein Lowering as a Therapeutic for Other Diseases?

Using our human CRP transgenic (CRPtg) and CRP knockout (CRP^{-/-}) mice we have investigated the contribution of CRP in a

wide variety of other diseases and, as in the case of CVD, have often observed a beneficial effect of CRP lowering. For example, in mice subjected to surgery-induced bilateral renal ischemia/reperfusion (I/R) injury, a procedure that faithfully mimics acute kidney injury (AKI) in humans receiving kidney transplants, we showed that CRP contributes to the pathogenesis of AKI. Essentially compared to wild type mice, CRPtg had worse outcomes after renal I/R whereas CRP^{-/-} were relatively resistant (106). Following I/R surgery CRPtg showed more disruption of their renal tubules and they had increased urine albumin and serum creatinine compared to wild type. To our surprise these exacerbating effects of CRP were accompanied by increased renal infiltration of myeloid derived cells with suppressor functions (MDSCs; primarily polymorphonuclear PMN-MDSCs) (106, 107). In contrast in the kidneys of CRP^{-/-} that had undergone renal I/R only a few PMN-MDSCs were found. In other experiments we established that CRP can selectively promote the expansion of MDSCs from mouse bone marrow progenitors and increase their suppressive function (i.e. their ability to inhibit T cell proliferation) (31). We conducted other experiments and found that MDSCs directly impact primary renal tubular epithelial cells (RTEC), i.e. in co-cultures MDSCs impaired the ability of RTECs to cycle through S-phase even in the absence of cell-cell contact (unpublished data). The combined findings suggest that CRP potentiates the expansion of MDSCs during AKI, likely by selectively promoting their expansion from bone marrow progenitors. By suppressing cell cycling, the kidney infiltrating MDSCs initiate a deleterious progression of events: the impairment of RTEC proliferation consequently impedes RTEC recovery and thus the restoration of normal tubular architecture, ultimately setting the stage for maladaptive repair and chronic kidney disease. Importantly, we also showed that treating CRPtg mice with the human CRP-specific ASO prior to I/R surgery lowered CRP, drastically reduced renal MDSC infiltration, and alleviated AKI (107). Subsequently we established that CRP also enables human neutrophils to manifest T cell suppressive actions (31). Based on these translational findings we think it is likely that therapeutic lowering of CRP might be of benefit in recipients of kidney transplants. We also tested the influence of CRP lowering therapy in the context of an animal model of rheumatoid arthritis (RA), i.e. collagen-induced arthritis (CIA). Thus Jones et al. (108) showed that development of CIA is delayed in CRPtg and accelerated in CRP^{-/-}, suggesting that during onset and development of disease CRP plays a protective role. On the other hand, when CRPtg with established CIA (clinical score of ≥ 2) were treated with the human CRP-specific ASO they showed less inflammation and improvement of CIA symptoms (100). The protective effect of CRP during onset of disease might reflect the ability of CRP to impair dendritic cell functions while at the same time promote MDSC suppressive activity (30, 31), culminating in a delayed autoimmune response. Conversely, the detrimental effect of CRP seen during active CIA is likely because of its complement activating potential (1). These observations underscore that if CRP lowering is used as a therapy, the timing of CRP lowering will likely be of

paramount importance. Indeed this 'timing effect' may be the reason why, despite effective CRP lowering, no improvement in symptoms was observed in patients with active RA treated with a CRP-specific ASO (109).

Something to Think About: Blood C-Reactive Protein Lowering in Cancer?

Despite the success of immunotherapies (e.g. checkpoint inhibitors) to treat solid tumors, there still is a significant fraction of cancer patients that experience no benefit or only a short remission after immunotherapy. Immunotherapy is based on a two pronged approach: 1) boosting the endogenous T cell anti-tumor response to overcome antigen escape and T cell exhaustion (110) and 2) modulating intra-tumoral MDSCs (111). Population studies have established that high CRP levels associate with increased cancer risk (112–114), increased cancer progression (8), and increased cancer mortality (115, 116), and in many cases CRP has been shown to be an independent prognostic factor for cancer (117–122). Given our finding that CRP reprograms myeloid cells we therefore think that cancer might represent a promising opportunity for beneficial CRP lowering, i.e. reducing blood CRP levels should result in fewer MDSCs and a less immunosuppressive tumor microenvironment, thereby potentiating anti-tumor T cell responses. As discussed earlier, our data showed that CRP is an enhancer of MDSC generation and suppressive function and CRP can also inhibit dendritic cell function; therefore we predict that compared to wild type mice, CRPtg should have more MDSCs and more tumor burden while the inverse should be seen in CRP^{-/-} mice. Indeed in pilot studies (unpublished data) using an E0771 orthotopic breast cancer model, we observed CRP^{-/-} had lower tumor burden and lower frequencies of tumor- and spleen-infiltrating MDSCs compared to CRPtg and wild type. These preliminary observations hint at the possibility that CRP contributes to MDSC generation and their eventual infiltration into tumors. Yet to be explored is whether CRP within the tumor microenvironment impedes the anti-tumor response directly (i.e. by inhibiting T cells per se) or indirectly (i.e. by promoting MDSCs). However, based on our previous observations that the CRP ASO decreased MDSC infiltration into I/R injured kidneys and that tumor challenged CRP^{-/-} mice had decreased intra-tumor MDSCs, we predict that a CRP-specific ASO should decrease CRP-driven MDSC infiltration into tumors. Targeted lowering of CRP should simultaneously allow for the maturation of tumor-reactive dendritic cells, which in turn would stimulate tumor-reactive T cell responses.

SUMMARY

Since its discovery nearly a century ago much has been explored and written about CRP's association with disease, and consequently CRP's role as a marker of inflammation is solidly established. That being said, CRP is more than just a marker of inflammation, i.e. it did not evolve nor was it maintained by natural selection to provide a faithful indication that something

is amiss with a patient. In the last decades evidence has steadily been building that CRP contributes to the maintenance of health and the propagation of some diseases, and for the first time specific CRP lowering is achievable. Therefore the time has come we think, for the biological activities of CRP and therapeutic lowering of CRP to be brought to the forefront in the clinical care setting. Like any therapy CRP lowering might have some untoward effects (e.g. it might increase the risk of infection),

nevertheless the time for a placebo-controlled double-blind case-controlled clinical trial of CRP lowering therapy may have finally arrived.

AUTHOR CONTRIBUTIONS

RJ and AS contributed equally to this work. All authors contributed to the article and approved the submitted version.

REFERENCES

- Volanakis JE. Human C-reactive protein: expression, structure, and function. *Mol Immunol* (2001) 38:189–97. doi: 10.1016/S0161-5890(01)00042-6
- Szalai AJ. The biological functions of C-reactive protein. *Vascu Pharmacol* (2002) 39(3):105–7. doi: 10.1016/s1537-1891(02)00294-x
- Szalai AJ, McCrory MA. Varied biologic functions of C-reactive protein: lessons learned from transgenic mice. *Immunol Res* (2002) 26(1-3):279–87. doi: 10.1385/IR.26:1-3:279
- Kushner I, Rzewnicki D, Samols D. What does minor elevation of C-reactive protein signify? *Am J Med* (2006) 119:166(2):e117–28. doi: 10.1016/j.amjmed.2005.06.057
- Lowe GD, Pepys MB. C-reactive protein and cardiovascular disease: weighing the evidence. *Curr Atheroscler Rep* (2006) 8(5):421–8. doi: 10.1007/s11883-006-0040-x
- Hage FG, Szalai AJ. C-reactive protein gene polymorphisms, C-reactive protein blood levels, and cardiovascular disease risk. *J Am Coll Cardiol* (2007) 50(12):1115–22. doi: 10.1016/j.jacc.2007.06.012
- Casas JP, Shah T, Hingorani AD, Danesh J, Pepys MB. C-reactive protein and coronary heart disease: a critical review. *J Intern Med* (2008) 264(4):295–314. doi: 10.1111/j.1365-2796.2008.02015.x
- Allin KH, Nordestgaard BG. Elevated C-reactive protein in the diagnosis, prognosis, and cause of cancer. *Crit Rev Clin Lab Sci* (2011) 48(4):155–70. doi: 10.3109/10408363.2011.599831
- Agrawal A, Gang TB, Rusinol AE. Recognition functions of pentameric C-reactive protein in cardiovascular disease. *Mediators Inflammation* (2014) 2014:319215. doi: 10.1155/2014/319215
- Lu J, Mold C, Du Clos TW, Sun PD. Pentraxins and Fc Receptor-Mediated Immune Responses. *Front Immunol* (2018) 9:2607. doi: 10.3389/fimmu.2018.02607
- Pepys MB. The Pentraxins 1975–2018: Serendipity, Diagnostics and Drugs. *Front Immunol* (2018) 9:2382. doi: 10.3389/fimmu.2018.02382
- Pathak A, Agrawal A. Evolution of C-Reactive Protein. *Front Immunol* (2019) 10:943. doi: 10.3389/fimmu.2019.00943
- Tillett WS, Francis T. Serological Reactions in Pneumonia with a Non-Protein Somatic Fraction of Pneumococcus. *J Exp Med* (1930) 52(4):561–71. doi: 10.1084/jem.52.4.561
- Abernethy TJ, Avery OT. The occurrence during acute infections of a protein not normally present in the blood: I. Distribution of the reactive protein in patients' sera and the effect of calcium on the flocculation reaction with C polysaccharide of pneumococcus. *J Exp Med* (1941) 73(2):173–82. doi: 10.1084/jem.73.2.173
- Macleod CM, Avery OT. The Occurrence during Acute Infections of a Protein Not Normally Present in the Blood: II. Isolation and Properties of the Reactive Protein. *J Exp Med* (1941) 73(2):183–90. doi: 10.1084/jem.73.2.183
- Macleod CM, Avery OT. The Occurrence during Acute Infections of a Protein Not Normally Present in the Blood: III. Immunological Properties of the C-Reactive Protein and Its Differentiation from Normal Blood Proteins. *J Exp Med* (1941) 73(2):191–200. doi: 10.1084/jem.73.2.191
- Kindmark CO. The concentration of C-reactive protein in sera from healthy individuals. *Scand J Clin Lab Invest* (1972) 29(4):407–11. doi: 10.3109/00365517209080258
- Claus DR, Osmand AP, Gewurz H. Radioimmunoassay of human C-reactive protein and levels in normal sera. *J Lab Clin Med* (1976) 87(1):120–8. doi: 10.5555/uri:pii:0022214376903371
- Siboo R, Kulisek E. A fluorescent immunoassay for the quantification of C-reactive protein. *J Immunol Methods* (1978) 23(1-2):59–67. doi: 10.1016/0022-1759(78)90109-6
- Shine B, de Beer FC, Pepys MB. Solid phase radioimmunoassays for human C-reactive protein. *Clin Chim Acta* (1981) 117(1):13–23. doi: 10.1016/0009-8981(81)90005-x
- WHO Expert Committee on Biological Standardization. World Health Organization Technical Report Series. In: *World Health Organization Technical Report Series*, 37 ed. Geneva, Switzerland: World Health Organization (1987).
- Hutchinson WL, Koenig W, Frohlich M, Sund M, Lowe GD, Pepys MB. Immunoradiometric assay of circulating C-reactive protein: age-related values in the adult general population. *Clin Chem* (2000) 46(7):934–8. doi: 10.1093/clinchem/46.7.934
- Greenfield JR, Samaras K, Jenkins AB, Kelly PJ, Spector TD, Gallimore JR, et al. Obesity is an important determinant of baseline serum C-reactive protein concentration in monozygotic twins, independent of genetic influences. *Circulation* (2004) 109(24):3022–8. doi: 10.1161/01.CIR.0000130640.77501.79
- Szalai AJ, Wu J, Lange EM, McCrory MA, Langefeld CD, Williams A, et al. Single-nucleotide polymorphisms in the C-reactive protein (CRP) gene promoter that affect transcription factor binding, alter transcriptional activity, and associate with differences in baseline serum CRP level. *J Mol Med (Berl)* (2005) 83(6):440–7. doi: 10.1007/s00109-005-0658-0
- Woloshin S, Schwartz LM. Distribution of C-reactive protein values in the United States. *N Engl J Med* (2005) 352(15):1611–3. doi: 10.1056/NEJM200504143521525
- Pearson TA, Mensah GA, Hong Y, Smith SC Jr, CDC, and AHA. CDC/AHA Workshop on Markers of Inflammation and Cardiovascular Disease: Application to Clinical and Public Health Practice: overview. *Circulation* (2004) 110(25):e543–44. doi: 10.1161/01.cir.0000148979.11121.6b
- Ridker PM, Danielson E, Fonseca FA, Genest J, Gotto AM Jr, Kastelein JJ, et al. Rosuvastatin to prevent vascular events in men and women with elevated C-reactive protein. *N Engl J Med* (2008) 359(21):2195–207. doi: 10.1056/NEJMoa0807646
- Ridker PM, Everett BM, Thuren T, MacFadyen JG, Chang WH, Ballantyne C, et al. Antiinflammatory Therapy with Canakinumab for Atherosclerotic Disease. *N Engl J Med* (2017) 377(12):1119–31. doi: 10.1056/NEJMoa1707914
- Luo X, Zhou W, Yan X, Guo T, Wang B, Xia H, et al. Prognostic value of C-reactive protein in patients with COVID-19. *Clin Infect Dis* (2020) 71(16):2174–9. doi: 10.1093/cid/ciaa641
- Jimenez RV, Wright TT, Jones NR, Wu J, Gibson AW, Szalai AJ. C-Reactive Protein Impairs Dendritic Cell Development, Maturation, and Function: Implications for Peripheral Tolerance. *Front Immunol* (2018) 9:372. doi: 10.3389/fimmu.2018.00372
- Jimenez RV, Kuznetsova V, Connelly AN, Hel Z, Szalai AJ. C-Reactive Protein Promotes the Expansion of Myeloid Derived Cells With Suppressor Functions. *Front Immunol* (2019) 10:2183. doi: 10.3389/fimmu.2019.02183
- Perlman E, Bullowa JG, Goodkind R. An Immunological and Electrophoretic Comparison of the Antibody to C Polysaccharide and the C-Reactive Protein of Acute Phase Serum. *J Exp Med* (1943) 77(2):97–110. doi: 10.1084/jem.77.2.97

33. Hedlund P, Frisk AR, Bucht H. The appearance of acute phase protein after induced fever in man. *Acta Med Scand* (1948) 131(5):417–21. doi: 10.1111/j.0954-6820.1948.tb04533.x
34. Osmand AP, Friedenson B, Gewurz H, Painter RH, Hofmann T, Shelton E. Characterization of C-reactive protein and the complement subcomponent C1t as homologous proteins displaying cyclic pentameric symmetry (pentraxins). *Proc Natl Acad Sci USA* (1977) 74(2):739–43. doi: 10.1073/pnas.74.2.739
35. Thompson D, Pepys MB, Wood SP. The physiological structure of human C-reactive protein and its complex with phosphocholine. *Structure* (1999) 7(2):169–77. doi: 10.1016/S0969-2126(99)80023-9
36. Thompson D, Pepys MB, Wood SP. 1B09. PDB. (2011). doi: 10.2210/pdb1B09/pdb
37. Rose AS, Bradley AR, Valasatava Y, Duarte JM, Prlic A, Rose PW. NGL viewer: web-based molecular graphics for large complexes. *Bioinformatics* (2018) 34(21):3755–8. doi: 10.1093/bioinformatics/bty419
38. Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, et al. The Protein Data Bank. *Nucleic Acids Res* (2000) 28(1):235–42. doi: 10.1093/nar/28.1.235
39. Berman H, Henrick K, Nakamura H. Announcing the worldwide Protein Data Bank. *Nat Struct Biol* (2003) 10(12):980. doi: 10.1038/nsb1203-980
40. Burley SK, Berman HM, Bhikadiya C, Bi C, Chen L, Di Costanzo L, et al. RCSB Protein Data Bank: biological macromolecular structures enabling research and education in fundamental biology, biomedicine, biotechnology and energy. *Nucleic Acids Res* (2019) 47(D1):D464–74. doi: 10.1093/nar/gky1004
41. Shrive AK, Cheetham GM, Holden D, Myles DA, Turnell WG, Volanakis JE, et al. Three dimensional structure of human C-reactive protein. *Nat Struct Biol* (1996) 3(4):346–54. doi: 10.1038/nsb0496-346
42. Gotschlich EC, Edelman GM. Binding properties and specificity of C-reactive protein. *Proc Natl Acad Sci USA* (1967) 57(3):706–12. doi: 10.1073/pnas.57.3.706
43. Kindmark CO. Stimulating effect of C-reactive protein on phagocytosis of various species of pathogenic bacteria. *Clin Exp Immunol* (1971) 8(6):941–8.
44. Anderson J, Stroud R, Volanakis J. “Studies on binding specificity of human C-reactive protein for phosphorylcholine”. *Fed Proceed: Fed Amer Soc Exp Biol* (1978) 9650(20814-3998):1495.
45. Gershov D, Kim S, Brot N, Elkon KB. C-Reactive protein binds to apoptotic cells, protects the cells from assembly of the terminal complement components, and sustains an antiinflammatory innate immune response: implications for systemic autoimmunity. *J Exp Med* (2000) 192(9):1353–64. doi: 10.1084/jem.192.9.1353
46. Du Clos TW. C-reactive protein reacts with the U1 small nuclear ribonucleoprotein. *J Immunol* (1989) 143(8):2553–9.
47. Pepys MB, Booth SE, Tennent GA, Butler PJ, Williams DG. Binding of pentraxins to different nuclear structures: C-reactive protein binds to small nuclear ribonucleoprotein particles, serum amyloid P component binds to chromatin and nucleoli. *Clin Exp Immunol* (1994) 97(1):152–7. doi: 10.1111/j.1365-2249.1994.tb06594.x
48. Du Clos TW. The interaction of C-reactive protein and serum amyloid P component with nuclear antigens. *Mol Biol Rep* (1996) 23(3-4):253–60. doi: 10.1007/BF00351177
49. Fujita Y, Kakino A, Nishimichi N, Yamaguchi S, Sato Y, Machida S, et al. Oxidized LDL receptor LOX-1 binds to C-reactive protein and mediates its vascular effects. *Clin Chem* (2009) 55(2):285–94. doi: 10.1373/clinchem.2008.119750
50. Wu Y, Potempa LA, El Kebir D, Filep JG. C-reactive protein and inflammation: conformational changes affect function. *Biol Chem* (2015) 396(11):1181–97. doi: 10.1515/hsz-2015-0149
51. Nehring SM, Goyal A, Bansal P, Patel BC. “C Reactive Protein (CRP)”. In: *StatPearls*. Treasure Island (FL: StatPearls Publishing LLC (2020).
52. Hutchinson WL, Coll AP, Gallimore JR, Tennent GA, Pepys MB. Is leptin an important physiological regulator of CRP? *Nat Med* (2007) 13(1):17–18; author reply 19–21. doi: 10.1038/nm0107-17
53. Muschel LH, Weatherwax RJ. Complement fixation in the C-reactive protein system. *Proc Soc Exp Biol Med* (1954) 87(1):191–3. doi: 10.3181/00379727-87-21330
54. Kaplan MH, Volanakis JE. Interaction of C-reactive protein complexes with the complement system. I. Consumption of human complement associated with the reaction of C-reactive protein with pneumococcal C-polysaccharide and with the choline phosphatides, lecithin and sphingomyelin. *J Immunol* (1974) 112(6):2135–47.
55. Volanakis JE, Kaplan MH. Interaction of C-reactive protein complexes with the complement system. II. Consumption of guinea pig complement by CRP complexes: requirement for human C1q. *J Immunol* (1974) 113(1):9–17.
56. Shih HH, Zhang S, Cao W, Hahn A, Wang J, Paulsen JE, et al. CRP is a novel ligand for the oxidized LDL receptor LOX-1. *Am J Physiol Heart Circ Physiol* (2009) 296(5):H1643–50. doi: 10.1152/ajpheart.00938.2008
57. Mold C, Nakayama S, Holzer TJ, Gewurz H, Du Clos TW. C-reactive protein is protective against *Streptococcus pneumoniae* infection in mice. *J Exp Med* (1981) 154(5):1703–8. doi: 10.1084/jem.154.5.1703
58. Yother J, Volanakis JE, Briles DE. Human C-reactive protein is protective against fatal *Streptococcus pneumoniae* infection in mice. *J Immunol* (1982) 128(5):2374–6.
59. Horowitz J, Volanakis JE, Briles DE. Blood clearance of *Streptococcus pneumoniae* by C-reactive protein. *J Immunol* (1987) 138(8):2598–603.
60. Szalai AJ, Briles DE, Volanakis JE. Human C-reactive protein is protective against fatal *Streptococcus pneumoniae* infection in transgenic mice. *J Immunol* (1995) 155(5):2557–63.
61. Szalai AJ. The antimicrobial activity of C-reactive protein. *Microbes Infect* (2002) 4(2):201–5. doi: 10.1016/s1286-4579(01)01528-3
62. Hurlimann J, Thorbecke GJ, Hochwald GM. The liver as the site of C-reactive protein formation. *J Exp Med* (1966) 123(2):365–78. doi: 10.1084/jem.123.2.365
63. Kushner I, Feldmann G. Control of the acute phase response. Demonstration of C-reactive protein synthesis and secretion by hepatocytes during acute inflammation in the rabbit. *J Exp Med* (1978) 148(2):466–77. doi: 10.1084/jem.148.2.466
64. Whitehead AS, Bruns GA, Markham AF, Colten HR, Woods DE. Isolation of human C-reactive protein complementary DNA and localization of the gene to chromosome 1. *Science* (1983) 221(4605):69–71. doi: 10.1126/science.6857266
65. Macintyre S, Samols D, Dailey P. Two carboxylesterases bind C-reactive protein within the endoplasmic reticulum and regulate its secretion during the acute phase response. *J Biol Chem* (1994) 269(39):24496–503. doi: 10.1016/S0021-9258(19)51111-5
66. Yue CC, Muller-Greven J, Dailey P, Lozanski G, Anderson V, Macintyre S. Identification of a C-reactive protein binding site in two hepatic carboxylesterases capable of retaining C-reactive protein within the endoplasmic reticulum. *J Biol Chem* (1996) 271(36):22245–50. doi: 10.1074/jbc.271.36.22245
67. Toniatti C, Arcone R, Majello B, Ganter U, Arpaia G, Ciliberto G. Regulation of the human C-reactive protein gene, a major marker of inflammation and cancer. *Mol Biol Med* (1990) 7(3):199–212.
68. Majello B, Arcone R, Toniatti C, Ciliberto G. Constitutive and IL-6-induced nuclear factors that interact with the human C-reactive protein promoter. *EMBO J* (1990) 9(2):457–65. doi: 10.1002/j.1460-2075.1990.tb08131.x
69. Toniatti C, Demartis A, Monaci P, Nicosia A, Ciliberto G. Synergistic trans-activation of the human C-reactive protein promoter by transcription factor HNF-1 binding at two distinct sites. *EMBO J* (1990) 9(13):4467–75. doi: 10.1002/j.1460-2075.1990.tb07897.x
70. Ramji DP, Vitelli A, Tronche F, Cortese R, Ciliberto G. The two C/EBP isoforms, IL-6DBP/NF-IL6 and C/EBP delta/NF-IL6 beta, are induced by IL-6 to promote acute phase gene transcription via different mechanisms. *Nucleic Acids Res* (1993) 21(2):289–94. doi: 10.1093/nar/21.2.289
71. Zhang D, Sun M, Samols D, Kushner I. STAT3 participates in transcriptional activation of the C-reactive protein gene by interleukin-6. *J Biol Chem* (1996) 271(16):9503–9. doi: 10.1074/jbc.271.16.9503
72. Ochriator JD, Harrison KA, Zahedi K, Mortensen RF. Role of STAT3 and C/EBP in cytokine-dependent expression of the mouse serum amyloid P-component (SAP) and C-reactive protein (CRP) genes. *Cytokine* (2000) 12:888–99. doi: 10.1006/cyto.2000.0668
73. Nishikawa T, Hagihara K, Serada S, Isobe T, Matsumura A, Song J, et al. Transcriptional complex formation of c-Fos, STAT3, and hepatocyte NF-1 alpha is essential for cytokine-driven C-reactive protein gene expression. *J Immunol* (2008) 180(5):3492–501. doi: 10.4049/jimmunol.180.5.3492
74. Ray S, Lee C, Hou T, Bhakat KK, Brasier AR. Regulation of signal transducer and activator of transcription 3 enhancosome formation by apurinic/

- aprimidinic endonuclease 1 in hepatic acute phase response. *Mol Endocrinol* (2010) 24(2):391–401. doi: 10.1210/me.2009-0319
75. Doumatey AP, Chen G, Tekola Ayele F, Zhou J, Erdos M, Shriner D, et al. C-reactive protein (CRP) promoter polymorphisms influence circulating CRP levels in a genome-wide association study of African Americans. *Hum Mol Genet* (2012) 21(13):3063–72. doi: 10.1093/hmg/dd133
 76. Kim Y, Noren Hooten N, Dluzen DF, Martindale JL, Gorospe M, Evans MK. Posttranscriptional Regulation of the Inflammatory Marker C-Reactive Protein by the RNA-Binding Protein HuR and MicroRNA 637. *Mol Cell Biol* (2015) 35(24):4212–21. doi: 10.1128/MCB.00645-15
 77. Nissen SE, Tuzcu EM, Schoenhagen P, Crowe T, Sasiela WJ, Tsai J, et al. Statin therapy, LDL cholesterol, C-reactive protein, and coronary artery disease. *N Engl J Med* (2005) 352(1):29–38. doi: 10.1056/NEJMoa042000
 78. Ridker PM, Cannon CP, Morrow D, Rifai N, Rose LM, McCabe CH, et al. C-reactive protein levels and outcomes after statin therapy. *N Engl J Med* (2005) 352(1):20–8. doi: 10.1056/NEJMoa042378
 79. Puri R, Nissen SE, Libby P, Shao M, Ballantyne CM, Barter PJ, et al. C-reactive protein, but not low-density lipoprotein cholesterol levels, associate with coronary atheroma regression and cardiovascular events after maximally intensive statin therapy. *Circulation* (2013) 128(22):2395–403. doi: 10.1161/CIRCULATIONAHA.113.004243
 80. Torzewski J, Torzewski M, Bowyer DE, Frohlich M, Koenig W, Waltenberger J, et al. C-reactive protein frequently localizes with the terminal complement complex in the intima of early atherosclerotic lesions of human coronary arteries. *Arterioscler Thromb Vasc Biol* (1998) 18(9):1386–92. doi: 10.1161/01.atv.18.9.1386
 81. Bhakdi S, Torzewski M, Klouche M, Hemmes M. Complement and atherogenesis: binding of CRP to degraded, nonoxidized LDL enhances complement activation. *Arterioscler Thromb Vasc Biol* (1999) 19(10):2348–54. doi: 10.1161/01.atv.19.10.2348
 82. Sun H, Koike T, Ichikawa T, Hatakeyama K, Shiomi M, Zhang B, et al. C-reactive protein in atherosclerotic lesions: its origin and pathophysiological significance. *Am J Pathol* (2005) 167(4):1139–48. doi: 10.1016/S0002-9440(10)61202-3
 83. Ji SR, Wu Y, Potempa LA, Liang YH, Zhao J. Effect of modified C-reactive protein on complement activation: a possible complement regulatory role of modified or monomeric C-reactive protein in atherosclerotic lesions. *Arterioscler Thromb Vasc Biol* (2006) 26(4):935–41. doi: 10.1161/01.ATV.0000206211.21895.73
 84. Meuwissen M, van der Wal AC, Niessen HW, Koch KT, de Winter RJ, van der Loos CM, et al. Colocalisation of intraplaque C reactive protein, complement, oxidised low density lipoprotein, and macrophages in stable and unstable angina and acute myocardial infarction. *J Clin Pathol* (2006) 59(2):196–201. doi: 10.1136/jcp.2005.027235
 85. Wolbink GJ, Brouwer MC, Buysmann S, ten Berge IJ, Hack CE. CRP-mediated activation of complement in vivo: assessment by measuring circulating complement-C-reactive protein complexes. *J Immunol* (1996) 157(1):473–9.
 86. Verma S, Devaraj S, Jialal I. Is C-reactive protein an innocent bystander or proatherogenic culprit? C-reactive protein promotes atherothrombosis. *Circulation* (2006) 113(17):2151. doi: 10.1161/CIRCULATIONAHA.105.611350
 87. Danenberg HD, Szalai AJ, Swaminathan RV, Peng L, Chen Z, Seifert P, et al. Increased thrombosis after arterial injury in human C-reactive protein-transgenic mice. *Circulation* (2003) 108(5):512–5. doi: 10.1161/01.CIR.0000085568.13915.1E
 88. Paul A, Ko KWS, Li L, Yechoor V, McCrory MA, Szalai AJ, et al. C-reactive protein accelerates the progression of atherosclerosis in apolipoprotein E-deficient mice. *Circulation* (2004) 109:647–55. doi: 10.1161/01.CIR.0000114526.50618.24
 89. Wang D, Oparil S, Chen YF, McCrory MA, Skibinski GA, Feng W, et al. Estrogen treatment abrogates neointima formation in human C-reactive protein transgenic mice. *Arterioscler Thromb Vasc Biol* (2005) 25(10):2094–9. doi: 10.1161/01.ATV.0000179602.85797.3f
 90. Xing D, Hage FG, Chen Y-F, McCrory MA, Feng W, Skibinski GA, et al. Exaggerated neointima formation in human C-reactive protein transgenic mice is IgG Fc receptor type I (FcγRI)-dependent. *Am J Pathol* (2008) 172:22–30. doi: 10.2353/ajpath.2008.070154
 91. Hirschfield GM, Gallimore JR, Kahan MC, Hutchinson WL, Sabin CA, Benson GM, et al. Transgenic human C-reactive protein is not proatherogenic in apolipoprotein E-deficient mice. *Proc Natl Acad Sci USA* (2005) 102(23):8309–14. doi: 10.1073/pnas.0503202102
 92. Tennent GA, Hutchinson WL, Kahan MC, Hirschfield GM, Gallimore JR, Lewin J, et al. Transgenic human CRP is not pro-atherogenic, pro-atherothrombotic or pro-inflammatory in apoE-/- mice. *Atherosclerosis* (2008) 196(1):248–55. doi: 10.1016/j.atherosclerosis.2007.05.010
 93. Hingorani AD, Shah T, Casas JP. Linking observational and genetic approaches to determine the role of C-reactive protein in heart disease risk. *Eur Heart J* (2006) 27(11):1261–3. doi: 10.1093/eurheartj/ehi852
 94. Jae SY, Fernhall B, Heffernan KS, Jeong M, Chun EM, Sung J, et al. Effects of lifestyle modifications on C-reactive protein: contribution of weight loss and improved aerobic capacity. *Metabolism* (2006) 55(6):825–31. doi: 10.1016/j.metabol.2006.02.010
 95. Ridker PM, MacFadyen JG, Everett BM, Libby P, Thuren T, Glynn RJ, et al. Relationship of C-reactive protein reduction to cardiovascular event reduction following treatment with canakinumab: a secondary analysis from the CANTOS randomised controlled trial. *Lancet* (2018) 391(10118):319–28. doi: 10.1016/s0140-6736(17)32814-3
 96. Pepys MB, Hirschfield GM, Tennent GA, Gallimore JR, Kahan MC, Bellotti V, et al. Targeting C-reactive protein for the treatment of cardiovascular disease. *Nature* (2006) 440(7088):1217–21. doi: 10.1038/nature04672
 97. de la Torre BG, Albericio F. The Pharmaceutical Industry in 2019. An Analysis of FDA Drug Approvals from the Perspective of Molecules. *Molecules* (2020) 25(3):745. doi: 10.3390/molecules25030745
 98. Ruger J, Ioannou S, Castanotto D, Stein CA. Oligonucleotides to the (Gene) Rescue: FDA Approvals 2017–2019. *Trends Pharmacol Sci* (2020) 41(1):27–41. doi: 10.1016/j.tips.2019.10.009
 99. Dhuri K, Bechtold C, Quijano E, Pham H, Gupta A, Vikram A, et al. Antisense Oligonucleotides: An Emerging Area in Drug Discovery and Development. *J Clin Med* (2020) 9(6). doi: 10.3390/jcm9062004
 100. Jones NR, Pegues MA, McCrory MA, Singleton W, Bethune C, Baker BF, et al. A selective inhibitor of human C-reactive protein translation is efficacious in vitro and in C-reactive protein transgenic mice and humans. *Mol Ther Nucleic Acids* (2012) 1:e52. doi: 10.1038/mtna.2012.44
 101. Noveck R, Stroes ES, Flaim JD, Baker BF, Hughes S, Graham MJ, et al. Effects of an antisense oligonucleotide inhibitor of C-reactive protein synthesis on the endotoxin challenge response in healthy human male volunteers. *J Am Heart Assoc* (2014) 3(4):1–11. doi: 10.1161/JAHA.114.001084
 102. Szalai AJ, McCrory MA, Xing D, Hage FG, Miller A, Oparil S, et al. Inhibiting C-reactive protein for the treatment of cardiovascular disease: promising evidence from rodent models. *Mediators Inflammation* (2014) 2014:353614. doi: 10.1155/2014/353614
 103. Mattecka S, Brunner P, Hahnel B, Kunze R, Vogt B, Sheriff A. PentraSorb C-Reactive Protein: Characterization of the Selective C-Reactive Protein Adsorber Resin. *Ther Apher Dial* (2019) 23(5):474–81. doi: 10.1111/1744-9987.12796
 104. Ries W, Heigl F, Garlich C, Sheriff A, Torzewski J. Selective C-reactive protein-apheresis in patients. *Ther Apher Dial* (2019) 23(6):570–4. doi: 10.1111/1744-9987.12804
 105. Torzewski J, Heigl F, Zimmermann O, Wagner F, Schumann C, Hettich R, et al. First-in-Man: Case Report of Selective C-Reactive Protein Apheresis in a Patient with SARS-CoV-2 Infection. *Am J Case Rep* (2020) 21:e925020. doi: 10.12659/AJCR.925020
 106. Pegues MA, McCrory MA, Zarjou A, Szalai AJ. C-reactive protein exacerbates renal ischemia-reperfusion injury. *Am J Physiol Renal Physiol* (2013) 304(11):F1358–65. doi: 10.1152/ajprenal.00476.2012
 107. Pegues MA, McWilliams IL, Szalai AJ. C-reactive protein exacerbates renal ischemia-reperfusion injury: are myeloid-derived suppressor cells to blame? *Am J Physiol Renal Physiol* (2016) 311(1):F176–181. doi: 10.1152/ajprenal.00107.2016
 108. Jones NR, Pegues MA, McCrory MA, Kerr SW, Jiang H, Sellati R, et al. Collagen-induced arthritis is exacerbated in C-reactive protein-deficient mice. *Arthritis Rheumatism* (2011) 63(9):2641–50. doi: 10.1002/art.30444
 109. Warren MS, Hughes SG, Singleton W, Yamashita M, Genovese MC. Results of a proof of concept, double-blind, randomized trial of a second generation antisense oligonucleotide targeting high-sensitivity C-reactive protein (hs-CRP) in rheumatoid arthritis. *Arthritis Res Ther* (2015) 17:80. doi: 10.1186/s13075-015-0578-5
 110. Brown CE, Mackall CL. CAR T cell therapy: inroads to response and resistance. *Nat Rev Immunol* (2019) 19(2):73–4. doi: 10.1038/s41577-018-0119-y

111. Fleming V, Hu X, Weber R, Nagibin V, Groth C, Altevogt P, et al. Targeting Myeloid-Derived Suppressor Cells to Bypass Tumor-Induced Immunosuppression. *Front Immunol* (2018) 9:398. doi: 10.3389/fimmu.2018.00398
112. Erlinger TP, Platz EA, Rifai N, Helzlsouer KJ. C-reactive protein and the risk of incident colorectal cancer. *JAMA* (2004) 291(5):585–90. doi: 10.1001/jama.291.5.585
113. Siemes C, Visser LE, Coebergh JW, Splinter TA, Witteman JC, Uitterlinden AG, et al. C-reactive protein levels, variation in the C-reactive protein gene, and cancer risk: the Rotterdam Study. *J Clin Oncol* (2006) 24(33):5216–22. doi: 10.1200/JCO.2006.07.1381
114. Allin KH, Bojesen SE, Nordestgaard BG. Baseline C-reactive protein is associated with incident cancer and survival in patients with cancer. *J Clin Oncol* (2009) 27(13):2217–24. doi: 10.1200/JCO.2008.19.8440
115. Suh SY, Ahn HY. A prospective study on C-reactive protein as a prognostic factor for survival time of terminally ill cancer patients. *Support Care Cancer* (2007) 15(6):613. doi: 10.1007/s00520-006-0208-5
116. Ko YJ, Kwon YM, Kim KH, Choi HC, Chun SH, Yoon HJ, et al. High-sensitivity C-reactive protein levels and cancer mortality. *Cancer Epidemiol Biomarkers Prev* (2012) 21(11):2076–86. doi: 10.1158/1055-9965.EPI-12-0611
117. Chen Z, Sun Y, Wang J, Shen X, Chen L, Zhuang Z. Prognostic and predictive value of serum C-reactive protein levels in patients with metastatic or locally recurrent gastric cancer. *Int J Biol Markers* (2016) 31(3):e294–9. doi: 10.5301/jbm.5000210
118. Kostner AH, Kersten C, Lowenmark T, Ydsten KA, Peltonen R, Isoniemi H, et al. The prognostic role of systemic inflammation in patients undergoing resection of colorectal liver metastases: C-reactive protein (CRP) is a strong negative prognostic biomarker. *J Surg Oncol* (2016) 114(7):895–9. doi: 10.1002/jso.24415
119. Li ZS, Yao K, Li YH, Chen JP, Deng CZ, Zhao Q, et al. Clinical significance of preoperative C-reactive protein and squamous cell carcinoma antigen levels in patients with penile squamous cell carcinoma. *BJU Int* (2016) 118(2):272–8. doi: 10.1111/bju.13379
120. Thomsen M, Kersten C, Sorbye H, Skovlund E, Glimelius B, Pfeiffer P, et al. Interleukin-6 and C-reactive protein as prognostic biomarkers in metastatic colorectal cancer. *Oncotarget* (2016) 7(46):75013–22. doi: 10.18632/oncotarget.12601
121. Li C, Xu Q, Chen L, Luo C, Ying J, Liu J. C-reactive protein (CRP) as a prognostic factor for colorectal cancer after surgical resection of pulmonary metastases. *Bull Cancer* (2017) 104(3):232–6. doi: 10.1016/j.bulcan.2016.11.016
122. Huang W, Wu L, Liu X, Long H, Rong T, Ma G. Preoperative serum C-reactive protein levels and postoperative survival in patients with esophageal squamous cell carcinoma: a propensity score matching analysis. *J Cardiothorac Surg* (2019) 14(1):167. doi: 10.1186/s13019-019-0981-0

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Jimenez and Szalai. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Identification of Neuronal Pentraxins as Synaptic Binding Partners of C1q and the Involvement of NP1 in Synaptic Pruning in Adult Mice

Réka Á. Kovács¹, Henrietta Vadászi¹, Éva Bulyáki¹, György Török^{2,3}, Vilmos Tóth^{1,4}, Dominik Mátyás^{1,4}, Judit Kun¹, Éva Hunyadi-Gulyás⁵, Flóra Zsófia Fedor^{1,6}, Ádám Csincsi⁷, Katalin Medzihradszky⁵, László Homolya², Gábor Juhász^{1,4}, Katalin A. Kékesi^{1,4,8}, Mihály Józsi⁷, Balázs A. Györfy^{1,4} and József Kardos^{1*}

¹ ELTE NAP Neuroimmunology Research Group, Department of Biochemistry, Institute of Biology, ELTE Eötvös Loránd University, Budapest, Hungary, ² Molecular Cell Biology Research Group, Institute of Enzymology, Research Center for Natural Sciences, Hungarian Academy of Sciences Centre of Excellence, Budapest, Hungary, ³ Department of Biophysics and Radiation Biology, Semmelweis University, Budapest, Hungary, ⁴ Laboratory of Proteomics, Institute of Biology, ELTE Eötvös Loránd University, Budapest, Hungary, ⁵ Laboratory of Proteomics Research, Biological Research Centre, Eötvös Loránd Research Network (ELKH), Szeged, Hungary, ⁶ Doctoral School of Chemical Engineering and Material Sciences, Pannon University, Veszprém, Hungary, ⁷ Complement Research Group, Department of Immunology, ELTE Eötvös Loránd University, Budapest, Hungary, ⁸ Department of Physiology and Neurobiology, Institute of Biology, ELTE Eötvös Loránd University, Budapest, Hungary

OPEN ACCESS

Edited by:

Barbara Bottazzi,
University of Milan, Italy

Reviewed by:

Elisabetta Menna,
Italian National Research Council, Italy
Maria-Grazia De Simoni,
Mario Negri Pharmacological
Research Institute (IRCCS), Italy

*Correspondence:

József Kardos
kardos@elte.hu

Specialty section:

This article was submitted to
Molecular Innate Immunity,
a section of the journal
Frontiers in Immunology

Received: 28 August 2020

Accepted: 21 December 2020

Published: 08 February 2021

Citation:

Kovács RÁ, Vadászi H, Bulyáki É, Török G, Tóth V, Mátyás D, Kun J, Hunyadi-Gulyás É, Fedor FZ, Csincsi Á, Medzihradszky K, Homolya L, Juhász G, Kékesi KA, Józsi M, Györfy BA and Kardos J (2021) Identification of Neuronal Pentraxins as Synaptic Binding Partners of C1q and the Involvement of NP1 in Synaptic Pruning in Adult Mice. *Front. Immunol.* 11:599771. doi: 10.3389/fimmu.2020.599771

Elements of the immune system particularly that of innate immunity, play important roles beyond their traditional tasks in host defense, including manifold roles in the nervous system. Complement-mediated synaptic pruning is essential in the developing and healthy functioning brain and becomes aberrant in neurodegenerative disorders. C1q, component of the classical complement pathway, plays a central role in tagging synapses for elimination; however, the underlying molecular mechanisms and interaction partners are mostly unknown. Neuronal pentraxins (NPs) are involved in synapse formation and plasticity, moreover, NP1 contributes to cell death and neurodegeneration under adverse conditions. Here, we investigated the potential interaction between C1q and NPs, and its role in microglial phagocytosis of synapses in adult mice. We verified *in vitro* that NPs interact with C1q, as well as activate the complement system. Flow cytometry, immunostaining and co-immunoprecipitation showed that synapse-bound C1q colocalizes and interacts with NPs. High-resolution confocal microscopy revealed that microglia-surrounded C1q-tagged synapses are NP1 positive. We have also observed the synaptic occurrence of C4 suggesting that activation of the classical pathway cannot be ruled out in synaptic plasticity in healthy adult animals. In summary, our results indicate that NPs play a regulatory role in the synaptic function of C1q. Whether this role can be intensified upon pathological conditions, such as in Alzheimer's disease, is to be disclosed.

Keywords: synaptic pruning, neuronal pentraxin, complement component C1q, synaptosome, flow cytometry, immunostaining, complement classical pathway, microglial phagocytosis

INTRODUCTION

Complement components and pentraxins represent major humoral factors of the innate immune system, that often in collaboration play important roles in host defense, removal of dead cells, and several other physiological processes (1–4). In addition to acting systemically, complement and pentraxins are integral part of the neuroimmune axis and are involved in neurodegenerative diseases.

In the healthy brain, synaptic pruning is tightly regulated, primarily guided by an inherent developmental program and neuronal activity, persistently sculpting the global synaptic network suitable for optimal functioning. Maladaptive synaptic connectivity is usually a core component in the pathology of central nervous system (CNS) diseases, such as autism spectrum disorders (5) and schizophrenia (6). Moreover, excessive synapse loss is the hallmark of Alzheimer's disease and other dementias (7, 8). A set of delicately regulated molecular machinery has evolved, having neuronal or non-neuronal origin, to tailor proper synaptogenesis and synapse elimination. Among them, a continuously growing body of evidence raises the role of the local complement system in synaptic pruning (9). Secreted microglial complement components C1q and C3b are deposited onto synapses to be eliminated leading to their recognition and engulfment by surrounding microglia (10, 11) in a yet not fully understood manner. Disturbances in complement-dependent synaptic pruning are in a causal relationship with the development of neurodegenerative disorders, schizophrenia, and epilepsy (5–8, 12), and recently reviewed by Tenner et al. (13), pointing out that properly functioning complement-mediated synapse elimination is indispensable for normal CNS functions. Even in adults, synapses are dynamically formed and eliminated as part of synaptic plasticity and linked to memory and learning. It is known that synaptic turnover can occur at high rates in adulthood (14). The average lifetime of synapses show large variety (15–17); synapses are constantly renewed, and thus it is relevant to carry out studies on adult mice.

Very little is currently known about the interaction partners of the initial complement component C1q in the CNS, especially on the synaptic surface that might drive synapse recognition. Earlier, we have shown in adult mice that C1q-tagging of synapses is linked to local apoptotic-like processes such as phosphatidylserine externalization (18), which has been recently pointed out as a key factor in developmental synaptic pruning by microglia (19). Nevertheless, members of the family of neuronal pentraxins (NPs) are suggested to be potential binding partners of C1q (20) because they share substantial structural homology with acute-phase immune proteins and well-known C1q binding partners: pentraxin 3 (PTX3) from the long-pentraxin family (21), the short-pentraxin C-reactive protein and serum amyloid P component (22). Intriguingly, mice lacking NP expression show defective synaptic refinement in the developing visual system (23), which is reminiscent of the deficits in input segregation described in C1q knock-out mice (10). NPs comprising NP1 and NP2, together with the neuronal pentraxin receptor (NPR) are abundantly present in the synaptic

compartment and play a vital role in maintaining synaptic transmission and synaptic strength. Previous studies have established that NPs act *via* forming a large complex with each other and the AMPA-type glutamate receptors, thereby clustering on postsynaptic membranes of excitatory synapses (24, 25). Through their AMPA-receptor binding capabilities, NPs have emerged as potent regulators of excitatory synaptogenesis (26), functional synapse conversion (27), and synaptic plasticity (25, 28). Presynaptic release of NP2 from excitatory axon terminals is neuronal activity-dependent (29–31), and its secretion aids the homeostatic fine-tuning of synaptic transmission in local neuronal networks (32). On the other hand, it has been reported that NP1 acts in neuronal activity-independent manner, and its hetero-oligomers with NP2 exhibit higher clustering activity on AMPA-receptor than NP1 homo-oligomers (25). Apparently in contrast to its neuronal activity-independent synaptogenic roles, NP1 also contributes to neuronal cell death and neurodegeneration under adverse conditions in an activation dependent manner. During low neuronal activity, increased NP1 expression is evoked, triggering apoptotic neuronal cell death (33) mediated by glycogen synthase kinase-3 (Gsk3) activity (34), and involved in the mitochondrial accumulation of the pro-apoptotic BAX (35). Similarly, NP1 promotes neuronal cell death under hypoxic-ischemic conditions (36) and might be an important player in the pathomechanism of neurotoxic amyloid-beta-induced synapse loss, neurite damage, and cell death (37).

In sum, NPs are involved in synapse formation and plasticity; moreover, NP1 also serves as a neuronal mediator of harmful external stimuli directing affected neurons to apoptosis. In spite of the huge impact of both the complement and NPs on the synaptic network and their assumed binding capabilities to each other, it remained elusive whether their synaptic functions converge. Therefore, in this study, we systematically examined the potential interaction between C1q and NPs, particularly in the synaptic compartment in adult, wild-type mice. Moreover, we studied whether their interaction plays a role in the microglial phagocytosis of C1q-tagged synapses.

MATERIALS AND METHODS

Animals

The experiments were conducted on in-house bred 6–8 months old male C57BL/6N mice. Animals were housed under standard laboratory conditions (12:12 h light–dark cycle with free access to water and food).

Antibodies Used in This Study

AB1: anti-mouse synaptophysin (#101 006, Synaptic Systems, Göttingen, Germany); AB2: anti-mouse cytochrome c oxidase subunit 4 (Cox4, #sc-58348, Santa Cruz Biotechnology, Dallas, TX, USA (Santa Cruz)); AB3: anti-mouse actin- β (#AC026, Abclonal, Woburn, MA, USA); AB4: anti-mouse postsynaptic density protein 95 (Psd95, #MA1-045, Thermo Fisher Scientific (Thermo)); AB5: anti-mouse L-lactate dehydrogenase B (Ldhd,

#PAB69Mu01, Cloude-Clone Corp.; Katy, TX, USA), AB6: anti-human and mouse neuronal pentraxin 1 (#20656-1-AP, Proteintech, Rosemont, IL, USA); AB7: anti-human and mouse neuronal pentraxin 2 (#sc-166035, Santa Cruz); AB8: anti-rabbit Alexa Fluor 488-conjugated (#711-545-152, Jackson ImmunoResearch Laboratories, West Grove, PA, USA (Jackson)); AB9: anti-mouse Alexa Fluor 647-conjugated (#715-605-151, Jackson); AB10: anti-human C1qb antibody (#H00000713-D01P, Abnova, Taipei, Taiwan); AB11: anti-mouse secondary antibody, HRP-conjugated (#715-035-150, Jackson), AB12: anti-His-tag antibody (#MA1-21315, Thermo), AB13: anti-human C4BP antibody (#MCA2609, Bio-Rad); AB14: anti-human C4 (A305, Quidel, San Diego, CA, USA); AB15: anti-goat HRP-conjugated (#P0449, Dako, Agilent, Santa Clara, CA, USA); AB16: anti-human neuronal pentraxin 1 (#STJ73037; St John's Laboratory, London, UK); AB17: anti-human and mouse C1qA (#PAD207Mu01; Cloud-Clone Corp.); AB18: anti-goat Alexa Fluor 488-conjugated (#705-545-147; Jackson); AB19: anti-mouse Alexa Fluor 488-conjugated (#715-545-151, Jackson); AB20: anti-rabbit Alexa Fluor 647-conjugated (1:500 dilution; catalogue number: 715-605-151; Jackson), AB21: anti-mouse C1qA (# AB172451; Abcam, Cambridge, UK); AB22: anti-mouse Iba1 (#234 004, Synaptic Systems); AB23: anti-mouse Alexa Fluor 594-conjugated (#715-585-150, Jackson); AB24: anti-rabbit Cy3-conjugated (#711-165-152; Jackson); AB25: anti-chicken Cy5-conjugated (#703-175-155; Jackson); AB26: anti-guinea pig Alexa Fluor 488-conjugated (#706-545-148; Jackson); AB27: anti-human and mouse neuronal pentraxin 2 (# 10889-1-AP; Proteintech); AB28: anti-mouse synaptophysin (#ab8049; Abcam); AB29: anti-mouse MAP2 (# ab5392; Abcam); AB30: anti-chicken Cy3-conjugated (#703-165-155; Jackson); AB31: rabbit isotype control (#02-6102, Thermo); AB32: mouse isotype control (Mopc, BioLegend); AB33: anti-C4 (#PAA888Mu01, Cloud-Clone Corp.). Dilutions are shown in the descriptions of the corresponding methods.

Primary Neuronal Culture

Primary neuronal cell culture was prepared from the cerebral cortices of mice on embryonic days 17–18 (E17–18). Subsequent to the euthanasia of the mother *via* cervical dislocation, E17–18 embryos were removed, and their cerebral cortices were collected free from meninges under aseptic conditions according to Mórotz et al. (38). After culturing the primary neurons for 14 days *in vitro*, the cell culture was subjected to immunofluorescence labeling (see the protocol below).

Isolated and Recombinant Proteins

Recombinant human His-tagged NP1 and NP2 proteins were purchased from R&D Systems (Minneapolis, MN, USA; catalog numbers: 7707-NP-050 and 7816-NP-050). Factor H (FH) (catalog number: 341274) and human C1 (catalog number: 204873) were purchased from Merck Millipore (Billerica, MA, USA). The C1q was isolated from mouse serum in-house according to Györfy et al. (18), and His-tagged human PTX3 was prepared in-house (see below).

Western Blotting

Protein samples were completed with an equal volume of reducing 2× Laemmli buffer, incubated at 96 °C for 5 min, and subjected to SDS-PAGE using 10% (w/v) polyacrylamide gels in all experiments. After separation, proteins were blotted to PVDF membranes at 100 mA for 1 h and then blocked with 5% (w/v) bovine serum albumin (BSA) in 0.1% (v/v) Tween-20, Tris-buffered saline (TBS-T) for 1 h at room temperature (RT). Membranes were incubated in blocking buffer overnight at 4 °C with primary antibodies as follows: either anti-Syp (AB1, 1:500), anti-Cox4 (AB2, 1:500), anti-actin-β (AB3, 1:10,000), anti-Psd95 (AB4, 1:1500), anti-Ldhd (AB5, 1:1,000), anti-NP1 (AB6, 1:1,000), or anti-NP2 (AB7, 1:1,000). Subsequently, blots were incubated in TBS-T for 2 h at RT with the proper secondary antibodies: either anti-rabbit Alexa Fluor 488-conjugated (AB8, 1:1,000) or, anti-mouse Alexa Fluor 647-conjugated (AB9, 1:1,000) antibody. Fluorescence of protein bands was detected with a Typhoon Trio+ scanner (Amersham Biosciences, Little Chalfont, UK).

Plasmid, Transfection, and Cell Culture

The entire coding sequence of the human PTX3 cDNA (1,146 bp) was cloned into the BamHI and EcoRI sites of pPTX plasmid (modified pcDNA3.1+). The PTX3 construct contained His₆-tag at the C terminus.

HEK-293 H cells, a kind gift of Árpád Mike (Eötvös Loránd University, Budapest, Hungary), were grown in ProCHO5 medium at 37 °C in a humidified incubator with 5% CO₂/95% air. To generate a stable cell line, we transfected cells with a plasmid containing the human pentraxin 3 gene using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA). Typically, 7 μg of DNA and 10 μl of Lipofectamine 3000 were used per T 25 cell culture flask. Geneticin (catalog number: 10131027, Thermo Fisher Scientific) was used as a selection marker. We started at 200 μg/ml protein concentration, and every second or third day, we duplicated the dose up to 1 mg/ml. PTX3 expression of the heterologous stable cell line was detected by Western blotting. Restriction and DNA modification enzymes, DNA and protein molecular weight standards were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Nickel Affinity and Anion Exchange Chromatography

PTX3 was purified by Ni²⁺-affinity chromatography (Profinity IMAC Resin, Bio-Rad, Hercules, CA, USA). The column was equilibrated with a buffer containing 20 mM Tris, 300 mM NaCl, and 10 mM imidazole, pH 8. After incubating the cell lysate on the column, PTX3 was eluted using the same buffer supplemented with 500 mM imidazole. After dialyzing PTX3-containing fractions against 20 mM Tris pH 8 buffer containing 300 mM NaCl, samples were loaded on anion-exchange column (HiTrap Q HP, GE Healthcare, Chicago, IL, USA) equilibrated with the same Tris buffer. The protein was eluted by a linear NaCl gradient (300–1000 mM). In each purification step, the PTX3 content of the fractions was tested by Western blotting.

Microtiter Plate Binding Assays

Costar microtiter plates (Corning, Corning, NY, USA) were coated with either NP1, NP2, or PTX3 (10 µg/ml) in phosphate-buffered saline (PBS) overnight at 4 °C. After blocking with 4% (w/v) BSA in 0.05% (v/v) Tween-20, PBS (PBS-T) for 1 h at RT, wells were incubated with C1q (5.6–45 µg/ml) diluted in PBS for 1 h at RT. The wells were washed with PBS-T and bound C1q was recognized *via* incubation with anti-C1qb antibody (AB10, 1:1000) in blocking solution for 1 h at RT. After washing with PBS-T, wells were incubated with HRP-conjugated anti-mouse secondary antibody (AB11, 1:8,000) in PBS-T for 45 min at RT. 3,3',5,5'-tetramethylbenzidine (TMB) substrate (catalog number: 34028, Thermo Fisher Scientific) level was measured at 450 nm. To investigate whether pentraxins can bind to the C1 complex, FH, or C4b-binding protein (C4BP), microtiter plate binding assays were performed essentially as described above. Wells were coated with either C1 (5 µg/ml) or FH (10 µg/ml) and, after the blocking, incubated with neuronal pentraxins. For C1, 3.7–30 µg/ml NP1 and NP2, and 1.8–15 µg/ml PTX3 were used and for FH, 2.5–20 µg/ml NP1 and NP2 and 1.2–10 µg/ml PTX3 were used. To detect bound pentraxins, wells were incubated with an anti-His-tag antibody (AB12, 1:1,000) in blocking solution for 1 h at RT, and the signal was detected as described above. For C4BP, equal concentrations (10 µg/ml) of NP1, NP2, and PTX3 were coated and were blocked with 4% (w/v) BSA in PBS-T for 1 h at RT. Then wells were incubated with 6.2–100 µg/ml C4BP. To detect bound C4BPs, wells were incubated with an anti-C4BP antibody (AB13, 1:1000) in blocking solution for 1 h at RT, and the signal was detected as described above.

Complement Activation Assays

We investigated complement cascade activation by neuronal pentraxins *via* the classical pathway. Nunc microplates (Thermo Fisher Scientific) were coated with either NP1, NP2, or PTX3 (10 µg/ml) and blocked with 4% (w/v) BSA in PBS-T for 1 h at RT. Next, wells were incubated with 2% (v/v) normal or C1q-depleted human serum (catalog number: 234401, Sigma-Aldrich, Saint Louis, MO, USA) in PBS containing $\text{Ca}^{2+}/\text{Mg}^{2+}$ (catalog number: 14040-091, Thermo Fisher Scientific) supplemented either with 20 mM EDTA or EDTA-free for 30 min at 37 °C. Complement activation was detected by quantifying the deposited C4b using anti-C4 primary (AB14, 1:1,000) and anti-goat HRP-conjugated (AB15, 1:2,000) secondary antibody.

Subcellular Fractionation

After urethane anesthesia, mice were transcardially perfused with ice-cold PBS to exclude potentially interfering effects of blood-derived C1q. Then mice were decapitated, and their brains were quickly removed. Fractionation of subcellular compartments was started immediately after brain removal. The fraction of cortical synaptosomes was prepared as described in Hahn et al., 2009 (39). Briefly, the cerebral cortex samples were mechanically homogenized in a sucrose-based, iso-osmotic medium.

Subsequently, a discontinuous density gradient was prepared, an ultracentrifugation step was applied, and the highly pure fraction of synaptosomes was collected from the interface between the two layers with different densities [validated in Györfy et al. (18)]. Synaptosome samples were either fractionated further immediately to obtain sub-synaptic compartments or kept at 4 °C for up to 16 h for immunolabeling.

A sub-synaptic fractionation procedure was applied to the synaptosomes prepared from the whole brains of mice in order to separate the compartments of synaptic plasma membrane, synaptic cytoplasm, and synaptic mitochondria in the required amounts of downstream investigations. Our aim was to unveil the distribution of NP1 and NP2 proteins among these sub-synaptic compartments. To this end, we performed the protocol described in Bermejo et al., 2014 with minor modifications (40). In brief, isolated synaptosomes were ruptured by exposure to hypo-osmotic conditions for 30 min at 4 °C and mechanical homogenization with a glass tissue homogenizer to enable complete lysis of the synaptosomes. After an ultracentrifugation step (25,000 × g, 20 min), the supernatant was collected designated as the cytoplasmic compartment comprising the soluble protein content and the synaptic vesicle fraction of the synaptosomes, and devoid of plasma membrane-attached insoluble supramolecular complexes (e.g., the post-synaptic density). The pellet was resuspended, layered on top of a discontinuous sucrose density gradient, and ultracentrifuged (150,000 × g, 2 h). The synaptic plasma membrane fraction was collected from the lowest interface, while the synaptic mitochondrial fraction was recovered from the pellet *via* solubilization in 2× Laemmli buffer. The synaptic plasma membrane was pelleted by ultracentrifugation (200,000 × g, 30 min) and solubilized in 2× Laemmli buffer as well. Finally, proteins of the synaptic cytoplasmic compartment were acetone-precipitated and solubilized in the same denaturing buffer. Fractions were stored at –80 °C until further use.

The purity of the sub-synaptic compartment fractions was evaluated using compartment-specific protein markers. We have chosen postsynaptic density protein 95 (Psd95) localized only in the insoluble, membrane-bound postsynaptic protein scaffold and showing marked enrichment in the synaptic plasma membrane fraction (40). L-lactate dehydrogenase B chain (Ldhd) and cytochrome c oxidase subunit 4 (Cox4) were used exclusively as cytoplasmic (41), and mitochondria-specific (42) proteins, respectively. In each fraction, Psd95, Ldhd, and Cox4 levels were quantified after Western blotting *via* densitometry, and the composition of every fraction was assessed based on their relative amount in the lysates. A system of linear equations was formulated where x , y , and z variables represented the synaptic plasma membrane, cytoplasm and mitochondrial content of each fraction, respectively, and experimentally detected NP fluorescence intensity levels were paired with the corresponding fractions. Solving the equations revealed the NP levels for each type of sub-synaptic compartment. Statistically significant differences in the levels of NPs between the different compartments were determined using one-way repeated measures ANOVA. In case of statistically significant difference between any of the investigated groups, the Bonferroni *post hoc* test was employed.

Immunolabeling of Synaptosomes and Flow Cytometry

Immunolabeling of synaptosome fraction and analysis *via* flow cytometry were carried out as described previously (18) with minor modifications. In brief, synaptosomes were gently fixed with 0.25% (w/v) formaldehyde in sucrose-Tris buffer (320 mM sucrose, 5 mM Tris, pH 7.4). This iso-osmotic buffer possessing low ionic strength is fundamental for preventing synaptosome-aggregation (43). On the other hand, we omitted EDTA from the traditional sucrose-EDTA-Tris buffer (18, 43) in order to prevent deterioration of target-binding capabilities of C1q (44). After blocking aspecific interactions with 1% (w/v) BSA in sucrose-Tris buffer, the samples were incubated with either anti-neuronal pentraxin 1 (AB 16, 1:100) or anti-neuronal pentraxin 2 (AB7, 1:100), with or without anti-C1qA (AB17, 1:100) primary antibodies for 60 min at RT. After washing, either anti-goat Alexa Fluor 488-conjugated (AB18, 1:500) or anti-mouse Alexa Fluor 488-conjugated (AB19, 1:500) with or without anti-rabbit Alexa Fluor 647 (AB20, 1:500) secondary antibodies were applied for 45 min at RT to detect NP1, NP2, and C1qA, respectively. Finally, samples were extensively washed and then filtered through a 5.0- μ m Durapore membrane filter (Merck Millipore).

Flow cytometry analysis of fluorescently immunolabeled synaptosomes was carried out as described earlier (18) using a BD FACSAria III sorter (BD Biosciences, San Jose, CA, USA) coupled with BD FACSDiva software (BD Biosciences). Gating was set the way it resulted 1% false positive for secondary control sample. For testing the viability of synaptosomes and validating the exclusive binding of antibodies to the synaptic surface, calcein-AM labeling (Thermo Fisher Scientific) was applied following the manufacturer's instructions. Calcein labeling was done separately, and the calcein-labeled samples went through the same procedure as the antibody-labeled samples.

Immunofluorescence Staining of Mouse Brain Sections

Urethane-anesthetized mice were transcardially perfused with ice-cold 0.1 M phosphate buffer (PB), pH 7.4, and subsequently, with 2% (w/v) formaldehyde, 0.1 M PB. After perfusion, the brains were removed and post-fixed for an additional 3 h using the same fixative solution at RT. After the washing steps with 0.1 M PB, 60 μ m-thick, sagittal brain sections were produced using a vibratome, which was followed by additional extensive washing with 0.1 M PB. For the immunostaining, sections were washed first with TBS and then incubated in a blocking buffer (150 mM NaCl, 50 mM Tris, 100 mM L-lysine, 3% (w/v) BSA, pH 7.4) for 45 min at RT. Subsequently, brain sections were incubated in primary antibody buffer (150 mM NaCl, 50 mM Tris, 100 mM L-lysine, 1% (w/v) BSA, pH 7.4) containing either anti-neuronal pentraxin 1 (AB16, 1:100) or anti-neuronal pentraxin 2 (AB7, 1:50) together with anti-C1qA (AB21, 1:1,000), and anti-synaptophysin (AB1, 1:500) primary antibodies for 3 days at 4 °C. A group of brain sections was also labeled with anti-Iba1 (AB22, 1:200) primary antibody, as well. After washing with TBS, sections were incubated for 3 h at RT with either anti-goat Alexa Fluor 488-conjugated (AB18, 1:400), anti-mouse Alexa Fluor 594-conjugated

(AB23, 1:400), together with anti-rabbit Cy3-conjugated (AB24, 1:400) and anti-chicken Cy5-conjugated (AB25, 1:400) secondary antibodies to detect NP1, NP2, C1q, and synaptophysin (Syp), respectively. For Iba1 (Ionized calcium-binding adapter molecule 1) detection, anti-guinea pig Alexa Fluor 488-conjugated (AB26, 1:400) secondary antibody was used. For C4 staining, brain sections were labeled with anti-C4 (AB33, 1:100) antibody and anti-synaptophysin (AB1, 1:500) primary antibodies for three days at 4 °C. Labeling with secondary antibodies was similar as above using anti-rabbit Alexa Fluor 488-conjugated (AB8, 1:400), together with anti-chicken Cy3-conjugated (AB30, 1:400) secondary antibodies to detect C4 and synaptophysin (Syp), respectively.

Immunostained sections were washed with TBS, mounted on glass slides using Aqua-Poly/Mount medium (catalog number: 18606-20; Polysciences, Warrington, PA, USA), and the slides were covered with cover glass (catalog number: CS2440100, Menzel Gläser, No. 1).

Immunofluorescence Staining of Mouse Primary Cortical Neurons

The glass coverslips with primary neurons were incubated in PBS and subsequently, fixed with 4% (w/v) formaldehyde at RT for 15 min and washed with PBS three times. We incubated the coverslips in blocking buffer (PBS containing 0.3% (v/v) Triton X-100, 3% (w/v) BSA, pH 7.4) at RT for 1 h. Subsequently, coverslips were incubated in primary antibody buffer (PBS containing 0.3% (v/v) Triton X-100, 3% (w/v) BSA, pH 7.4) containing either anti-neuronal pentraxin 1 (AB16, 1:1,000) or anti-neuronal pentraxin 2 (AB27, 1:100) together with anti-synaptophysin (AB28, 1:100) and anti-MAP2 (AB29, 1:10,000) O/N at 4 °C. After extensive washing with PBS, coverslips were incubated for 1.5 h at RT with either anti-goat Alexa Fluor 488-conjugated (AB18, 1:800) or anti-rabbit Alexa Fluor 488-conjugated (AB8, 1:800), together with anti-chicken Cy3-conjugated (AB30, 1:800), and anti-mouse Alexa Fluor 647-conjugated (AB9, 1:800) secondary antibodies to detect NP1, NP2, MAP2 (microtubule-associated protein 2), and Syp, respectively. Immunostained coverslips were washed with PBS and mounted on glass slides using Aqua-Poly/Mount medium (catalog number: 18606-20; Polysciences).

High-Resolution Confocal Microscopy and Image Analysis

Leica HyVolution 2 pseudo-super-resolution imaging was performed by a Leica TCS SP8 STED microscope using a Leica HC PL APO 100 $\times$ STED white (1.4 NA) objective (Leica Microsystems, Wetzlar, Germany). The fluorescence of each dye was detected sequentially by using a hybrid detector and spectral detections. When three-labeled samples were imaged, the fluorescence of Alexa Fluor 488, Cy3, and Cy5 dyes were detected between 500–544 nm, 562–602 nm and 650–700 nm wavelengths at 488, 552, and 638 nm excitations, respectively. In the case of the samples with four labels, we used 488 nm (for Alexa Fluor 488), 552 nm (for Cy3 and Alexa Fluor 594) as well as 638 nm (for Alexa Fluor 647) wavelength lasers, and 504–546 nm, 589–614 nm, 630–652 nm, and 658–700 nm emission filters,

respectively. Huygens Pro deconvolution software was used for image restoration and Leica LAS X 3.1.1 software was used for image analysis. The lateral dimensions of the acquired images were $\sim 50 \times 50 \mu\text{m}$, whereas the axial dimension was $\sim 3 \mu\text{m}$. To make the investigation unbiased, the $50 \times 50 \mu\text{m}$ fields were randomly selected in the neuropil of the approximately $1,500 \times 1,500 \mu\text{m}$ large somatosensory area. Eighteen 3D-images were collected from nine sections of three animals; each image was stacked in 15–25 planes.

Colocalization analysis was carried out using an automated high-throughput workflow specifically tailored to aid the reliable identification of immunolabeled proteins and assessment of the degree of their colocalization. This approach is based on the Fiji image-analysis platform [an extended version of the ImageJ program (45)] and on a combination of its suitable plugins. The herein described protocol enables the colocalization analysis of C1q, NP1/2, and Syp proteins, all of which are distributed on the brain sections as discrete spots (diameters $< 1 \mu\text{m}$ in the XY plane). Images were split according to the fluorescence channels, and individual images were subjected to image segmentation *via* a two-step process. First, local maxima of individual spots (peaks of fluorescence intensity within a predefined radius in the X, Y, and Z planes) were identified using the 3D Maxima Finder plugin. Subsequently, segmentation of spots was carried out employing the 3D Spot Segmentation plugin. In brief, after applying a “watershed” process on the original images, individual spots were segmented using both the original and local maxima images based on the “local mean” thresholding method. The threshold values were set between 5,000 and 7,000 in the 16 bit (0–65,535) intensity scale. Pairwise colocalization analyses between C1q, NP1/2, and Syp proteins were carried out using their corresponding segmented images. Objects-based analyses were performed using the JACoP [“Just Another Colocalization Plugin” (46)], working on distances between objects’ geometrical centers (centroids). Briefly, an ellipsoid was calculated around every centroid with the maximal lateral and axial sizes of 200 nm and 500 nm, respectively (approximating the point spread function of the object). Particles were considered colocalizing if their corresponding ellipsoids were overlapping in the 3D space. After the pairwise analyses, evaluation of colocalization was also implemented between those C1q and NP1/2 centroids that simultaneously showed colocalization with Syp centroids, as well. The latter examination revealed the degree of colocalization between synaptic C1q and NP1/2 proteins. We also tested whether the close proximity of synaptic C1q and NP1/2 proteins in overall was solely accidental consequently to their high abundance in the synapse-rich cerebral cortex. For this, we utilized the Distance Analysis (DiAna) plugin (47) to randomly shuffle in the 3D space the centroids of one of the channels before the pairwise comparison of the two channels and the calculation of the cumulative frequency distribution of the minimal center-to-center distances for an image. The Monte Carlo simulation was automatically performed 100-times, and the mean cumulative frequency distribution and its 95% confidence interval envelope were calculated for the shuffled images. As

the cumulative frequency distribution of the observed minimal center-to-center distances was not normally distributed, we compared the difference between the observed distributions with the mean shuffled distribution using a non-parametric statistical approach. Thus, we employed the Wilcoxon signed-rank test, and compared the median of the observed cumulative frequency distribution with that of the corresponding mean shuffled distribution.

C4, NP1, and Syp colocalization analysis was performed by using the same method as described above.

Finally, we assessed the degree of colocalization of NP1/2 with C1q-colocalizing Syp spots within the microglia to address whether C1q-tagged eliminated synapses preferentially contain NPs or not. This analysis involved the identification of C1q- and NP1/2-colocalizing Syp centroids using the scheme described above. Microglial cells were reconstructed using microglial Iba1 staining and pixel intensity threshold-based image segmentation in Fiji. 3D-rendered reconstruction of the same stack of images created only for visualization purposes, using the 3D Viewer plugin of Fiji, displayed using its “Surface” function with a threshold set to either 100 (for C1q, NP1/2, and Syp) or 50 (for Iba1). Subsequently, the number of those C1q-colocalized Syp centroids that were overlapping with the Iba1 staining with or without NP1/2 proteins were evaluated using JACoP plugin’s centers-particles coincidence function.

Co-Immunoprecipitation

Co-immunoprecipitation experiments were performed to identify the direct or indirect interaction partners of synaptic NP1 and C1q. Cortical synaptosomes were lysed *via* mechanical homogenization with Sample Grinding Kit (catalog number: 80-6483-37, GE Healthcare), according to the manufacturer’s instructions, in TBS containing 1% (v/v) Triton X-100, 1 mM CaCl_2 , 1 mM MgCl_2 , supplemented with protease and phosphatase inhibitor cocktails (catalog numbers: P8340 and P5726, respectively, Sigma-Aldrich, Saint Louis, MO, USA) at 4 °C. Subsequently, lysates were pre-cleared with ProteinA/G Sepharose beads (catalog number: ab193262, Abcam) strictly following the manufacturer’s instructions, and incubated overnight at 4 °C with one of the primary antibodies as follows, depending on the experiment: anti-NP1 (AB6, 2 $\mu\text{g}/\text{sample}$), anti-C1qA (AB17, 2 $\mu\text{g}/\text{sample}$), rabbit isotype control (AB31, 2 $\mu\text{g}/\text{sample}$), mouse isotype control (AB32, 8 $\mu\text{g}/\text{sample}$). On the next day, samples were incubated with the beads for 1 h at 4 °C. Unbound proteins were completely removed after consecutive centrifugation steps, and then, captured proteins were eluted by incubating the beads at 96 °C for 5 min in 2× Laemmli buffer. The samples were run on SDS-PAGE, and the whole lanes were subjected to protein identification.

Mass Spectrometry-Based Protein Identification

Protein identification by mass spectrometry was carried out as described previously (48). Briefly, each gel-lane was cut to five pieces, and the proteins were reduced, alkylated, and in-gel digested with trypsin (sequencing grade modified, side chain protected porcine trypsin, Promega, Madison, WI, USA)

following the protocol by Shevchenko et al. (49). The HPLC-MS/MS analysis of the tryptic peptide mixtures was performed using a nanoflow UHPLC system (Waters M-Class UPLC) coupled to an LTQ-Orbitrap Elite (Thermo Fisher Scientific, Bremen, Germany) fitted with a nanospray ion source. 5 μ l of tryptic peptides was injected into a trap column and then separated on a reverse-phase nano column. The elution of the peptides to the emitter tip was achieved using a flow rate of 250 nl/min and a 36 min long gradient increasing from 10 to 40% of solvent B (solvent A was water containing 0.1% formic acid, and solvent B was acetonitrile containing 0.1% formic acid).

RESULTS

Neuronal Pentraxins Bind to C1q and C1 and Activate the Classical Complement Pathway

The presumed interaction between NPs and C1q was first examined *in vitro* using recombinant proteins in a microtiter plate binding assay. NP1, NP2, and the positive control PTX3 were coated and then incubated with purified C1q. Confirming our initial assumption, C1q showed direct interaction with both NP1 and NP2 in a dose-dependent manner, comparable to the positive control PTX3 (Figure 1A). Our additional, reverse experiments showed the same binding characteristics between pentraxins and C1q when purified C1q was coated (Supplementary Datasheet 2). We addressed whether NPs could also bind to the entire C1 complex consisting of C1q and serine proteases C1r and C1s. Similar to the results above, dose-dependent binding of NPs and PTX3, as well, to C1 was observed using ELISA (Figure 1B). We further investigated if the demonstrated interactions are functional and strong enough to trigger activation of the classical pathway of the complement system. Thus, we coated NPs and conducted a complement

activation assay using 2% normal and C1q-depleted human sera. According to Seelen et al., the 2% serum is sufficient to activate the classical pathway exclusively through C1q (50). Our results show that NPs are able to activate the classical pathway, measured as deposited C4 fragments in the presence of the C1 complex similar to the positive control Immunglobulin G (IgG) and PTX3. On the other hand, NPs, IgG, and PTX3 equally failed to activate the complement cascade in the absence of C1q from the serum indicating their contribution to complement activation *via* the classical pathway instead of the alternative or lectin routes (Figure 1C). Note that full recovery of complement activation was not observed using C1q-depleted serum with externally added C1q, in the assay utilizing neither the NPs, nor the positive control IgG and PTX3 proteins. Nevertheless, the added C1q substantially facilitated complement activation using all the investigated pentraxins and the IgG protein compared to the C1q-lacking condition.

Neuronal Pentraxins Bind C4BP and Factor H

Several host ligands bind C1q and thus activate complement, but soluble complement regulatory proteins C4BP and factor H bound at the same time limit complement activation to the C3 level (51). PTX3 is known to bind C4BP, an inhibitor of the classical pathway (52). We investigated the interaction of NPs with C4BP and found a dose-dependent binding profile similar to that of PTX3 (Supplementary Datasheet 2). Factor H, an inhibitor of the alternative pathway also bound NPs and PTX3 when it was coated to ELISA plate (Supplementary Datasheet 2).

NP1 Co-Immunoprecipitates With C1q in the Synaptic Lysate

After demonstrating the binding C1q to NPs *in vitro*, we investigated if the interaction could occur *in vivo* in the synaptic environment using co-immunoprecipitation followed

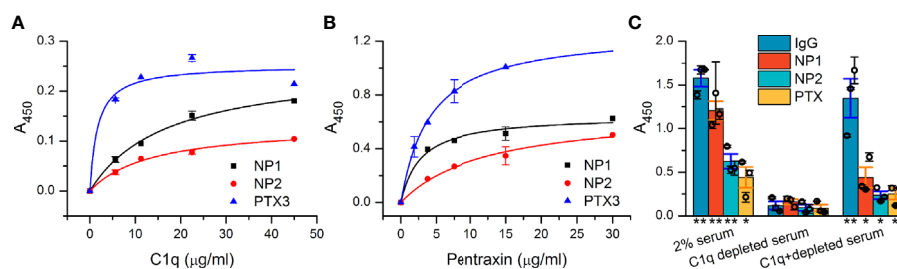


FIGURE 1 | Interaction of neuronal pentraxins with C1q and C1 *in vitro* and activation of the complement classical pathway. **(A)** In an ELISA assay, NP1, NP2, and the positive control PTX3 were coated, and then, wells were incubated with purified C1q, and binding was detected with anti-C1q antibody. C1q showed direct interaction with both NP1 and NP2 in a dose-dependent manner, comparable to PTX3. Means $\pm$ SEM; $n = 5$. **(B)** Assay results with C1 complex immobilized. Recombinant NPs were detected with anti-His antibody and exhibited dose-dependent binding (representative experiment, Means $\pm$ SEM; $n = 4$). Plots in **(A, B)** were fitted with hyperbolic function. **(C)** NP-dependent activation of the complement classical pathway was examined using microtiter plate binding assay. Neuronal pentraxins were able to activate the cascade in the presence of 2% serum, similarly to the positive controls (IgG and PTX3), as indicated by the deposition of C4 fragments, detected with anti-C4 antibody. In C1q-depleted serum, activation of the classical pathway by any of the investigated activators was dropped to baseline level. Supplementation of C1q-depleted 2% serum with purified C1q in the physiological concentration range (1.4 μ g/ml) restored the activation. Columns represent the overall means $\pm$ SEM. Three biological replicates (each is a mean of two technical parallels) are also presented with hollow circles. The statistical analysis was two-sample t-test on normally distributed samples compared to the C1q-depleted respective samples (* $p < 0.05$, ** $p < 0.01$, $n = 3$ biological replicates with two technical parallels).

by mass spectrometry-based protein identification. Using NP1-specific primary antibody, NP1 was pulled down together with NP2, all three chains of C1q (C1qA, C1qB, and C1qC), the pentraxin receptor (NPTXR), and in some samples, glutamate receptors Gria2 and Grm3. Co-immunoprecipitation using anti-C1qA antibody captured the other C1q chains C1qB and C1qC, while experiments using rabbit and mouse isotype control antibodies showed trace amounts of C1qB and C1qC chains (the results of mass spectrometry is attached as **Supplementary Datasheet 1**). Our results suggest that NP1 and C1q might be actual *in vivo* binding partners. The interaction of NP1 with NP2, NPTXR and the AMPA glutamate receptors detected in these experiments corroborates with earlier findings (24, 25).

Neuronal Pentraxins Are Present in Synapses of the Primary Neurons

To visualize the location of neuronal pentraxins along the neurons, we immunostained mouse cerebral cortical primary neurons. Pyramidal cell dendrites were visualized with MAP2, while individual synapses with Syp labeling. Experiments identified both NP1 and NP2 colocalizing with synapses in the primary neuronal cell culture. On the other hand, the multiple immunostaining also revealed that some synapses are not NP-positive along the dendrite. The distribution of these proteins suggests a differential function of NPs (**Figure 2**). It is known that NP1 and NP2 are mainly or even exclusively localized to excitatory synapses (26–28, 53, 54).

C1q Is Exclusively Present on the Surface of NP1/2 Positive Synaptosomes

To test whether C1q and NPs colocalize *in vivo* in the intact synapse, we performed double immunolabeling of isolated synaptosomes for C1q and NP1/2 and carried out their subsequent flow cytometry analysis. Flow cytometry was conducted by following our rigorously validated and currently published protocol (18). Our data demonstrated that $10.85 \pm 0.81\%$ (mean $\pm$ SEM) of synaptosomes were tagged with C1q, and $\sim 97\%$ of them ($10.49 \pm 0.82\%$ of the recorded synaptosomes) were labeled with the anti-NP1 antibody as well (**Figure 3**). In contrast, out of the $42.38 \pm 3.20\%$ NP1 positive synaptosomes,

only one fourth ($\sim 24\%$) were C1q-positive ($10.49\% \pm 0.82\%$ of the synaptosomes were double-positive). In case of NP2, multiparametric analysis indicated a similar labeling pattern: $13.9 \pm 0.5\%$ (mean $\pm$ SEM) of synaptosomes were tagged with C1q, and $\sim 97\%$ of them ($13.53 \pm 0.5\%$ of the synaptosomes) were labeled with the anti-NP2 antibody, as well (**Figure 3**). Summarizing our data, nearly all of the C1q-tagged synaptosomes contained extracellular NP1 and NP2, further strengthening our hypothesis that they can interact *in vivo* on the synaptic surface. On the other hand, a considerably high portion of NP1/2-positive synaptosomes ($\sim 75\%$) appeared C1q-untagged that could be explained by C1q-independent extracellular functions of NPs. **Supplementary Datasheet 2** shows control measurements on non-labeled, only secondary antibody-labeled, only primary antibody-labeled samples, demonstrating that there was no spillover between the channels APC and FITC.

Neuronal Pentraxin-1/2 Colocalize With C1q in the Synapses *In Vivo*

The high degree of the simultaneous synaptic presence does not necessarily imply that C1q and NPs are present close enough to each other to assume their physical binding. Thus, our next objective was to verify their *in vivo* synaptic colocalization at higher resolution *via* immunostaining of mouse cortical brain sections combined with pseudo-super-resolution confocal microscopy and automated image analysis. This approach permitted us to systematically and reliably evaluate the colocalization of C1q and NPs with the synaptic marker Syp, and ultimately, the level of colocalization between Syp-colocalized (synaptic) C1q and NPs. The first step of the image analysis was segmentation of C1q, NP1/2, and Syp fluorescent signals that resulted in 3D spot-like objects. Subsequently, positions of the objects were computationally identified based on the exact location of their centroids. Ellipsoids having 200 nm and 500 nm maximal lateral and axial sizes, respectively, were automatically drawn around every centroid, and objects with overlapping ellipsoids were considered colocalizing. Image analysis revealed that $\sim 67\%$ and $\sim 44\%$ of synaptic C1q colocalize with synaptic NP1 and NP2, respectively (**Figure 4**), supporting our prior results on their direct interaction. Importantly, it seems

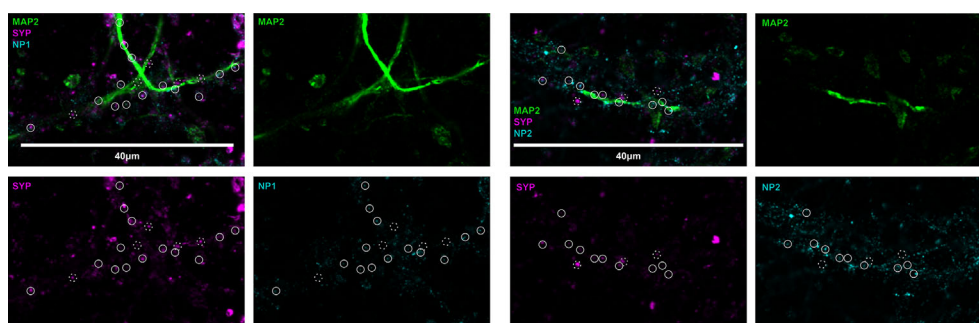


FIGURE 2 | Representative image of neuronal pentraxins along the axons. Mouse primary cortical neurons were immunostained for MAP2, Syp, and either NP1 or NP2. On the representative images, synaptic NPs are clearly apparent, whereas synapses without NP-staining are also visible. Solid circles show synaptophysin signals colocalized with either NP1 or NP2, while dashed circles show synapses, which do not contain the neuronal pentraxins.

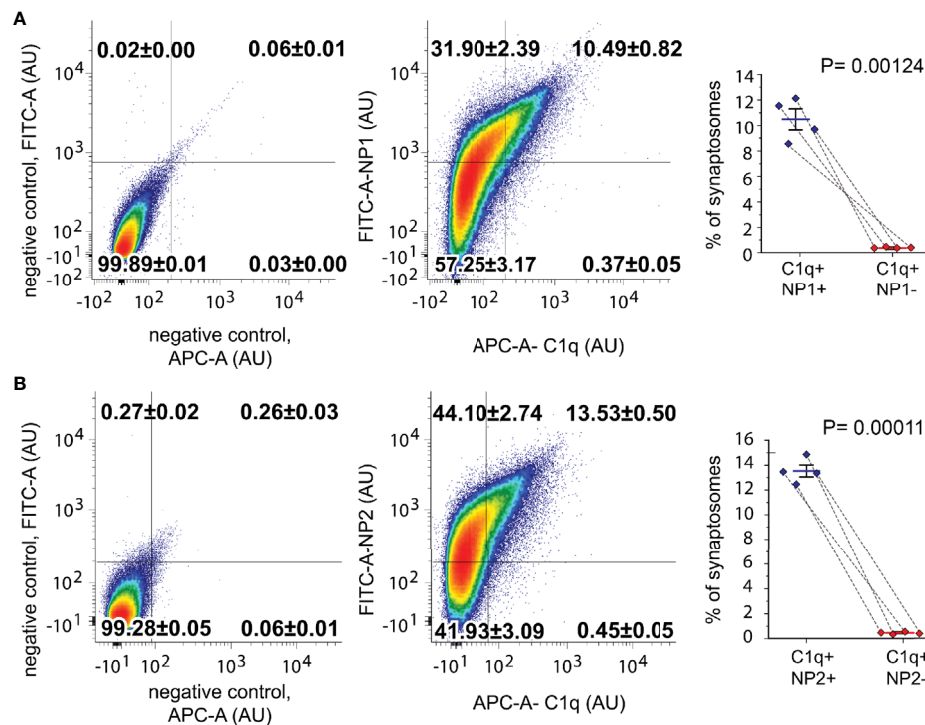


FIGURE 3 | Co-occurrence of the C1q-tag with NP1/2 on synaptosomes examined by flow cytometry. According to the gating criteria, C1q-labeled synaptosomes were almost exclusively (~97%) positive for NP1 (**A**) and NP2 (**B**). In contrast to C1q labeling, only ~24% of NP-labeled synaptosomes were positive for C1q. Density plots show representative measurements where the percentages of synaptosomes that belong to each quadrant were also indicated. The secondary antibody controls went through the same procedure as the fully labeled samples. Statistically significant differences were determined with two-tailed Student's *t*-test of paired samples (Means $\pm$ SEM; *n* = 4 mice). To test the viability of synaptosomes, calcein-AM labeling was applied separately (**Supplementary Datasheet 2**).

unlikely that merely the high abundance of synaptic C1q and NPs in the neuropil accounts for their strong colocalization, as segmented C1q objects shuffled randomly failed to exhibit a similarly close proximity to NPs (**Figures 4C, D**). Comparing the two NP species, it is interesting to note that microscopy data implies that rather the synaptic NP1 but not NP2 could be the preferable target of C1q. Although we observed a particularly high degree of synaptic colocalization between C1q and NPs with this approach, the results fall short compared to the flow cytometry data showing externally located NPs on ~97% of C1q-positive synaptosomes. This result may support the idea that not all the NPs detected on synaptosomal surfaces were C1q-bound because they possess C1q-independent functions (24, 25, 32, 55). In addition, flow cytometry analysis presumably showed a bias towards excitatory instead of inhibitory synapses as synaptosome fractions are generally enriched in excitatory synapses, which detach and reseal easier during the isolation process compared to inhibitory ones (10). In immunostaining of brain sections, there is no such difference. This methodical difference might also explain the dissimilarities between flow cytometry and immunostaining results, taking into account that NPs are exclusively located in the excitatory synapses (53) in contrast to C1q which likely tags inhibitory synapses as well (56).

Neuronal Pentraxin 1 Is Predominantly Located in the Synaptic Plasma Membrane Fraction

In spite of the abundant colocalization between C1q and NP1 and to a lesser extent NP2 in brain sections, the question emerges, to what extent NPs are exposed on the synaptic surfaces enabling C1q-binding or present intracellularly in the synapse. Unfortunately, light microscopy of tissue sections cannot distinguish between intra- and extracellular protein localizations. Thus, we sought to complement microscopy data with a detailed assessment of the subcellular distribution of NPs by subcellular fractionation. Synaptosomes were fractionated to consecutively purify the synaptic cytoplasm, synaptic mitochondria, and synaptic plasma membrane using a previously described protocol (40). The purity of the fractions was tested with selective subcellular protein markers and their compositions were analyzed (**Figure 5A**) revealing the subcellular fraction-specific corrected levels of NPs. One-way ANOVA tests pointed out statistically significant differences for both NP1 ($F(2,6) = 78.418$, $P = 0.00005$) and NP2 ($F(2,6) = 15.940$, $P = 0.00397$) between their levels in the different compartments. We found that synaptic NP1 is primarily enriched in the synaptic plasma membrane fraction, while the majority of NP2 is localized in the synaptic cytoplasm (**Figure**

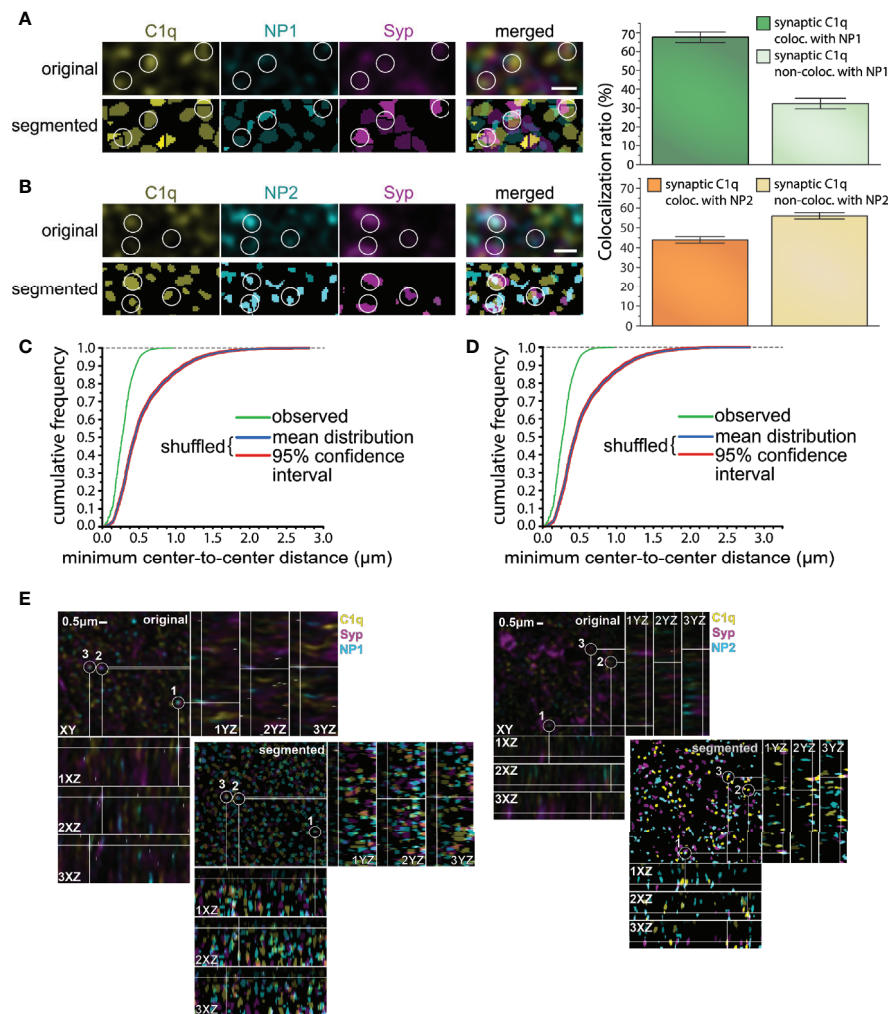


FIGURE 4 | Colocalization of synaptic C1q with NPs in mouse brain sections. **(A, B)** High-resolution confocal microscopy images of triple immunostained cerebral cortical sections were segmented to identify individual C1q, NP1/2, and Syp spots. White circles indicate several triple-colocalizing spots automatically identified according to the predefined criterion. Analyses demonstrated extensive colocalization of synaptic C1q with synaptic NPs, particularly with NP1. (T-test results: (NP1) $P = 2,19007\text{E-}10$, (NP2) $P = 4,71338\text{E-}06$ on normally distributed samples.) **(C, D)** Close proximity of synaptic C1q with NP1 **(C)** and NP2 **(D)** is not the mere consequence of their high abundance. Medians of the minimal center-to-center distance's cumulative frequency distributions significantly differ between the observed and randomly shuffled samples ($P = 0.00019$, Wilcoxon signed-rank test). Means $\pm$ SEM are shown; $n = 18$ 3D-images recorded from sections of three mice. Scale bar = $0.5 \mu\text{m}$. **(E)** The lateral view of the colocalized proteins.

5B). Therefore, it is plausible that the abundant C1q-colocalized synaptic NP1 proteins observed in mouse brain sections are primarily located in the synaptic surface instead of the synaptic intracellular space, assuming their physical binding. On the other hand, the largest pool of synaptic NP2 is accumulated intracellularly implying that C1q-colocalized synaptic NP2 molecules observed by microscopy are, in fact, partly separated from extracellular C1q by the synaptic plasma membrane.

The Majority of the Engulfed C1q-Tagged Synapses in Microglia Is NP1-Positive

Our experiments have provided strong evidence using multiple methods for the direct interaction between C1q and NPs, with particular emphasis placed on NP1. Nevertheless, it remained

unclear whether synaptic C1q–NP1/2 interactions influence microglial phagocytosis of synapses directly *via* the local complement system. Therefore, we carried out additional investigations to assess the levels of synaptic NPs in engulfed, C1q-tagged synaptic material inside the cytoplasm of the microglia. Similarly to our prior examinations, synaptic C1q and NPs in brain sections were initially identified by high-resolution confocal microscopy and image analyses. Then we reconstructed microglial processes based on immunostaining for the microglia marker Iba1. Finally, synaptic C1q and NP1/2 spots completely surrounded by microglia were assessed, and the ratio of C1q-tagged synapses with and without NP1/2 inside the microglia was evaluated. It has been demonstrated that both NPs and Syp derive mostly from neurons, lacking microglial

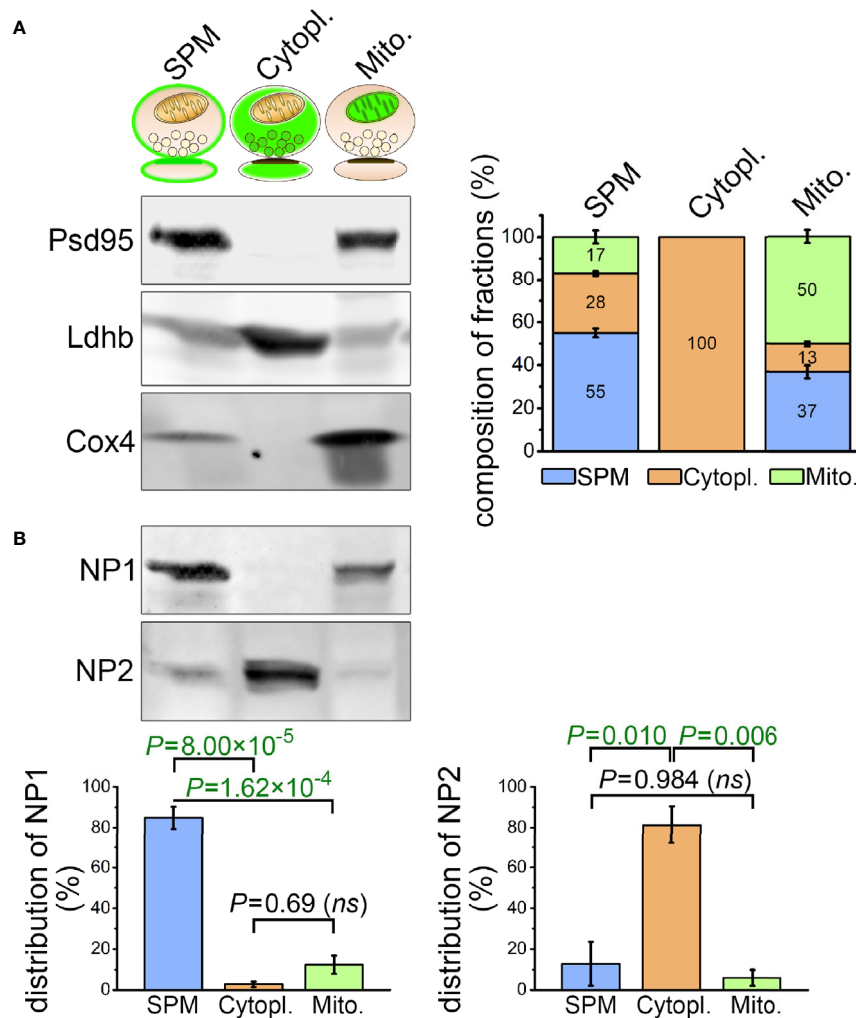


FIGURE 5 | Differential sub-synaptic localization of NP1 and NP2. **(A)** Characterization of the synaptic plasma membrane (SPM), synaptic cytoplasm (Cytopl.), and synaptic mitochondria (Mito.) fractions by western blot technique. The representative western blot image (left) demonstrates the enrichment of postsynaptic density protein 95 (Psd95), L-lactate dehydrogenase B chain (Ldhb), and cytochrome c oxidase subunit 4 (Cox4) protein markers in their respective compartments, while the stacked bar graphs (right) depict the results of the quantification. **(B)** NP1 is predominantly located in the SPM fraction, while NP2 is enriched in the synaptic cytoplasm. Means $\pm$ S.E.M. are shown; $n = 4$ mice. Statistically significant differences were identified using one-way repeated measures ANOVA, followed by Bonferroni *post hoc* test.

expression [e.g., the Brain RNA-Seq open-access database, <https://www.brainrnaseq.org/> (57)], implying that Syp-colocalized NPs detected inside the microglia likely belong to phagocytosed but not yet digested synaptic material. In contrast, C1q-expression in the cerebral cortex is strictly limited to the microglia (58). Therefore, we have only taken into account those intracellular C1q spots within the microglia that showed strong colocalization with Syp indicating that they were secreted, synaptically deposited, and likely phagocytosed as part of the removed synapse by microglia. Our experiments demonstrated that the majority of C1q-tagged, microglia-surrounded synapses (~65%) show colocalization with NP1 as well (Figure 6), supporting the hypothesis that microglia predominantly eliminate in a complement-dependent manner those synapses that expose NP1 on their surfaces. On the contrary, NP2 was

present in only ~28% of the microglia-surrounded C1q-tagged synapses (Supplementary Datasheet 2), suggesting that it is less involved in complement-mediated synaptic pruning. Taken together, our data support the hypothesis that the direct binding of C1q to synaptic NP1 is important for synapse elimination on the microglia–complement axis. Further studies are needed to prove this hypothesis and clarify the role of NP1 by investigating synaptic C1q and NP1 colocalization with phagocytic markers.

Synaptic Presence of C4

There is an intriguing question if C1q action is independent from the complement cascade activation in our system. Beth Stevens and Steven McCarroll showed that gene variants that boosted C4 expression the most were associated with higher schizophrenia

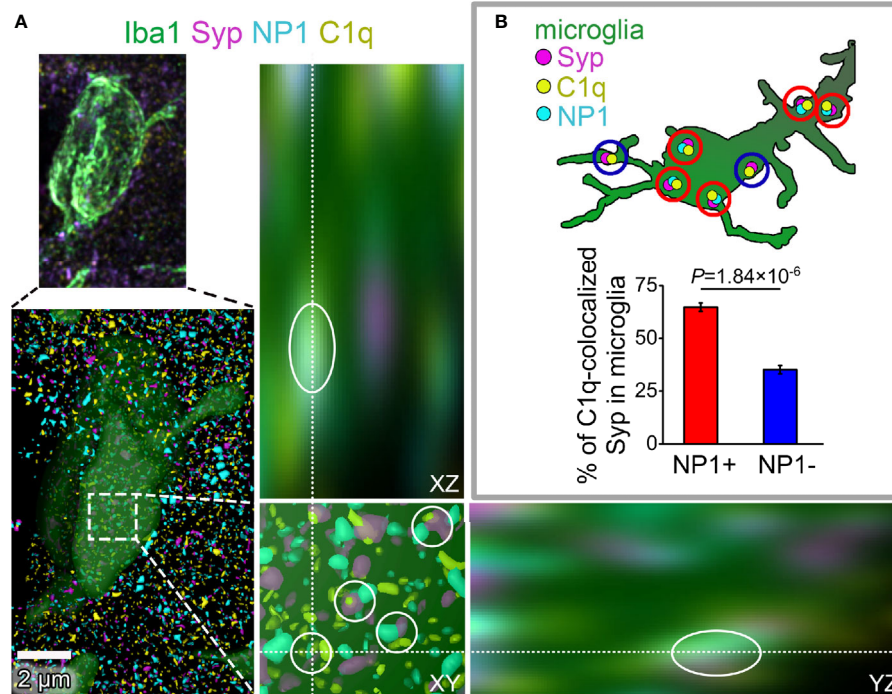


FIGURE 6 | The presence of NP1 in microglial engulfed C1q-tagged synapses. **(A)** Microglia were reconstructed from confocal image stacks using Iba1-staining (green) and engulfed, C1q- and Syp-colocalized NP1 spots (yellow, magenta, and cyan, respectively) were identified on mouse brain sections (white cycles). Orthogonal views (XY, YZ) demonstrate that microglia completely surround one of the phagocytosed C1q-tagged synaptic material with NP1 content. **(B)** Image analyses revealed that $64.80 \pm 1.97\%$ of C1q-tagged microglial Syp proteins are NP1 positive as well. (Means $\pm$ SEM are shown; $n = 16$ images recorded from brain sections of three mice. Statistically significant difference between groups was identified using two-tailed Student *t*-test of paired samples.)

risk, suggesting that high levels of the protein could promote excess pruning (59). On the other hand, Stephan and co-workers showed that the level of C1q in the brain is significantly increased with normal aging, more than that of the other components, such as C3 (56). This would suggest that C1q might have an independent function in the brain. We investigated the presence and synaptic localization of C4 *via* immunostaining of mouse cortical brain sections combined with pseudo-super-resolution confocal microscopy and automated image analysis. We clearly observed the presence of C4 in the brain sections of the adult mice. The results showed that 24% of C4 is synaptically localized (**Figure 7**). This high-level presence of synaptic-localized C4 suggests that the classical complement pathway might play a role in the synaptic functions of the complement, and C1q is not a single player. To discover the exact mechanism and pathways will require more investigations.

DISCUSSION

Generally, synaptic connections are not rigid but undergo constant remodeling in response to external and internal stimuli. Proper operation of this structural synaptic plasticity relies on the equilibrium between synaptogenesis and synaptic pruning even in the adult brain, which is the target of our investigations. However, its distortion, such as extensive synapse

loss in neurodegenerative conditions, can lead to severe functional disturbances. Several molecular partners are known to regulate this balance (60); however, we are still far from the detailed mechanistic understanding of synaptogenesis and selective synapse removal. Recently, complement proteins came into the focus of interest because accumulating evidence pointed out their role in synaptic pruning. Among them, probably C1q is responsible for the selectivity in synapse recognition (20), but the exact role of the complement cascade in the CNS in this mechanism is still elusive. Although little is known about the synaptic interaction partners of C1q in the CNS, NPs appear to be potential targets as suggested by their structural homology to peripheral C1q binding partners of the pentraxin protein family (20–22) and their previously described role in synaptic refinement in the developing visual system (23). Our present study systematically addressed this hypothesis and revealed NP1 and NP2 as two novel synaptic C1q binding partners in the adult brain. Moreover, we hypothesize that NP1 is linked to the selective elimination of C1q-tagged synapses by the microglia. We have to note that our results are valid for synaptic pruning in the adult mice and would need further investigations to verify the applicability for the young developing brain.

Our investigations incorporated both *in vitro* and *in vivo* experiments to unambiguously prove the physical interaction of NPs with C1q and to reveal its potential synaptic importance. To overcome the inherent limitations and obstacles of each

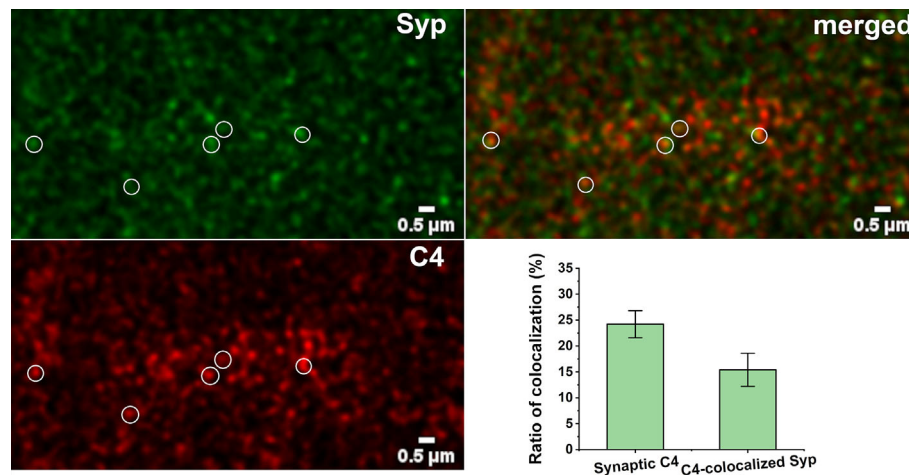


FIGURE 7 | Synaptic presence of C4. High-resolution confocal microscopy images of double immunostained cerebral cortical sections were analyzed for colocalization of C4 and synaptophysin (Syp). $24.2 \pm 2.6\%$ of C4 showed synaptic localization. $15.4 \pm 3.2\%$ of synaptophysin colocalized with C4. Some of the colocalizing spots are highlighted with white circles. Means $\pm$ SEM are shown; $n =$ nine images recorded from sections of three mice. Scale bar = $0.5 \mu\text{m}$.

technique, we strove for employing mutually complementary methods. Initially, we addressed whether NPs mimic the C1q-binding abilities of PTX3 surmised by their remarkable structural homology. Our *in vitro* experiments verified the direct binding of isolated NPs to C1q, which was strong enough to elicit downstream complement activation (**Figure 1**). This result is in agreement with the well-known binding of immobilized PTX3 to C1q in the periphery leading to the activation of the classical complement pathway on the surfaces of apoptotic cells (21, 61). Nevertheless, it was previously reported that pre-incubation of PTX3 with C1q in the fluid-phase acts oppositely and causes inhibition of complement activation (21). Contrary to this behavior, our microtiter plate binding assays demonstrated that NP1/2–C1q binding results complement activation in both the fluid and immobilized phases (**Figure 1**). As PTX3 mediates complement activation in the periphery, NPs might be capable of influencing the possible activation of the classical pathway in the brain, which idea is strengthened by our complement activation results.

Currently, the *in vivo* function of C1q–NPs binding in the extracellular milieu without being immobilized to the cell surface is unknown. Theoretically, their extracellular interaction can either serve as a means to deplete deposition of the “eat-me” signal complement components onto the cell surface or can aid complement accumulation *via* their tethering by membrane-bound NP receptors. Therefore, further studies are warranted to verify the assembly of the C1q–NP1/2 complex in the extracellular space *in vivo* and clarify its possible role.

PTX3 interacts with the classical and lectin pathway regulator C4BP and the alternative pathway regulator Factor H preventing exaggerated complement activation that would otherwise lead to inflammation and host tissue damage (52, 62, 63). According to our results, NPs can bind to both C4BP and Factor H (**Supplementary Datasheet 2**). The phenomenon may stem from the structural homology of pentraxins, and in the future,

it may be worth exploring whether these connections have a significance in the brain.

Our data suggest that C1q–NP1/2 complex is present in the synapses. Importantly, the great extent of their colocalization was demonstrated by co-immunoprecipitation, flow cytometry, and high-resolution confocal microscopy investigations, ruling out the possibility that the formation of their synaptic complex is merely artificial (**Figures 3, 4**). The techniques we used allowed for specifically studying synaptically bound C1q–NP1/2 complexes, and the obtained data indicate that the C1q–NP1 complex is present on the synaptic surface. Thus, the question may arise where the synaptic C1q–NP1 complex could be formed within the synapse. The classically known C1q, built-up by 18 subunits is an outstandingly large protein with a size that almost exceeds the average size of the synaptic cleft (~ 20 to 40 nm) (64). Taking into account this size limitation, the pool of secreted C1q that mediates selective pruning of synapses is likely attached to perisynaptic regions instead of direct binding into the synaptic junction. Previous data showing primarily perisynaptic C1q binding *via* immunogold electron microscopy examinations also supports this notion (56). Considering that NPs within the synaptic junction are well-known binding partners of AMPA receptor (AMPA) subunits, we hypothesize that C1q binds to a pool of perisynaptic and therefore possibly not AMPAR-bound NPs. In agreement with the literature, we confirmed the presence of NPs in the synapses *via* immunocytochemistry, flow cytometry, and immunohistochemistry. However, conspicuously, NPs were not uniformly present in all synapses, which might imply that, despite the fact that NPs are considered common synaptic proteins, functional differences between synapses may cause a heterogeneous distribution of NPs. Regarding synaptic functional differences, NP2 is an immediate early gene translated by neuronal activation (29), while the expression of NP1 is increased under reduced activity (33). Moreover, NP1 and NP2 are also known to form

heterocomplexes (25). Thus, the apparent contradiction between their expression pattern and assembly needs to be clarified.

Our current results repeatedly demonstrated the high but certainly not complete colocalization between C1q and NPs (**Figures 3, 4**). This observation implies the critical role of yet unknown rules that determine selective recognition of certain synaptic NPs by C1q. Although synaptic NPs are capable of complement binding (**Figure 1**), they probably have independent functions in parallel that explains their uneven C1q-binding pattern. As already described, NPs are involved in AMPAR clustering at the excitatory synapses, and by this mechanism, they can regulate excitatory synaptogenesis, functional synaptic plasticity, and synapse formation (24–27). Moreover, NP1 plays a role in neuronal cell death under hypoxic–ischemic conditions (36) and mediates the accumulation of the pro-apoptotic BAX protein on the mitochondrion's surface (35). On a theoretical basis, several explanations could be offered to clarify the partial recognition of NPs by the synaptic C1q. First, the ratio of NP1 and NP2 within their heterooligomeric complex can vary, influencing, e.g., their AMPAR binding characteristics (25). A particular subunit composition of the perisynaptic NP complex that does not favor AMPAR binding might, instead, facilitate synaptic C1q deposition. Second, posttranslational modifications of NPs could also influence their C1q-binding characteristics. It has been reported that NP1 is subjected to glycosylation (65), and we previously assumed its phosphorylation as well (18) in agreement with prior screening data [PhosphoSitePlus database (66)]. Taking into account that sialic acid in the neuronal glycocalyx prevents C1q deposition (67), and PTX3 can possess sialic acid residues modulating its C1q-binding capabilities (68), the presence of a unique NP glycosylation pattern with or without sialic acid might determine the strength of the NP–C1q interaction on the synaptic surface as well.

Our subcellular fractionation experiment demonstrated the differential localization of NPs within the synapse (**Figure 5**). Consistent with its AMPAR clustering function, NP1 is enriched in the synaptic plasma membrane with detectable levels in the synaptic mitochondrial fraction as well. Its mitochondrial identification is in accordance with prior data showing the role of NP1 in the intrinsic mitochondrial pathway of apoptosis (33, 69). In contrast, the majority of NP2 was identified in the synaptic cytoplasm fraction and, to a lesser extent, in the SPM. Although NP2 is primarily known as a synaptic protein, which binds AMPARs, our results demonstrate their prominent intracellular accumulation as well. Considering that NP2 is an immediate early gene (32), it can be hypothesized that the presence of its cytoplasmic pool is the result of its persistent activity-dependent translation without reaching secretion.

In addition to describing the C1q–NP binding, we investigated the biological role of this interaction in synapse elimination. Our results raises the probability that C1q-tagged, primarily NP1-positive synapses are engulfed by microglia (**Figure 6**). Thus, NP1 could facilitate the binding of C1q to

synapses to be removed. On the other hand, NP2 alone is unlikely to serve as an “eat-me” signal for complement components in the course of synapse phagocytosis (**Supplementary Datasheet 2**). Although the exact subunit composition of the NP assembly that is involved in synapse elimination remains elusive, our results imply that the complex consists predominantly of NP1 instead of NP2. It is already known that C1q is deposited onto synapses with reduced activity (11) and where apoptotic-like mechanisms are triggered (18). In line with these data, the initiation of local synaptic apoptotic mechanisms is preceded by a decrease in synaptic transmission (70). It was assumed that in a very similar manner to C1q, NPs are also involved in the elimination of low-activity synapses during synaptic pruning at early ontogenesis (23). Moreover, members of the pentraxin family at the periphery play key role in the elimination of apoptotic cells *via* phagocytosis, which process is regulated *via* the interaction between pentraxins and complement components, such as C1q and complement pathway inhibitors (22, 52, 61, 63, 71). Combining these prior data and our current results, we propose that NP1 aids the removal of apoptotic synapses with reduced synaptic strength *via* the complement–microglia pathway. The presence of synaptic C4 suggests that activation of the classical pathway might occur in this adult mouse model which is in line with previous reports on the role of C4 in synapse elimination (59). We propose that preventing excessive synaptic exposition of the C1q-binding form of NP1 or blocking the C1q–NP1 interaction could be beneficial to arrest increased complement-dependent synapse loss, which is a major component in the pathogenesis of AD and other neurodegenerative diseases (13).

Finally, accumulating evidence implies that NPs might be promising cerebrospinal fluid (CSF) and blood biomarkers of early AD (72–74). In addition, amyloid- β -induced increase in NP1 expression has been linked to neuronal toxicity in AD (37). In contrast, NP2 is downregulated in *post mortem* human AD brain specimens, which correlates with the reduction of the AMPAR subunit GluA4. Moreover, reduced NP2 levels were identified in the CSF of AD patients, and NP2 amounts showed a robust positive correlation with cognitive performance and hippocampal volume (74). Therefore, both NP1 and NP2 can be regarded as important CSF or blood biomarkers of AD, but the background of their different levels will have to be clarified in future studies.

In summary, our results clearly demonstrate the interaction between C1q and NPs on the synapse using a wide array of *in vitro* and *in vivo* examinations. Moreover, we hypothesize the preferential engulfment of NP1-containing synapses *via* the complement–microglia axis.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee for animal studies of Eötvös Loránd University, Budapest, Hungary. The care and treatment of all animals conformed to guidelines approved by Council Directive 86/609/EEC and the Hungarian Act of Animal Care and Experimentation (1998, XXVIII) as well as with local regulations for the care and use of animals for research. All efforts were taken to minimize the animals' pain and suffering and to reduce the number of animals used.

AUTHOR CONTRIBUTIONS

RK, HV, ÉB, VT, DM, JuK, GT, FF, EG, ÁC, and BG performed experiments. RK, VT, ÉH-G, BG, and Jók analyzed results. KM, LH, MJ, GJ, KK, BG, and Jók designed the work and supervised experiments. RK, KK, BG, and Jók wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by the National Research, Development and Innovation Office of Hungary (grants

KTIA_NAP_13-2-2014-0017 and 2017-1.2.1-NKP-2017-00002, FIEK_16-1-2016-0005, VEKOP-2.3.3-15-2016-00007, GINOP-2.3.2-15-2016-00020, and K128123) and the Ministry of Human Capacities (grant NTP-NFTÖ-19-B-0057 and *Institutional Excellence Program, ELTE ID: D11206*), and the EU's Horizon 2020 research and innovation program under grant agreement No. 739593.

ACKNOWLEDGMENTS

We appreciate the help of Mátyás Pajkos in colocalization analysis of high-resolution confocal microscopy images. We thank for the usage of MTA Cloud (<https://cloud.mta.hu/>). We thank the Single Cell Omics Advanced Core Facility staff of the HCEMM and Biological Research Centre for help with their resources and their support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2020.599771/full#supplementary-material>

REFERENCES

- Bottazzi B, Doni A, Garlanda C, Mantovani A. An integrated view of humoral innate immunity: Pentraxins as a paradigm. *Annu Rev Immunol* (2010) 28:157–83. doi: 10.1146/annurev-immunol-030409-101305
- Haapasalo K, Meri S. Regulation of the Complement System by Pentraxins. *Front Immunol* (2019) 10:1750. doi: 10.3389/fimmu.2019.01750
- Ma YJ, Garred P. Pentraxins in Complement Activation and Regulation. *Front Immunol* (2018) 9:3046. doi: 10.3389/fimmu.2018.03046
- Hajishengallis G, Reis ES, Mastellos DC, Ricklin D, Lambris JD. Novel mechanisms and functions of complement. *Nat Immunol* (2017) 18:1288–98. doi: 10.1038/ni.3858
- Gilbert J, Man H-Y. Fundamental Elements in Autism: From Neurogenesis and Neurite Growth to Synaptic Plasticity. *Front Cell Neurosci* (2017) 11:359. doi: 10.3389/fncel.2017.00359
- McGlashan TH. Schizophrenia as a Disorder of Developmentally Reduced Synaptic Connectivity. *Arch Gen Psychiatry* (2000) 57:637–48. doi: 10.1001/archpsyc.57.7.637
- Berchtold NC, Coleman PD, Cribbs DH, Rogers J, Gillen DL, Cotman CW. Synaptic genes are extensively downregulated across multiple brain regions in normal human aging and Alzheimer's disease. *Neurobiol Aging* (2013) 34:1653–61. doi: 10.1016/j.neurobiolaging.2012.11.024
- Terry RD, Masliah E, Salmon DP, Butters N, DeTeresa R, Hill R, et al. Physical basis of cognitive alterations in alzheimer's disease: Synapse loss is the major correlate of cognitive impairment. *Ann Neurol* (1991) 30:572–80. doi: 10.1002/ana.410300410
- Stephan AH, Barres BA, Stevens B. The Complement System: An Unexpected Role in Synaptic Pruning During Development and Disease. *Annu Rev Neurosci* (2012) 35:369–89. doi: 10.1146/annurev-neuro-061010-113810
- Fourgeaud L, Boulanger LM. Synapse Remodeling, Compliments of the Complement System. *Cell* (2007) 131:1034–6. doi: 10.1016/j.cell.2007.11.031
- Schafer DP, Lehrman EK, Kautzman AG, Koyama R, Mardinly AR, Yamasaki R, et al. Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner. *Neuron* (2012) 74:691–705. doi: 10.1016/j.neuron.2012.03.026
- Salin P, Tseng G, Hoffman S, Parada I, Prince D. Axonal sprouting in layer V pyramidal neurons of chronically injured cerebral cortex. *J Neurosci* (1995) 15:8234–45. doi: 10.1523/JNEUROSCI.15-12-08234.1995
- Tenner AJ, Stevens B, Woodruff TM. New tricks for an ancient system: Physiological and pathological roles of complement in the CNS. *Mol Immunol* (2018) 102:3–13. doi: 10.1016/j.molimm.2018.06.264
- Trachtenberg JT, Chen BE, Knott GW, Feng G, Sanes JR, Welker E, et al. Long-term in vivo imaging of experience-dependent synaptic plasticity in adult cortex. *Nature* (2002) 420:788–94. doi: 10.1038/nature01273
- Holtmaat AJGD, Trachtenberg JT, Wilbrecht L, Shepherd GM, Zhang X, Knott GW, et al. Transient and persistent dendritic spines in the neocortex in vivo. *Neuron* (2005) 45:279–91. doi: 10.1016/j.neuron.2005.01.003
- Knott G, Holtmaat A. Dendritic spine plasticity-Current understanding from in vivo studies. *Brain Res Rev* (2008) 58:282–9. doi: 10.1016/j.brainresrev.2008.01.002
- Acker D, Paradis S, Miller P. Stable memory and computation in randomly rewiring neural networks. *J Neurophysiol* (2019) 122:66–80. doi: 10.1152/jn.00534.2018
- Györfy BA, Kun J, Török G, Bulváki É, Borhegyi Z, Gulyácssy P, et al. Local apoptotic-like mechanisms underlie complement-mediated synaptic pruning. *Proc Natl Acad Sci U S A* (2018) 115:6303–8. doi: 10.1073/pnas.1722613115
- Scott-Hewitt N, Perrucci F, Morini R, Erreni M, Mahoney M, Witkowska A, et al. Local externalization of phosphatidylserine mediates developmental synaptic pruning by microglia. *EMBO J* (2020) 39:1–20. doi: 10.1525/emboj.2020105380
- Perry VH, O'Connor V. C1q: the perfect complement for a synaptic feast? *Nat Rev Neurosci* (2008) 9:807–11. doi: 10.1038/nrn2394
- Nauta AJ, Bottazzi B, Mantovani A, Salvatori G, Kishore U, Schwaebler WJ, et al. Biochemical and functional characterization of the interaction between pentraxin 3 and C1q. *Eur J Immunol* (2003) 33:465–73. doi: 10.1002/immu.200310022
- Du Clos TW, Mold C. Pentraxins (CRP, SAP) in the process of complement activation and clearance of apoptotic bodies through Fcγ receptors. *Curr Opin Organ Transplant* (2011) 16:15–20. doi: 10.1097/MOT.0b013e32832834253c7

23. Bjartmar L, Huberman AD, Ullian EM, Renteria RC, Liu X, Xu W, et al. Neuronal Pentraxins Mediate Synaptic Refinement in the Developing Visual System. *J Neurosci* (2006) 26:6269–81. doi: 10.1523/jneurosci.4212-05.2006
24. O'Brien RJ, Xu D, Petralia RS, Steward O, Huganir RL, Worley P. Synaptic Clustering of AMPA Receptors by the Extracellular Immediate-Early Gene Product Narp. *Neuron* (1999) 23:309–23. doi: 10.1016/S0896-6273(00)80782-5
25. Xu D, Hopf C, Reddy R, Cho RW, Guo L, Lanahan A, et al. Narp and NP1 form heterocomplexes that function in developmental and activity-dependent synaptic plasticity. *Neuron* (2003) 39:513–28. doi: 10.1016/S0896-6273(03)00463-X
26. Sia G-M, Béique J-C, Rumbaugh G, Cho R, Worley PF, Huganir RL. Interaction of the N-Terminal Domain of the AMPA Receptor GluR4 Subunit with the Neuronal Pentraxin NP1 Mediates GluR4 Synaptic Recruitment. *Neuron* (2007) 55:87–102. doi: 10.1016/j.neuron.2007.06.020
27. Farhy-Tselnick I, van Casteren ACM, Lee A, Chang VT, Aricescu AR, Allen NJ. Astrocyte-Secreted Glypican 4 Regulates Release of Neuronal Pentraxin 1 from Axons to Induce Functional Synapse Formation. *Neuron* (2017) 96:428–45.e13. doi: 10.1016/j.neuron.2017.09.053
28. Figueiro-Silva J, Gruart A, Clayton KB, Podlesniy P, Abad MA, Gasull X, et al. Neuronal pentraxin 1 negatively regulates excitatory synapse density and synaptic plasticity. *J Neurosci* (2015) 35:5504–21. doi: 10.1523/JNEUROSCI.2548-14.2015
29. Tsui CC, Copeland NG, Gilbert DJ, Jenkins NA, Barnes C, Worley PF. Narp, a novel member of the pentraxin family, promotes neurite outgrowth and is dynamically regulated by neuronal activity. *J Neurosci* (1996) 16:2463–78. doi: 10.1523/JNEUROSCI.16-08-02463.1996
30. Kirkpatrick LL, Matzuk MM, Dodds DC, Perin MS. Biochemical Interactions of the Neuronal Pentraxins. *J Biol Chem* (2000) 275:17786–92. doi: 10.1074/jbc.M002254200
31. Gerrow K, El-Husseini A. Receptors look outward: revealing signals that bring excitation to synapses. *Sci STKE* (2007) 2007:pe56. doi: 10.1126/stke.4082007pe56
32. Chang MC, Park JM, Pelkey KA, Grabenstatter HL, Xu D, Linden DJ, et al. Narp regulates homeostatic scaling of excitatory synapses on parvalbumin-expressing interneurons. *Nat Neurosci* (2010) 13:1090–7. doi: 10.1038/nn.2621
33. DeGregorio-Rocasolano N, Gasull T, Trullas R. Overexpression of neuronal pentraxin 1 is involved in neuronal death evoked by low K(+) in cerebellar granule cells. *J Biol Chem* (2001) 276:796–803. doi: 10.1074/jbc.M007967200
34. Enguita M, DeGregorio-Rocasolano N, Abad A, Trullas R. Glycogen synthase kinase 3 activity mediates neuronal pentraxin 1 expression and cell death induced by potassium deprivation in cerebellar granule cells. *Mol Pharmacol* (2005) 67:1237–46. doi: 10.1124/mol.104.007062
35. Clayton KB, Podlesniy P, Figueiro-Silva J, Lopez-Domenech G, Benitez L, Enguita M, et al. NP1 Regulates Neuronal Activity-Dependent Accumulation of BAX in Mitochondria and Mitochondrial Dynamics. *J Neurosci* (2012) 32:1453–66. doi: 10.1523/JNEUROSCI.4604-11.2012
36. Russell JC, Kishimoto K, O'Driscoll C, Hossain MA. Neuronal pentraxin 1 induction in hypoxic-ischemic neuronal death is regulated via a glycogen synthase kinase-3 α /3 β dependent mechanism. *Cell Signal* (2011) 23:673–82. doi: 10.1016/j.cellsig.2010.11.021
37. Abad MA, Enguita M, DeGregorio-Rocasolano N, Ferrer I, Trullas R. Neuronal Pentraxin 1 Contributes to the Neuronal Damage Evoked by Amyloid- and Is Overexpressed in Dystrophic Neurites in Alzheimer's Brain. *J Neurosci* (2006) 26:12735–47. doi: 10.1523/JNEUROSCI.0575-06.2006
38. Mórotz GM, De Vos KJ, Vagnoni A, Ackerley S, Shaw CE, Miller CCJ. Amyotrophic lateral sclerosis-associated mutant VAPBP56s perturbs calcium homeostasis to disrupt axonal transport of mitochondria. *Hum Mol Genet* (2012) 21:1979–88. doi: 10.1093/hmg/dds011
39. Hahn C-G, Banerjee A, Macdonald ML, Cho D-S, Kamins J, Nie Z, et al. The post-synaptic density of human postmortem brain tissues: an experimental study paradigm for neuropsychiatric illnesses. *PLoS One* (2009) 4:e5251. doi: 10.1371/journal.pone.0005251
40. Bermejo MK, Milenkovic M, Salahpour A, Ramsey AJ. Preparation of synaptic plasma membrane and postsynaptic density proteins using a discontinuous sucrose gradient. *J Vis Exp* (2014) (91):e51896. doi: 10.3791/51896
41. Johnson M, Whittaker V. Lactate dehydrogenase as a cytoplasmic marker in brain. *Biochem J* (1963) 88:404–9. doi: 10.1042/bj0880404
42. Capaldi RA. Structure and Function of Cytochrome c Oxidase. *Annu Rev Biochem* (1990) 59:569–96. doi: 10.1146/annurev.bi.59.070190.003033
43. Choi SW, Gerencser AA, Nicholls DG. Bioenergetic analysis of isolated cerebrocortical nerve terminals on a microgram scale: spare respiratory capacity and stochastic mitochondrial failure. *J Neurochem* (2009) 109:1179–91. doi: 10.1111/j.1471-4159.2009.06055.x
44. Roumenina LT, Kantardjiev AA, Atanasov BP, Waters P, Gadjeva M, Reid KBM, et al. Role of Ca²⁺ in the electrostatic stability and the functional activity of the globular domain of human C1q. *Biochemistry* (2005) 44:14097–109. doi: 10.1021/bi051186n
45. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. *Nat Methods* (2012) 9:676–82. doi: 10.1038/nmeth.2019
46. Bolte S, Cordelières FP. A guided tour into subcellular colocalization analysis in light microscopy. *J Microsc* (2006) 224:213–32. doi: 10.1111/j.1365-2818.2006.01706.x
47. Gilles J-F, Dos Santos M, Boudier T, Bolte S, Heck N, DiAna, an ImageJ tool for object-based 3D co-localization and distance analysis. *Methods* (2017) 115:55–64. doi: 10.1016/j.jymeth.2016.11.016
48. Than NG, Romero R, Tarca AL, Kekesi KA, Xu Y, Xu Z, et al. Integrated systems biology approach identifies novel maternal and placental pathways of preeclampsia. *Front Immunol* (2018) 9:1661. doi: 10.3389/fimmu.2018.01661
49. Shevchenko A, Tomas H, Havliš J, Olsen JV, Mann M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat Protoc* (2007) 1:2856–60. doi: 10.1038/nprot.2006.468
50. Seelen MA, Roos A, Wieslander J, Molnes TE, Sjöholm AG, Wurzner R, et al. Functional analysis of the classical, alternative, and MBL pathways of the complement system: Standardization and validation of a simple ELISA. *J Immunol Methods* (2005) 296:187–98. doi: 10.1016/j.jim.2004.11.016
51. Sjöberg AP, Trouw LA, Blom AM. Complement activation and inhibition: a delicate balance. *Trends Immunol* (2009) 30:83–90. doi: 10.1016/j.it.2008.11.003
52. Braunschweig A, Józsi M. Human Pentraxin 3 Binds to the Complement Regulator C4b-Binding Protein. *PLoS One* (2011) 6:e23991. doi: 10.1371/journal.pone.0023991
53. Loh KH, Stawski PS, Draycott AS, Udeshi ND, Lehrman EK, Wilton DK, et al. Proteomic Analysis of Unbounded Cellular Compartments: Synaptic Clefs. *Cell* (2016) 166:1295–307.e21. doi: 10.1016/j.cell.2016.07.041
54. Thatipamula S, Hossain MA. Critical role of extracellularly secreted neuronal pentraxin 1 in ischemic neuronal death. *BMC Neurosci* (2014) 15:1–11. doi: 10.1186/s12868-014-0133-3
55. Pelkey KA, Barksdale E, Craig MT, Yuan X, Sukumaran M, Vargish GA, et al. Pentraxins coordinate excitatory synapse maturation and circuit integration of parvalbumin interneurons. *Neuron* (2015) 85:1257–72. doi: 10.1016/j.neuron.2015.02.020
56. Stephan AH, Madison DV, Mateos JM, Fraser DA, Lovellett EA, Coutellier L, et al. A dramatic increase of C1q protein in the CNS during normal aging. *J Neurosci* (2013) 33:13460–74. doi: 10.1523/JNEUROSCI.1333-13.2013
57. Zhang Y, Chen K, Sloan SA, Bennett ML, Scholze AR, O'Keefe S, et al. An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex. *J Neurosci* (2014) 34:11929–47. doi: 10.1523/JNEUROSCI.1860-14.2014
58. Fonseca MI, Chu S-H, Hernandez MX, Fang MJ, Modarresi L, Selvan P, et al. Cell-specific deletion of C1qa identifies microglia as the dominant source of C1q in mouse brain. *J Neuroinflamm* (2017) 14:48. doi: 10.1186/s12974-017-0814-9
59. Sekar A, Bialas AR, De Rivera H, Davis A, Hammond TR, Kamitaki N, et al. Schizophrenia risk from complex variation of complement component 4. *Nature* (2016) 530:177–83. doi: 10.1038/nature16549
60. Südhof TC. Towards an Understanding of Synapse Formation. *Neuron* (2018) 100:276–93. doi: 10.1016/j.neuron.2018.09.040
61. Nauta AJ, Daha MR, Van Kooten C, Roos A. Recognition and clearance of apoptotic cells: A role for complement and pentraxins. *Trends Immunol* (2003) 24:148–54. doi: 10.1016/S1471-4906(03)00030-9
62. Inforzato A, Doni A, Barajon I, Leone R, Garlanda C, Bottazzi B, et al. PTX3 as a paradigm for the interaction of pentraxins with the Complement system. *Semin Immunol* (2013) 25:79–85. doi: 10.1016/j.smim.2013.05.002

63. Baruah P, Dumitriu IE, Peri G, Russo V, Mantovani A, Manfredi AA, et al. The tissue pentraxin PTX3 limits C1q-mediated complement activation and phagocytosis of apoptotic cells by dendritic cells. *J Leukoc Biol* (2006) 80:87–95. doi: 10.1189/jlb.0805445
64. Bally I, Ancelet S, Moriscot C, Gonnet F, Mantovani A, Daniel R, et al. Expression of recombinant human complement C1q allows identification of the C1r/C1s-binding sites. *Proc Natl Acad Sci* (2013) 110:8650–5. doi: 10.1073/pnas.1304894110
65. Hooper AWM, Alamilla JF, Venier RE, Gillespie DC, Igldoura SA. Neuronal pentraxin 1 depletion delays neurodegeneration and extends life in Sandhoff disease mice. *Hum Mol Genet* (2017) 26:661–73. doi: 10.1093/hmg/ddw422
66. Hornbeck PV, Zhang B, Murray B, Kornhauser JM, Latham V, Skrzypek E. PhosphoSitePlus, 2014: Mutations, PTMs and recalibrations. *Nucleic Acids Res* (2015) 43:D512–20. doi: 10.1093/nar/gku1267
67. Linnartz B, Kopatz J, Tenner AJ, Neumann H. Sialic Acid on the Neuronal Glycocalyx Prevents Complement C1 Binding and Complement Receptor-3-Mediated Removal by Microglia. *J Neurosci* (2012) 32:946–52. doi: 10.1523/jneurosci.3830-11.2012
68. Inforzato A, Peri G, Doni A, Garlanda C, Mantovani A, Bastone A, et al. Structure and function of the long pentraxin PTX3 glycosidic moiety: Fine-tuning of the Interaction with C1q and Complement Activation. *Biochemistry* (2006) 45:11540–51. doi: 10.1021/bi0607453
69. Al Rahim M, Thatipamula S, Hossain MA. Critical role of neuronal pentraxin 1 in mitochondria-mediated hypoxic-ischemic neuronal injury. *Neurobiol Dis* (2013) 50:59–68. doi: 10.1016/j.nbd.2012.10.003
70. Sheng M, Ertürk A. Long-term depression: A cell biological view. *Philos Trans R Soc B Biol Sci* (2014) 369:20130138. doi: 10.1098/rstb.2013.0138
71. Rovere P, Peri G, Fazzini F, Bottazzi B, Doni A, Bondanza A, et al. The long pentraxin PTX3 binds to apoptotic cells and regulates their clearance by antigen-presenting dendritic cells. *Blood* (2000) 96:4300–6. doi: 10.1182/blood.V96.13.4300
72. Ma Q-L, Teng E, Zuo X, Jones M, Teter B, Zhao EY, et al. Neuronal pentraxin 1: A synaptic-derived plasma biomarker in Alzheimer's disease. *Neurobiol Dis* (2018) 114:120–8. doi: 10.1016/j.nbd.2018.02.014
73. Begcevic I, Tsolaki M, Brinc D, Brown M, Martinez-Morillo E, Lazarou I, et al. Neuronal pentraxin receptor-1 is a new cerebrospinal fluid biomarker of Alzheimer's disease progression. *F1000Research* (2018) 7:1012. doi: 10.12688/f1000research.15095.1
74. Xiao MF, Xu D, Craig MT, Pelkey KA, Chien CC, Shi Y, et al. NPTX2 and cognitive dysfunction in Alzheimer's Disease. *Elife* (2017) 6:1–27. doi: 10.7554/eLife.23798

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Kovács, Vadász, Bulyáki, Török, Tóth, Mátyás, Kun, Hunyadi-Gulyás, Fedor, Csinci, Medzihradsky, Homolya, Juhász, Kékesi, Józsi, Györfy and Kardos. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



C-Reactive Protein Triggers Cell Death in Ischemic Cells

Ahmed Sheriff^{1,2*}, Stefan Kayser¹, Patrizia Brunner³ and Birgit Vogt¹

¹ Pentacor GmbH, Hennigsdorf, Germany, ² Medizinische Klinik m.S. Gastroenterologie/Infektiologie/Rheumatologie, Charité Universitätsmedizin, Berlin, Germany, ³ Adsorb GmbH, Berlin, Germany

OPEN ACCESS

Edited by:

Kenji Daigo,
Nippon Medical School, Japan

Reviewed by:

Michael Torzewski,
Robert-Bosch-Hospital, Germany
Blanca Molins,
Institut de Recerca Biomèdica August
Pi i Sunyer (IDIBAPS), Spain

*Correspondence:

Ahmed Sheriff
ahmed.sheriff@charite.de

Specialty section:

This article was submitted to
Molecular Innate Immunity,
a section of the journal
Frontiers in Immunology

Received: 17 November 2020

Accepted: 22 January 2021

Published: 10 February 2021

Citation:

Sheriff A, Kayser S, Brunner P and
Vogt B (2021) C-Reactive Protein
Triggers Cell Death in Ischemic Cells.
Front. Immunol. 12:630430.
doi: 10.3389/fimmu.2021.630430

C-reactive protein (CRP) is the best-known acute phase protein. In humans, almost every type of inflammation is accompanied by an increase of CRP concentration. Until recently, the only known physiological function of CRP was the marking of cells to initiate their phagocytosis. This triggers the classical complement pathway up to C4, which helps to eliminate pathogens and dead cells. However, vital cells with reduced energy supply are also marked, which is useful in the case of a classical external wound because an important substrate for pathogens is disposed of, but is counterproductive at internal wounds (e.g., heart attack or stroke). This mechanism negatively affects clinical outcomes since it is established that CRP levels correlate with the prognosis of these indications. Here, we summarize what we can learn from a clinical study in which CRP was adsorbed from the bloodstream by CRP-apheresis. Recently, it was shown that CRP can have a direct effect on blood pressure in rabbits. This is interesting in regard to patients with high inflammation, as they often become tachycardic and need catecholamines. These two physiological effects of CRP apparently also occur in COVID-19. Parts of the lung become ischemic due to intra-alveolar edema and hemorrhage and in parallel CRP increases dramatically, hence it is assumed that CRP is also involved in this ischemic condition. It is meanwhile considered that most of the damage in COVID-19 is caused by the immune system. The high amounts of CRP could have an additional influence on blood pressure in severe COVID-19.

Keywords: CRP–C-reactive protein, ischemia/reperfusion injury, cardiovascular, COVID-19, inflammation

INTRODUCTION

Inflammation in humans is deeply evolutionary rooted. A quick and intense inflammatory response is required for the efficient eradication of injury and was highly beneficial in times where external wounds or life-threatening infections where the main—if not only—cause of damage to the body (1). Inflammation as a weapon against environmental risks and triggers is unfortunately a two-edged sword, because it is dangerous when turned against the own body. In modern times, an elevated inflammatory function is thought to be associated with higher risk to develop atherosclerosis, diabetes and other age-related diseases, which are not caused by pathogens (2). But an enhanced immune system can not only facilitate cardiovascular disease, it also exacerbates acute incidents, which are “sterile.” In order to heal internal wounds, the body needs the inflammatory reaction to eliminate dead cells. At the same time activating proliferation and repair mechanisms as well as restoring tissue homeostasis is essential. This immune response is, however, since centuries specialized on the thorough eradication of cells around a wound to minimize the risk of infection.

Even if these cells are only energy deprived but still viable. In the setting of an internal wound, as e.g., myocardial infarction or stroke, this effect is often destructive and threatening as it only aggravates deterioration and involves severe collateral damage (3, 4).

Likewise, an excessive immune reaction not justified by its external trigger induces more negative than positive effects on the body (5). During a systemic inflammatory response syndrome or sepsis the inflammation is so enormous and disproportionate that it causes widespread tissue injury and might finally result in multiple organ failure (6). Here, the whole body is often affected by inflammation showing hard to control hemodynamic instability.

Although a multitude of proteins are involved in inflammation, most of them do not actively participate in the elimination of pathogens or human cells (1). One of the acute-phase mediators directly involved in these pro-inflammatory processes is C-reactive protein (CRP) which was discovered by Tillett and Francis (7). CRP is well-established as one of the most reliable markers of inflammation, rising dramatically during any type of inflammation.

There are several very good and extensive reviews published, summarizing the role of CRP as unspecific inflammatory marker and its history from discovery to world-wide used lab marker (8–11). Even though CRP has been investigated in numerous clinical studies and its association and correlation with the progress of certain diseases is evidently clear (12–19), evidence that it is a mediator of the respective disease in humans was missing while strong evidence exists for different animal species (16, 20–22). In addition, CRP can dissociate into monomers physiologically, although it is still under debate if it thereby exerts different molecular functions than the pentameric form (23). The transition of pentameric CRP to monomeric CRP was described in specific inflammatory microenvironments (24, 25). Pro-inflammatory isoforms of pentameric and monomeric CRP were reported (26). The circulating CRP is pentameric and the actual source of all further processes. Despite this extensive in-depth knowledge, the widespread opinion is still that in humans CRP is only an unspecific biomarker.

We want to focus on the controversy/debate that CRP also in humans is not only a marker but an active pro-inflammatory protein, which contributes causally to the severity of tissue damage and the outcome of various diseases (27).

CRP IS AN ACTIVE INFLAMMATORY PROTEIN

CRP is secreted by the liver into the blood circulation where it efficiently detects and opsonizes bacteria upon their infiltration (28, 29). By marking these pathogens, it initiates their phagocytosis via activation of complement. This mechanism is mainly caused by the binding of CRP to the phosphorylcholine groups in the membrane of bacteria. These groups, however, are also present in all human cells, albeit not accessible on healthy cells. Cells that are apoptotic, necrotic, energy-depleted or simply exposed to inflammatory environments, often being acidic and hypoxic, undergo conformational and

biochemical changes in their membrane (30). One of these being the partial hydrolyzation of phosphatidylcholine (PC) to lyso-phosphatidylcholine (LPC) by another acute-phase-protein, namely the secretory phospholipase A2 type IIa (sPLA2 IIa) (31–34). This makes the phosphorylcholine group accessible to the binding of CRP. Hence, CRP irreversibly marks dying, dead, damaged or hypoxic/ischemic cells. Subsequently, the classical complement pathway is activated and the CRP-marked cells are disposed by phagocytosis (35–40). See **Figure 1** for the hypothesized pathomechanism of CRP after an acute phase response caused by inflamed or hypoxic/ischemic tissue.

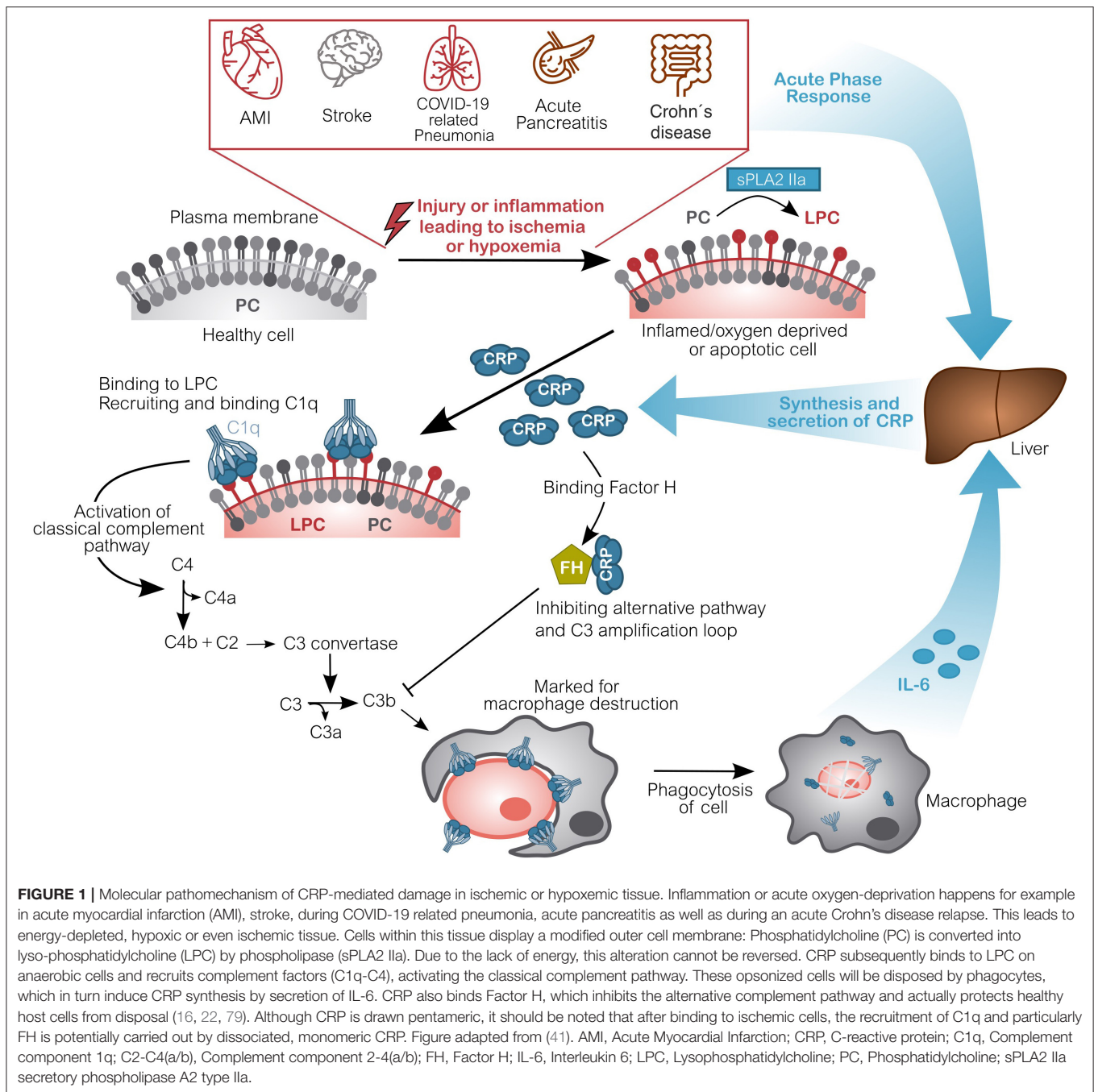
In the setting of an internal wound this generates a vicious cycle: The primary inflammation triggered by e.g., ischemia (e.g., acute myocardial infarction or stroke) activates a switch to anaerobic metabolism and a striking synthesis and secretion of CRP mediated by IL-6. CRP is circulated to the wound, where it mediates the disposal of dead and dying cells. High CRP concentrations cause more cells to be marked, including still viable cells, which could have regenerated their membrane after restoration of the oxygen flow and switching back to aerobic metabolism. The phagocytosis of these cells in turn produces IL-6, inducing the synthesis of additional CRP, subsequently amplifying the immune response. Thereby, CRP causally contributes to the tissue damage and scarring after an incident [**Figure 1**, adapted from (41)] (22, 42–44). It should be noted that CRP is synthesized and secreted as a pentamer by the liver, however, it can dissociate into monomers within the microenvironment of the inflamed/ischemic tissue and might exert the drawn functions as monomer (**Figure 1**).

Although not every step of this molecular mechanism has been proven and shown in detail, there have been convincing proof-of-concept studies in animals substantiating this hypothesis. A large body of data obtained either in rats, porcine models or *in vitro* in the infarcted myocardium of humans has demonstrated that CRP plays an active role in exacerbating ischemia and reperfusion-induced damage (16, 22, 45–50).

Recent studies exceedingly revealed that CRP modulates signaling cascades besides the classical complement pathway (51–54). This shows that CRP has direct physiological effects on not only inflammation but also the function of e.g., endothelial cells, be it their metabolism, differentiation or migration (55–57). In the context of endothelial cells, it is also discussed if CRP might have protective effects in atherosclerotic lesions. It was shown that, although CRP induces complement activation, it protects the bound cells from the formation of final complement components (58, 59), mainly by recruiting complement factor H (60). The role of CRP in atherosclerosis is however still under debate and cannot be compared to acute ischemic incidents regarding the circulating concentration of CRP.

CRP HAS AN EFFECT ON BLOOD PRESSURE

One of these recent studies showed a direct, quick and extreme effect of CRP on blood pressure in rabbits (61). Human CRP was intravenously injected *in vivo* to reach a level of 50 mg/L. It



dramatically reduced the arterial blood pressure within minutes, while the heart rate remained the same and did not counteract as expected and necessary to maintain the oxygen supply of the organism. The effect persisted for more than 17 min.

While it has been shown that blood pressure and heart rate as well as adrenergic receptor (AR) signaling can affect CRP concentrations (62–64), a direct influence of CRP on hemodynamic variables has hardly been investigated so far. Other *in vivo* studies administering CRP were performed in rats and

humans (65–67). However, the recombinant CRP used in the studies never achieved concentrations of 50 mg/l and blood pressure was measured not directly but only hours after injection (65, 67). Hence, the acute effects might have been overlooked.

The direct and acute effect of high CRP concentrations on blood pressure gives a first hint, why critically ill patients, suffering from e.g., sepsis or acute pancreatitis, can develop hardly controllable hemodynamic variables with preceding elevated CRP levels (68).

After seeing such a dramatic drop in blood pressure the influence of CRP on AR signal transduction was investigated *in vitro* (61). ARs signal via calcium (Ca^{2+}) as second messenger was measured in real-time. CRP triggered calcium signaling in a dose-dependent manner in two different human cell lines, expressing either α - or β -adrenergic receptors. Further, CRP induced an additional calcium increase that came on top of AR agonists phenylephrine or isoprenaline. This indicates a molecular mechanism that is independent of adrenoceptor activation.

Effects of CRP on endothelial cells have mostly been attributed to its effect on endothelial nitric oxide synthase (eNOS), although studies have to be interpreted with care, as contaminating products in commercial CRP solutions, such as sodium azide, were possibly often causally involved (69–71). eNOS is activated by an increase in intracellular calcium, leading to vasodilatation (72, 73), which could explain the drop of blood pressure *in vivo*. The mechanism underlying CRP's induction of calcium influx is still undiscovered and should be investigated in future studies.

Therefore, it was demonstrated that one molecule of the inflammation cascade has an influence on blood pressure. The direct influence of other inflammatory mediators needs to be investigated.

WHAT WE CAN LEARN FROM STUDIES USING CRP AS A THERAPEUTIC TARGET MOLECULE

Many reports following the hypothesis that CRP has pathological effects suffer from a mean to shut down its activity. Knock-out mice do not represent appropriate models, because CRP in mice fulfills different functions than in humans and does not act as an acute-phase protein (74). Other animal models like e.g., transgenic rabbits expressing human CRP have been used but here most of the investigations focused on atherosclerosis and not on acute incidents (75).

In humans the use of CRP-lowering drugs has so far not been successful, since CRP as acute-phase protein increases drastically within hours and its circulating levels need to be lowered quickly in an acute setting. This cannot be achieved by targeting its synthesis or using approaches, which take several days to affect CRP levels (16, 21, 76).

Selective immuno-adsorption of CRP from the serum avoids these problems and has been shown to efficiently reduce CRP concentrations by ~60% within hours (77–79). The elimination of pathogenic substances from the blood by means of extracorporeal apheresis is an established therapy in the clinical routine of numerous diseases.

CRP apheresis aims to remove CRP from the blood plasma after an infarction to reduce acute tissue damage and ischemic reperfusion damage (41). It has most extensively been utilized after acute myocardial infarction (AMI).

CRP Depletion After Myocardial Infarction

Patients recovering from a heart attack often suffer from a reduced quality of life and a very high risk of subsequent

serious complications (e.g., heart failure, arrhythmias, second heart attack, death), which imposes an enormous burden on the healthcare system. It was observed that this risk correlates significantly with the size of myocardial injury and scarring (80, 81).

It has long been known that inflammation, mainly mediated by the innate immune system, expands myocardial injury. However, anti-inflammatory strategies to mitigate myocardial necrosis have so far failed, perhaps because these processes are also necessary for the healing and repair of the heart (3, 4, 82, 83). Whereas, baseline CRP values are recognized as a determinant of the incidence of cardiovascular disease (12, 14), serum CRP concentration after AMI correlates with the clinical outcome (19, 42, 44, 84–87). It is textbook knowledge that high CRP peaks in the first 72 h after AMI correlate with larger infarct size and higher mortality and the incidence of additional adverse cardiovascular events (42–44). This has been documented for four decades and is consistent with the described pathological function of CRP to eliminate cells in the area at risk (27, 30, 88, 89). This region comprises cells that might recover after revascularization and reperfusion, but are eventually destroyed by immune-mediated mechanisms, as explained above and shown in detail in numerous experimental approaches specifically focusing on AMI (20, 46, 48, 85, 90, 91).

It has therefore been suggested earlier to target CRP in AMI, but the therapeutic approaches were not clinically relevant or sufficiently rapid (16, 92–94). This changed when it was shown that specific extracorporeal removal of CRP by CRP apheresis resulted in a significant reduction of the infarct area and stabilization of the left ventricular ejection fraction (LVEF) in a preclinical trial in pigs (22, 50). An interesting observation is that the scar morphology of the animals after CRP apheresis was completely different from controls, supporting the hypothesis that CRP is directly involved in tissue destruction and scarring after the incident (22). Consequently, CRP apheresis has been used in one clinical trial and two case reports in patients with ST-elevation myocardial infarction (STEMI) (77, 79, 95).

In the CAMI-1 trial it was investigated whether specific depletion of CRP can reduce the size of myocardial infarction in humans. Eighty-three patients were included and the treatment was safe and well-tolerated (79). The extent of increase of CRP concentration during the first 32 h after STEMI significantly correlated with the infarct size in control patients. Patients with similar initial CRP increase who subsequently underwent CRP apheresis, had smaller infarct sizes and better LVEF and wall motion (strains) when compared to control patients. Surprisingly, some patients treated with CRP apheresis had not even minor infarct scars and a normal LVEF (96).

It is possible that the supply bottleneck in ischemic tissue does not immediately lead to tissue necrosis, but rather to a conversion of the energy metabolism into anaerobic glycolysis, which leads to a significant lack of energy of the individual cardiomyocytes (97). Afterwards, the cardiomyocytes go into stunning until the metabolism switches back to the aerobic one which eliminates the energy deficiency. This suggests that the cardiomyocytes only

survive if they are not marked by CRP and thus disposed of by phagocytes.

CRP in Stroke

This effect can possibly be applied to other ischemia-mediated injuries as for example stroke. Here, similar mechanisms to AMI take place and inflammation plays a crucial role during the occlusion but also subsequent therapeutic reperfusion of the tissue (98, 99). The inflammatory response after stroke has been discovered to be a key prognostic factor for patients (100, 101). High CRP concentrations during the first 48 h after the incident significantly predict immediate and long-term mortality as well as the overall prognosis (13, 101, 101–103). This is supported by pre-clinical evidence in a stroke model in rats and subsequent application of high amounts of human CRP (49). This strongly supports the hypothesis that CRP plays a similar pathological role as in AMI.

CRP MAY INCREASE DESTRUCTION OF TISSUE IN COVID-19

SARS-CoV-2 can lead to COVID-19 and induces pulmonary fibrosis and cardiac complications in a minor percentage of infected individuals, among other organ deterioration (104). A major therapeutic approach focuses on the treatment of acute respiratory distress syndrome, as it is the leading cause of mortality, followed by cardiac and septic complications. In the severe course of the disease a massive increase in the CRP concentration accompanied by an initial cytokine storm is followed by pulmonary fibrosis (105, 106). Intra-alveolar edema and hemorrhage is a common observation in the lungs of COVID-19 patients which leads to ischemic alveolar tissue. It may be assumed that CRP itself triggers tissue damage by binding to these ischemic cells and is thus also causally involved in the enlargement of the destroyed tissue and contributes to irreversible tissue destruction (22, 79). Both IL-6 and CRP increase dramatically in the course of clinical manifestation of COVID-19 (107) and rising CRP levels were shown to significantly predict the respiratory decline in patients (105). CRP levels also correlate with CT findings of COVID-19 patients (108). These findings further support the hypothesis that a significant increase in CRP is a signal of lung deterioration and disease progression.

Complement deposits were found by pathologists in the lungs of deceased COVID-19 patients. Among them was especially C1q. Since C1q also inhibits antiviral CD8⁺ effector T-cell responses, a higher frequency of CD8⁺PD1⁺ T-cells was found, possibly indicating T-cell exhaustion (109). Despite the depletion of the T cells, massive destruction in the lungs is found along with the extreme levels of CRP in the aggravated COVID-19 patients. C1q is known to bind CRP after CRP binds the lysophosphatidylcholine of ischemic cells (22).

In addition, cardiac involvement was observed by MRI analysis in 78% of patients, and persistent myocardial inflammation was observed in 60% of patients with recent COVID-19 disease, regardless of pre-existing conditions, the severity and overall course of the acute disease and the time from initial diagnosis (110). Myocardial inflammation was suggested as the underlying mechanism (111, 112).

CRP apheresis provides a therapeutic approach to rapidly decrease the high CRP levels in COVID-19 patients before lung deterioration can progress (113). Until now, the therapeutic option of reducing the extremely high amount of CRP has been used once in the early phase of COVID-19 and in end-stage patients with one case being reported (113, 114). A clinical study in this indication would be beneficial and is currently planned.

CONCLUSION AND OUTLOOK

The understanding of CRP has undergone two basic transitions. First, CRP has been established as a general biomarker of inflammation and infection in clinical practice. Then, its role as a stable and highly useful prognostic factor for cardiovascular and cerebral disease in healthy individuals has been widely acknowledged and utilized (17, 115). However, the characterization of CRP as not only a biomarker but also a mediator or even trigger of destruction of tissue in humans is still widely ignored (27, 46, 49).

CRP as an archaic protein of the innate immune system physiologically disposes cells and responds to almost every change in tissue homeostasis. From the perspective of the body's energy balance, one has to ask oneself why CRP is produced in large quantities by the liver in situations where it seems wiser to keep a proper energy reserve. Certainly not to provide us with a meaningful biomarker. It is more logical to recognize that it is provided in potentially septic wounds during enemy defense, to eliminate further cells, so that they do not serve the enemy for propagation. This function has unfortunately a negative effect on typically aseptic inner wounds. Further, the demonstration of a direct effect of CRP on blood pressure shows us that the molecular functions of CRP are still not comprehensively described and that the role of this protein is largely underestimated in critically ill patients.

Accepting CRP as an active inflammatory protein offers the promising possibility to therapeutically target CRP whenever the inflammatory reaction is too extensive or not beneficial. Ongoing and future clinical trials will illuminate whether this therapeutic approach will continue to prove its value.

AUTHOR CONTRIBUTIONS

AS and PB conceptualized and wrote the first draft. SK and BV made substantial corrections.

REFERENCES

1. Netea MG, Balkwill F, Chonchol M, Cominelli F, Donath MY, Giamarellos-Bourboulis EJ, et al. A guiding map for inflammation. *Nat Immunol.* (2017) 18:826–31. doi: 10.1038/ni.3790
2. Ridker PM. On evolutionary biology, inflammation, infection, and the causes of atherosclerosis. *Circulation.* (2002) 105:2–4. doi: 10.1161/circ.105.1.2
3. Anzai T. Inflammatory mechanisms of cardiovascular remodeling. *Circul J.* (2018) 82:629–35. doi: 10.1253/circj.CJ-18-0063
4. Ong S-B, Hernández-Reséndiz S, Crespo-Avilan GE, Mukhametshina RT, Kwek X-Y, Cabrera-Fuentes HA, et al. Inflammation following acute myocardial infarction: multiple players, dynamic roles, and novel therapeutic opportunities. *Pharmacol Therap.* (2018) 186:73–87. doi: 10.1016/j.pharmthera.2018.01.001
5. Neher MD, Weckbach S, Flierl MA, Huber-Lang MS, Stahel PF. Molecular mechanisms of inflammation and tissue injury after major trauma—is complement the “bad guy”? *J Biomed Sci.* (2011) 18:90–90. doi: 10.1186/1423-0127-18-90
6. Day JRS, Taylor KM. The systemic inflammatory response syndrome and cardiopulmonary bypass. *Int J Surg.* (2005) 3:129–40. doi: 10.1016/j.ijssu.2005.04.002
7. Tillett WS, Francis T. Serological reactions in pneumonia with a non-protein somatic fraction of pneumococcus. *J Exp Med.* (1930) 52:561–71.
8. Szalai AJ, Agrawal A, Greenhough TJ, Volanakis JE. C-reactive protein: structural biology and host defense function. *Clin Chem Lab Med.* (1999) 37:265–70. doi: 10.1515/CCLM.1999.046
9. Volanakis JE. Human C-reactive protein: expression, structure, and function. *Mol Immunol.* (2001) 38:189–97. doi: 10.1016/s0161-5890(01)00042-6
10. Verma S, Szmítko PE, Ridker PM. C-reactive protein comes of age. *Nat Clin Pract Cardiovasc Med.* (2005) 2:29–36. doi: 10.1038/ncpcardio0074
11. Casas JP, Shah T, Hingorani AD, Danesh J, Pepys MB. C-reactive protein and coronary heart disease: a critical review. *J Intern Med.* (2008) 264:295–314. doi: 10.1111/j.1365-2796.2008.02015.x
12. Koenig W, Sund M, Frohlich M, Fischer HG, Lowel H, Doring A, et al. C-Reactive protein, a sensitive marker of inflammation, predicts future risk of coronary heart disease in initially healthy middle-aged men: results from the MONICA (Monitoring Trends and Determinants in Cardiovascular Disease) Augsburg Cohort Study, 1984 to 1992. *Circulation.* (1999) 99:237–42.
13. Arenillas JF, Alvarez-Sabin J, Molina CA, Chacon P, Montaner J, Rovira A, et al. C-reactive protein predicts further ischemic events in first-ever transient ischemic attack or stroke patients with intracranial large-artery occlusive disease. *Stroke.* (2003) 34:2463–8. doi: 10.1161/01.STR.0000089920.93927.A7
14. Danesh J, Wheeler JG, Hirschfield GM, Eda S, Eiriksdottir G, Rumley A, et al. C-reactive protein and other circulating markers of inflammation in the prediction of coronary heart disease. *N Engl J Med.* (2004) 350:1387–97. doi: 10.1056/NEJMoa032804
15. Ridker PM, Cannon CP, Morrow D, Rifai N, Rose LM, McCabe CH, et al. C-reactive protein levels and outcomes after statin therapy. *N Engl J Med.* (2005) 352:20–8. doi: 10.1056/NEJMoa042378
16. Pepys MB, Hirschfield GM, Tennent GA, Gallimore JR, Kahan MC, Bellotti V, et al. Targeting C-reactive protein for the treatment of cardiovascular disease. *Nature.* (2006) 440:1217–21. doi: 10.1038/nature04672
17. Peters SA, Visseren FL, Grobbee DE. Biomarkers. Screening for C-reactive protein in CVD prediction. *Nat Rev Cardiol.* (2013) 10:12–4. doi: 10.1038/nrcardio.2012.164
18. Ridker PM, MacFadyen JG, Everett BM, Libby P, Thuren T, Glynn RJ, et al. Relationship of C-reactive protein reduction to cardiovascular event reduction following treatment with canakinumab: a secondary analysis from the CANTOS randomised controlled trial. *Lancet.* (2018) 391:319–28. doi: 10.1016/S0140-6736(17)32814-3
19. Mani P, Puri R, Schwartz GG, Nissen SE, Shao M, Kastelein JJP, et al. Association of initial and serial c-reactive protein levels with adverse cardiovascular events and death after acute coronary syndrome: a secondary analysis of the VISTA-16 trial. *JAMA Cardiol.* (2019) 4:314–20. doi: 10.1001/jamacardio.2019.0179
20. Barrett TD, Hennen JK, Marks RM, Lucchesi BR. C-reactive-protein-associated increase in myocardial infarct size after ischemia/reperfusion. *J Pharmacol Exp Ther.* (2002) 303:1007–13. doi: 10.1124/jpet.102.040600
21. Szalai AJ, McCrory MA, Xing D, Hage FG, Miller A, Oparil S, et al. Inhibiting C-reactive protein for the treatment of cardiovascular disease: promising evidence from rodent models. *Mediators Inflamm.* (2014) 2014:353614. doi: 10.1155/2014/353614
22. Sheriff A, Schindler R, Vogt B, Abdel-Aty H, Unger JK, Bock C, et al. Selective apheresis of C-reactive protein: a new therapeutic option in myocardial infarction? *J Clin Apher.* (2015) 30:15–21. doi: 10.1002/jca.21344
23. Boncler M, Wu Y, Watala C. The multiple faces of c-reactive protein—physiological and pathophysiological implications in cardiovascular disease. *Molecules.* (2019) 24:2062. doi: 10.3390/molecules24112062
24. Braig D, Nero TL, Koch H-G, Kaiser B, Wang X, Thiele JR, et al. Transitional changes in the CRP structure lead to the exposure of proinflammatory binding sites. *Nat Commun.* (2017) 8:14188. doi: 10.1038/ncomm14188
25. McFadyen JD, Kiefer J, Braig D, Loseff-Silver J, Potempa LA, Eisenhardt SU, et al. Dissociation of c-reactive protein localizes and amplifies inflammation: evidence for a direct biological role of c-reactive protein and its conformational changes. *Front Immunol.* (2018) 9:1351. doi: 10.3389/fimmu.2018.01351
26. Thiele JR, Zeller J, Bannasch H, Stark GB, Peter K, Eisenhardt SU. Targeting C-reactive protein in inflammatory disease by preventing conformational changes. *Med Inflamm.* (2015) 2015:372432. doi: 10.1155/2015/372432
27. Kunze R. C-reactive protein: from biomarker to trigger of cell death? *Ther Apher Dial.* (2019) 23:494–6. doi: 10.1111/1744-9987.12802
28. Szalai AJ, Briles DE, Volanakis JE. Role of complement in C-reactive-protein-mediated protection of mice from *Streptococcus pneumoniae*. *Infect Immunity.* (1996) 64:0–4853.
29. Mold C, Rodic-Polic B, Du Clos TW. Protection from Streptococcus pneumoniae infection by c-reactive protein and natural antibody requires complement but not Fcγ receptors. *J Immunol.* (2002) 168:6375–81. doi: 10.4049/jimmunol.168.12.6375
30. Sparkes BL, Woods K, Roth M, Welti R, and Fleming SD. (2009) Phospholipase A2 alters membrane lipid composition during ischemia/reperfusion (39.55). *The Journal of Immunology* 182(1 Supplement), 39.55–39.55
31. Nijmeijer R, Lagrand WK, Baidoshvili A, Lubbers YT, Hermens WT, Meijer CJ, et al. Secretory type II phospholipase A2 binds to ischemic myocardium during myocardial infarction in humans. *Cardiovasc Res.* (2002) 53:138–46. doi: 10.1016/s0008-6363(01)00480-1
32. Nijmeijer R, Willemsen M, Meijer CJ, Visser CA, Verheijen RH, Gottlieb RA, et al. Type II secretory phospholipase A2 binds to ischemic flip-flopped cardiomyocytes and subsequently induces cell death. *Am J Physiol Heart Circ Physiol.* (2003) 285:H2218–24. doi: 10.1152/ajpheart.00887.2002
33. Murakami M, Taketomi Y, Sato H, Yamamoto K. Secreted phospholipase A2 revisited. *J Biochem.* (2011) 150:233–55. doi: 10.1093/jb/mvr088
34. Yagami T, Yamamoto Y, Koma H. The role of secretory phospholipase A(2) in the central nervous system and neurological diseases. *Mol Neurobiol.* (2014) 49:863–76. doi: 10.1007/s12035-013-8565-9
35. Kushner I, Kaplan MH. Studies of acute phase protein: I. An immunohistochemical method for the localization of cx-reactive protein in rabbits association with necrosis in local inflammatory lesions. *J Exp Med.* (1961) 114:961–74. doi: 10.1084/jem.114.6.961
36. Kushner I, Rakita L, Kaplan MH. Studies of acute-phase protein. II. Localization of Cx-reactive protein in heart in induced myocardial infarction in rabbits. *J Clin Invest.* (1963) 42:286–92. doi: 10.1172/jci104715
37. Narkates AJ, Volanakis JE. C-reactive protein binding specificities: artificial and natural phospholipid bilayers. *Ann N Y Acad Sci.* (1982) 389:172–82. doi: 10.1111/j.1749-6632.1982.tb22135.x
38. Vogt B, Fuhrnrohr B, Muller R, Sheriff A. CRP and the disposal of dying cells: consequences for systemic lupus erythematosus and rheumatoid arthritis. *Autoimmunity.* (2007) 40:295–8. doi: 10.1080/08916930701358925
39. Goda T, Miyahara Y. Calcium-independent binding of human C-reactive protein to lysophosphatidylcholine in supported planar phospholipid monolayers. *Acta Biomaterialia.* (2017) 48:206–14. doi: 10.1016/j.actbio.2016.10.043
40. Sproston NR, Ashworth JJ. Role of C-reactive protein at sites of inflammation and infection. *Front Immunol.* (2018) 9:754–4. doi: 10.3389/fimmu.2018.00754
41. Kayser S, Brunner P, Althaus K, Dorst J, Sheriff A. Selective apheresis of c-reactive protein for treatment of indications with elevated

- CRP concentrations. *J Clin Med.* (2020)9:2947. doi: 10.3390/jcm902947
42. Dimitrijević O, Stojcevski BD, Ignjatović S, Singh NM. Serial measurements of C-reactive protein after acute myocardial infarction in predicting one-year outcome. *Int Heart J.* (2006) 47:833–42. doi: 10.1536/ihj.47.833
43. Reindl M, Reinstadler SJ, Feistritz HJ, Klug G, Tiller C, Mair J, et al. Relation of inflammatory markers with myocardial and microvascular injury in patients with reperfused ST-elevation myocardial infarction. *Eur Heart J Acute Cardiovasc Care.* (2017) 6:640–9. doi: 10.1177/2048872616661691
44. Stumpf C, Sheriff A, Zimmermann S, Schaeffner L, Schlundt C, Raaz D, et al. C-reactive protein levels predict systolic heart failure and outcome in patients with first ST-elevation myocardial infarction treated with coronary angioplasty. *Arch Med Sci.* (2017) 13:1086–93. doi: 10.5114/aoms.2017.69327
45. Hack CE, Wolbink GJ, Schalkwijk C, Speijer H, Hermens WT, van den Bosch H. A role for secretory phospholipase A2 and C-reactive protein in the removal of injured cells. *Immunol Today.* (1997) 18:111–5. doi: 10.1016/j.imm.1997.09.013
46. Griselli M, Herbert J, Hutchinson WL, Taylor KM, Sohail M, Krausz T, et al. C-reactive protein and complement are important mediators of tissue damage in acute myocardial infarction. *J Exp Med.* (1999) 190:1733–40. doi: 10.1084/jem.192.9.1353
47. Gershov D, Kim S, Brot N, Elkun KB. C-Reactive protein binds to apoptotic cells, protects the cells from assembly of the terminal complement components, and sustains an antiinflammatory innate immune response: implications for systemic autoimmunity. *J Exp Med.* (2000) 192:1353–64. doi: 10.1084/jem.192.9.1353
48. Nijmeijer R, Lagrand WK, Lubbers YT, Visser CA, Meijer CJ, Niessen HW, et al. C-reactive protein activates complement in infarcted human myocardium. *Am J Pathol.* (2003) 163:269–75. doi: 10.1016/S0002-9440(10)63650-4
49. Gill R, Kemp JA, Sabin C, Pepys MB. Human C-reactive protein increases cerebral infarct size after middle cerebral artery occlusion in adult rats. *J Cereb Blood Flow Metab.* (2004) 24:1214–8. doi: 10.1097/01.Wcb.0000136517.61642.99
50. Slagman AC, Bock C, Abdel-Aty H, Vogt B, Gebauer F, Janelt G, et al. Specific removal of C-reactive protein by apheresis in a porcine cardiac infarction model. *Blood Purif.* (2011) 31:9–17. doi: 10.1159/000320763
51. You YK, Huang XR, Chen HY, Lyu XF, Liu HF, Lan HY. C-reactive protein promotes diabetic kidney disease in db/db mice via the CD32b-Smad3-mTOR signaling pathway. *Sci Rep.* (2016) 6:26740. doi: 10.1038/srep26740
52. Newling M, Sritharan L, van der Ham AJ, Hoepel W, Fiechter RH, de Boer L, et al. C-reactive protein promotes inflammation through fcyr-induced glycolytic reprogramming of human macrophages. *J Immunol.* (2019) 203:225–35. doi: 10.4049/jimmunol.1900172
53. Sun W, Wu Y, Gao M, Tian Y, Qi P, Shen Y, et al. C-reactive protein promotes inflammation through TLR4/NF- κ B/TGF- β pathway in HL-1 cells. *Biosci Rep.* (2019) 39:BSR20190888. doi: 10.1042/bsr20190888
54. Zhang L, Shen ZY, Wang K, Li W, Shi JM, Osoro EK, et al. C-reactive protein exacerbates epithelial-mesenchymal transition through Wnt/ β -catenin and ERK signaling in streptozotocin-induced diabetic nephropathy. *Faseb J.* (2019) 33:6551–63. doi: 10.1096/fj.201801865RR
55. Schneeweis C, Gräfe M, Bungenstock A, Spencer-Hänsch C, Fleck E, Goetze S. Chronic CRP-exposure inhibits VEGF-induced endothelial cell migration. *J Atheroscler Thromb.* (2010) 17:203–12. doi: 10.5551/jat.3004
56. Del Giudice M, Gangestad SW. Rethinking IL-6 and CRP: Why they are more than inflammatory biomarkers, and why it matters. *Brain Behav Immun.* (2018) 70:61–75. doi: 10.1016/j.bbi.2018.02.013
57. Rajab IM, Hart PC, Potempa LA. How C-reactive protein structural isoforms with distinctive bioactivities affect disease progression. *Front Immunol.* (2020) 11:2126. doi: 10.3389/fimmu.2020.02126
58. Bhakdi S, Torzewski M, Paprotka K, Schmitt S, Barsom H, Suriyaphol P, et al. Possible protective role for C-reactive protein in atherogenesis: complement activation by modified lipoproteins halts before detrimental terminal sequence. *Circulation.* (2004) 109:1870–6. doi: 10.1161/01.Cir.0000124228.08972.26
59. Torzewski M, Bhakdi S. Complement and atherosclerosis—united to the point of no return? *Clin Biochem.* (2013) 46:20–5. doi: 10.1016/j.clinbiochem.2012.09.012
60. Haapasalo K, Meri S. Regulation of the complement system by pentraxins. *Front Immunol.* (2019) 10:1750. doi: 10.3389/fimmu.2019.01750
61. Bock C, Vogt B, Mattheck S, Yapici G, Brunner P, Fimpel S, et al. C-reactive protein causes blood pressure drop in rabbits and induces intracellular calcium signaling. *Front Immunol.* (2020) 11:1978. doi: 10.3389/fimmu.2020.01978
62. Abramson JL, Weintraub WS, Vaccarino V. Association between pulse pressure and C-reactive protein among apparently healthy US adults. *Hypertension.* (2002) 39:197–202. doi: 10.1161/hy0202.104270
63. Anzai T, Yoshikawa T, Takahashi T, Maekawa Y, Okabe T, Asakura Y, et al. Early use of beta-blockers is associated with attenuation of serum C-reactive protein elevation and favorable short-term prognosis after acute myocardial infarction. *Cardiology.* (2003) 99:47–53. doi: 10.1159/000068449
64. Whelton SP, Nara V, Blaha MJ, Nasir K, Blumenthal RS, Jenny NS, et al. Association between resting heart rate and inflammatory biomarkers (high-sensitivity C-reactive protein, interleukin-6, and fibrinogen) (from the Multi-Ethnic Study of Atherosclerosis). *Am J Cardiol.* (2014) 113:644–9. doi: 10.1016/j.amjcard.2013.11.009
65. Bisioendial R, Birjmohun R, Keller T, van Leuven S, Levels H, Levi M, et al. In vivo effects of C-reactive protein (CRP)-infusion into humans. *Circ Res.* (2005) 97:e115–116. doi: 10.1161/01.RES.0000196746.75724.8b
66. Clapp BR, Hirschfield GM, Storry C, Gallimore JR, Stidwill RP, Singer M, et al. Inflammation and endothelial function: direct vascular effects of human C-reactive protein on nitric oxide bioavailability. *Circulation.* (2005) 111:1530–6. doi: 10.1161/01.CIR.0000159336.31613.31
67. Bisioendial RJ, Kastelein JJ, Peters SL, Levels JH, Birjmohun R, Rotmans JJ, et al. Effects of CRP infusion on endothelial function and coagulation in normocholesterolemic and hypercholesterolemic subjects. *J Lipid Res.* (2007) 48:952–60. doi: 10.1194/jlr.P600014-JLR200
68. Balk RA. Systemic inflammatory response syndrome (SIRS): where did it come from and is it still relevant today? *Virulence.* (2014) 5:20–6. doi: 10.4161/viru.27135
69. Berg CWVD, Taylor KE, Lang D. C-reactive protein-induced in vitro vasorelaxation is an artefact caused by the presence of sodium azide in commercial preparations. *Arterioscl Thromb Vasc Biol.* (2004) 24:e168–71. doi: 10.1161/01.ATV.0000142807.92781.d9
70. Lafuente N, Azcutia V, Matesanz N, Cercas E, Rodríguez-Mañas L, Sánchez-Ferrer CF, et al. Evidence for sodium azide as an artifact mediating the modulation of inducible nitric oxide synthase by c-reactive protein. *J Cardiovasc Pharmacol.* (2005) 45:193–6. doi: 10.1097/01.fjc.0000154371.95907.bd
71. Liu C, Wang s, deb a, nath ka, katusic zs, mcconnell jp, et al. proapoptotic, antimigratory, antiproliferative, and antiangiogenic effects of commercial c-reactive protein on various human endothelial cell types in vitro. *Circ Res.* (2005) 97:135–43. doi: 10.1161/01.RES.0000174612.90094.fd
72. Fleming I, Busse R. Signal transduction of eNOS activation. *Cardiovasc Res.* (1999) 43:532–41. doi: 10.1016/s0008-6363(99)00094-2
73. Fleming I, Fisslthaler B, Dimpeler S, Kemp BE, Busse R. Phosphorylation of Thr(495) regulates Ca(2+)/calmodulin-dependent endothelial nitric oxide synthase activity. *Circ Res.* (2001) 88:E68–75. doi: 10.1161/hh1101.092677
74. Torzewski M, Waqar AB, Fan J. Animal models of C-reactive protein. *Mediators Inflamm.* (2014) 2014:683598. doi: 10.1155/2014/683598
75. Koike T, Kitajima S, Yu Y, Nishijima K, Zhang J, Ozaki Y, et al. Human C-reactive protein does not promote atherosclerosis in transgenic rabbits. *Circulation.* (2009) 120:2088–94. doi: 10.1161/circulationaha.109.872796
76. Jones NR, Pegues MA, McCrory MA, Singleton W, Bethune C, Baker BF, et al. A selective inhibitor of human c-reactive protein translation is efficacious in vitro and in c-reactive protein transgenic mice and humans. *Mol Ther Nucleic Acids.* (2012) 1:e52. doi: 10.1038/mtna.2012.44
77. Ries W, Sheriff A, Heigl F, Zimmermann O, Garlischs CD, Torzewski J. “First in Man”: Case report of selective c-reactive protein apheresis in a patient with acute st segment elevation myocardial infarction. *Case Rep Cardiol.* (2018) 2018:4767105. doi: 10.1155/2018/4767105
78. Mattheck S, Brunner P, Hänel B, Kunze R, Vogt B, Sheriff A. PentraSorb C-reactive protein: characterization of the selective c-reactive protein adsorber resin. *Therapeutic Apheresis and Dialysis.* (2019) 23:474–81. doi: 10.1111/1744-9987.12796
79. Ries W, Heigl F, Garlischs C, Sheriff A, Torzewski J. Selective C-Reactive Protein-Apheresis in Patients. *Ther Apher Dial.* (2019) 23:570–4. doi: 10.1111/1744-9987.12804

80. Stone GW, Selker HP, Thiele H, Patel MR, Udelson JE, Ohman EM, et al. Relationship between infarct size and outcomes following primary pci: patient-level analysis from 10 randomized trials. *J Am Coll Cardiol.* (2016) 67:1674–83. doi: 10.1016/j.jacc.2016.01.069
81. de Waha S, Patel MR, Granger CB, Ohman EM, Maehara A, Eitel I, et al. Relationship between microvascular obstruction and adverse events following primary percutaneous coronary intervention for ST-segment elevation myocardial infarction: an individual patient data pooled analysis from seven randomized trials. *Eur Heart J.* (2017) 38:3502–10. doi: 10.1093/eurheartj/ehx414
82. Frangogiannis NG, Smith CW, Entman ML. The inflammatory response in myocardial infarction. *Cardiovasc Res.* (2002) 53:31–47. doi: 10.1016/s0008-6363(01)00434-5
83. Frangogiannis NG. Regulation of the inflammatory response in cardiac repair. *Circ Res.* (2012) 110:159–73. doi: 10.1161/CIRCRESAHA.111.243162
84. Pietila K, Harmoinen A, Hermens W, Simoons ML, Van de Werf F, Verstraete M. Serum C-reactive protein and infarct size in myocardial infarct patients with a closed versus an open infarct-related coronary artery after thrombolytic therapy. *Eur Heart J* (1993) 14:915–919. doi: 10.1093/eurheartj/14.7.915
85. Beranek JT. C-reactive protein and complement in myocardial infarction and postinfarction heart failure. *Eur Heart J.* (1997) 18:1834–6. doi: 10.1093/oxfordjournals.eurheartj.a015180
86. Suleiman M, Khatib R, Agmon Y, Mahamid R, Boulos M, Kapeliovich M, et al. Early inflammation and risk of long-term development of heart failure and mortality in survivors of acute myocardial infarction predictive role of C-reactive protein. *J Am Coll Cardiol.* (2006) 47:962–8. doi: 10.1016/j.jacc.2005.10.055
87. Liu D, Qi X, Li Q, Jia W, Wei L, Huang A, et al. Increased complements and high-sensitivity C-reactive protein predict heart failure in acute myocardial infarction. *Biomed Rep.* (2016) 5:761–5. doi: 10.3892/br.2016.793
88. Pepys MB. C-reactive protein fifty years on. *Lancet.* (1981) 1:653–7. doi: 10.1016/s0140-6736(81)91565-8
89. Mevorach D, Mascarenhas JO, Gershov D, Elkon KB. Complement-dependent clearance of apoptotic cells by human macrophages. *J Exp Med.* (1998) 188:2313–20.
90. Lagrand WK, Niessen HW, Wolbink GJ, Jaspars LH, Visser CA, Verheugt FW, et al. C-reactive protein colocalizes with complement in human hearts during acute myocardial infarction. *Circulation.* (1997) 95:97–103.
91. Valtchanova-Matchouganska A, Gondwe M, Nadar A. The role of C-reactive protein in ischemia/reperfusion injury and preconditioning in a rat model of myocardial infarction. *Life sciences.* (2004) 75:901–10. doi: 10.1016/j.lfs.2003.12.029
92. Heinecke JW. Chemical knockout of C-reactive protein in cardiovascular disease. *Nat Chem Biol.* (2006) 2:300–1. doi: 10.1038/nchembio0606-300
93. Kitsis RN, Jialal I. Limiting myocardial damage during acute myocardial infarction by inhibiting C-reactive protein. *N Engl J Med.* (2006) 355:513–5. doi: 10.1056/NEJMcibr063197
94. Krijnen PA, Meischl C, Nijmeijer R, Visser CA, Hack CE, Niessen HW. Inhibition of sPLA2-IIA, C-reactive protein or complement: new therapy for patients with acute myocardial infarction? *Cardiovasc Hematol Disord Drug Targets.* (2006) 6:113–23. doi: 10.2174/187152906777441830
95. Boljevic D, Nikolic A, Rusovic S, Lakcevic J, Bojic M, Balint B. A promising innovative treatment for st-elevation myocardial infarction: the use of c-reactive protein selective apheresis: case report. *Blood Purification.* (2020) 49:753–7. doi: 10.1159/000506176
96. Ries W, Heigl F, Garlachs C, Sheriff A, Torzewski J. *Die CRP-Apherese: Eine neue Therapiemöglichkeit bei Inflammation.* Medicom: Nephro-News (2019).
97. Reinstadler SJ, Stiermaier T, Fuernau G, de Waha S, Desch S, Metzler B, et al. The challenges and impact of microvascular injury in ST-elevation myocardial infarction. *Expert Review of Cardiovascular Therapy.* (2016) 14:431–43. doi: 10.1586/14779072.2016.1135055
98. Muir KW, Tyrrell P, Sattar N, Warburton E. Inflammation and ischaemic stroke. *Curr Opin Neurol.* (2007) 20:334–42. doi: 10.1097/WCO.0b013e32813ba151
99. Anrather J, Iadecola C. Inflammation and Stroke: An Overview. *Neurotherapeutics.* (2016) 13:661–70. doi: 10.1007/s13311-016-0483-x
100. Winbeck K, Poppert H, Etgen T, Conrad B, Sander D. Prognostic relevance of early serial C-reactive protein measurements after first ischemic stroke. *Stroke.* (2002) 33:2459–64. doi: 10.1161/01.str.0000029828.51413.82
101. Montaner J, Fernandez-Cadenas I, Molina CA, Ribo M, Huertas R, Rosell A, et al. Poststroke C-reactive protein is a powerful prognostic tool among candidates for thrombolysis. *Stroke.* (2006) 37:1205–10. doi: 10.1161/01.STR.0000217744.89208.4e
102. Muir KW, Weir CJ, Alwan W, Squire IB, Lees KR. C-reactive protein and outcome after ischemic stroke. *Stroke.* (1999) 30:981–5. doi: 10.1161/01.str.30.5.981
103. Elkind MS, Tai W, Coates K, Paik MC, Sacco RL. High-sensitivity C-reactive protein, lipoprotein-associated phospholipase A2, and outcome after ischemic stroke. *Arch Intern Med.* (2006) 166:2073–80. doi: 10.1001/archinte.166.19.2073
104. Wadman M, Couzin-Frankel J, Kaiser J, Maticic C. A rampage through the body. *Science.* (2020) 368:356–60. doi: 10.1126/science.368.6489.356
105. Mueller AA, Tamura T, Crowley CP, DeGrado JR, Haider H, Jezmir JL, et al. Inflammatory biomarker trends predict respiratory decline in COVID-19 patients. *Cell Rep Med.* (2020) 1:100144. doi: 10.1016/j.xcrm.2020.100144
106. Ruan Q, Yang K, Wang W, Jiang L, Song J. Clinical predictors of mortality due to COVID-19 based on an analysis of data of 150 patients from Wuhan, China. *Intensive Care Med.* (2020) 46:846–8. doi: 10.1007/s00134-020-05991-x
107. Chen G, Wu D, Guo W, Cao Y, Huang D, Wang H, et al. Clinical and immunological features of severe and moderate coronavirus disease 2019. *J Clin Invest.* (2020) 130:2620–9. doi: 10.1172/JCI137244
108. Tan C, Huang Y, Shi F, Tan K, Ma Q, Chen Y, et al. C-reactive protein correlates with computed tomographic findings and predicts severe COVID-19 early. *J Med Virol.* (2020) 92:856–62. doi: 10.1002/jmv.25871
109. Nienhold R, Ciani Y, Koelzer VH, Tzankov A, Haslbauer JD, Menter T, et al. Two distinct immunopathological profiles in autopsy lungs of COVID-19. *Nat Commun.* (2020) 11:5086. doi: 10.1038/s41467-020-18854-2
110. Puntmann VO, Carerj ML, Wieters I, Fahim M, Arendt C, Hoffmann J, et al. Outcomes of cardiovascular magnetic resonance imaging in patients recently recovered from coronavirus disease 2019 (COVID-19). *JAMA Cardiol.* (2020) 5:1265–73. doi: 10.1001/jamacardio.2020.3557
111. Inciardi RM, Lupi L, Zacccone G, Italia L, Raffo M, Tomasoni D, et al. Cardiac involvement in a patient with coronavirus disease 2019 (COVID-19). *JAMA Cardiology.* (2020) 5:819–24. doi: 10.1001/jamacardio.2020.1096
112. Xu Z, Shi L, Wang Y, Zhang J, Huang L, Zhang C, et al. Pathological findings of COVID-19 associated with acute respiratory distress syndrome. *Lancet Respir Med.* (2020) 8:420–2. doi: 10.1016/S2213-2600(20)30076-X
113. Kayser S, Kunze R, Sheriff A. Selective C-reactive protein apheresis for Covid-19 patients suffering from organ damage. *Ther Apher Dial.* (2020) doi: 10.1111/1744-9987.13532
114. Torzewski J, Heigl F, Zimmermann O, Wagner F, Schumann C, Hettich R, et al. First-in-man: case report of selective c-reactive protein apheresis in a patient with sars-cov-2 infection. *Am J Case Rep.* (2020) 21:e925020. doi: 10.12659/AJCR.925020
115. Di Napoli M, Schwaninger M, Cappelli R, Ceccarelli E, Di Gianfilippo G, Donati C, et al. Evaluation of C-reactive protein measurement for assessing the risk and prognosis in ischemic stroke: a statement for health care professionals from the CRP Pooling Project members. *Stroke.* (2005) 36:1316–29. doi: 10.1161/01.STR.0000165929.78756.ed

Conflict of Interest: AS was CEO and shareholder of Pentracor GmbH. SK was employed by Pentracor GmbH. BV was shareholder and employee of Pentracor GmbH. PB was employed by iAdsorb GmbH.

Copyright © 2021 Sheriff, Kayser, Brunner and Vogt. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Anti-Pentraxin Antibodies in Autoimmune Diseases: Bystanders or Pathophysiological Actors?

Benoit Brilland^{1,2*}, Emeline Vinatier^{2,3}, Jean-François Subra^{1,2}, Pascale Jeannin^{2,3}, Jean-François Augusto^{1,2} and Yves Delneste^{2,3}

¹ CHU Angers, Service de Néphrologie-Dialyse-Transplantation, Angers, France, ² Université d'Angers, INSERM, CRCINA, Angers, France, ³ CHU Angers, Service d'Immunologie et Allergologie, Angers, France

OPEN ACCESS

Edited by:

Barbara Bottazzi,
University of Milan, Italy

Reviewed by:

Angelo A. Manfredi,
Vita-Salute San Raffaele University,
Italy

Peter A. Ward,
University of Michigan, United States

*Correspondence:

Benoit Brilland
benoit.brilland@chu-angers.fr

Specialty section:

This article was submitted to
Cytokines and Soluble
Mediators in Immunity,
a section of the journal
Frontiers in Immunology

Received: 05 November 2020

Accepted: 29 December 2020

Published: 16 February 2021

Citation:

Brilland B, Vinatier E, Subra J-F, Jeannin P, Augusto J-F and Delneste Y (2021) Anti-Pentraxin Antibodies in Autoimmune Diseases: Bystanders or Pathophysiological Actors? *Front. Immunol.* 11:626343. doi: 10.3389/fimmu.2020.626343

Pentraxins are soluble innate immunity receptors involved in sensing danger molecules. They are classified as short (CRP, SAP) and long pentraxin subfamilies, including the prototypic long pentraxin PTX3. Pentraxins act mainly as bridging molecules favoring the clearance of microbes and dead cells. They are also involved in many other biological processes, such as regulation of complement activation, inflammation and tissue homeostasis. Autoantibodies directed against pentraxins have been reported in various autoimmune diseases, especially in systemic lupus erythematosus and ANCA-associated vasculitis. In this review, we review the main biological characteristics and functions of pentraxins and summarize data concerning autoantibodies directed against pentraxins in the context of autoimmune diseases and discuss their potential pathological role.

Keywords: pentraxins, autoimmunity, systemic lupus erythematosus, ANCA-associated vasculitis, anti-pentraxin autoantibodies

INTRODUCTION

The innate immune system is involved in many physiological processes, including antimicrobial defence, inflammation, initiation and regulation of adaptive immunity and maintenance of tissue homeostasis (1). This system has a cellular arm, composed principally of myeloid cells and innate lymphoid cells, and a humoral arm, including soluble innate immunity receptors, also known as soluble pattern recognition molecules (sPRM) (2). The innate immune system specializes in sensing of so-called “danger signals” that originate from (i) non-self, such as microbes; the microbial motifs recognized by innate cells are called pathogen-associated molecular patterns (PAMPs) or (ii) from modified (or altered) self, mainly dying cells and molecules expressed by damaged cells/tissues or accidentally released in the extracellular environment; these motifs are called damage-associated molecular patterns (DAMPs).

Abbreviations: AAV, ANCA-associated vasculitis; Ab, antibody; ANCA, anti-neutrophil cytoplasm antibody; CRP, C-reactive protein; DNA, deoxyribonucleic acid; MHC, major histocompatibility complex; MPO, myeloperoxidase; PAPS, primary antiphospholipid syndrome; PR3, proteinase 3; PTX, pentraxin; RA, rheumatoid arthritis; SAP, serum amyloid P; SLE, systemic lupus erythematosus; SLEDAI, SLE activity index; SS, Sjögren syndrome; SSA, Sjögren's syndrome-related antigen A; SSc, systemic sclerosis.

The detection of danger signals by innate immunity receptors is crucial in initiating appropriate immune responses that are fine-tuned to the motifs encountered (tolerance to self, protection against non-self). These receptors are highly conserved molecules called Pattern Recognition Receptors (PRR). PRR recognize a wide variety of ligands, including proteins, lipids, carbohydrates, and nucleic acids. In addition to innate immune cells (of myeloid and lymphoid origin), PRR are expressed by a wide variety of cell types, including endothelial cells, epithelial cells, and adaptive immune cells. Most PRR are involved in the initiation of inflammation and those expressed by professional antigen-presenting cells are critical in initiating antigen-specific immune responses.

PRR can be associated to cells (expressed at the surface or localized intracellularly) or released in the extracellular milieu (sPRM). According to their localization, cell-associated PRR can be classified in three groups (3): (i) membrane receptors, including membrane Toll-like receptors (TLR1, TLR2, TLR4, TLR5, and TLR6), scavenger receptors and C-type lectin receptors, (ii) endosomal receptors, including TLR3, TLR7, TLR8, and TLR9, and (iii) cytoplasmic receptors, including nucleotide-binding oligomerization domain (NOD)-like receptors (NLR), retinoic acid-inducible gene I (RIGI)-like receptors (RLR), and AIM2-like receptors (ALR). Membrane PRR are involved in the clearance of danger signals (i.e., phagocytosis of particulate motifs) and/or cell activation, while intracellular PRR are mainly involved in the detection of nucleic acids.

Soluble PRM act as bridging molecules, linking extracellular DAMPs and PAMPs with cell associated PRR. They include collectins, ficolins, some complement family proteins, soluble forms of membrane PRR (such as soluble scavenger receptors and C-type lectins released after shedding of the membrane forms), and by pentraxins. As their membrane counterparts, sPRM are highly conserved and bind a variety of ligands expressed by microbes and altered/modified self. Soluble PRM recognize the same molecular motifs that cell-associated PRR. They have a role in agglutination, complement activation and opsonisation (i.e., facilitating the recognition and elimination of danger motifs by phagocytes). By reference to their bridging activity, sPRM are usually viewed as ancestors of antibodies (4) and are sometimes referred to as opsonins. They also play a pivotal role in determining the nature of the immune responses induced by the motifs encountered.

A tolerance breakdown to sPRM, evidenced by the presence of autoantibodies, has been reported in different autoimmune diseases, especially autoantibodies targeting members of the pentraxin family. Such autoantibodies have been particularly reported in systemic lupus erythematosus (SLE) and anti-neutrophil cytoplasmic autoantibody (ANCA)-associated vasculitis. In this review, we present the main characteristics and functions of pentraxins and data on anti-pentraxin autoantibodies. Finally, we raise hypothesis on the potential pathological role(s) of anti-pentraxin autoantibodies.

THE PENTRAXIN FAMILY

Structure of Pentraxins

Pentraxins are characterized by a C-terminal “pentraxin domain” of about 200 amino acids, containing a highly conserved HxCxS/TWxS motif (where x represents any amino acid) called the “pentraxin signature” (5, 6). They are organized in a cyclic pentameric structure (4, 7). The pentraxin family is divided into two subfamilies, the short and long pentraxins, depending on the length of their amino acid sequences.

Short pentraxins are characterized by a short N-terminal domain (approximately 20 amino acids). This subfamily contains the acute phase proteins C-reactive protein (CRP), also known as PTX1, and serum amyloid P component (SAP), also known as PTX2.

Long pentraxins are characterized by a long N-terminal domain (178 amino acids). In addition to the prototypic long pentraxin PTX3, this group contains neuronal pentraxin 1 (NP1), NP2, neuronal pentraxin receptor (NPR) and PTX4 (4, 8). Contrary to PTX3, only few data are available on these other members of the long pentraxin subfamily and will thus not be further mentioned in this review.

Ligands and Immune Functions of Pentraxins

The Short Pentraxins CRP and SAP

The short pentraxins CRP and SAP are assembled as multimers of five or ten 25 kDa subunits of 204 and 205 amino acids, respectively. CRP and SAP are the main acute phase proteins in human and mouse, respectively. They are mainly produced by the liver in response to pro-inflammatory cytokines, such as IL-1 and IL-6. Under inflammatory situations, such as bacterial sepsis, sterile inflammation (such as gout) or massive tissue destruction (a process associated with the release of inflammatory endogenous molecules such as HSP or HMGB1), the circulating levels of CRP can rise up to 1,000-time their basal concentrations in human (3 mg/L) (9).

Short pentraxins recognize a large variety of ligands in a calcium-dependent manner. Of note, the first ligand described for CRP was the C-polysaccharide of *Streptococcus pneumoniae*, hence the name. SAP takes its name from the fact that it is one of the universal component of the amyloid deposits found in amyloidosis and Alzheimer's disease.

CRP and SAP promote microbe opsonisation, thus facilitating their elimination by phagocytes. The functions of short pentraxins are not limited to bacterial clearance as they can also bind many other ligands such as apoptotic cells, amyloid fibrils (10), and endogenous danger molecules, such as histones and nucleic acids released by damaged cells (11, 12).

Short pentraxins are potent regulators of complement pathways. They can activate the classical complement pathway by linking to the complement factor C1q (13) and the lectin complement pathway by binding to ficolins (14, 15). In contrast, they can inhibit the alternative pathway amplification loop by binding to factor H (16). Short pentraxins can also bind to IgG receptors (FcγR). This binding can either inhibit immune

complex-mediated phagocytosis or activate Fc γ R-mediated phagocytosis and cytokine secretion (17–20).

The Prototypic Long Pentraxin PTX3

PTX3 is assembled as multimers of 45 kDa (381 amino acids) subunits and is organized as an octamer with monomers linked by disulfide bonds. Unlike short pentraxins, PTX3 is rapidly neo-synthesized by many cell types (4, 5) upon activation by a diversity of signals, including microbes and microbial moieties and inflammatory cytokines (4, 21). Contrary to short pentraxins, PTX3 is constitutively expressed, with circulating levels of $\approx$ 200 ng/ml in healthy subjects. The levels of PTX3 are strongly and rapidly increased during inflammatory responses (800–1,000 ng/ml). PTX3 is also preformed in neutrophils and rapidly released upon activation (22, 23).

PTX3 binds to the same ligands that short pentraxins, but in a calcium-independent manner (5). Like short pentraxins, PTX3 opsonizes and facilitates the clearance of numerous bacteria (such as *Pseudomonas aeruginosa* and *Salmonella typhimurium*) (24, 25), virus (such as influenza virus) (26), fungi (such as *Aspergillus fumigatus*) (27), and apoptotic cells (4, 5) by phagocytes. As for CRP and SAP, PTX3 can recruit Fc γ R to induce phagocytosis in a way similar to IgG (17, 18, 20, 28, 29). PTX3 also modulates the complement cascade through its capacity to bind to proteins of the classical (C1q) and lectin (mannose-binding lectin, ficolins) complement pathways (13, 15). PTX3 thus facilitates the tissue deposition of iC3b, thereby promoting complement receptor 3 (CR3)-driven phagocytosis (28). It also increases the binding of activated complement on microbes initiated by ficolin-1. PTX3 interacts with the complement factor H, the main alternative pathway regulatory protein but without modulating its complement inhibitory activity (30). PTX3 also acts as a nodal point for the assembly of the cumulus oophorus hyaluronan-rich extracellular matrix.

Nevertheless, studies have reported that PTX3 may exhibit a pro-inflammatory activity, underlying its “dual complexity” (31). Indeed, PTX3-deficient mice or mice injected with PTX3 develop less severe phenotypes in various models of sterile inflammation (32–34). Especially, a direct role of PTX3 on endothelial cell functions has been described (i.e., dysfunction and morphological changes in the endothelial layer through a P-selectin/matrix metalloproteinase-1 pathway) (35, 36). Considering its diversity of functions, PTX3 is considered as a pivotal sPRM at the crossroad between innate immunity, inflammation, matrix deposition and female fertility (4).

Pentraxins, Cell Death, and Immune Responses

The management of dying cell clearance is critical to maintain tolerance and to avoid the initiation of auto-immune responses. Schematically, the dichotomy “tolerance versus auto-immunity” is determined by the type of cell death (apoptosis versus necrosis) and the absence or presence of inflammation (either induced by PAMPs or DAMPs) at the time of dying cell clearance.

Apoptosis is characterized by coordinated (programmed) processes allowing dying cells to be eliminated without release

of potentially harmful and inflammatory endogenous DAMPs. The clearance of early apoptotic cells is classically viewed as a dynamic tolerogenic process allowing maintaining or inducing regulatory T cell responses. In contrast, necrosis, which usually occurs following a severe aggression, whether sterile or not, is a passive, accidental cell death process accompanied by the release of intracellular components into the extracellular environment, among which are found potent inflammatory and cytotoxic molecules (such as histones and HMGB1) (37). Importantly, the processing of dying cell-derived antigens in an inflammatory context may lead to the activation/maturation of professional antigen-presenting cells and, ultimately, to the activation of self-reactive T cells.

Phagocytosis of apoptotic cells, also called efferocytosis, is a fine-tuned process, involving at least three molecular partners (38): (i) eat-me molecules (i.e., neo-expressed motifs allowing discriminating live and dying cells), (ii) sensing and internalization receptors expressed by phagocytes, such as complement component receptor (which recognize collectins), scavenger receptors, integrins, and other receptors (e.g., MER and CR3/CR4), and (iii) sPRM acting as bridging molecules between dying cells and phagocytes.

The pivotal role of opsonins in the clearance of apoptotic cells has been illustrated in C1q- and SAP-deficient mice. C1q-deficient mice spontaneously develop signs of autoimmunity (39) and mice with targeted deletion of the SAP gene spontaneously develop antinuclear autoimmunity and severe glomerulonephritis, a phenotype resembling human SLE (40).

While CRP and SAP have a facilitating role (11, 41, 42), the role dedicated to PTX3 remains unclear. Indeed, our team has demonstrated that preformed PTX3 contained within neutrophil granules is relocalized at the surface of dying neutrophils, thereby acting as an “eat-me” molecule to mediate their capture by phagocytes (43). In contrast, a study reported that PTX3 binds selectively to late apoptotic cells and inhibits their capture by phagocytes (42, 44) suggesting that short and long pentraxins, in the extracellular milieu, may have opposite impacts on apoptotic cells clearance.

It remains also difficult to reconcile *in vitro* and *in vivo* data. On the one hand, and contrary to what would have been suspected based on *in vitro* data, SLE-prone mice supplemented with CRP and CRP-transgenic mice develop a mild kidney disease (45–47). On the other hand, and in agreement with *in vitro* data, C57/BL6 SAP-deficient mice can produce autoantibodies and develop immune complex glomerulonephritis (40, 48). It is important to note that the genetic background is important in enabling autoimmune phenotype expression. Indeed, SAP^{-/-} C57/BL6 but not SAP^{-/-} 129/Sv mice, spontaneously develop autoantibodies (48). Moreover, the authors also reported that SAP^{-/-} and SAP^{-/-} human SAP transgenic mice exhibit a similar autoimmune profile, suggesting that differences may exist between the human and mouse SAP in their capacity to interact with DAMPs. A similar complexity has been reported in C1q-deficient mice, as C1q^{-/-} C57/BL6 $\times$ 129Sv but not C1q^{-/-} C57/BL6 mice spontaneously develop autoimmunity and

glomerulonephritis (39). These studies underline the important of genetic background of mice in their susceptibility to develop autoimmunity, suggesting that tolerance breakdown could be strain dependent or dependent on the strategy to knock out target gene expression (impact of genetic recombination on the expression of other key genes) and/or that differences exist between human and mouse sPRM in their capacity to interact with DAMPs. To the best of our knowledge, PTX3-deficient mice do not spontaneously develop signs of autoimmunity. However, they develop a more severe phenotype in a ischemia reperfusion injury model (49) but exhibit a reduced inflammation in an acute arthritis model (50), even though both models are associated with a strong inflammation and, certainly, consecutive or associated to cell death. Moreover, PTX3-deficient mice bred with SLE prone mice have a decreased ability to clear apoptotic cells and tissue damages are aggravated in lung but not in kidney. Collectively, these results suggest that PTX3 deficiency may favor tissue injury associated to the initiation of an autoimmune response (51).

The potential role of pentraxins during auto-immune diseases has been reinforced by translational studies. CRP has been found decreased during SLE flares whereas inflammation markers were elevated (52, 53), suggesting that low levels of CRP may be associated with an impaired efferocytosis (54). Moreover, we reported that the levels of PTX3 are lower in patients with SLE during active disease as compared to healthy subjects (55). Whether these decreases impact the efferocytosis process remains undetermined. Nevertheless, these results suggest that a decrease of pentraxin levels which may lead to a reduced efferocytosis, is associated with an autoimmune signature that can be induced by the processing of dying cells in an inflammatory environment.

Collectively, and although the predominant roles of pentraxins seem to be host-protective, detrimental effects were observed in certain experimental settings. This so-called “yin-yang” effect may be inherent to the multi-functional properties of pentraxins, the complexity of efferocytosis, the redundancy between sPRM and the potential interaction of pentraxins with different DAMPs released by dying cells (56).

ANTI-PENTRAXIN ANTIBODIES IN AUTOIMMUNE DISEASES

Antibodies directed against CRP, SAP, and PTX3 have been detected in various autoimmune diseases, especially in SLE and ANCA-associated vasculitis (AAV). Their potential roles in the pathogenesis of autoimmune diseases are described thereafter.

Anti-Pentraxin Autoantibodies in SLE

SLE is an autoimmune disease with a very heterogeneous clinical presentation. The prognosis is mainly dependent on renal impairment, affecting 50% to 70% of patients. SLE is characterized by the production of autoantibodies directed against a large panel of self-antigens, including mainly nuclear antigens (DNA, soluble nuclear antigens (Sm), ribonucleoproteins

(RNP) and histones) but also anti-pentraxin Abs. SLE is also characterized by the activation of complement and the deposition of immune complexes that lead to systemic tissue damages (especially renal, vascular, hematological, cutaneous, and articular lesions) (57). The emergence of autoantibodies is determined by environmental, hormonal, and genetic factors suspected to be involved in the dysregulation of the innate and adaptive immune systems. In a mechanistic point of view, SLE is driven by a strong activation of dendritic cells and the production of type I interferons and is associated to an altered efferocytosis (57, 58).

Anti-CRP Antibodies in SLE

Autoantibodies directed against CRP bind to the monomeric form but not the pentameric form (59). The dissociation of a pentameric to a monomeric form of CRP has been reported in inflammatory situations (60) and monomeric CRP accumulates at the surface of various cell types (B cells, NK cells, platelets) and in inflamed tissues (61).

Anti-CRP Abs have been initially reported in SLE (**Table 1**) (76). They are present in a significant proportion of patients, ranging from 22% to 78%. Only one study (65) did not found an increased prevalence of anti-CRP Abs in SLE patients compared to healthy subjects. This conflicting result may be due to different methods of Abs determination.

The correlation between the presence of anti-CRP Abs and clinical presentation at disease onset was not systematically evaluated (59, 62). In a study including 137 patients with SLE, Figueredo et al. showed that patients with anti-CRP Abs also had elevated levels of anti-double strand (ds) DNA and anti-phospholipids Abs and low levels of the complement component C3. The frequency of anti-CRP Abs was also more frequent in patients with nephritis (27% and 13% in patients with or without nephritis, respectively) (63). Nevertheless, this result was not confirmed in another study (69). In patients with nephritis, anti-CRP Abs were also more frequent (66). Moreover, patients with anti-CRP Abs also had a higher SLEDAI score (66, 70) and were more likely to present with acute renal injury (66). However, these results must be nuanced as no relation between disease activity and presence of anti-CRP Abs was found by others (67, 72). Focusing on renal involvement, a significant correlation was found between anti-CRP Abs and acute renal tubulointerstitial injury (inflammation and fibrosis) but not with specific glomerular lesions (66). Collectively, these data suggest that anti-CRP Abs reflect SLE activity rather than a specific pattern of SLE nephritis.

Anti-SAP Antibodies in SLE

To the best of our knowledge, only two studies reported the prevalence of anti-SAP Abs in SLE (65, 73) (**Table 1**). In two studies performed by the same team, Zandman et al (73). included 328 patients with SLE and found anti-SAP Abs in 44% of patients, slightly above the 20% found in the second study, as compared with 2% in healthy subjects (65). As for anti-CRP Abs, anti-SAP Abs were not associated with a specific disease phenotype (73), despite an association with anti-

TABLE 1 | Prevalence of anti-pentraxin antibodies in different autoimmune diseases.

HS	SLE	PAPS	SSc	SS	RA	AAV	Reference
Anti-CRP Abs, n (%)							
1/40 (2.5%)	39/50 (78%)	–	2/20 (10%)	–	–	–	(59)
5/100 (5%)	13/27 (48%)	–	–	2/16 (12.5%)	0/15 (0%)	–	(62)
4/80 (5%)	77/137 (51%)	68/127 (54%)	–	–	–	–	(63)
–	43/190 (22.6%)	–	–	–	23/103 (22.3%)	–	(64)
2/45 (4.4%)	12/150 (8%)	–	–	–	–	–	(65)
0/60 (0%)	20/49 (40.8%)*	–	–	–	–	–	(66)
	56/96 (59.3%)**						
1/34 (2.9%)	18/81 (22.2%)	–	–	–	–	–	(67)
0/60 (0%)	–	–	–	2/7 (28.6%)	–	1/20 (5%)	(68)
–	26/100 (26%)	–	–	–	–	–	(69)
21.01 ± 14.32	35.6 ± 35.1	–	–	–	–	–	(70)
5/92 (5.4%)	–	–	–	–	–	11/120 (9.2%)	(71)
10/36 (27.8%)	18/34 (52.9%)	–	–	–	–	–	(72)
Anti-SAP Abs, n (%)							
3/124 (2.4%)	145/328 (44%)	–	–	–	–	–	(73)
1/45 (2.2%)	20/100 (20%)	–	–	–	–	–	(65)
3/93 (3.2%)	–	–	–	–	–	21/120 (17.5%)	(71)
Anti-PTX3 Abs, n (%)							
4/93 (4.3%)	18/36 (50%)	–	–	–	1/40 (2.5%)	–	(55)
8/130 (6.2%)	60/130 (46.2%)	–	1/26 (3.8%)	2/26 (7.7%)	2/27 (7.1%)	–	(74)
12/227 (5.3%)	–	–	–	–	–	56/150 (37.3%)	(71)
2/100 (2%)	38/196 (19.4%)*	–	–	–	–	–	(75)
	61/150 (40.7%)*						

HS, healthy subjects; SLE, systemic lupus erythematosus; PAPS, primary antiphospholipid syndrome; SSc, systemic sclerosis; SS, Sjögren syndrome; RA, rheumatoid arthritis; AAV, ANCA-associated vasculitis.

*SLE patients without lupus nephritis.

**SLE patients with lupus nephritis.

dsDNA titers and disease severity index. The levels of auto-Abs lowered under treatment.

Anti-PTX3 Antibodies in SLE

Anti-PTX3 autoantibodies were first described in SLE patients by our team (55). Their strong prevalence ($\approx 50\%$ of patients, as compared to 4% in healthy subjects) was later confirmed in an independent study (74). In our cohort, we observed an association between anti-PTX3 Ab levels and disease activity (assessed by the SLEDAI score). Antibodies titers were also correlated with classical SLE activity biomarkers, such as anti-dsDNA Abs, anti-nuclear Abs and C3/C4 complement components levels (55). However, the study by Bassi et al. did not found any association between anti-PTX3 antibody positivity and SLE activity (74). The authors assessed disease activity using the ECLAM (European Consensus Lupus Activity Measurement) score, making it difficult to compare the two studies. Interestingly, in this study, the authors suggested that anti-PTX3 autoantibodies might provide protection from renal involvement as they represent an independent factor negatively associated with kidney injury. Similar results were reported in a recent study evaluating the prevalence of anti-PTX3 Abs in SLE (75). In this large cohort study, including 246 SLE patients and 100 healthy subjects, Yuan et al. found that patients with SLE nephritis had a lower prevalence of anti-PTX3 Abs compared with patients without nephritis (20% versus 40%, respectively). Moreover, the serum levels of anti-PTX3 Abs were negatively correlated with proteinuria in lupus nephritis. The levels of proteinuria, serum creatinine and the prevalence of thrombotic

microangiopathy were also higher in patients without anti-PTX3 Abs. Although it did not reach statistical significance, patients with anti-PTX3 antibodies were less likely to reach end stage kidney disease. PTX3 deposition and renal fibrosis were also significantly lower in anti-PTX3 Ab-positive patients than negative patients (74, 77). These results are consistent with data in animal models. Indeed, lupus prone NZB/NZW mice immunized with PTX3 produce anti-PTX3 Abs and have a delayed occurrence of nephritogenic Abs, a decreased proteinuria, and an increased survival (78).

These results suggest that the balance between the levels of serum PTX3 and anti-PTX3 autoantibodies might influence the nephritic process (progression or attenuation). Anti-PTX3 Abs are not only markers of the inflammatory status in SLE but may also be protective by counteracting the deleterious effects of tissue deposition of PTX3 or complement activation, especially in kidneys. In support, the occurrence of anti-dsDNA and anti-C1q Abs in PTX3-immunized mice was significantly delayed and their levels increased when anti-PTX3 autoantibodies were decreasing. Hence, anti-PTX3 antibodies may prevent the proinflammatory deposition of PTX3 in target organs, decreasing the amount of available autoantigens in inflamed kidneys. This suggests that anti-PTX3 Abs might inhibit the secretion of nephritogenic Abs in lupus nephritis (78).

Anti-Pentraxin Antibodies in AAV

ANCA-associated vasculitis (AAV) include granulomatosis with polyangiitis (GPA), micropolyangiitis (MPA) and granulomatosis with polyangiitis and hypereosinophilia

(EGPA). AAV are systemic pathologies related to small vessel inflammation whose prognosis is mainly linked to renal (rapidly progressive glomerulonephritis) and pulmonary (intra-alveolar hemorrhage) damages (79). These conditions can rapidly become life-threatening or lead to irreversible functional impairment of the affected organ if they are not promptly treated. Renal or pulmonary impairment can be associated with skin, ear-nose-throat, joint, or neurological damages. These autoimmune diseases are frequently associated with the presence of ANCA, which are diagnostic markers in 80%–90% of patients (80). ANCA-negative vasculitis have been described, especially in patients with EGPA (up to 50%) but also in patients with GPA or MPA (up to 10%) (81–83), raising the possibility of other autoantibodies which remain to be identified. The identification of these autoantibodies should be useful for the formal diagnosis of AAV, as early diagnosis is essential to initiate appropriate treatment in order to limit irreversible organ injuries and favor long-term patient survival (84). Some ANCA targeting “minor antigens” have been described (directed against bactericidal/permeability increasing protein, elastase, and cathepsin G) in various autoimmune diseases, especially connective tissue diseases, but their pathogenic role in AAV remains to be demonstrated (85, 86). Antibodies against pentraxins have been described in AAV patients.

Anti-CRP Antibodies in AAV

As reported by our group and by Tan et al. (68, 71), anti-CRP Abs were found more frequently in AAV patients as compared to healthy controls, but the levels were similar levels in both groups. To our knowledge, no other study addressed this issue.

Anti-SAP Antibodies in AAV

In our study, the only one to our knowledge that evaluated the prevalence of anti-SAP Abs in AAV patients (71), anti-SAP Ab titers were higher in AAV patients than in healthy subjects. Moreover, 17.5% of AAV patients were positive for anti-SAP Abs compared with 3.2% in the control groups. Among the anti-SAP-positive patients, two were ANCA negative, suggesting that anti-SAP Abs could represent a diagnostic marker in this group of patients.

Anti-PTX3 Antibodies in AAV

The fact that PTX3 is constitutively expressed by fully differentiated (mature) neutrophils suggests that it may represent a potential target for ANCA. Unlike short pentraxins which are synthesized in the liver (9), PTX3 is stored into neutrophil-specific granules (87). Moreover, PTX3 can be localized in neutrophil extra-cellular traps (NETs) with other intracellular proteins targeted by these autoantibodies, such as MPO and PR3 (56, 88). NETs result from a particular type of neutrophil death called NETosis (89), which is characterized by the release of the intracellular content, including genomic DNA. Extracellular DNA thus forms a network to which are associated intracellular molecules such as PR3 and MPO. NETs are microbicide, contributing to fight extracellular infectious agents. However, NETs can also be cytotoxic for endothelial cells and tissues during sterile inflammation. NETosis favors tolerance breakdown, promoting autoimmunity as evidenced by

the generation of ANCA through dendritic cell-mediated antigen presentation (90, 91). Moreover, ANCA can induce NETs, which can, in turn, activate the complement alternative pathway (92–94), leading to an amplification loop of neutrophil activation and death. Indeed, C5a will further attracts and primes neutrophils, rendering them more sensitive to ANCA-mediated activation.

Anti-PTX3 Abs were assessed in the serums of 150 AAV patients by our team (71). Nearly 40% of AAV patients had anti-PTX3 Abs. Moreover, Abs titers were higher in patients with active than in those with inactive AAV. In our cohort, 14 patients (6 EGPA, 6 MPA, and 2 GPA) were positive for indirect immunofluorescence (IIF) ANCA screening, but negative for major (MPO, PR3) and minor (lactoferrin, BPI, elastase, and cathepsin G) ANCA antigens. Interestingly half of them (7/14) had detectable anti-PTX3 Abs. Moreover, anti-PTX3 Abs were found to have a unique IIF pattern known as “small cytoplasmic” ANCA characterized by small-size cytoplasmic granules in methanol- and ethanol-fixed neutrophils, and less intense in formal-fixed neutrophils.

Nevertheless, we did not find any clue for a pathological role of these Abs. Indeed, no difference of renal outcome between anti-PTX3 autoantibody-positive and -negative patients was observed and no relation between anti-PTX3 Ab levels and kidney function at the time of AAV diagnosis was observed (personal unpublished data). It would probably be interesting to evaluate the prevalence of anti-PTX3 Abs in a larger cohort of ANCA-negative patients before to conclude on their clinical relevance and predictive value.

We may wonder whether anti-PTX3 Abs are really ANCA. Indeed, anti-PTX3 Abs were found positive by immunofluorescence on fixed neutrophils and anti-PTX3 Ab positivity was observed in ANCA-negative AAV patients. Finally, the biological role(s) of anti-PTX3 Abs remain(s) to be evaluated. The purification of anti-PTX3 Abs from the serum of patients without other detectable ANCA would allow analyzing the *in vitro* biological activity of these autoantibodies.

Anti-Pentraxin Antibodies in Other Autoimmune Systemic Diseases

Anti-Short Pentraxin Antibodies

Anti-CRP Abs have been identified in other autoimmune diseases, such as systemic sclerosis (SSc), rheumatoid arthritis (RA), and Sjögren syndrome (SS) patients, although at a lower prevalence than in SLE patients (**Table 1**). Interestingly, a high prevalence of anti-CRP Abs (54%) has been reported in patients with primary antiphospholipid syndrome (PAPS) (63). Patients with PAPS and anti-CRP Abs were more likely to present thrombosis or fetal loss (55% vs 20%). More studies are needed to evaluate if anti-CRP Abs could be used as a biomarker for thrombosis risk assessment.

To the best of our knowledge, the presence of anti-SAP Abs in disease other than SLE or AAV has not been reported.

Anti-PTX3 Antibodies

Anti-PTX3 Abs have been detected in other autoimmune diseases such as SSc, SS and RA, but at a frequency similar to healthy subjects (74).

ANTI-PENTRAXIN ANTIBODIES: PATHOGENIC ACTORS OR BYSTANDER MARKERS?

The presence of anti-pentraxin autoantibodies has been reported mainly in SLE and AAV. Interestingly, antibody levels are generally high at disease onset and decline during remission phases, suggesting that they may represent, at least, diagnostic markers.

It should be noted that, given the strong homology between pentraxins, and in particular between the short pentraxins CRP and SAP, the potential cross-reactivity of autoantibodies directed against pentraxins can be a source of error on the nature of the target as well as on their potential pathological roles.

At this time, we do not know whether these antibodies reflect only tolerance breakdown, like many other autoantibodies reported in these pathologies, or whether they play a pathophysiological role. A potential pathological role for autoantibodies is supported by data on MPO-ANCA for which a pathogenic role is highly suspected. In addition to their capacity to activate neutrophils, MPO-ANCA induce glomerulonephritis when injected in mice (95) and vasculitis in newborns after placental transmission (96). Although neonatal lupus with congenital atrioventricular block after placental transmission anti-Sjögren's-syndrome-related antigen A (SSA) Abs has been described (97), the pathogenicity of antinuclear Abs remains a matter of debate.

To date, no experimental *in vitro* or *in vivo* data supports a pathogenic role for anti-pentraxin Abs. Purifying anti-pentraxin Abs may allow to evaluate their potential pathological role, as previously described for anti-MPO and anti-PR3 Abs. However, the cross-reactivity of anti-pentraxin antibodies, which is more than likely due to strong sequence (especially between CRP, SAP, and the C-terminal domain of PTX3) and structure homologies (accessibility to shared conformational epitopes exposed at the surface of pentraxins), makes it difficult to draw a conclusion. Moreover, different epitopes of each pentraxin could be recognized by different antibodies, and the resulting functional outcomes may have different consequences. The isotype of the anti-pentraxin autoantibodies, the site of production (tissue versus mucosal barriers) and the immune status of the tissue at the time of efferocytosis (such as chronic inflammation) can also have important consequences on the regulatory versus activating potential of these autoantibodies. Such complexity could lead to misinterpretation of the roles of anti-PTX Abs in the pathogenesis of autoimmune diseases.

Nevertheless, and based on the role of pentraxins in efferocytosis, it is tempting to speculate that these autoantibodies could alter the efferocytosis process and, consequently, the outcome of immune responses against self-antigens.

On the one hand, anti-pentraxin Abs could inhibit or delay apoptotic cells engulfment by professional antigen-presenting cells, leading to the evolution of dying cells from early to late apoptotic cells. Importantly, late apoptotic/secondary necrotic cells can release potent inflammatory DAMPs that can activate

antigen-presenting cells and, consequently, induce the initiation of humoral and cellular specific immune responses. On the other hand, anti-pentraxin Abs may induce the phagocytosis of PTX-opsonized dying cells *via* FcγR, a process that favors antigen-presenting cell activation and antigen processing that, ultimately, favors the initiation of MHC-I and MHC-II immune responses (98, 99). Moreover, while the interaction of apoptotic cells with pentraxins usually has an opsonizing role, a reduced internalization of apoptotic cells by dendritic cells has been reported for PTX3. The authors described this mechanism as “PTX3 censorship”, i.e., masking of “eat-me” signals rendering them not detectable by phagocytes (100). Antibodies directed against PTX3 could therefore re-confer immunogenicity to apoptotic cells by promoting the presentation of apoptotic cell-associated antigens by professional APCs and, consequently, the priming and/or expansion of self-reactive T cells. Even though speculative, both mechanisms, which are not exclusive and could occur concomitantly, may favor/maintain the development of autoimmune responses directed against dying cell-associated antigens (**Figure 1**). Additional studies need to be performed to elucidate the complex dialog between soluble and membrane associated PTX3 (soluble versus) with anti-PTX3 Abs and consequences on sensing and processing of apoptotic cells. Finally, a lower frequency of anti-PTX3 Abs have been reported in patients with lupus nephritis, suggesting that these autoantibodies could also be protecting against PTX3 or complement deposition in kidneys, further enhancing the complexity of the potential pathogenic role of anti-pentraxin auto-antibodies.

Nevertheless, we cannot exclude a protective role for anti-pentraxin Ab, notably in kidney disorders associated with anti-pentraxin Abs. Indeed, anti-pentraxin Abs could inhibit the pentraxin deposition in tissue, thereby preventing complement binding and activation. Whether this process may occur *in vivo* remains undetermined. Another original mechanism has been suggested for anti-SAP Abs. Bickerstaff M et al have reported that SAP inhibits the generation of pathogenic anti-DNA autoantibodies, probably by delaying chromatin degradation (40). This process could be inhibited by anti-SAP Abs, thereby favoring or amplifying the generation of autoantibodies and inflammation.

Nevertheless, it is important to underline that these processes are based on the hypothesis that anti-pentraxin antibodies exhibit mostly similar biological properties. These scenarios can be very different, both in terms of magnitude and nature of the immune response induced, depending on the diversity of autoantibodies produced (isotype, levels) and the presence or absence of each of these pentraxins. The scenario may be even more complicated, particularly due to the involvement of other sPRM and PRR involved in the recognition and removal of danger signals.

CONCLUDING REMARKS

Pentraxins are soluble PRM involved in antimicrobial defense and efferocytosis. A tolerance breakdown to pentraxins has been

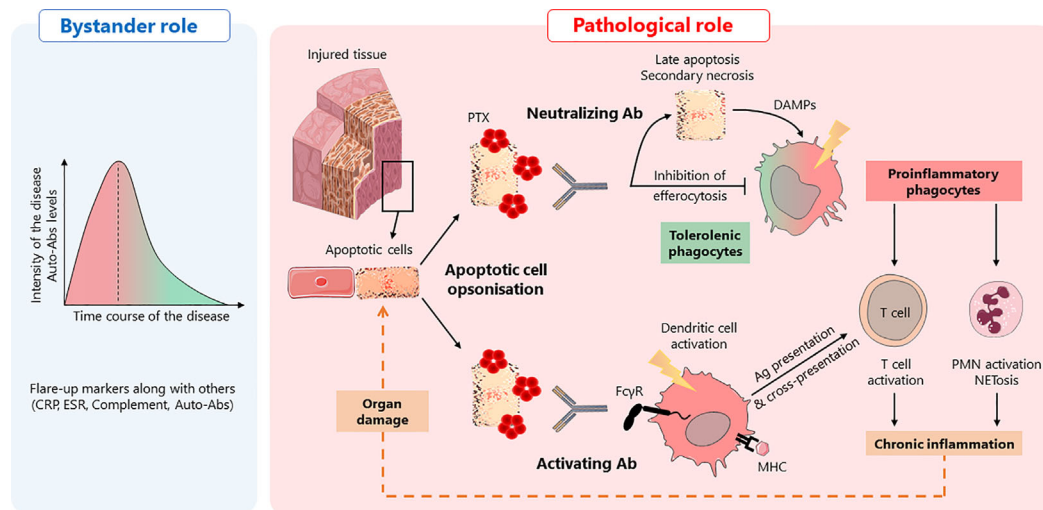


FIGURE 1 | Anti-pentraxin antibodies in autoimmunity: a hypothetical model. Anti-pentraxin antibodies are found elevated at autoimmune disease onset and decline during remission phases, suggesting that they are, at least, diagnostic markers, playing a “bystander role” (left panel). Based on the role of pentraxins in efferocytosis, two hypotheses can be drawn about a hypothetical “pathological role” of anti-pentraxin Abs (right panel). In a physiological context, apoptotic cells are opsonized by pentraxins (PTX) that facilitate their engulfment. This process promotes tolerogenic phagocytes and prevents inflammation. The presence of anti-pentraxin Abs can lead to the initiation of adaptive immune responses against dying cell antigens and NETosis, a pro-inflammatory process associated with the release of autoantigens. First, anti-PTX Ab may behave as neutralizing antibodies, inhibiting apoptotic cells engulfment by professional antigen-presenting cells, leading to the evolution of dying cells from early to late apoptotic/necrotic cells. These dying cells release potent inflammatory DAMPs inducing the activation of antigen-presenting cells. Second, anti-PTX Ab can be considered as activating Ab, inducing the phagocytosis of dying cells via FcγR. This process also induces the activation of antigen-presenting cells, favoring the initiation of MHC-I and MHC-II immune responses through antigen presentation and cross-presentation. Whatever the “function” of anti-pentraxin Ab, they initiate an inflammatory process leading to a deleterious amplification loop responsible for tissue damages.

evidenced during various autoimmune diseases. Especially, anti-PTX3 autoantibodies are frequent in SLE and AAV and may be of interest for diagnosis, disease activity, response to treatment and outcomes. Moreover, anti-PTX3 Abs share some characteristics with ANCAs and are associated with a specific indirect immunofluorescence neutrophil staining, suggesting that they differ from other ANCA. The prevalence of anti-PTX3 Abs appears to be high during ANCA-negative AAV and could constitute a useful marker in these settings. Prospective longitudinal clinical studies are required to confirm the potential of anti-PTX3 Abs as diagnosis and prognosis markers. In a mechanistic point of view, studies are required to determine the potential pathogenic role of anti-PTX3 and whether they should be targeted if they are more than just bystanders in severe autoimmune diseases.

AUTHOR CONTRIBUTIONS

BB wrote the first draft of the manuscript. YD and J-FA provided critical revision of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. This work was realized in the context of (i) the LabEX IGO program (National Research Agency; ANR-11-LABX-0016-01) and (ii) the University Hospital of Angers - University of Angers joint program (project 3I-Impact).

REFERENCES

- Janeway CA, Medzhitov R. Innate immune recognition. *Annu Rev Immunol* (2002) 20:197–216. doi: 10.1146/annurev.immunol.20.083001.084359
- Locati M, Curtale G, Mantovani A. Diversity, Mechanisms, and Significance of Macrophage Plasticity. *Annu Rev Pathol* (2020) 15:123–47. doi: 10.1146/annurev-pathmechdis-012418-012718
- Jeannin P, Jaillon S, Delneste Y. Pattern recognition receptors in the immune response against dying cells. *Curr Opin Immunol* (2008) 20:530–7. doi: 10.1016/j.coi.2008.04.013
- Garlanda C, Bottazzi B, Bastone A, Mantovani A. Pentraxins at the crossroads between innate immunity, inflammation, matrix deposition, and female fertility. *Annu Rev Immunol* (2005) 23:337–66. doi: 10.1146/annurev.immunol.23.021704.115756
- Deban L, Jaillon S, Garlanda C, Bottazzi B, Mantovani A. Pentraxins in innate immunity: lessons from PTX3. *Cell Tissue Res* (2011) 343:237–49. doi: 10.1007/s00441-010-1018-0
- Gewurz H, Zhang XH, Lint TF. Structure and function of the pentraxins. *Curr Opin Immunol* (1995) 7:54–64. doi: 10.1016/0952-7915(95)80029-8
- Guillon C, Bigouagou UM, Folio C, Jeannin P, Delneste Y, Gouet P. A staggered decameric assembly of human C-reactive protein stabilized by zinc ions revealed by X-ray crystallography. *Protein Pept Lett* (2014) 22:248–55. doi: 10.2174/0929866522666141231111226

8. Martinez de la Torre Y, Fabbri M, Jaillon S, Bastone A, Nebuloni M, Vecchi A, et al. Evolution of the pentraxin family: the new entry PTX4. *J Immunol Baltim Md 1950* (2010) 184:5055–64. doi: 10.4049/jimmunol.0901672
9. Pepys MB, Hirschfield GM. C-reactive protein: a critical update. *J Clin Invest* (2003) 111:1805–12. doi: 10.1172/JCI18921
10. Botto M, Hawkins PN, Bickerstaff MC, Herbert J, Bygrave AE, McBride A, et al. Amyloid deposition is delayed in mice with targeted deletion of the serum amyloid P component gene. *Nat Med* (1997) 3:855–9. doi: 10.1038/nm0897-855
11. Gershov D, Kim S, Brot N, Elkon KB. C-Reactive protein binds to apoptotic cells, protects the cells from assembly of the terminal complement components, and sustains an antiinflammatory innate immune response: implications for systemic autoimmunity. *J Exp Med* (2000) 192:1353–64. doi: 10.1084/jem.192.9.1353
12. Agrawal A, Singh PP, Bottazzi B, Garlanda C, Mantovani A. Pattern recognition by pentraxins. *Adv Exp Med Biol* (2009) 653:98–116. doi: 10.1007/978-1-4419-0901-5_7
13. Inforzato A, Doni A, Barajon I, Leone R, Garlanda C, Bottazzi B, et al. PTX3 as a paradigm for the interaction of pentraxins with the complement system. *Semin Immunol* (2013) 25:79–85. doi: 10.1016/j.smim.2013.05.002
14. Ng PML, Le Saux A, Lee CM, Tan NS, Lu J, Thiel S, et al. C-reactive protein collaborates with plasma lectins to boost immune response against bacteria. *EMBO J* (2007) 26:3431–40. doi: 10.1038/sj.emboj.7601762
15. Mantovani A, Valentino S, Gentile S, Inforzato A, Bottazzi B, Garlanda C. The long pentraxin PTX3: a paradigm for humoral pattern recognition molecules. *Ann N Y Acad Sci* (2013) 1285:1–14. doi: 10.1111/nyas.12043
16. Okemefuna AI, Nan R, Miller A, Gor J, Perkins SJ. Complement factor H binds at two independent sites to C-reactive protein in acute phase concentrations. *J Biol Chem* (2010) 285:1053–65. doi: 10.1074/jbc.M109.044529
17. Bharadwaj D, Mold C, Markham E, Du Clos TW. Serum amyloid P component binds to Fc gamma receptors and opsonizes particles for phagocytosis. *J Immunol Baltim Md 1950* (2001) 166:6735–41. doi: 10.4049/jimmunol.166.11.6735
18. Mold C, Baca R, Du Clos TW. Serum amyloid P component and C-reactive protein opsonize apoptotic cells for phagocytosis through Fc gamma receptors. *J Autoimmun* (2002) 19:147–54. doi: 10.1006/jaut.2002.0615
19. Mold C, Gresham HD, Du Clos TW. Serum amyloid P component and C-reactive protein mediate phagocytosis through murine Fc gamma Rs. *J Immunol Baltim Md 1950* (2001) 166:1200–5. doi: 10.4049/jimmunol.166.2.1200
20. Lu J, Marnell LL, Marjon KD, Mold C, Du Clos TW, Sun PD. Structural recognition and functional activation of Fc gamma R by innate pentraxins. *Nature* (2008) 456:989–92. doi: 10.1038/nature07468
21. Basile A, Sica A, d'Aniello E, Breviaro F, Garrido G, Castellano M, et al. Characterization of the Promoter for the Human Long Pentraxin PTX3: ROLE OF NF- κ B IN TUMOR NECROSIS FACTOR- α AND INTERLEUKIN-1 β REGULATION. *J Biol Chem* (1997) 272:8172–8. doi: 10.1074/jbc.272.13.8172
22. Jaillon S, Peri G, Delneste Y, Frémaux I, Doni A, Moalli F, et al. The humoral pattern recognition receptor PTX3 is stored in neutrophil granules and localizes in extracellular traps. *J Exp Med* (2007) 204:793–804. doi: 10.1084/jem.20061301
23. Lominadze G, Powell DW, Luerman GC, Link AJ, Ward RA, McLeish KR. Proteomic analysis of human neutrophil granules. *Mol Cell Proteomics MCP* (2005) 4:1503–21. doi: 10.1074/mcp.M500143-MCP200
24. Thakur R, Shankar J. In silico Analysis Revealed High-risk Single Nucleotide Polymorphisms in Human Pentraxin-3 Gene and their Impact on Innate Immune Response against Microbial Pathogens. *Front Microbiol* (2016) 7:192. doi: 10.3389/fmicb.2016.00192
25. Moalli F, Paroni M, Véliz Rodriguez T, Riva F, Polentarutti N, Bottazzi B, et al. The therapeutic potential of the humoral pattern recognition molecule PTX3 in chronic lung infection caused by *Pseudomonas aeruginosa*. *J Immunol Baltim Md 1950* (2011) 186:5425–34. doi: 10.4049/jimmunol.1002035
26. Reading PC, Bozza S, Gilbertson B, Tate M, Moretti S, Job ER, et al. Antiviral activity of the long chain pentraxin PTX3 against influenza viruses. *J Immunol Baltim Md 1950* (2008) 180:3391–8. doi: 10.4049/jimmunol.180.5.3391
27. Bozza S, Campo S, Arseni B, Inforzato A, Ragnar L, Bottazzi B, et al. PTX3 binds MD-2 and promotes TRIF-dependent immune protection in aspergillosis. *J Immunol Baltim Md 1950* (2014) 193:2340–8. doi: 10.4049/jimmunol.1400814
28. Moalli F, Doni A, Deban L, Zelante T, Zagarella S, Bottazzi B, et al. Role of complement and Fc{gamma} receptors in the protective activity of the long pentraxin PTX3 against *Aspergillus fumigatus*. *Blood* (2010) 116:5170–80. doi: 10.1182/blood-2009-12-258376
29. Mold C, Rodriguez W, Rodic-Polic B, Du Clos TW. C-reactive protein mediates protection from lipopolysaccharide through interactions with Fc gamma R. *J Immunol Baltim Md 1950* (2002) 169:7019–25. doi: 10.4049/jimmunol.169.12.7019
30. Deban L, Jarva H, Lehtinen MJ, Bottazzi B, Bastone A, Doni A, et al. Binding of the long pentraxin PTX3 to factor H: interacting domains and function in the regulation of complement activation. *J Immunol Baltim Md 1950* (2008) 181:8433–40. doi: 10.4049/jimmunol.181.12.8433
31. Magrini E, Mantovani A, Garlanda C. The Dual Complexity of PTX3 in Health and Disease: A Balancing Act? *Trends Mol Med* (2016) 22:497–510. doi: 10.1016/j.molmed.2016.04.007
32. Souza DG, Amaral FA, Fagundes CT, Coelho FM, Arantes RME, Sousa LP, et al. The Long Pentraxin PTX3 Is Crucial for Tissue Inflammation after Intestinal Ischemia and Reperfusion in Mice. *Am J Pathol* (2009) 174:1309–18. doi: 10.2353/ajpath.2009.080240
33. Souza DG, Soares AC, Pinho V, Torloni H, Reis LFL, Martins MT, et al. Increased Mortality and Inflammation in Tumor Necrosis Factor-Stimulated Gene-14 Transgenic Mice after Ischemia and Reperfusion Injury. *Am J Pathol* (2002) 160:1755–65. doi: 10.1016/S0002-9440(10)61122-4
34. Real JM, Spilborghs GMGT, Morato-Marques M, de Moura RP, Negri EM, Camargo AA, et al. Pentraxin 3 accelerates lung injury in high tidal volume ventilation in mice. *Mol Immunol* (2012) 51:82–90. doi: 10.1016/j.molimm.2012.02.113
35. Suzuki S, Shishido T, Funayama A, Netsu S, Ishino M, Kitahara T, et al. Long Pentraxin PTX3 Exacerbates Pressure Overload-Induced Left Ventricular Dysfunction. *PLoS One* (2013) 8:e53133. doi: 10.1371/journal.pone.0053133
36. Carrizzo A, Lenzi P, Procaccini C, Damato A, Biagioni F, Ambrosio M, et al. Pentraxin 3 Induces Vascular Endothelial Dysfunction Through a P-selectin/Matrix Metalloproteinase-1 Pathway. *Circulation* (2015) 131:1495–505. doi: 10.1161/CIRCULATIONAHA.114.014822
37. D'Arcy MS. Cell death: a review of the major forms of apoptosis, necrosis and autophagy. *Cell Biol Int* (2019) 43:582–92. doi: 10.1002/cbin.11137
38. Morioka S, Maueröder C, Ravichandran KS. Living on the Edge: Efferocytosis at the Interface of Homeostasis and Pathology. *Immunity* (2019) 50:1149–62. doi: 10.1016/j.immuni.2019.04.018
39. Mitchell DA, Pickering MC, Warren J, Fossati-Jimack L, Cortes-Hernandez J, Cook HT, et al. C1q deficiency and autoimmunity: the effects of genetic background on disease expression. *J Immunol Baltim Md 1950* (2002) 168:2538–43. doi: 10.4049/jimmunol.168.5.2538
40. Bickerstaff MCM, Botto M, Hutchinson WL, Herbert J, Tennent GA, Bybee A, et al. Serum amyloid P component controls chromatin degradation and prevents antinuclear autoimmunity. *Nat Med* (1999) 5:694–7. doi: 10.1038/9544
41. Mihlan M, Stippa S, Józsi M, Zipfel PF. Monomeric CRP contributes to complement control in fluid phase and on cellular surfaces and increases phagocytosis by recruiting factor H. *Cell Death Differ* (2009) 16:1630–40. doi: 10.1038/cdd.2009.103
42. van Rossum AP, Fazzini F, Limburg PC, Manfredi AA, Rovere-Querini P, Mantovani A, et al. The prototypic tissue pentraxin PTX3, in contrast to the short pentraxin serum amyloid P, inhibits phagocytosis of late apoptotic neutrophils by macrophages. *Arthritis Rheum* (2004) 50:2667–74. doi: 10.1002/art.20370
43. Jaillon S, Jeannin P, Hamon Y, Frémaux I, Doni A, Bottazzi B, et al. Endogenous PTX3 translocates at the membrane of late apoptotic human neutrophils and is involved in their engulfment by macrophages. *Cell Death Differ* (2009) 16:465–74. doi: 10.1038/cdd.2008.173
44. Baruah P, Dumitriu IE, Peri G, Russo V, Mantovani A, Manfredi AA, et al. The tissue pentraxin PTX3 limits C1q-mediated complement activation and phagocytosis of apoptotic cells by dendritic cells. *J Leukoc Biol* (2006) 80:87–95. doi: 10.1189/jlb.0805445

45. Rodriguez W, Mold C, Marnell LL, Hutt J, Silverman GJ, Tran D, et al. Prevention and reversal of nephritis in MRL/lpr mice with a single injection of C-reactive protein. *Arthritis Rheum* (2006) 54:325–35. doi: 10.1002/art.21556
46. Szalai AJ. C-Reactive Protein (CRP) and Autoimmune Disease: Facts and Conjectures. *Clin Dev Immunol* (2004) 11:221–6. doi: 10.1080/17402520400001751
47. Szalai AJ, Weaver CT, McCrory MA, van Ginkel FW, Reiman RM, Kearney JF, et al. Delayed lupus onset in (NZB × NZW)F₁ mice expressing a human C-reactive protein transgene: Delayed Lupus Onset in Mice Expressing Human CRP. *Arthritis Rheum* (2003) 48:1602–11. doi: 10.1002/art.11026
48. Gillmore JD, Hutchinson WL, Herbert J, Bybee A, Mitchell DA, Hasslerjian RP, et al. Autoimmunity and glomerulonephritis in mice with targeted deletion of the serum amyloid P component gene: SAP deficiency or strain combination? *Immunology* (2004) 112:255–64. doi: 10.1111/j.1365-2567.2004.01860.x
49. de Oliveira THC, Souza DG, Teixeira MM, Amaral FA. Tissue Dependent Role of PTX3 During Ischemia-Reperfusion Injury. *Front Immunol* (2019) 10:1461. doi: 10.3389/fimmu.2019.01461
50. Batista NV, Barbagallo M, Oliveira VLS, Castro-Gomes T, Oliveira RDR, Louzada-Junior P, et al. The Long Pentraxin 3 Contributes to Joint Inflammation in Gout by Facilitating the Phagocytosis of Monosodium Urate Crystals. *J Immunol* (2019) 202:1807–14. doi: 10.4049/jimmunol.1701531
51. Lech M, Römmele C, Kulkarni OP, Susanti HE, Migliorini A, Garlanda C, et al. Lack of the Long Pentraxin PTX3 Promotes Autoimmune Lung Disease but not Glomerulonephritis in Murine Systemic Lupus Erythematosus. *PLoS One* (2011) 6:e20118. doi: 10.1371/journal.pone.0020118
52. Firooz N, Albert D, Wallace D, Ishimori M, Berel D, Weisman M. High-sensitivity C-reactive protein and erythrocyte sedimentation rate in systemic lupus erythematosus. *Lupus* (2011) 20:588–97. doi: 10.1177/0961203310393378
53. Linares LF, Gomez-Reino JJ, Carreira PE, Morillas L, Ibero I. C-reactive protein (CRP) levels in systemic lupus erythematosus (SLE). *Clin Rheumatol* (1986) 5:66–9. doi: 10.1007/BF02030970
54. Kruse K, Janko C, Urbonaviciute V, Mierke CT, Winkler TH, Voll RE, et al. Inefficient clearance of dying cells in patients with SLE: anti-dsDNA autoantibodies, MFG-E8, HMGB-1 and other players. *Apoptosis* (2010) 15:1098–113. doi: 10.1007/s10495-010-0478-8
55. Augusto J-F, Onno C, Blanchard S, Dubuquoi S, Mantovani A, Chevailler A, et al. Detection of anti-PTX3 autoantibodies in systemic lupus erythematosus. *Rheumatology* (2009) 48:442–4. doi: 10.1093/rheumatology/ken507
56. Daigo K, Hamakubo T. Host-protective effect of circulating pentraxin 3 (PTX3) and complex formation with neutrophil extracellular traps. *Front Immunol* (2012) 3:378. doi: 10.3389/fimmu.2012.00378
57. Tsokos GC. Systemic Lupus Erythematosus. *N Engl J Med* (2011) 365:2110–21. doi: 10.1056/NEJMr1100359
58. Brilland B, Scherlinger M, Khoryati L, Goret J, Duffau P, Lazaro E, et al. Platelets and IgE: shaping the innate immune response in systemic lupus erythematosus. *Clin Rev Allergy Immunol* (2019) 58(2):194–212. doi: 10.1007/s12016-019-08744-x
59. Bell SA, Faust H, Schmid A, Meurer M. Autoantibodies to C-reactive protein (CRP) and other acute-phase proteins in systemic autoimmune diseases. *Clin Exp Immunol* (1998) 113:327–32. doi: 10.1046/j.1365-2249.1998.00655.x
60. Potempa LA, Zeller JM, Fiedel BA, Kinoshita CM, Gewurz H. Stimulation of human neutrophils, monocytes, and platelets by modified C-reactive protein (CRP) expressing a neoantigenic specificity. *Inflammation* (1988) 12:391–405. doi: 10.1007/BF00915774
61. Eisenhardt SU, Thiele JR, Bannasch H, Stark GB, Peter K. C-reactive protein: How conformational changes influence inflammatory properties. *Cell Cycle* (2009) 8:3885–92. doi: 10.4161/cc.8.23.10068
62. Sjöwall C, Eriksson P, Almer S, Skogh T. Autoantibodies to C-reactive Protein is a Common Finding in SLE, but not in Primary Sjögren's Syndrome, Rheumatoid Arthritis or Inflammatory Bowel Disease. *J Autoimmun* (2002) 19:155–60. doi: 10.1006/jaut.2002.0608
63. Figueredo MA, Rodriguez A, Ruiz-Yagüe M, Romero M, Fernandez-Cruz A, Gomez-de la Concha E, et al. Autoantibodies against C-reactive protein: clinical associations in systemic lupus erythematosus and primary antiphospholipid syndrome. *J Rheumatol* (2006) 33:1980–6.
64. Rosenau BJ. Antibodies to C reactive protein. *Ann Rheum Dis* (2006) 65:674–6. doi: 10.1136/ard.2005.037895
65. Shoenfeld Y, Kravitz MS, Witte T, Doria A, Tsutsumi A, Tatsuya A, et al. Autoantibodies against Protective Molecules C1q, C-Reactive Protein, Serum Amyloid P, Mannose-Binding Lectin, and Apolipoprotein A1: Prevalence in Systemic Lupus Erythematosus. *Ann N Y Acad Sci* (2007) 1108:227–39. doi: 10.1196/annals.1422.025
66. Tan Y, Yu F, Yang H, Chen M, Fang Q, Zhao M. Autoantibodies against monomeric C-reactive protein in sera from patients with lupus nephritis are associated with disease activity and renal tubulointerstitial lesions. *Hum Immunol* (2008) 69:840–4. doi: 10.1016/j.humimm.2008.09.006
67. O'Neill SG, Giles I, Lambrianides A, Manson J, D'Cruz D, Schrieber L, et al. Antibodies to apolipoprotein A-I, high-density lipoprotein, and C-reactive protein are associated with disease activity in patients with systemic lupus erythematosus. *Arthritis Rheum* (2010) 62:845–54. doi: 10.1002/art.27286
68. Tan Y, Yu F, Qu Z, Su T, Xing G-Q, Wu L-H, et al. Modified C-Reactive Protein Might be a Target Autoantigen of TINU Syndrome. *Clin J Am Soc Nephrol* (2011) 6:93–100. doi: 10.2215/CJN.09051209
69. Pradhan V, Rajadhyaksha A, Yadav K, Surve P, Patwardhan M, Dhavale N, et al. Anti-C reactive protein antibodies in Indian patients with systemic lupus erythematosus. *Indian J Nephrol* (2013) 23:434. doi: 10.4103/0971-4065.120341
70. Jung J-Y, Koh B-R, Kim H-A, Jeon J-Y, Suh C-H. Autoantibodies to C-Reactive Protein in Incomplete Lupus and Systemic Lupus Erythematosus. *J Invest Med* (2014) 62:890–3. doi: 10.1097/JIM.0000000000000094
71. Simon A, Subra J-F, Guilpain P, Jeannin P, Pignon P, Blanchard S, et al. Detection of Anti-Pentraxin-3 Autoantibodies in ANCA-Associated Vasculitis. *PLoS One* (2016) 11:e0147091. doi: 10.1371/journal.pone.0147091
72. Son C-N, Lee T-H, Bang J-H, Jeong H-J, Chae J-N, Lee W-M, et al. The relationship between anti-C-reactive protein and disease activity in patients with systemic lupus erythematosus. *Korean J Intern Med* (2018) 33:823–8. doi: 10.3904/kjim.2016.065
73. Zandman-Goddard G. Anti-serum amyloid component P antibodies in patients with systemic lupus erythematosus correlate with disease activity. *Ann Rheum Dis* (2005) 64:1698–702. doi: 10.1136/ard.2005.035832
74. Bassi N, Ghirardello A, Blank M, Zampieri S, Sarzi-Puttini P, Mantovani A, et al. IgG anti-pentraxin 3 antibodies in systemic lupus erythematosus. *Ann Rheum Dis* (2010) 69:1704–10. doi: 10.1136/ard.2009.117804
75. Yuan M, Tan Y, Pang Y, Li Y, Song Y, Yu F, et al. Anti-pentraxin 3 autoantibodies might be protective in lupus nephritis: a large cohort study. *Ren Fail* (2017) 39:465–73. doi: 10.1080/0886022X.2017.1308258
76. Robey FA, Jones KD, Steinberg AD. C-reactive protein mediates the solubilization of nuclear DNA by complement in vitro. *J Exp Med* (1985) 161:1344–56. doi: 10.1084/jem.161.6.1344
77. Bassi N, Del Prete D, Ghirardello A, Gatto M, Ceol M, Zen M, et al. PTX3, Anti-PTX3, and Anti-C1q Autoantibodies in Lupus Glomerulonephritis. *Clin Rev Allergy Immunol* (2015) 49:217–26. doi: 10.1007/s12016-015-8476-9
78. Gatto M, Ghirardello A, Luisetto R, Bassi N, Fedrigo M, Valente M, et al. Immunization with pentraxin 3 (PTX3) leads to anti-PTX3 antibody production and delayed lupus-like nephritis in NZB/NZW F1 mice. *J Autoimmun* (2016) 74:208–16. doi: 10.1016/j.jaut.2016.07.002
79. Jennette JC, Falk RJ, Bacon PA, Basu N, Cid MC, Ferrario F, et al. revised International Chapel Hill Consensus Conference Nomenclature of Vasculitides. *Arthritis Rheum* (2013) 65:1–11. doi: 10.1002/art.37715
80. Jennette JC, Nachman PH. ANCA Glomerulonephritis and Vasculitis. *Clin J Am Soc Nephrol* (2017) 12:1680–91. doi: 10.2215/CJN.02500317
81. Jennette JC, Falk RJ, Hu P, Xiao H. Pathogenesis of Antineutrophil Cytoplasmic Autoantibody-Associated Small-Vessel Vasculitis. *Annu Rev Pathol Mech Dis* (2013) 8:139–60. doi: 10.1146/annurev-pathol-011811-132453
82. Comarmond C, Pagnoux C, Khellaf M, Cordier J-F, Hamidou M, Viallard J-F, et al. Eosinophilic granulomatosis with polyangiitis (Churg-Strauss): Clinical characteristics and long-term followup of the 383 patients enrolled in the French Vasculitis Study Group cohort. *Arthritis Rheum* (2013) 65:270–81. doi: 10.1002/art.37721

83. Geetha D, Jefferson JA. ANCA-Associated Vasculitis: Core Curriculum 2020. *Am J Kidney Dis* (2020) 75:124–37. doi: 10.1053/j.ajkd.2019.04.031
84. Flossmann O, Berden A, de Groot K, Hagen C, Harper L, Heijl C, et al. Long-term patient survival in ANCA-associated vasculitis. *Ann Rheum Dis* (2011) 70:488–94. doi: 10.1136/ard.2010.137778
85. Beauvillain C, Delneste Y, Renier G, Jeannin P, Subra JF, Chevailler A. Antineutrophil cytoplasmic autoantibodies: how should the biologist manage them? *Clin Rev Allergy Immunol* (2008) 35:47–58. doi: 10.1007/s12016-007-8071-9
86. Talor MV, Stone JH, Stebbing J, Barin J, Rose NR, Burek CL. Antibodies to selected minor target antigens in patients with anti-neutrophil cytoplasmic antibodies (ANCA): Minor target antigens in ANCA-associated vasculitis. *Clin Exp Immunol* (2007) 150:42–8. doi: 10.1111/j.1365-2249.2007.03453.x
87. Jaillon S, Bonavita E, Gentile S, Rubino M, Laface I, Garlanda C, et al. The long pentraxin PTX3 as a key component of humoral innate immunity and a candidate diagnostic for inflammatory diseases. *Int Arch Allergy Immunol* (2014) 165:165–78. doi: 10.1159/000368778
88. Daigo K, Yamaguchi N, Kawamura T, Matsubara K, Jiang S, Ohashi R, et al. The proteomic profile of circulating pentraxin 3 (PTX3) complex in sepsis demonstrates the interaction with azurocidin 1 and other components of neutrophil extracellular traps. *Mol Cell Proteomics* (2012) 11:M111.015073. doi: 10.1074/mcp.M111.015073
89. Berthelot J-M, Le Goff B, Neel A, Maugars Y, Hamidou M. NETosis: At the crossroads of rheumatoid arthritis, lupus, and vasculitis. *Joint Bone Spine* (2017) 84:255–62. doi: 10.1016/j.jbspin.2016.05.013
90. Kessenbrock K, Krumbholz M, Schönermarck U, Back W, Gross WL, Werb Z, et al. Netting neutrophils in autoimmune small-vessel vasculitis. *Nat Med* (2009) 15:623–5. doi: 10.1038/nm.1959
91. Sangaletti S, Tripodo C, Chiodoni C, Guarnotta C, Cappetti B, Casalini P, et al. Neutrophil extracellular traps mediate transfer of cytoplasmic neutrophil antigens to myeloid dendritic cells toward ANCA induction and associated autoimmunity. *Blood* (2012) 120:3007–18. doi: 10.1182/blood-2012-03-416156
92. Söderberg D, Segelmark M. Neutrophil Extracellular Traps in ANCA-Associated Vasculitis. *Front Immunol* (2016) 7:256. doi: 10.3389/fimmu.2016.00256
93. Wang H, Wang C, Zhao M-H, Chen M. Neutrophil extracellular traps can activate alternative complement pathways: NETs activate complement in AAV. *Clin Exp Immunol* (2015) 181:518–27. doi: 10.1111/cei.12654
94. Brilland B, Garnier A-S, Chevailler A, Jeannin P, Subra J-F, Augusto J-F. Complement alternative pathway in ANCA-associated vasculitis: Two decades from bench to bedside. *Autoimmun Rev* (2020) 19:102424. doi: 10.1016/j.autrev.2019.102424
95. Xiao H, Heeringa P, Hu P, Liu Z, Zhao M, Aratani Y, et al. Antineutrophil cytoplasmic autoantibodies specific for myeloperoxidase cause glomerulonephritis and vasculitis in mice. *J Clin Invest* (2002) 110:955–63. doi: 10.1172/JCI200215918
96. Schlieben DJ, Korbet SM, Kimura RE, Schwartz MM, Lewis EJ. Pulmonary-renal syndrome in a newborn with placental transmission of ANCAs. *Am J Kidney Dis* (2005) 45:758–61. doi: 10.1053/j.ajkd.2005.01.001
97. Brucato A, Frassi M, Franceschini F, Cimaz R, Faden D, Pisoni MP, et al. Risk of congenital complete heart block in newborns of mothers with anti-Ro/SSA antibodies detected by counterimmunoelectrophoresis: a prospective study of 100 women. *Arthritis Rheum* (2001) 44:1832–5. doi: 10.1002/1529-0131(200108)44:8<1832::AID-ART320>3.0.CO;2-C
98. Mantegazza AR, Magalhaes JG, Amigorena S, Marks MS. Presentation of phagocytosed antigens by MHC class I and II. *Traffic Cph Den* (2013) 14:135–52. doi: 10.1111/tra.12026
99. Junker F, Gordon J, Qureshi O. Fc Gamma Receptors and Their Role in Antigen Uptake, Presentation, and T Cell Activation. *Front Immunol* (2020) 11:1393. doi: 10.3389/fimmu.2020.01393
100. Rovere P, Peri G, Fazzini F, Bottazzi B, Doni A, Bondanza A, et al. The long pentraxin PTX3 binds to apoptotic cells and regulates their clearance by antigen-presenting dendritic cells. *Blood* (2000) 96:4300–6. doi: 10.1182/blood.V96.13.4300.h8004300_4300_4306

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Brilland, Vinatier, Subra, Jeannin, Augusto and Delneste. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Endogenous Long Pentraxin 3 Exerts a Protective Role in a Murine Model of Pulmonary Fibrosis

Federica Maccarinelli^{1†}, Mattia Bugatti^{1,2†}, Ander Churrua Schuind¹, Sara Ganzerla², William Vermi^{1,2}, Marco Presta^{1*} and Roberto Ronca^{1*}

¹ Department of Molecular and Translational Medicine, University of Brescia, Brescia, Italy, ² ASST Spedali Civili di Brescia, Brescia, Italy

OPEN ACCESS

Edited by:

Barbara Bottazzi,
University of Milan, Italy

Reviewed by:

Taruna Madan,
National Institute for Research in
Reproductive Health (ICMR), India
Remo Castro Russo,
Federal University of Minas Gerais,
Brazil

*Correspondence:

Roberto Ronca
roberto.ronca@unibs.it
Marco Presta
marco.presta@unibs.it

[†]These authors share first authorship

Specialty section:

This article was submitted to
Molecular Innate Immunity,
a section of the journal
Frontiers in Immunology

Received: 15 October 2020

Accepted: 11 January 2021

Published: 18 February 2021

Citation:

Maccarinelli F, Bugatti M,
Churrua Schuind A, Ganzerla S,
Vermi W, Presta M and Ronca R
(2021) Endogenous Long Pentraxin 3
Exerts a Protective Role in a Murine
Model of Pulmonary Fibrosis.
Front. Immunol. 12:617671.
doi: 10.3389/fimmu.2021.617671

Pulmonary fibrosis is a progressive scarring disease of the lungs, characterized by inflammation, fibroblast activation, and deposition of extracellular matrix. The long pentraxin 3 (PTX3) is a member of the pentraxin family with non-redundant functions in innate immune responses, tissue repair, and haemostasis. The role played in the lungs by PTX3 during the fibrotic process has not been elucidated. In this study, the impact of PTX3 expression on lung fibrosis was assessed in an intratracheal bleomycin (BLM)-induced murine model of the disease applied to wild type animals, transgenic mice characterized by endothelial overexpression and stromal accumulation of PTX3 (Tie2-PTX3 mice), and genetically deficient *Ptx3*^{-/-} animals. Our data demonstrate that PTX3 is produced during BLM-induced fibrosis in wild type mice, and that PTX3 accumulation in the stroma compartment of Tie2-PTX3 mice limits the formation of fibrotic tissue in the lungs, with reduced fibroblast activation and collagen deposition, and a decrease in the recruitment of the immune infiltrate. Conversely, *Ptx3*-null mice showed an exacerbated fibrotic response and decreased survival in response to BLM treatment. These results underline the protective role of endogenous PTX3 during lung fibrosis and pave the way for the study of novel PTX3-derived therapeutic approaches to the disease.

Keywords: long pentraxin-3, lung fibrosis, bleomycin, stroma, fibroblast, immune infiltrate

INTRODUCTION

Pulmonary fibrosis (PF) includes more than 200 different pathological conditions characterized by inflammation and scar tissue formation in the lungs. PF can be grouped in five categories: drug-induced, radiation-induced, environmental, autoimmune, and occupational (1). However, the most common type of PF is represented by the “Idiopathic Pulmonary Fibrosis” (IPF), whose etiology remains unknown. In the last years, the incidence of IPF has increased over time in most countries worldwide, with approximately 50,000 new cases diagnosed each year in the U.S (2).

Starting symptoms of IPF, such as shortness of breath, dry hacking cough and fatigue, get significantly worse when the deposition of scar tissue augments, increases in stiffness and causes an irreversible loss of pulmonary functionality (3).

Despite the unknown cause(s) of IPF, aging (IPF is rare before 50 years of age), cigarette smoking, and genetic predisposition may represent relevant risk factors (4, 5). At histopathological

level, IPF appears as a progressive scarring disease in the lungs, characterized by injury and hyperplasia of alveolar epithelial cells and fibroblasts, accumulation of inflammatory cells, consistent deposition of extracellular matrix (ECM), and formation of scars (6). Particular attention has been given to the onset of an inflammatory response and to the presence of an immune infiltrate that establishes and sustains a damaging and fibrotic context in the lungs (7). Even though little is known about the role of monocytes and tissue-resident macrophages in lung fibrosis (8), macrophages are persistently increased in the lung in close proximity to collagen-producing fibroblasts, in keeping with the role exerted by monocytes in the development of experimental fibrosis (9, 10). In particular, M2 macrophages accumulating in the lungs during fibrogenesis have been identified as the major source of several pro-fibrotic mediators, which stimulate fibroblast proliferation and collagen synthesis/deposition (11–14).

Different *in vivo* animal models of PF have been developed to understand the evolution of fibrotic responses in the lungs and in other organs (15), allowing the identification of cell types, mediators, and processes that are likely involved also in the human disease. To date, bleomycin (BLM)-induced pulmonary fibrosis in mice represents the best characterized murine model to study PF and IPF (15, 16). BLM is an antibiotic efficacious for the treatment of squamous cell carcinomas and skin tumors, but with limiting and dose-dependent pulmonary toxicity that results in progressive fibrosis (17). In mice, a single (or multiple, according to the specific schedule) intratracheal administration of BLM causes lung injury, resulting in pulmonary fibrosis that can be observed by day 14 (15, 18–20). The initial direct damage to alveolar epithelial cells is followed by dense ECM deposition associated with vessel remodeling and intense inflammatory infiltration, mimicking what is observed in patients (15).

The soluble pattern recognition receptor long pentraxin-3 (PTX3) is a member of the pentraxin family and a component of the humoral arm of the innate immunity. PTX3 expression is normally low in tissues and serum under physiological conditions, but the levels of PTX3 quickly rise in the presence of inflammatory and/or infectious stimuli due to its local production by different cell types, such as infiltrating immune cells, endothelial cells, and other stromal components (21). PTX3 exerts its function by binding to different ligands, including growth factors, microbial moieties, complement components, and ECM proteins (22–24). PTX3 has been reported to be upregulated and to play a protective role in various lung diseases (21). In lung infections, the protective function of PTX3 has been described in different pathological settings, including aspergillosis (25), pneumonia (26), and severe acute respiratory syndrome (27). In acute lung injury, which is strictly associated with activation of innate immune responses in the lungs, PTX3 deficiency in *Ptx3* null mice results in increased sensitivity to lung tissue damage after exposition to LPS (28). Finally, PTX3 has been proposed as a possible biomarker of disease in acute lung injury and other pulmonary disorders, including asthma, and lung carcinoma (28–30).

In the present study, we investigated the impact of endogenous PTX3 in a BLM-induced murine model of lung

fibrosis. Our data show that pulmonary PTX3 expression is upregulated during lung fibrosis. In addition, by using both transgenic PTX3-overexpressing mice and *Ptx3* null animals, we demonstrate that endogenous PTX3 exerts a protective effect in BLM-induced lung fibrosis.

MATERIALS AND METHODS

Bleomycin-Induced Pulmonary Fibrosis

Animal experiments were approved by the local animal ethics committee (OPBA, Organismo Preposto al Benessere degli Animali, Università degli Studi di Brescia, Italy) and were performed in accordance with national guidelines and regulations. Procedures involving animals and their care conformed with institutional guidelines that comply with national and international laws and policies (EEC Council Directive 86/609, OJ L 358, 12 December 1987) and with “ARRIVE” guidelines (Animals in Research Reporting *In Vivo* Experiments). Eight weeks old wild type (WT) C57BL/6, *Ptx3*^{−/−} (31) and Tie2-PTX3 (32) male mice received a single, slow intratracheal injection of 4.0 mg/kg bleomycin (B2434 Sigma-Aldrich) dissolved in 30 μ l of phosphate-buffered saline (PBS). Body weight variations were monitored throughout the whole experimental period (**Supplementary Figure S1**). Mice were sacrificed at different time points (14, 21, and 28 days after treatment), and lungs collected and prepared for histopathological analysis. For each time point 5–8 mice were used for each strain (WT, Tie2-PTX3, and *Ptx3*^{−/−} animals).

Histopathological Analysis

The left lung was fixed overnight in 10% formalin (05-01004F BioOptica), dehydrated in a graded ethanol series, embedded in paraffin and cut into 4- μ m sections. Pulmonary fibrosis was analyzed using digital microscopy. For the analysis of the fibrosis area, sections were stained with Masson’s trichrome, digitalized by AperioScanScope CS Slide Scanner (Leica Biosystem, New Castle Ltd, UK) at 40x magnification and submitted to ImageScope software (Leica).

Immunohistochemistry

Paraffin-embedded lung tissues were prepared as previously described (33). Four- μ m sections were deparaffinized with xylene, incubated with 3% H₂O₂ in methanol for 30 min to inhibit endogenous peroxidase activity and then re-hydrated. Immunostaining was performed upon microwave or thermostat bath oven epitope retrieval in ethylene diamine tetra-acetic acid (EDTA) buffer (pH 8.00). The following primary antibodies were used: rabbit polyclonal anti-PTX3 (kind gift of B. Bottazzi, Humanitas Clinical Institute-Milan 1:100), mouse monoclonal anti- α -SMA antibody (clone 1A4, 1:300, Biocare, # CM001), rat monoclonal anti-CD45 (clone 30-F11, 1:100, BD Pharmingen, #553076), rabbit polyclonal anti-IBA1 (1:300, Wako, 019-19741), rat monoclonal anti-Ly6G (clone 1A8, 1:400, Cederlane, #AB-F118UD), mouse monoclonal anti-CD3 (clone SP7, 1:70, Leica, #565-LCE) and rabbit monoclonal PECAM-1 (clone M-20,

1:200, Santa Cruz, #SC-1506). Immunoreaction was revealed by using EnVision+ System-HRP Labelled Polymer anti-mouse or anti-rabbit (Dako) or using Rat-on-Mouse HRP-Polymer (Biocare Medical) followed by DAB as chromogen: sections were counterstained with hematoxylin. Immunostained slides were digitalized as described above and evaluated using Positive Pixel Count v9 9.0 Algorithm (ImageScope, Leica Biosystem). Staining was graded for positive pixel density (0 indicating $< 25,000/\text{mm}^2$; 1, $< 50,000/\text{mm}^2$; 2, $< 100,000/\text{mm}^2$; 3 $> 100,000/\text{mm}^2$ positive pixel). It must be pointed out that physiological α -SMA immuno-reactivity in perivascular and peri-bronchiolar areas was excluded in the evaluation of the samples.

Hydroxyproline Quantification in Lungs

The right upper lung lobes were homogenized in ddH₂O (10 mg of tissue in 100 μl). Then an aliquot was hydrolyzed in HCl 6M at 105°C for 3 h and centrifuged at 13,000 rpm for 5 min. 5 μl of the supernatant were pipetted in triplicate onto a 96 well plate and incubated at 60°C for 1 h. Collagen content was assessed using a Hydroxyproline Colorimetric Assay Kit (MAK008 Sigma-Aldrich) according to the manufacturer's instructions.

RNA Extraction and qPCR

Total RNA was isolated from right lower lung using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. Two μg of total RNA were retro-transcribed with MMLV reverse transcriptase (Invitrogen) using random primers. cDNA was analyzed by quantitative real-time polymerase chain reaction (qPCR) analysis. Beta-Actin (*ActB*) was used as housekeeping gene for normalization. Primers used: Mm_*Ptx3* forward: 5'-GACCTCGGATGACTACGAG-3', reverse: 5'-CTC CGAGTGCTCCTGGCG-3'; *Col1A1* for: 5'-TGCTCCTCTTAG GGGCCACT-3', rev: 5'-ATTGGGGACCCTTAGGCCATT-3'; *Col1A2* for: 5'-GGTGAGCCTGGTCAAACGG-3', rev: 5'-ACT GTGTCCTTTACGCCTTT-3'; *Col3A1* for: 5'-CTGTAACATG GAAACTGGGGAAA-3', rev: 5'-CCATAGCTGAACTGAAA ACCACC-3'; *Acta2* for: 5'-TGCTGACAGAGGCACCACTG AA-3', rev: 5'-CAGTTGTACGTCCAGAGGCATAG-3'; *ActB* for: 5'-CTGTCGAGTCGCGTCCACC-3', rev: 5'-ATCGT CATCCATGGCGAACTG-3'.

Statistical Analyses

T-test for unpaired data (2-tailed) was used to test the probability of significant differences between two groups of samples. For the statistical analysis the WT group at day 0 was used as reference. The following abbreviations were used for the indication of significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

RESULTS

PTX3 Is Modulated During BLM-Induced Lung Fibrosis

Intratracheal administration of BLM induces peri-bronchial lung fibrosis in mice, detectable 14 days after treatment and progressing up to day 21, to decrease at/after day 28 (15).

On this basis, in order to evaluate the modulation of PTX3 expression in the lungs during fibrosis onset, C57BL/6 male mice were treated intratracheal with BLM and sacrificed after 14, 21, and 28 days. Then, harvested lungs were processed for immunohistochemical (IHC) and qPCR analyses. In keeping with its role as an "early" response to damaging and inflammatory stimuli (21), PTX3 immuno-reactivity was significantly increased in fibrotic lung sections 14 days after BLM treatment, to decrease to basal levels at day 28 (**Figures 1A, B**). These data were confirmed by qPCR analysis that demonstrated a significant upregulation of *Ptx3* expression at day 14 and its gradual decrease thereafter (**Figure 1C**).

PTX3 Exerts a Protective Role in Lung Fibrosis

Previous observations had shown a potential role of PTX3 as a protective factor in different pathological settings in the lungs (21). On this basis, we evaluated the impact of PTX3 overexpression during the onset and progression of the fibrotic process in the lungs by taking advantage of a transgenic Tie2-PTX3 murine model. In this model the overexpression of the human *PTX3* gene (**Supplementary Figure S2**) is driven by the endothelial-specific *Tie2* promoter, leading to the stromal accumulation of high levels of the PTX3 protein in all tissues, including the lungs, without any significant impact on normal development of organs and tissues (32, 34). Wild type (WT) and

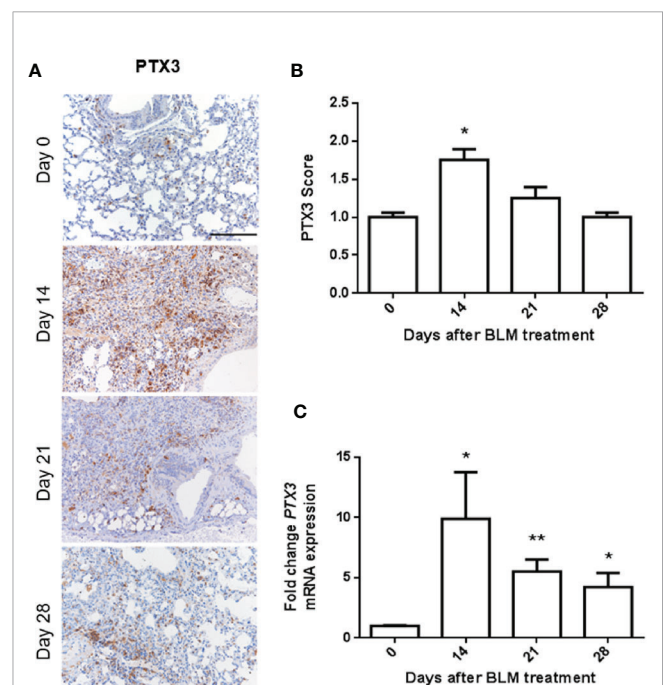


FIGURE 1 | PTX3 expression is induced in BLM-induced lung fibrosis. PTX3 expression in the lungs of C57BL/6 wild type mice treated with bleomycin (BLM) and sacrificed at day 0, 14, 21, and 28 after treatment was detected by immunohistochemistry (**A**) and quantified by scoring the immunostaining (**B**) or by qPCR (**C**). $N = 5-8$ mice/group; scale bar = 200 μm ; * $P < 0.05$, ** $p < 0.01$.

transgenic Tie2-PTX3 mice were treated intratracheal with BLM and the progression of lung fibrosis was investigated. As shown in **Figures 2A, B**, quantification of fibrotic/collagen positive areas after trichrome staining of lung sections revealed an increase of fibrotic tissue in WT animals from day 14 to day 21, with a partial regression at day 28. When compared to WT mice, fibrotic areas were significantly reduced in Tie2-PTX3 mice at all the time points investigated, close to the levels measured in untreated animals. Accordingly, the levels of hydroxyproline, a marker for the presence of collagen in the ECM, were significantly higher in WT lungs when compared with those measured in Tie2-PTX3 animals (**Figure 2C**).

Further characterization of BLM-treated lungs showed a significant increase of areas with alpha-smooth muscle actin (α SMA⁺) positive fibroblasts in WT mice when compared with Tie2-PTX3 animals (**Figures 3A, B**). Related to this fibroblastic reaction, upregulation of the expression of *Colla1*, *Colla2*, and *Col3a1* collagen subunits and of the α SMA-encoding *Acta2* gene was detected by qPCR in the lungs of WT but not of Tie2-PTX3 mice (**Figure 3C**). In addition, foci of angiogenesis, represented by CD31⁺ endothelial cells, were detectable in the fibrotic areas of BLM-treated mice with no apparent difference between WT and Tie2-PTX3 groups (**Supplementary Figure S3**).

The inflammatory infiltrate plays a pivotal role during the response to tissue damage that drives fibrosis (7, 8). In order to quantify the overall immune infiltrate in BLM-treated lungs, CD45 immunostaining was performed on samples from the different groups. As shown in **Figure 4A**, a vast CD45⁺ cell

infiltrate was detected in WT lungs, while a significant reduction of CD45⁺ cells was observed in Tie2-PTX3 lungs at all the time points investigated. These observations are in line with the limited fibrotic response occurring in Tie2-PTX3 animals. Accordingly, in keeping with the pivotal role played by macrophages in BLM-induced fibrosis (7), IBA1⁺ macrophages were widely present in fibrotic WT lungs throughout the whole experimental period and consistently reduced in Tie2-PTX3 lungs that showed a detectable macrophagic infiltrate only at day 14 (**Figure 4B**). In addition, a further characterization of the immune infiltrate at the time points considered demonstrate that CD3⁺ lymphocytes increase in both WT and Tie2-PTX3 at day 14 after treatment, to decrease more rapidly in PTX3 overexpressing animals in respect to controls (**Supplementary Figure S4A**). Finally, very few Ly6G⁺ neutrophils were present in the lungs of treated mice, with no significant differences between the WT and Tie2-PTX3 animals (**Supplementary Figure S4B**).

Together, these data indicate that PTX3 overexpression exerts a protective impact on BLM-induced fibrosis in transgenic Tie2-PTX3 mice. These findings, together with the observation that a significant upregulation of PTX3 expression occurs in WT animals during BLM-induced fibrosis (see above), prompted us to assess the role, if any, of endogenous PTX3 on this process. To this aim, we took advantage from genetically deficient *Ptx3*^{-/-} mice that lack endogenous PTX3 expression in all organs, including lungs, and are characterized by a normal pre and post-natal development with no signs of inherited pathological conditions with the exception of subfertility in

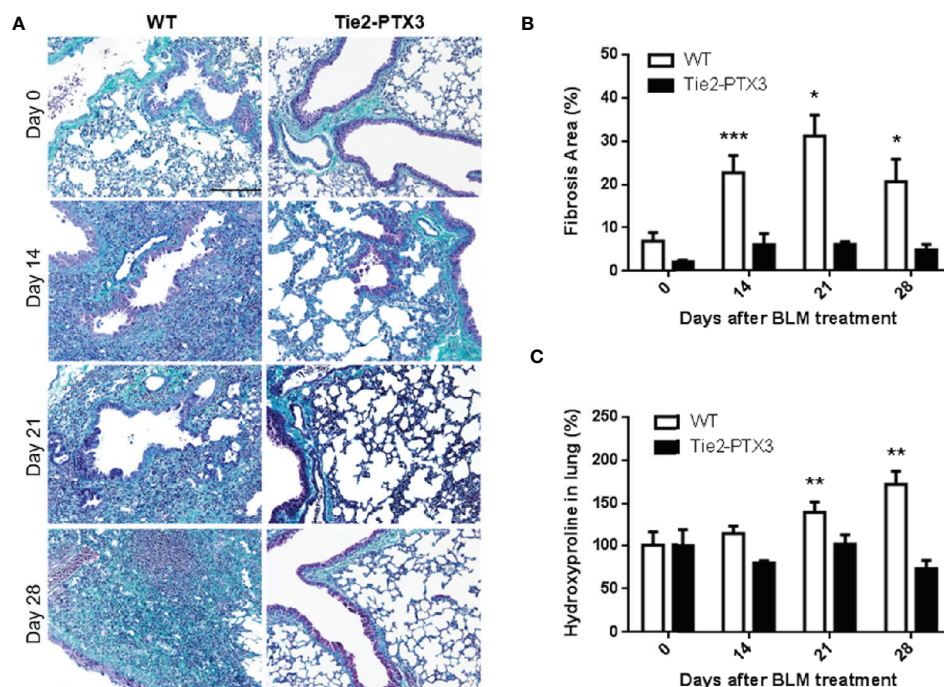


FIGURE 2 | Stromal PTX3 overexpression prevents lung fibrosis in BLM-treated mice. Fibrosis in lungs of wild type (WT) and transgenic Tie2-PTX3 mice treated with bleomycin (BLM) was determined by Masson's trichrome staining (**A**), subsequent quantification of fibrotic area (**B**), and quantification of hydroxyproline in lungs extracts (**C**). *N* = 5–8 mice/group; scale bar = 200 μ m; **P* < 0.05, ***p* < 0.01, ****p* < 0.001.

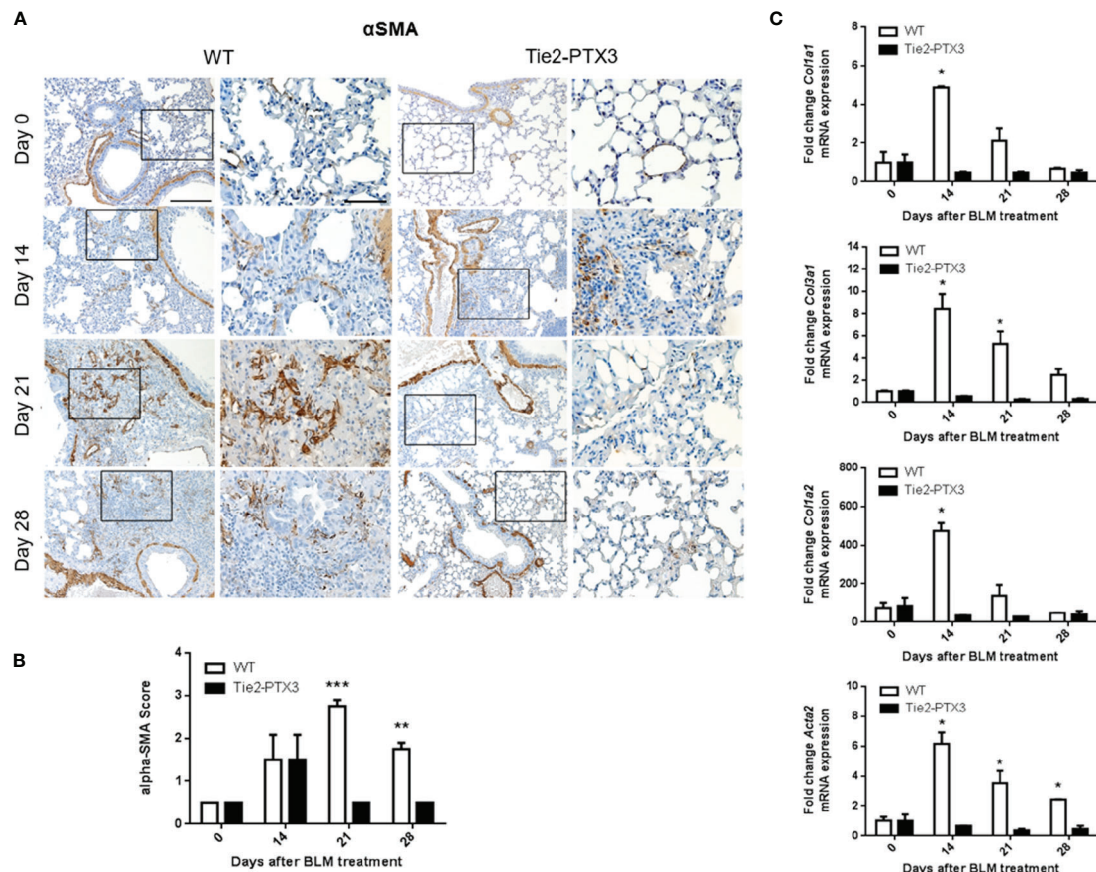


FIGURE 3 | Stromal PTX3 overexpression reduces fibroblast activation in BLM-induced lung fibrosis. **(A)** Immunohistochemical analysis of α SMA expression in the lungs of wild type (WT) and transgenic Tie2-PTX3 mice treated with bleomycin (BLM). For each image, the boxed area is shown at higher magnification in the right panel. Scale bar = 100 μ m; scale bar of magnified images = 200 μ m. **(B)** Score quantification of the α SMA immunostaining. α SMA immuno-reactivity in perivascular and peri-bronchiolar areas was excluded from the quantification. **(C)** qPCR analysis on lung extracts for the expression of collagen subunits and *Acta2* mRNA. $N = 5$ –8 mice/group; * $P < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

female animals (31). On the other hand, these mice may show different responses under defined experimental conditions characterized by the involvement of the innate immune arm [reviewed in (23, 35, 36)]. BLM was administered intratracheal to *Ptx3* null mice and lung fibrosis was followed in parallel to that occurring in WT and Tie2-PTX3 animals. As shown in **Figure 5A**, BLM treatment caused a dramatic decrease of the lifespan of *Ptx3*^{−/−} mice, with 4 out of 8 mice dying at day 14 and no survival occurring at day 21 after treatment. At variance, no animal death was observed for BLM-treated WT and Tie2-PTX3 mice. Accordingly, trichrome staining of the lung sections and quantification of fibrotic areas confirmed a significant increase of fibrotic/scar tissue at day 14 in *PTX3*^{−/−} mice when compared to WT and Tie2-PTX3 animals (**Figures 5B, C**).

DISCUSSION

Hyper-proliferating fibroblasts/myofibroblasts and augmented deposition of ECM are typical features of IPF and the main

cause of lung architecture alterations underlying the loss of respiratory function (6). This is paralleled by an inflammatory response whose contribution to IPF has long been debated (37) and in some cases remains controversial (38, 39). For instance, treatment of IPF patients with steroids reduces inflammation in the lung but does not improve survival nor clinical outcome (40, 41), and a clinical trial based on immune-suppression was discontinued due to increased hospitalization and death (42).

PTX3 is a component of the humoral arm of the innate immunity. Even though its expression in human tissues is usually associated with inflammation, both pro-inflammatory and inflammation-limiting properties have been reported in preclinical models of disease, and a possible dual role has been unveiled in physiological and pathological settings (35, 43, 44). On one side, PTX3 generally exhibits protective antibody-like functions and promotes tissue repair *via* matrix remodeling and modulation of the inflammatory response. On the other hand, potential damaging effects have been ascribed to PTX3 due to its capacity to induce endothelial dysfunction (45), exacerbated complement activation and inflammation (44, 46, 47).

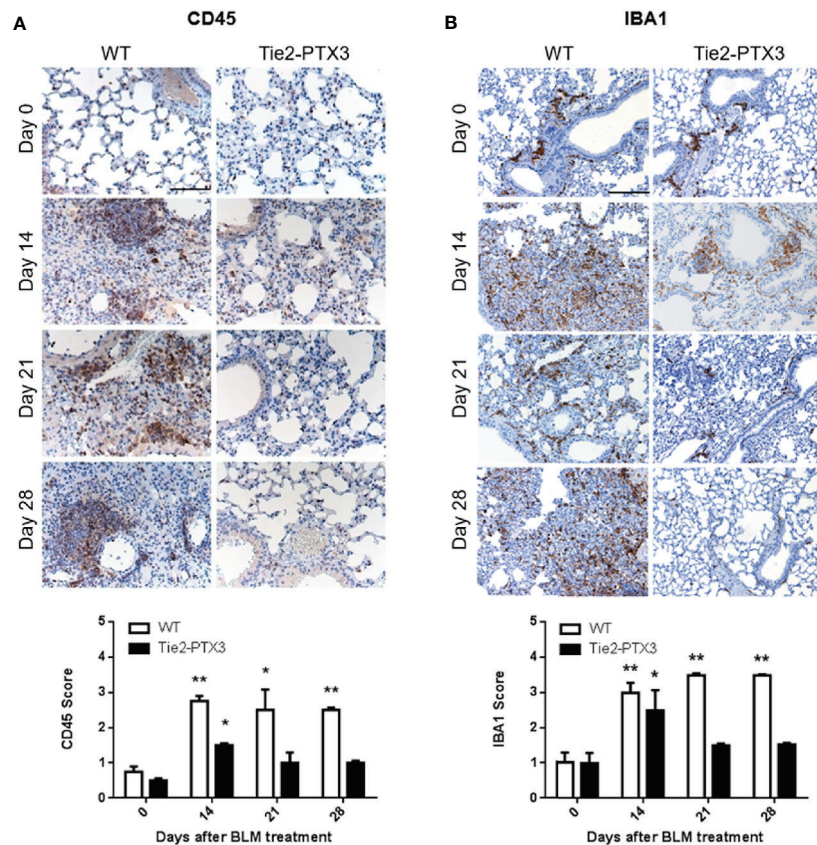


FIGURE 4 | Stromal PTX3 overexpression prevents immune infiltration in BLM-induced lung fibrosis. CD45 (A) and IBA1 (B) levels in the lungs of wild type (WT) and transgenic Tie2-PTX3 mice treated with bleomycin (BLM) were assessed by immunohistochemistry and quantified by scoring the immunostaining. $N = 5-8$ mice/group; scale bar = 100 μm (A), 200 μm (B); * $P < 0.05$; ** $p < 0.01$.

In inflammatory/injury settings, neutrophils are among the first leukocytes to be recruited. Once activated, they secrete their content of “ready to release” PTX3 that partially remains associated with neutrophil extracellular traps (NETs) (48, 49). On the other hand, PTX3 locally released by activated leukocytes may impair leukocyte rolling on endothelium, thus attenuating the recruitment of neutrophils, regulating inflammation, and reducing tissue damage in murine models of acute lung injury, pleurisy, and mesenteric inflammation (50).

Under physiological conditions, the expression levels of PTX3 are low in the lungs of healthy mice and can be modulated depending on the severity and duration of the damaging agent (31, 51–53). In this study we investigated the expression of PTX3 in a BLM-induced murine model of lung fibrosis with the aim to define its role in this pathological context.

In keeping with previous observations in preclinical models of lung fibrosis and in lung tissue from IPF patients (54), PTX3 expression rapidly increases in the lungs of BLM-treated animals both at mRNA and protein levels. This upregulation is transient, reaches a peak at 14 days after treatment to decrease to physiological levels thereafter. Thus, PTX3 upregulation represents an early event in response to the fibrotic insult,

unable to restrain and control the onset of BLM-induced fibrosis in the lungs, that continues till day 28 after treatment.

Two distinct experimental approaches pointed to a protective role for PTX3 in this model of PF. In a first set of experiments, PTX3 overexpression in transgenic Tie2-PTX3 animals, which results in the accumulation of PTX3 in the lung stroma (32), was able to limit the formation of fibrotic tissue in the lungs, with reduced activation of fibroblasts and ECM deposition. Moreover, the infiltration of immune CD45⁺ cells was reduced in Tie2-PTX3 lungs that showed a dramatic decrease in infiltrating IBA1⁺ macrophages when compared to WT animals, with moderate or no major differences in infiltrating neutrophils and T lymphocytes at the experimental points investigated. These observations are in line with the critical regulatory activities exerted by macrophages during all the steps of fibrosis onset and repair (55, 56). The protective effect of PTX3 on lung fibrosis was confirmed in a second set of experiments performed on *Ptx3*^{-/-} animals in which PTX3 deficiency caused an increased deposition of fibrotic tissue and a rapid animal death following BLM administration.

Even though the molecular mechanisms at the basis of this impact of PTX3 on PF remain to be elucidated, previous

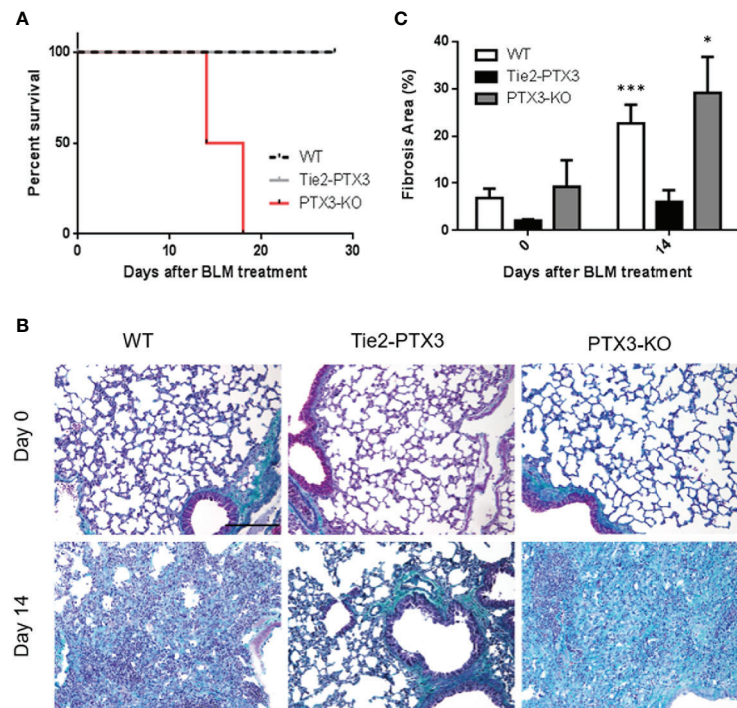


FIGURE 5 | Ptx3 knockdown increases the deposition of fibrotic tissue and a rapid animal death in BLM-induced lung fibrosis. **(A)** Kaplan-Meier survival curve of wild type (WT) and transgenic Tie2-PTX3 and *Ptx3*^{-/-} (KO) mice treated with bleomycin (BLM). Masson's trichrome staining **(B)** and quantification of the fibrotic area **(C)** performed on lungs at day 0 and day 14 after treatment. *N* = 5–8 mice/group; scale bar = 100 μ m; **P* < 0.05, ****p* < 0.001.

experimental evidences suggest that its accumulation in the pulmonary stroma may modulate different biological processes, including the recruitment of immune cells (50, 57, 58) and fibroblast activation (22, 36). This occurs *via* the interaction of PTX3 with growth factors, complement components, the haemostatic system and the fibrinolytic cascade. Indeed, PTX3 can bind to fibroblast growth factors (FGFs) and impair the FGF/FGF receptor system (22, 24) which plays a relevant role in fibrosis (59) as well as in immune cell recruitment (33, 60). *In vitro*, the interaction of PTX3 with Fc γ RI has been shown to favor the differentiation of human and murine monocytes into fibrocytes, fibroblast-like cells expressing both haematopoietic and stromal cell markers frequently found in fibrotic lesions (54, 61). In the same context, the serum amyloid P component (SAP) was shown to play a fibrocyte-inhibitory activity stronger than PTX3 (54), and injections of SAP inhibited fibrosis in various mouse models of the disease (62, 63). Interestingly, SAP expression was almost absent in fibrotic areas, while PTX3 was widespread, thus suggesting that relative levels of SAP and PTX3 may have a significant role in fibrocyte differentiation at fibrotic sites (54).

In the acute lung injury, a strong activation of the innate immune system in the lungs is accompanied by the expression of PTX3. This pathological context is characterized by infiltration of neutrophils and increased production of nitric oxide and of tissue factor. It has been suggested that high levels

of PTX3 activate the local innate immune system and play a protective role against lung insults (21, 51). Accordingly, *Ptx3*^{-/-} mice are more susceptible to tissue damage after exposure to LPS (28). In addition, PTX3 can interact with fibrin and plasminogen under acidic conditions in damaged/inflamed tissues, fostering the remodeling of the fibrin-rich matrix and tissue repair (64).

Overall, in this study we have reported the observational results of the effect of endogenous PTX3 modulation on lung fibrosis onset and phenotype. Intravenous or intraperitoneal treatment with recombinant PTX3 has resulted in beneficial outcomes in various experimental models of inflammatory diseases (25, 31, 48, 65–67). Thus, in a therapeutic perspective, it will be of importance to assess the effect of recombinant PTX3 to prevent fibrosis in BLM-treated mice. Our results and these future studies will pave the way for the characterization of the molecular players modulated by PTX3 in PF and for the design of PTX3-derived approaches to be used for the treatment of PF patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by OPBA University of Brescia.

AUTHOR CONTRIBUTIONS

FM performed *in vivo* experiments. MB performed immunohistochemistry. AS, WV, and SG gave technical assistance. MP conceived the experimental plan and revised the paper. RR conceived and supervised the experiments, and wrote the paper. All authors contributed to the article and approved the submitted version.

REFERENCES

- Bartczak K, Bialas AJ, Kotecki MJ, Gorski P, Piotrowski WJ. More than a Genetic Code: Epigenetics of Lung Fibrosis. *Mol Diagn Ther* (2020) 24 (6):665–81. doi: 10.1007/s40291-020-00490-7
- Saulea J, Nunez B, Sala E, Soriano JB. Idiopathic Pulmonary Fibrosis: Epidemiology, Natural History, Phenotypes. *Med Sci (Basel)* (2018) 6(4). doi: 10.3390/medsci6040110
- Diridollou T, Sohler L, Rousseau C, Angibaud A, Chauvin P, Gaignon T, et al. Idiopathic pulmonary fibrosis: Significance of the usual interstitial pneumonia (UIP) CT-scan patterns defined in new international guidelines. *Respir Med Res* (2020) 77:72–8. doi: 10.1016/j.resmer.2020.02.004
- Raghu G, Collard HR, Egan JJ, Martinez FJ, Behr J, Brown KK, et al. An official ATS/ERS/JRS/ALAT statement: idiopathic pulmonary fibrosis: evidence-based guidelines for diagnosis and management. *Am J Respir Crit Care Med* (2011) 183(6):788–824. doi: 10.1164/rccm.2009-040GL
- Lederer DJ, Martinez FJ. Idiopathic Pulmonary Fibrosis. *N Engl J Med* (2018) 379(8):797–8. doi: 10.1056/NEJMc1807508
- Kuhn C, Mason RJ. Immunolocalization of SPARC, tenascin, and thrombospondin in pulmonary fibrosis. *Am J Pathol* (1995) 147(6):1759–69.
- Laskin DL, Malaviya R, Laskin JD. Role of Macrophages in Acute Lung Injury and Chronic Fibrosis Induced by Pulmonary Toxicants. *Toxicol Sci* (2019) 168(2):287–301. doi: 10.1093/toxsci/kfy309
- Misharin AV, Morales-Nebreda L, Reyfman PA, Cuda CM, Walter JM, McQuattie-Pimentel AC, et al. Monocyte-derived alveolar macrophages drive lung fibrosis and persist in the lung over the life span. *J Exp Med* (2017) 214(8):2387–404. doi: 10.1084/jem.20162152
- Larson-Casey JL, Deshane JS, Ryan AJ, Thannickal VJ, Carter AB. Macrophage Akt1 Kinase-Mediated Mitophagy Modulates Apoptosis Resistance and Pulmonary Fibrosis. *Immunity* (2016) 44(3):582–96. doi: 10.1016/j.immuni.2016.01.001
- Gibbons MA, MacKinnon AC, Ramachandran P, Dhaliwal K, Duffin R, Phythian-Adams AT, et al. Ly6Chi monocytes direct alternatively activated profibrotic macrophage regulation of lung fibrosis. *Am J Respir Crit Care Med* (2011) 184(5):569–81. doi: 10.1164/rccm.201010-1719OC
- Bickelhaupt S, Erbel C, Timke C, Wirkner U, Dadrich M, Flechsig P, et al. Effects of CTGF Blockade on Attenuation and Reversal of Radiation-Induced Pulmonary Fibrosis. *J Natl Cancer Inst* (2017) 109(8). doi: 10.1093/jnci/djw339
- Prasse A, Pechkovsky DV, Toews GB, Jungraithmayr W, Kollert F, Goldmann T, et al. A vicious circle of alveolar macrophages and fibroblasts perpetuates pulmonary fibrosis via CCL18. *Am J Respir Crit Care Med* (2006) 173(7):781–92. doi: 10.1164/rccm.200509-1518OC
- Pulichino AM, Wang IM, Caron A, Mortimer J, Auger A, Boie Y, et al. Identification of transforming growth factor beta1-driven genetic programs of acute lung fibrosis. *Am J Respir Cell Mol Biol* (2008) 39(3):324–36. doi: 10.1165/rcmb.2007-0186OC
- Shvedova AA, Kisin ER, Mercer R, Murray AR, Johnson VJ, Potapovich AI, et al. Unusual inflammatory and fibrogenic pulmonary responses to single-walled carbon nanotubes in mice. *Am J Physiol Lung Cell Mol Physiol* (2005) 289(5):L698–708. doi: 10.1152/ajplung.00084.2005

FUNDING

This work was supported by Associazione Italiana per la Ricerca sul Cancro (IG 2019 no. 23116 to MP and IG 2019 no. 23151 to RR). FM was supported by Fondazione Veronesi Fellowship.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2021.617671/full#supplementary-material>

- Moore BB, Hogaboam CM. Murine models of pulmonary fibrosis. *Am J Physiol Lung Cell Mol Physiol* (2008) 294(2):L152–60. doi: 10.1152/ajplung.00313.2007
- Izbicki G, Segel MJ, Christensen TG, Conner MW, Breuer R. Time course of bleomycin-induced lung fibrosis. *Int J Exp Pathol* (2002) 83(3):111–9. doi: 10.1046/j.1365-2613.2002.00220.x
- Muggia FM, Louie AC, Sikic BI. Pulmonary toxicity of antitumor agents. *Cancer Treat Rev* (1983) 10(4):221–43. doi: 10.1016/0305-7372(83)90012-9
- Phan SH, Thrall RS, Ward PA. Bleomycin-induced pulmonary fibrosis in rats: biochemical demonstration of increased rate of collagen synthesis. *Am Rev Respir Dis* (1980) 121(3):501–6. doi: 10.1164/arrd.1980.121.3.501
- Snider GL, Hayes JA, Korthy AL. Chronic interstitial pulmonary fibrosis produced in hamsters by endotracheal bleomycin: pathology and stereology. *Am Rev Respir Dis* (1978) 117(6):1099–108. doi: 10.1164/arrd.1978.117.6.1099
- Thrall RS, McCormick JR, Jack RM, McReynolds RA, Ward PA. Bleomycin-induced pulmonary fibrosis in the rat: inhibition by indomethacin. *Am J Pathol* (1979) 95(1):117–30.
- Balhar J, Koussih L, Zhang J, Gounni AS. Pentraxin 3: an immuno-regulator in the lungs. *Front Immunol* (2013) 4:127. doi: 10.3389/fimmu.2013.00127
- Giacomini A, Ghedini GC, Presta M, Ronca R. Long pentraxin 3: A novel multifaceted player in cancer. *Biochim Biophys Acta* (2018) 1869(1):53–63. doi: 10.1016/j.bbcan.2017.11.004
- Doni A, Stravalaci M, Inforzato A, Magrini E, Mantovani A, Garlanda C, et al. The Long Pentraxin PTX3 as a Link Between Innate Immunity, Tissue Remodeling, and Cancer. *Front Immunol* (2019) 10:712. doi: 10.3389/fimmu.2019.00712
- Presta M, Foglio E, Churrua Schuind A, Ronca R. Long Pentraxin-3 Modulates the Angiogenic Activity of Fibroblast Growth Factor-2. *Front Immunol* (2018) 9:2327. doi: 10.3389/fimmu.2018.02327
- Moalli F, Doni A, Deban L, Zelante T, Zagarella S, Bottazzi B, et al. Role of complement and Fc{gamma} receptors in the protective activity of the long pentraxin PTX3 against *Aspergillus fumigatus*. *Blood* (2010) 116(24):5170–80. doi: 10.1182/blood-2009-12-258376
- Deban L, Jarva H, Lehtinen MJ, Bottazzi B, Bastone A, Doni A, et al. Binding of the long pentraxin PTX3 to factor H: interacting domains and function in the regulation of complement activation. *J Immunol* (2008) 181(12):8433–40. doi: 10.4049/jimmunol.181.12.8433
- Han B, Ma X, Zhang J, Zhang Y, Bai X, Hwang DM, et al. Protective effects of long pentraxin PTX3 on lung injury in a severe acute respiratory syndrome model in mice. *Lab Investigation J Tech Methods Pathol* (2012) 92(9):1285–96. doi: 10.1038/labinvest.2012.92
- Okutani D, Han B, Mura M, Waddell TK, Keshavjee S, Liu M. High-volume ventilation induces pentraxin 3 expression in multiple acute lung injury models in rats. *Am J Physiol Lung Cell Mol Physiol* (2007) 292(1):L144–53. doi: 10.1152/ajplung.00002.2006
- Zhang J, Shan L, Koussih L, Redhu NS, Halayko AJ, Chakir J, et al. Pentraxin 3 (PTX3) expression in allergic asthmatic airways: role in airway smooth muscle migration and chemokine production. *PLoS One* (2012) 7(4):e34965. doi: 10.1371/journal.pone.0034965

30. Diamandis EP, Goodglick L, Planque C, Thornquist MD. Pentraxin-3 is a novel biomarker of lung carcinoma. *Clin Cancer Res* (2011) 17(8):2395–9. doi: 10.1158/1078-0432.CCR-10-3024
31. Garlanda C, Hirsch E, Bozza S, Salustri A, De Acetis M, Nota R, et al. Non-redundant role of the long pentraxin PTX3 in anti-fungal innate immune response. *Nature* (2002) 420(6912):182–6. doi: 10.1038/nature01195
32. Ronca R, Giacomini A, Di Salle E, Coltrini D, Pagano K, Ragona L, et al. Long-Pentraxin 3 Derivative as a Small-Molecule FGF Trap for Cancer Therapy. *Cancer Cell* (2015) 28(2):225–39. doi: 10.1016/j.ccell.2015.07.002
33. Ronca R, Tamma R, Coltrini D, Ruggieri S, Presta M, Ribatti D. Fibroblast growth factor modulates mast cell recruitment in a murine model of prostate cancer. *Oncotarget* (2017) 8(47):82583–92. doi: 10.18632/oncotarget.19773
34. Matarazzo S, Melocchi L, Rezzola S, Grillo E, Maccarinelli F, Giacomini A, et al. Long Pentraxin-3 Follows and Modulates Bladder Cancer Progression. *Cancers* (2019) 11(9). doi: 10.3390/cancers11091277
35. Mantovani A, Valentino S, Gentile S, Inforzato A, Bottazzi B, Garlanda C. The long pentraxin PTX3: a paradigm for humoral pattern recognition molecules. *Ann N Y Acad Sci* (2013) 1285:1–14. doi: 10.1111/nyas.12043
36. Doni A, Garlanda C, Mantovani A. Innate immunity, hemostasis and matrix remodeling: PTX3 as a link. *Semin Immunol* (2016) 28(6):570–7. doi: 10.1016/j.smim.2016.10.012
37. McLean-Tooke A, Moore I, Lake F. Idiopathic and immune-related pulmonary fibrosis: diagnostic and therapeutic challenges. *Clin Trans Immunol* (2019) 8(11):e1086. doi: 10.1002/cti2.1086
38. Gauldie J. Pro: Inflammatory mechanisms are a minor component of the pathogenesis of idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* (2002) 165(9):1205–6. doi: 10.1164/rccm.2202054
39. Strieter RM. Con: Inflammatory mechanisms are not a minor component of the pathogenesis of idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* (2002) 165(9):1206–7; discussion 7–8. doi: 10.1164/rccm.2202055
40. Grijm K, Verberne HJ, Krouwels FH, Weller FR, Jansen HM, Bresser P. Semiquantitative ⁶⁷Ga scintigraphy as an indicator of response to and prognosis after corticosteroid treatment in idiopathic interstitial pneumonia. *J Nucl Med* (2005) 46(9):1421–6.
41. Pereira CA, Malheiros T, Coletta EM, Ferreira RG, Rubin AS, Otta JS, et al. Survival in idiopathic pulmonary fibrosis-cytotoxic agents compared to corticosteroids. *Respir Med* (2006) 100(2):340–7. doi: 10.1016/j.rmed.2005.05.008
42. Raghu G, Anstrom KJ, King TE Jr., Lasky JA, Martinez FJ. Prednisone, azathioprine, and N-acetylcysteine for pulmonary fibrosis. *N Engl J Med* (2012) 366(21):1968–77. doi: 10.1056/NEJMoa1113354
43. Bonacina F, Baragetti A, Catapano AL, Norata GD. Long pentraxin 3: experimental and clinical relevance in cardiovascular diseases. *Mediators Inflamm* (2013) 2013:725102. doi: 10.1155/2013/725102
44. Magrini E, Mantovani A, Garlanda C. The Dual Complexity of PTX3 in Health and Disease: A Balancing Act? *Trends Mol Med* (2016) 22(6):497–510. doi: 10.1016/j.molmed.2016.04.007
45. Foo SS, Chen W, Taylor A, Sheng KC, Yu X, Teng TS, et al. Role of pentraxin 3 in shaping arthritogenic alphaviral disease: from enhanced viral replication to immunomodulation. *PLoS Pathog* (2015) 11(2):e1004649. doi: 10.1371/journal.ppat.1004649
46. Inforzato A, Doni A, Barajon I, Leone R, Garlanda C, Bottazzi B, et al. PTX3 as a paradigm for the interaction of pentraxins with the complement system. *Semin Immunol* (2013) 25(1):79–85. doi: 10.1016/j.smim.2013.05.002
47. Souza DG, Soares AC, Pinho V, Torloni H, Reis LF, Teixeira MM, et al. Increased mortality and inflammation in tumor necrosis factor-stimulated gene-14 transgenic mice after ischemia and reperfusion injury. *Am J Pathol* (2002) 160(5):1755–65. doi: 10.1016/s0002-9440(10)61122-4
48. Jaillon S, Peri G, Delneste Y, Fremaux I, Doni A, Moalli F, et al. The humoral pattern recognition receptor PTX3 is stored in neutrophil granules and localizes in extracellular traps. *J Exp Med* (2007) 204(4):793–804. doi: 10.1084/jem.20061301
49. Daigo K, Yamaguchi N, Kawamura T, Matsubara K, Jiang S, Ohashi R, et al. The proteomic profile of circulating pentraxin 3 (PTX3) complex in sepsis demonstrates the interaction with azurocidin 1 and other components of neutrophil extracellular traps. *Mol Cell Proteomics MCP* (2012) 11(6):M111015073. doi: 10.1074/mcp.M111.015073
50. Deban L, Russo RC, Sironi M, Moalli F, Scanziani M, Zambelli V, et al. Regulation of leukocyte recruitment by the long pentraxin PTX3. *Nat Immunol* (2010) 11(4):328–34. doi: 10.1038/ni.1854
51. He X, Han B, Liu M. Long pentraxin 3 in pulmonary infection and acute lung injury. *Am J Physiol Lung Cell Mol Physiol* (2007) 292(5):L1039–49. doi: 10.1152/ajplung.00490.2006
52. Pauwels NS, Bracke KR, Maes T, Van Pottelberge GR, Garlanda C, Mantovani A, et al. Cigarette smoke induces PTX3 expression in pulmonary veins of mice in an IL-1 dependent manner. *Respir Res* (2010) 11:134. doi: 10.1186/1465-9921-11-134
53. Han B, Mura M, Andrade CF, Okutani D, Lodyga M, dos Santos CC, et al. TNFalpha-induced long pentraxin PTX3 expression in human lung epithelial cells via JNK. *J Immunol* (2005) 175(12):8303–11. doi: 10.4049/jimmunol.175.12.8303
54. Pilling D, Cox N, Vakil V, Verbeek JS, Gomer RH. The long pentraxin PTX3 promotes fibrocyte differentiation. *PLoS One* (2015) 10(3):e0119709. doi: 10.1371/journal.pone.0119709
55. Wynn TA, Barron L. Macrophages: master regulators of inflammation and fibrosis. *Semin Liver Dis* (2010) 30(3):245–57. doi: 10.1055/s-0030-1255354
56. Wynn TA, Vannella KM. Macrophages in Tissue Repair, Regeneration, and Fibrosis. *Immunity* (2016) 44(3):450–62. doi: 10.1016/j.immuni.2016.02.015
57. Bonavita E, Gentile S, Rubino M, Maina V, Papait R, Kunderfranco P, et al. PTX3 is an extrinsic oncosuppressor regulating complement-dependent inflammation in cancer. *Cell* (2015) 160(4):700–14. doi: 10.1016/j.cell.2015.01.004
58. Annes T, Ronca R, Tamma R, Giacomini A, Ruggieri S, Grillo E, et al. PTX3 Modulates Neovascularization and Immune Inflammatory Infiltrate in a Murine Model of Fibrosarcoma. *Int J Mol Sci* (2019) 20(18). doi: 10.3390/ijms20184599
59. Guzy RD, Li L, Smith C, Dorry SJ, Koo HY, Chen L, et al. Pulmonary fibrosis requires cell-autonomous mesenchymal fibroblast growth factor (FGF) signaling. *J Biol Chem* (2017) 292(25):10364–78. doi: 10.1074/jbc.M117.791764
60. Presta M, Andres G, Leali D, Dell'Era P, Ronca R. Inflammatory cells and chemokines sustain FGF2-induced angiogenesis. *Eur Cytokine Netw* (2009) 20(2):39–50. doi: 10.1684/ecm.2009.0155
61. Reilkoff RA, Bucala R, Herzog EL. Fibrocytes: emerging effector cells in chronic inflammation. *Nat Rev Immunol* (2011) 11(6):427–35. doi: 10.1038/nri2990
62. Pilling D, Roife D, Wang M, Ronkainen SD, Crawford JR, Travis EL, et al. Reduction of bleomycin-induced pulmonary fibrosis by serum amyloid P. *J Immunol* (2007) 179(6):4035–44. doi: 10.4049/jimmunol.179.6.4035
63. Murray LA, Rosada R, Moreira AP, Joshi A, Kramer MS, Hesson DP, et al. Serum amyloid P therapeutically attenuates murine bleomycin-induced pulmonary fibrosis via its effects on macrophages. *PLoS One* (2010) 5(3):e9683. doi: 10.1371/journal.pone.0009683
64. Doni A, Musso T, Morone D, Bastone A, Zambelli V, Sironi M, et al. An acidic microenvironment sets the humoral pattern recognition molecule PTX3 in a tissue repair mode. *J Exp Med* (2015) 212(6):905–25. doi: 10.1084/jem.20141268
65. Gaziano R, Bozza S, Bellocchio S, Perruccio K, Montagnoli C, Pitzurra L, et al. Anti-Aspergillus fumigatus efficacy of pentraxin 3 alone and in combination with antifungals. *Antimicrobial Agents Chemother* (2004) 48(11):4414–21. doi: 10.1128/AAC.48.11.4414-4421.2004
66. Paroni M, Moalli F, Nebuloni M, Pasqualini F, Bonfield T, Nonis A, et al. Response of CFTR-deficient mice to long-term chronic *Pseudomonas aeruginosa* infection and PTX3 therapy. *J Infect Dis* (2013) 208(1):130–8. doi: 10.1093/infdis/jis636
67. Moalli F, Paroni M, Veliz Rodriguez T, Riva F, Polentarutti N, Bottazzi B, et al. The therapeutic potential of the humoral pattern recognition molecule PTX3 in chronic lung infection caused by *Pseudomonas aeruginosa*. *J Immunol* (2011) 186(9):5425–34. doi: 10.4049/jimmunol.1002035

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Maccarinelli, Bugatti, Churrua Schuind, Ganzerla, Vermi, Presta and Ronca. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Increased Pentraxin 3 Levels Correlate With IVIG Responsiveness and Coronary Artery Aneurysm Formation in Kawasaki Disease

Toshiyuki Kitoh^{1,2*}, Tsuyoshi Ohara¹, Taichiro Muto², Akihisa Okumura², Reizo Baba^{2,3}, Yusuke Koizumi⁴, Yuka Yamagishi⁴, Hiroshige Mikamo⁴, Kenji Daigo⁵ and Takao Hamakubo⁵

¹ Laboratory of Pediatrics, School of Pharmacy, Aichi Gakuin University, Nagoya, Japan, ² Department of Pediatrics, School of Medicine, Aichi Medical University, Nagakute, Japan, ³ Department of Lifelong Sports and Health Sciences, College of Life and Health Sciences, Chubu University, Kasugai, Japan, ⁴ Department of Clinical Infectious Diseases, School of Medicine, Aichi Medical University, Nagakute, Japan, ⁵ Department of Protein-protein Interaction Research, Institute for Advanced Medical Sciences, Nippon Medical School, Tokyo, Japan

OPEN ACCESS

Edited by:

Annalisa Del Prete,
University of Brescia, Italy

Reviewed by:

Linda Wagner-Weiner,
The College, University of
Chicago, United States
Angelo A. Manfredi,
Vita-Salute San Raffaele
University, Italy

*Correspondence:

Toshiyuki Kitoh
tkitoh@dpc.agu.ac.jp

Specialty section:

This article was submitted to
Cytokines and Soluble
Mediators in Immunity,
a section of the journal
Frontiers in Immunology

Received: 01 November 2020

Accepted: 17 March 2021

Published: 12 April 2021

Citation:

Kitoh T, Ohara T, Muto T, Okumura A, Baba R, Koizumi Y, Yamagishi Y, Mikamo H, Daigo K and Hamakubo T (2021) Increased Pentraxin 3 Levels Correlate With IVIG Responsiveness and Coronary Artery Aneurysm Formation in Kawasaki Disease. *Front. Immunol.* 12:624802. doi: 10.3389/fimmu.2021.624802

Kawasaki disease (KD) is a febrile disease of childhood characterized by systemic vasculitis that can lead to coronary artery lesions (CAL). This was a prospective cohort study to determine the levels of the pentraxin 3 (PTX3), soluble CD24-Subtype (Presepsin) and N-terminal pro-brain natriuretic peptide (NT-pro BNP) in consecutive KD patients. From January 2013 to March 2015, all patients with KD admitted to Aichi Medical University Hospital who provided consent had their plasma saved before IVIG administration. In total, 97 cases were registered. 22 cases of incomplete KD were excluded from the outcome analysis. The total 75 cases were used for statistical analyses. A PTX3 threshold of >7.92 ng/ml provided a specificity of 88.5 %, a sensitivity of 94.4 %, and a likelihood ratio as high as 15.92 for the diagnosis of KD compared with febrile non-KD controls. Although an echocardiographic diagnosis of CAL in the early course of the disease was confirmed in 24 cases, it was not in the remaining 51 cases. Neither NT-proBNP nor Presepsin had statistical significance for the prediction of the echocardiographic CAL diagnosis. Only PTX3 was significantly predictive of the echocardiographic CAL diagnosis ($p=0.01$). The PTX3 level was significantly higher in the intravenous immunoglobulin (IVIG) non-responders (45.9 ± 7.45) than in the IVIG responders (17.0 ± 1.46 ng/ml) ($p < 0.001$). The PTX3 level also correlated with the number of IVIG treatment courses needed to resolve fever ($R^2 = 0.64$). Persistent CAL (pCAL) formation was observed in three cases; one of aneurysm only and two aneurysms with dilatations. The patients with pCAL had significantly higher PTX3 levels (85 ± 8.4 ng/ml) than patients without pCAL (22 ± 2.2 ng/ml) ($p < 0.0001$). In terms of pCAL prediction, the area under the curve (AUC) of receiver operating characteristic ROC curve of PTX3 was 0.99, and it was significantly greater than that of Presepsin (0.67) or NT-proBNP (0.75). PTX3 is a soluble pattern recognition molecule that acts as a main component of the innate immune system. These data suggest that PTX3 can be utilized as a definitive

biomarker for the prediction of IVIG resistance and subsequent CAL formation in patients with KD.

Keywords: pentraxin 3, Kawasaki disease, coronary aneurysm, intravenous immunoglobulin therapy, N-terminal pro-brain natriuretic peptide, presepsin, systemic vasculitis, coronary artery abnormalities

INTRODUCTION

Kawasaki disease (KD) is an acute inflammatory disorder of unknown origin associated with medium-sized vessel vasculitis (1). However, inflammation of medium-sized arteries throughout the body, particularly of the coronary arteries, can occur during the acute phase of KD (2), and coronary artery aneurysms develop in the subacute phase (3). Coronary artery lesions (CAL), including dilations and aneurysms, respectively manifesting in the acute and transient form or persistent chronic form (designated as persistent CAL; pCAL), constitute the most serious complication. PCALs are the most common cause of acquired heart disease in children in developed countries (4, 5). Coronary artery aneurysms develop in 15–25% of untreated children and may lead to myocardial infarction, sudden death, or ischemic heart disease (6). Without treatment, mortality may approach 1%, usually within six weeks of onset (7).

Timely initiation of treatment with intravenous immunoglobulin (IVIG) has reduced the incidence of coronary artery aneurysms from 25% to 4% (8, 9). Even with treatment in the acute phase of KD with IVIG and aspirin, up to 5% of patients will develop serious and potentially life-threatening cardiac complications (10). Several scoring systems have been developed to identify children at highest risk for coronary artery abnormalities (11–13) and has been confirmed as a powerful predictor of coronary artery aneurysms in various Japanese studies. In other countries, however, it is not followed due to the imperfect performance of scoring systems (14).

Although the most appropriate treatment for patients who fail to respond to IVIG remains unclear, several suggestive trials have been reported. Severe KD that is resistant to IVIG may benefit from intravenous pulse corticosteroid therapy (15), infliximab infusion, or plasma exchange (PE) (16, 17). If a reliable biomarker were to be identified that would make it possible to distinguish the patients with KD at high risk for CAL formation, prompt initiation of second line therapy would lessen the risk of subsequent pCAL formation.

We hypothesized that the plasma concentrations of pentraxin 3 (PTX3), N-terminal pro-brain natriuretic peptide (NT-proBNP) or the soluble CD14 subtype (Presepsin) in patients with KD might be increased and thus reflect the severity of KD. We have expected that at least one of these might serve as a useful biological marker of CAL formation and prove useful as an additional part of the scoring systems. PTX3 is one of the pathogen recognition receptors (18). NT-proBNP is conventionally utilized as a biomarker to predict CAL

formation. Presepsin represents a newly emerging sepsis marker. We examined the correlation between the measured values of these candidate markers and the rate of IVIG resistance as well as the incidence of coronary artery lesions.

MATERIALS AND METHODS

Study Population and Laboratory Evaluation

The study was carried out in accordance with the principles of the Declaration of Helsinki and approved by the Institutional Review Board of the Aichi Medical University Hospital. Written informed consent was obtained from the subjects or their parents. All children recruited in this study were admitted to Aichi Medical University (AMU) in the period between January 2013 and March 2015. The children were evaluated by experienced clinicians at AMU and fulfilled the diagnostic criteria for KD as endorsed by the Diagnostic Guidelines for Kawasaki Disease (5th revision) (19). All of the KD patients were treated with oral aspirin (30 mg/kg per day) and IVIG (2 g/kg per day). 115 cases were registered in total. 18 cases that presented with fever, but with symptoms that were not compatible with KD, were excluded. 22 cases of incomplete KD were also excluded from the outcome analysis. 41 boys and 34 girls fulfilling the standard diagnostic criteria for KD were analyzed in the study. The plasma samples for research were collected from a total of 75 patients in three phases: 75 patients at the time of KD diagnosis/disease onset and prior to IVIG treatment (acute phase), 60 patients at 1 to 2 weeks following IVIG treatment (sub-acute phase), and 54 patients at 3 weeks to 4 months following disease onset (convalescent phase). There were 61 patients whose samples were obtained in all three phases. Along with these parameters, additional clinical laboratory data [i.e., ESR, CRP, the white blood cell (WBC) count, absolute lymphocyte count (ALC), absolute neutrophil count (ANC), platelet (PLT) count, D-dimer and alanine aminotransferase (ALT)] were evaluated for each phase of the disease. Samples from 18 children with fever, but with symptoms incompatible with KD, were used as febrile non-KD controls. Control plasma was also obtained from 20 non-febrile healthy control subjects during the course of the pre-operative evaluation.

Echocardiogram Measurements and Failure to Respond to IVIG

A complete echocardiogram was conducted as part of the standard diagnostic evaluation of KD for all patients in each phase of the disease according to the “Guidelines for Diagnosis and Management of Cardiovascular Sequelae in Kawasaki Disease” (JCS 2013) (20).

Abbreviations: AUC, area under curve; CAA, coronary artery aneurysm; CAL, coronary artery lesion; IVIG, intravenous gamma-globulin; KD, Kawasaki disease; ROC curve, receiver operating characteristic curve.

Quantification of Biomarker Candidates

Venous blood samples were collected in sterile tubes containing EDTA-potassium and centrifuged at 2000 g for 10 minutes at 4°C. The plasma samples were extracted from the aliquots and were immediately frozen and stored at −80°C until analysis. The plasma PTX3 and N-terminal pro-brain natriuretic peptide (NT-proBNP) levels were determined by enzyme-linked immunosorbent assay (ELISA) (Perseus Proteomics Inc. Tokyo, Japan) (21) and Biomedica Medizin produkte GmbH & Co KG, Wien, Germany), respectively following the manufacturers' instructions. Plates were analyzed in duplicate on a Spark™ 10M multimode microplate reader (Tecan Trading AG, Switzerland). The plasma Presepsin level was determined using a chemiluminescent enzyme immunoassay (CLEIA) (Pathfast™, Chemical Medience Corporation, Tokyo, Japan) (22).

STATISTICAL ANALYSIS

Statistical analysis was performed using the statistical software package Prism 8.0 for Windows (GraphPad, San Diego, CA, USA). Student's t-test was used for comparison between normally distributed continuous data. One-way or two-way ANOVA followed by Bonferroni's comparison was used as a post hoc test to evaluate the statistical difference between more than two groups. The t-test analysis was used to evaluate the statistical difference between two groups. Non-parametric analysis using the Mann–Whitney U test was utilized for continuous data that did not follow a normal distribution. The relationship between quantitative variables was assessed using the Spearman correlation coefficient. Statistical significance was taken as $p < 0.05$. To compare the power of plasma biomarker candidates to predict IVIG unresponsiveness or CAL formation, receiver-operating curves (ROCs) were plotted and area under the curves (AUCs) were calculated. The optimal cut-off points were determined using ROC curves to maximize the sensitivity and specificity.

RESULTS

Clinicoepidemiological Characteristics of the KD Patients

The plasma samples used for research were collected from 75 children enrolled during the study period. One boy had two episodes of KD and one girl had three episodes of KD. Seven cases of KD received no IVIG; 45 cases, one dose; 19 cases, two doses; 4 cases, three doses (**Table 1**). Among the 68 IVIG-treated KD patients, 46 cases were IVIG responders and 22 cases were IVIG non-responders. Infliximab was administered to two patients after two doses of IVIG. Plasma exchange was performed in two patients after three doses of IVIG. An elective second line use of steroids was not employed in this cohort. Of the 75 patients, 24 had transient coronary artery lesions detected by echocardiography. Ultimately, three patients had persistent CAL sequelae according to echocardiography and 72 did not.

TABLE 1 | Clinical characteristics of patients with Kawasaki disease.

Characteristic	No. of patients (%)
Total	75
Age (months)	
Median	27
Range	3–114
Gender (male/female)	39/36
Intravenous gamma globulin	
Yes	68 (90.7)
one dose of IVIG	45
two doses of IVIG	19
three doses of IVIG	4
No	7 (9.3)
IVIG treated	68
IVIG responder	46 (67.6)
IVIG non-responder	22 (36.4)
acute coronary artery abnormalities	24 (32.0)
chronic coronary artery abnormalities	3 (4.0)
Aneurysms	3 (4.0)
Aneurysms & Dilatations	2 (2.7)
Follow up (months)	
Median	18
Range	5–34
Outcome	
Complete recovery without sequelae	72 (96.0)
Persistent aneurysm	3 (4.0)

Elevated Levels of PTX-3, NT-proBNP During Acute KD

The KD patients were evaluated for the respective circulating levels of PTX3, NT-proBNP and Presepsin throughout the clinical course of the disease. We set three sampling time points during the disease course: at the time of onset of KD or before IVIG (Pre-IVIG), after IVIG (Post-IVIG) and during convalescence period after resolution of the fever (Conv). The PTX3 and NT-proBNP levels were significantly higher in the before-IVIG group compared with febrile non-KD controls (**Figures 1A, B**). Both were significantly decreased in the KD patients after IVIG treatment. The levels of Presepsin in the patients with KD were not significantly higher than in the febrile non-KD controls (**Figure 1C**). Plasma Presepsin was not upregulated during the acute phase.

The PTX3 levels of healthy controls and febrile non KD patients on admission were 3.8 ± 0.35 ng/ml ($n=20$) and 11.8 ± 2.2 ng/ml ($n=18$), respectively. The PTX3 levels in the KD patients (33.4 ± 7.3 ng/ml) were significantly higher than those in the non-KD febrile controls (11.8 ± 2.2 ng/ml; $p < 0.001$). The PTX3 threshold of >7.92 ng/ml provided a specificity of 88.5 %, a sensitivity of 94.4 %, and a likelihood ratio as high as 15.92 for the diagnosis of KD compared with the febrile non-KD controls. The high levels of PTX3 noted before IVIG treatment decreased after treatment (Pre-IVIG, 33.4 ± 7.3 ng/ml; Post-IVIG, 9.64 ± 3.61 ng/ml; **Figure 1A**).

Plasma PTX3, NT-proBNP and Presepsin Levels and Coronary Artery Lesions in Acute Phase KD

In the acute phase of KD, the high echo density of the perivascular area of the coronary artery or coronary artery

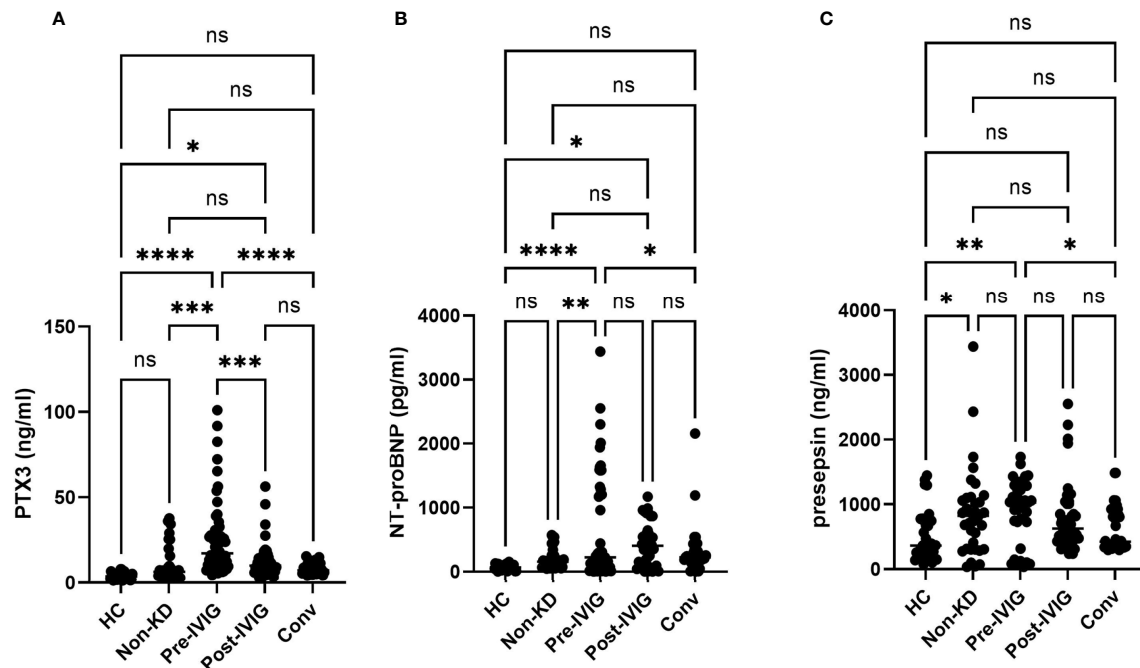


FIGURE 1 | Plasma levels of PTX3, NT-proBNP and Presepsin during the disease course of KD. Unpaired Student t-test. Comparison of the level of PTX3 (A), NT-proBNP (B), and Presepsin (C) in healthy non-febrile controls, non-KD febrile controls, and KD. Between-group differences were determined using ANOVA and logical regression analysis. A: Circulating levels of PTX3 throughout KD. B: Circulating levels of NT-proBNP throughout KD. C: Circulating levels of Presepsin throughout KD. ns, not significant; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$. HC, healthy controls; Non-KD, non KD febrile disease; at the time of onset of KD or before IVIG (Pre-IVIG), after IVIG (Post-IVIG); Conv, convalescent.

dilatation (echocardiographic CAL diagnosis; echoCAL) was detected in 23 cases by echocardiography (Table 2). There were no significant differences in the age or the ratio of males to females between the echoCAL (-) and echoCAL (+) groups. No significant difference was found for the day of illness at the time of IVIG administration (5.4 [3-8] and 5.3 [3-12] day) and the sampling day of the illness (4.5[1-7] and 4.7 [2-12] day). There were no significant differences in any other of the examined variables, such as WBC count, serum sodium, CRP, D-dimer, ALT and Albumin levels between the 2 groups. The result of using an independent t-test indicated that the p-values of NT-proBNP and Presepsin were both 0.05 or higher, and there was thus no statistical significance. The p-value of PTX3 was considered significant at 0.01

The PTX3, NT-proBNP and Presepsin Levels Used to Predict IVIG Resistance/Non-Responders

IVIG resistance was defined as a persistent or recurrent fever for more than 48 h after completion of the initial IVIG treatment. Failure to respond is typically defined as persistent or recrudescence fever ≥ 24 hours after completion of the initial IVIG infusion. Plasma levels were determined in the IVIG responders ($n = 46$) and IVIG non-responders with KD ($n = 22$) in evaluating the IVIG response. Twenty-two of the 68 IVIG-

treated patients were non-responders (IVIG res (-)) (Table 3). There was no significant difference in sex, the day of illness at the time of IVIG administration, nor the sampling day of illness between the IVIG res (+) and IVIG res (-). The number of patients who developed CAL was not significantly higher in the IVIG res (+) group (41% in IVIG res (+) vs 50% in IVIG res (-) group, $p=0.49$). In addition, no significant differences were observed for any of the other variables examined, including the WBC count, serum sodium level, CRP level, ALT and serum albumin level between the 2 groups. Analyses with an unpaired t test showed that the P value of NT-proBNP, Presepsin and PTX3 for IVIG resistance vs. responsiveness was 0.19, 0.04 and 0.001, respectively. PTX3 and Presepsin were significant. PTX3 exhibited a higher degree of significance than either NT-proBNP or Presepsin. The PTX3 values in the IVIG non-responders ($n = 22$) were significantly higher when compared to the IVIG responders ($n = 46$) (37.29 ± 31.3 vs. 18.2 ± 12.5 ; $p=0.001$). The threshold of >33.2 ng/ml PTX3 provided a sensitivity of 64.3% (95% CI: 53.35 to 81.83%), a specificity of 83.3% (95% CI: 54.35 to 95.95%), and a likelihood ratio as high as 12.2 for the diagnosis of "IVIG non-responder".

Correlation of the PTX3, NT-proBNP and Presepsin Levels With IVIG Resistance

Figure 2 shows the plasma PTX3 value before IVIG treatment in patients who required 0, 1, 2, or 3 doses of IVIG to resolve the

TABLE 2 | Demographic, laboratory characteristics of patients at the time of diagnosis in the groups without CAL and with CAL.

	CAL(-) (n = 51)	CAL(+) (n = 24)	P value
Age (m)	20.95±2.9 [3-72]	19.38±4.1 [2-78]	0.45
Male sex (%)*	0.4	0.4	0.92
Day of illness at the time of IVIG administration (d)	5.4±1.9 [3-8]	5.3±1.2 [3-12]	0.91
Sampling day of illness (d)	4.5±1.3 [1-7]	4.7±1.8 [2-12]	0.61
IVIG non-responder (%)*	12±0.24	10±0.42	0.04
WBC count (103/mL)	13329±4400 [3090-24900]	13225±3716 [6000-23900]	0.92
Sodium (mEq/L)	136.5±2.89 [130-144]	135.8±2.93 [129-141]	0.23
CRP (mg/dL)	5.13±3.31 [0.36-12.88]	6.22±4.24 [0.99-15.88]	0.23
D-Dimer (μg/mL)	2.07±2.26 [0.9-9.03]	2.20±1.09 [1.01-5.21]	0.85
ALT	62.2±9.6 [7-422]	135.8±18.5 [7-807]	0.27
ALB	3.82±0.33 [2.9-4.5]	3.76±0.30 [3.1-4.5]	0.42
NT-proBNP (pg/mL)	453.2±199.6 [7.4-1659]	831±443.5 [6.2-3437]	0.05
Presepsin (pg/mL)	482.7±237.9 [74-1943]	818.2±455.6 [103-3437]	0.04
PTX3 (ng/mL)	18.16±11.6 [3.22-5383]	33.21±14.9 [4.4-101]	0.01

Presented as the mean ±SD with the 5th and 95th percentiles in square brackets. Mann-Whitney U test. *2-sided χ^2 test. Echo CAL, high echo density of the perivascular area of the coronary artery or coronary artery dilatation.

KD. The correlation of the IVIG dose number was obtained using the IVIG correlation coefficient which is a test of such a correlation. No significant correlation was found between the NT-proBNP or Presepsin level and the number of doses of IVIG needed to resolve inflammation in KD. The PTX3 levels in KD patients who required zero, one, two, or three doses of IVIG to resolve the KD were 12.5 ± 3.5 , 21.0 ± 7.8 , 35.6 ± 27.5 , and 72.2 ± 32.8 ng/ml, respectively (significantly different according to one-way ANOVA, $p < 0.05$). The PTX3 level was also significantly correlated with the number of IVIG treatments needed to resolve fever. ($p < 0.001$). There was a strong correlation between the PTX3 value and the number of doses administered IVIG; $y = 22.18x - 4.27$, $R^2 = 0.64$.

PTX3 and Coronary Abnormalities

The patients with pCAL had higher PTX3 levels than those without pCAL (85 ± 8.4 vs. 22 ± 2.2 ng/ml, $p < 0.0001$). The NT-proBNP and Presepsin levels in KD patients with pCAL were not significantly higher than those without pCAL. All three cases with pCAL formation were IVIG non-responders (**Figure 3A**). The plasma PTX3 values on admission of these patients were 72.4, 91.8, and 101 ng/ml, the highest values in this study. **Figure 3B** shows the AUC and predictive power for pCAL of the candidate biomarker levels at the time of admission. The respective AUC value of PTX3,

TABLE 3 | Demographic, laboratory characteristics of patients at the time of diagnosis in the IVIG responsive group, and the IVIG resistant group.

	IVIG res(+) (n = 46)	IVIG res(-) (n = 22)	P value
Age (m)	29.05±23.09 [3-108]	21.19±21.93 [2-96]	0.17
Male sex (%)*	0.50	0.67	0.18
Day of illness at the time of IVIG administration (d)	4.6±1.5 [3-12]	4.8±1.0 [3-7]	0.33
Sampling day of illness (d)	4.6±1.5 [2-12]	4.0±1.3 [1-6]	0.09
Complicated with CAL (%)*	0.50	0.41	0.49
WBC count (103/mL)	13790±3674 [5500-24900]	13405±4623 [6000-23900]	0.70
Sodium (mEq/L)	136.3±2.97 [130-144]	135.3±2.67 [129-139]	0.29
CRP (mg/dL)	6.71±4.15 [1.26-20.55]	7.32±3.24 [1.7-15.64]	0.55
D-Dimer (μg/mL)	2.01±0.85 [1-4.33]	2.53±1.49 [0.82-5.13]	0.22
ALT	75.8±15.1 [7-422]	116.2±39.0 [8-807]	0.24
ALB	3.81±0.33 [2.9-4.5]	3.80±0.30 [3.1-4.3]	0.87
NT-proBNP (pg/mL)	520.8±417.3 [7.4-3437]	785.6±360.1 [6.2-2010]	0.19
Presepsin (pg/mL)	489±217.6 [103-2552]	943.8±463.3 [131-3437]	0.04
PTX3 (ng/mL)	18.2±12.5 [3.22-65.3]	37.29±31.3 [5.56-101.0]	0.001

Presented as the mean ±SD with the 5th and 95th percentiles in square brackets. Mann-Whitney U test. *2-sided χ^2 test. CAL means echo CAL, high echo density of the perivascular area of the coronary artery or coronary artery dilatation.

NT-proBNP and Presepsin was 0.99 ± 0.014 (95% CI: 0.96 to 1.00, $p = 0.005$), 0.86 ± 0.054 (95% CI: 0.75 to 0.96, $p = 0.039$), and 0.67 ± 0.15 (95% CI: 0.37 to 0.96, $p = 0.33$). The cut-off value of 60.8 ng/ml that was used to predict subsequent pCAL had a sensitivity of 1.00 (95% CI: 29.24 to 100.0%), a specificity of 0.98 (95% CI: 88.09 to 99.58%), and a likelihood ratio of 29.0.

DISCUSSION

Prediction of CAL risk has become an important issue in KD because of recent advances in the therapeutic strategies available, such as prednisolone therapy (15) or treatment with a tumor necrosis factor- α antagonist (23), advances that enable stratification of the initial treatment for the patients with a high level of predicted risk. Thus, we carried out this collateral analysis to ascertain the diagnostic accuracy of PTX3, NT-proBNP and Presepsin in detecting CAL before the initial IVIG treatment.

NT-proBNP has been suggested to be a potential diagnostic biomarker for KD (24). NT-proBNP as a risk predictor of CAL after first being reported by Kaneko et al. (25), was suggested to be associated with the development of CAL. It was shown by a meta-analysis performed to ascertain the diagnostic value of

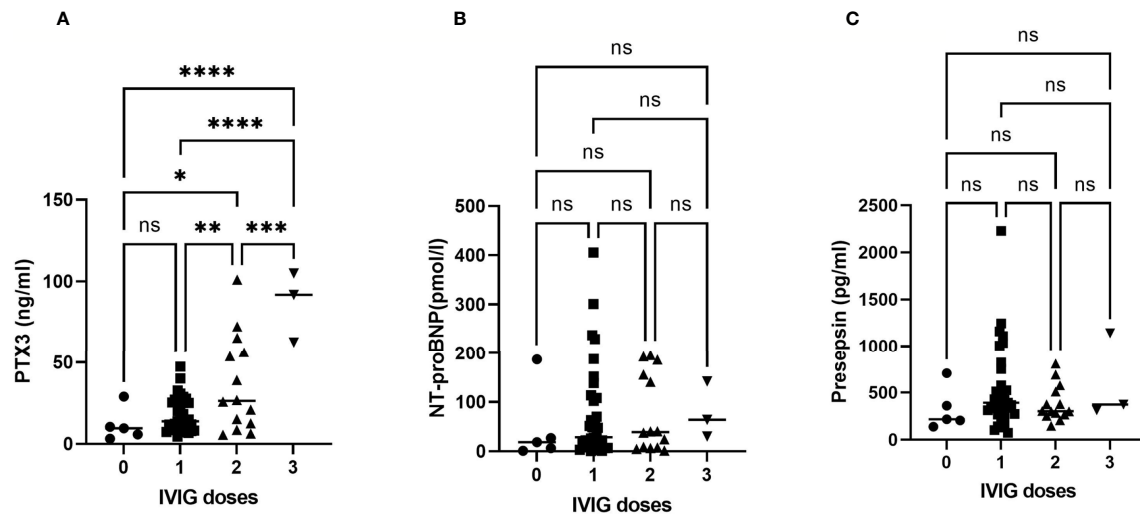


FIGURE 2 | Correlation curve showing the dose of IVIG needed to resolve KD versus PTX3, NT-proBNP and Presepsin levels. The plasma PTX3 (A), NT-proBNP (B) and Presepsin (C) levels are plotted against the dose of IVIG needed to resolve the KD. Zero indicates cases treated with aspirin only (non-IVIG). The PTX3 levels exhibited a significant correlation with IVIG doses required to resolve KD ($y = 22.177x - 4.2684$, $R^2 = 0.6402$). ns, not significant; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$.

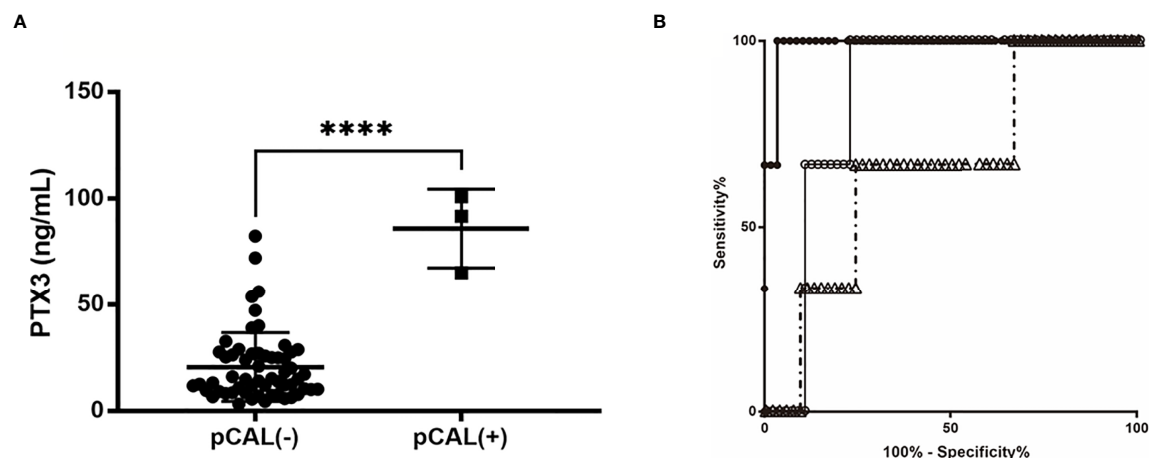


FIGURE 3 | AUC and predictive power of the biomarker candidate levels at the time of admission. (A) Unpaired Student t-test. Comparison of the PTX3 level in patients with or without pCAL. **** $p < 0.001$. (B) ROC curves comparing the sensitivity and specificity of certain variables and CAL formation; the AUC values of PTX3, NT-proBNP and Presepsin were respectively 0.9885 ± 0.014 (95% confidence interval [CI]: 0.961 to 1.00, $p = 0.005$), 0.8563 ± 0.054 (95% CI: 0.7513 to 0.9614, $p = 0.039$), and 0.6667 ± 0.148 (95% CI: 0.3765 to 0.9568, $p = 0.33$). The cut-off value of 69 ng/ml to predict subsequent CAL had a specificity of 1.00, sensitivity of 0.98, and a likelihood ratio of 49.0 PTX3, filled circles •; NT-proBNP, open circles ○; Presepsin, open triangles △.

NT-proBNP in detecting CAL in KD that the overall diagnostic sensitivity and specificity were 0.84 (95% CI: 0.78-0.89) and 0.71 (95% CI: 0.68-0.75), respectively. The area under the summary receiver operating characteristic curves (SROC) value was 0.8582 ± 0.0531 (24). The AUC value in our study was 0.6316 ± 0.08 (95% CI: 0.47 to 0.79), which is comparable to that of SROC in terms of meta-analysis. Both AUC values are still inferior to that of our PTX3 data of 0.9885 ± 0.014 (95% CI: 0.96 to 1.00, $p = 0.005$).

Presepsin is also known as a soluble cluster of differentiation (CD)14 subtype, which is a humoral risk stratification marker for systemic inflammatory response syndrome and sepsis. The results of many clinical studies have indicated that Presepsin is a useful biomarker not only for early diagnosis, but also for risk stratification and prognosis prediction for sepsis in adults (26) as well as children (27). The present study shows that Presepsin was, as the case in sepsis (28), inferior to PTX3 in predicting IVIG resistance and/or CAL formation.

The pathophysiology of the acute phase of Kawasaki disease is characterized by excessive activation of the innate immune system and is accompanied by an increase in inflammatory cytokines and chemokines (29). Furthermore, it is thought that these factors trigger the induction of the fever, acute phase protein production in the blood, neutrophil increase, and vasculitis. PTX3 is a prototypic soluble pattern recognition receptor in the innate immune system that is expressed at sites of inflammation and is involved in regulation of the tissue homeostasis (18). PTX3 has also been studied as a diagnostic biomarker for sepsis. PTX3 levels are low under normal conditions (<2 ng/ml in adults, 1.24 ng/ml [0.87–2.08] in children) (30), but the plasma PTX3 concentrations increase in certain conditions such as sepsis, small-vessel vasculitis (31), and acute myocardial infarction (32). In sepsis, PTX3 may serve as an early marker of the severity and outcome of the disease. PTX3 is correlated with the severity of sepsis in febrile patients presenting with the disease in hospital emergency rooms (33). PTX3 levels also elevated in patients with systemic inflammation (median = 71.3 ng/ml) (34). A role for PTX3 as a sensitive marker of vascular inflammation has also been proposed (18, 35). The innate immune system, which is considered to be involved in the pathogenesis of KD, includes a humoral arm such as PTX3 that is complementary to cellular recognition and effector function (18).

In this study, we observed the following in patients with KD. a) The plasma PTX3 and NT-proBNP values were significantly increased compared with these values in non KD febrile patients determined before IVIG. Using 25.7 ng/ml as the optimal cut-off, we identified PTX3 as an excellent biomarker for the purpose of differentiating KD cases from non-KD cases, including subjects highly suspected of KD. b) Patients with acute early coronary lesions presented with a greater increase in the PTX3 plasma values than patients without any acute early coronary artery lesions. c) Patients who had PTX3 plasma values exceeding 33.2 ng/ml exhibited increased risk of IVIG resistance. d) A significant correlation was found between the levels of PTX3 and the corresponding number of doses of IVIG treatment needed to resolve the inflammation caused by KD ($p < 0.01$). e) The cut-off value of 60.8 ng/ml to predict subsequent CAL had a specificity of 1.00, sensitivity of 0.98, and a likelihood ratio of 29.0.

Katsube et al. reported the PTX3 level in 56 patients with KD. They determined that the PTX3 levels in unresponsive cases ($n=7$) were significantly higher than in responsive cases ($n=49$) (55.3 vs. 18.9; $p<0.01$). There were two cases of CAL among the unresponsive cases, and the PTX3 values in these cases were 63.7 and 89.3 ng/ml (36). These PTX3 values are similar to those of the three CAL cases in our study. The sensitivity and specificity of PTX3 in predicting IVIG unresponsiveness were 85.7 and 95.9 % when the PTX3 cut-off value was set at 40 ng/ml (36). In our study, the cut-off value of 60.8 ng/ml used to predict subsequent CAL had a specificity of 1.00, sensitivity of 0.98, and a likelihood ratio of 29.0. A very similar predictive power was observed in an independent report (37). We also observe that the plasma PTX3 level was elevated in KD before IVIG to 33.4 ± 7.3 , more than 10 times higher than the normal value in controls (2.7 ± 1.0) (30),

and returned to an improved but still abnormal level of 9.64 ± 3.61 during convalescence. Katsube et al. also reported the sustained PTX3 elevation that implies continuing silent vasculitis even in one month after the onset of KD.

Recently, Ching et al. reported that significantly higher serum PTX3 levels in patients with CAL compared to patients without CAL, as well as IVIG resistance during the acute phase of KD. They concluded PTX3 as a sensitive marker for coronary artery dilation in KD rather than IVIG resistance (38). Our observation is consistent with them regarding to coronary artery dilatation and IVIG resistance. In this study, we found that the elevated PTX3 level correlated with the number of doses administered IVIG. In our cohort, there were three cases of pCAL formation with highly elevated plasma PTX3 level. These results suggested that the PTX3 is a good marker for pCAL formation and the severity of KD. Our results further strengthen their recommendation to utilize PTX3 in assessing KD patients.

The limitations of this study include limited sample size, particularly within the pCAL group. However, the results clearly demonstrate a significant elevation of the PTX3 level in KD patients that is correlated with required IVIG doses for responsiveness. These data further suggest a specific role for PTX3 in KD disease severity as well as the treatment response. Future studies should focus on investigating the underlying mechanisms by which PTX3 and other innate immunity proteins are involved in KD pathogenesis and CAL development.

CONCLUSIONS

It is demonstrated that PTX3 is highly useful in the diagnosis of KD. The PTX3 level before IVIG was a strong and sensitive predictor of IVIG non-responsiveness and subsequent CAL formation, and may help identify high-risk patients requiring additional second-line therapy other than repeated IVIG treatment alone. According to these results, the prognostic value of PTX3 as a single biomarker is the best available when compared with the currently used risk scoring systems (e.g. the Kobayashi Score) (11), or other proposed predictors such as NT-proBNP or Presepsin. A particularly high level of PTX3 is also an independent predictor of CAL formation after fever resolution.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available from the corresponding author TK on request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of the Aichi Medical University Hospital. Written informed consent to participate in

this study was obtained from the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

TK participated in the design of the study, collected data, interpreted the data, did the statistical analysis, and wrote the initial draft report. TO performed the NT-pro BNP assay, collected data, interpreted data, and revised the report. AO supervised his assay. RB performed and read echocardiography and defined the final outcome of the KD patient for any coronary artery lesions. YK performed the PTX3 and Presepsin assay. YY and HM supervised his assay. KD and TH participated in the design of the study and reviewed the manuscript, and approved the final manuscript as submitted. All authors contributed to the article and approved the submitted version.

REFERENCES

1. Kawasaki T. Acute febrile mucocutaneous syndrome with lymphoid involvement with specific desquamation of the fingers and toes in children. *Arerugi* (1967) 16(3):178–222.
2. Rowley AH, Baker SC, Orenstein JM, Shulman ST. Searching for the cause of Kawasaki disease — cytoplasmic inclusion bodies provide new insight. *Nat Rev Microbiol* (2008) 6(5):394–401. doi: 10.1038/nrmicro1853
3. Saadoun D, Vautier M, Cacoub P. Medium- and Large-Vessel Vasculitis. *Circulation* (2021) 143(3):267–82. doi: 10.1161/circulationaha.120.046657
4. Dietz SM, Van Stijn D, Burgner D, Levin M, Kuipers IM, Hutten BA, et al. Dissecting Kawasaki disease: a state-of-the-art review. *Eur J Pediatr* (2017) 176(8):995–1009. doi: 10.1007/s00431-017-2937-5
5. Singh S, Vignesh P, Burgner D. The epidemiology of Kawasaki disease: a global update. *Arch Dis Child* (2015) 100(11):1084–8. doi: 10.1136/archdischild-2014-307536
6. Kato H, Sugimura T, Akagi T, Sato N, Hashino K, Maeno Y, et al. Long-term consequences of Kawasaki disease. A 10- to 21-year follow-up study of 594 patients. *Circulation* (1996) 94(6):1379–85. doi: 10.1161/01.cir.94.6.1379
7. Kawasaki T, Kosaki F, Okawa S, Shigematsu I, Yanagawa H. A New Infantile Acute Febrile Mucocutaneous Lymph Node Syndrome (MLNS) Prevailing in Japan. *Pediatrics* (1974) 54(3):271–6.
8. Furusho K, Kamiya T, Nakano H, Kiyosawa N, Shinomiya K, Hayashidera T, et al. High-dose intravenous gammaglobulin for Kawasaki disease. *Lancet* (1984) 2(8411):1055–8. doi: 10.1016/S0140-6736(84)91504-6
9. Newburger JW, Takahashi M, Beiser AS, Burns JC, Bastian J, Chung KJ, et al. A Single Intravenous Infusion of Gamma Globulin as Compared with Four Infusions in the Treatment of Acute Kawasaki Syndrome. *New Engl J Med* (1991) 324(23):1633–9. doi: 10.1056/NEJM199106063242305
10. Dajani AS, Taubert KA, Takahashi M, Bierman FZ, Freed MD, Ferrieri P, et al. Guidelines for long-term management of patients with Kawasaki disease. Report from the Committee on Rheumatic Fever, Endocarditis, and Kawasaki Disease, Council on Cardiovascular Disease in the Young, American Heart Association. *Circulation* (1994) 89(2):916–22. doi: 10.1161/01.cir.89.2.916
11. Kobayashi T, Inoue Y, Takeuchi K, Okada Y, Tamura K, Tomomasa T, et al. Prediction of Intravenous Immunoglobulin Unresponsiveness in Patients With Kawasaki Disease. *Circulation* (2006) 113(22):2606–12. doi: 10.1161/circulationaha.105.592865
12. Egami K, Muta H, Ishii M, Suda K, Sugahara Y, Iemura M, et al. Prediction of resistance to intravenous immunoglobulin treatment in patients with Kawasaki disease. *J Pediatr* (2006) 149(2):237–40. doi: 10.1016/j.jpeds.2006.03.050
13. Sano T, Kurotobi S, Matsuzaki K, Yamamoto T, Maki I, Miki K, et al. Prediction of non-responsiveness to standard high-dose gamma-globulin

FUNDING

This work was supported by Tokai Foundation for Technology (to TK) Toyohashi, Aichi, Japan, and Research grants project number AS242Z02588P (to TK and TH) from the New Energy and Industrial Technology Development Organization, Minister of Economy, Trade, and Industry, Japan. This work was supported by JSPS KAKENHI grant number JP16K08422 (to TK, TM, HM, KD, and TH).

ACKNOWLEDGMENTS

We thank Dr. Kenta Murotani, Biostatistics Center, Graduate School of Medicine, Kurume University, Kurume, Japan who checked our final statistics analysis. Pacific Edit reviewed the manuscript prior to submission.

- therapy in patients with acute Kawasaki disease before starting initial treatment. *Eur J Pediatr* (2007) 166(2):131–7. doi: 10.1007/s00431-006-0223-z
14. Newburger JW, Takahashi M, Gerber MA, Gewitz MH, Tani LY, Burns JC, et al. Diagnosis, Treatment, and Long-Term Management of Kawasaki Disease. *Circulation* (2004) 110(17):2747–71. doi: 10.1161/01.cir.0000145143.19711.78
15. Kobayashi T, Saji T, Otani T, Takeuchi K, Nakamura T, Arakawa H, et al. Efficacy of immunoglobulin plus prednisolone for prevention of coronary artery abnormalities in severe Kawasaki disease (RAISE study): a randomised, open-label, blinded-endpoints trial. *Lancet* (2012) 379(9826):1613–20. doi: 10.1016/S0140-6736(11)61930-2
16. Newburger JW, Takahashi M, Gerber MA, Gewitz MH, Tani LY, Burns JC, et al. Diagnosis, Treatment, and Long-Term Management of Kawasaki Disease: A Statement for Health Professionals From the Committee on Rheumatic Fever, Endocarditis and Kawasaki Disease, Council on Cardiovascular Disease in the Young, American Heart Association. *Circulation* (2004) 110(17):2747–71. doi: 10.1161/01.cir.0000145143.19711.78
17. Shimada S, Matsuoka D, Murase T, Hachiya A, Motoki N, Nakazawa Y. Impact of infliximab administration before plasma exchange therapy on patients with Kawasaki disease. *Ther Apher Dial* (2020) 24(6):718–24. doi: 10.1111/1744-9987.13486
18. Garlanda C, Bottazzi B, Magrini E, Inforzato A, Mantovani A. PTX3, a Humoral Pattern Recognition Molecule, in Innate Immunity, Tissue Repair, and Cancer. *Physiol Rev* (2018) 98(2):623–39. doi: 10.1152/physrev.00016.2017
19. Ayusawa M, Sonobe T, Uemura S, Ogawa S, Nakamura Y, Kiyosawa N, et al. Revision of diagnostic guidelines for Kawasaki disease (the 5th revised edition). *Pediatr Int* (2005) 47(2):232–4. doi: 10.1111/j.1442-200x.2005.02033.x
20. Group JCSJW. Guidelines for Diagnosis and Management of Cardiovascular Sequelae in Kawasaki Disease (JCS 2013)– Digest Version –. *Circ J* (2014) 78(10):2521–62. doi: 10.1253/circj.CJ-66-0096
21. Inoue K, Sugiyama A, Reid PC, Ito Y, Miyauchi K, Mukai S, et al. Establishment of a high sensitivity plasma assay for human pentraxin3 as a marker for unstable angina pectoris. *Arterioscler Thromb Vasc Biol* (2007) 27(1):161–7. doi: 10.1161/01.ATV.0000252126.48375.d5
22. Sato M, Takahashi G, Shibata S, Onodera M, Suzuki Y, Inoue Y, et al. Clinical Performance of a New Soluble CD14-Subtype Immunochromatographic Test for Whole Blood Compared with Chemiluminescent Enzyme Immunoassay: Use of Quantitative Soluble CD14-Subtype Immunochromatographic Tests for the Diagnosis of Sepsis. *PLoS One* (2015) 10(12):e0143971. doi: 10.1371/journal.pone.0143971
23. Miura M, Kobayashi T, Igarashi T, Hamada H, Iwata N, Sasaki Y, et al. Real-world Safety and Effectiveness of Infliximab in Pediatric Patients With Acute Kawasaki Disease: A Postmarketing Surveillance in Japan (SAKURA Study). *Pediatr Infect Dis J* (2020) 39(1):41–7. doi: 10.1097/INF.0000000000002503

24. Zheng X, Zhang Y, Liu L, Yue P, Wang C, Zhou K, et al. N-terminal pro-brain natriuretic peptide as a biomarker for predicting coronary artery lesion of Kawasaki disease. *Sci Rep* (2020) 10(1):5130. doi: 10.1038/s41598-020-62043-6
25. Kaneko K, Yoshimura K, Ohashi A, Kimata T, Shimo T, Tsuji S. Prediction of the risk of coronary arterial lesions in Kawasaki disease by brain natriuretic peptide. *Pediatr Cardiol* (2011) 32(8):1106–9. doi: 10.1007/s00246-011-9986-8
26. Masson S, Caironi P, Spanuth E, Thoma R, Panigada M, Sangiorgi G, et al. Presepsin (soluble CD14 subtype) and procalcitonin levels for mortality prediction in sepsis: data from the Albumin Italian Outcome Sepsis trial. *Crit Care* (2014) 18(1):R6. doi: 10.1186/cc13183
27. Nakanishi K, Kasashima S, Ishii Y, Shimoeda H, Kawashima A. Clinical Use of Presepsin Compared with That of Procalcitonin in Children with Sepsis. *Rinsho Byori Jpn* (2016) 64(10):1128–33.
28. El Gendy FM, El-Mekkawy MS, Saleh NY, Habib MSE, Younis FE. Clinical study of Presepsin and Pentraxin3 in critically ill children. *J Crit Care* (2018) 47:36–40. doi: 10.1016/j.jcrc.2018.06.004
29. Hara T, Nakashima Y, Sakai Y, Nishio H, Motomura Y, Yamasaki S. Kawasaki disease: a matter of innate immunity. *Clin Exp Immunol* (2016) 186(2):134–43. doi: 10.1111/cei.12832
30. Yamasaki K, Kurimura M, Kasai T, Sagara M, Kodama T, Inoue K. Determination of physiological plasma pentraxin 3 (PTX3) levels in healthy populations. *Clin Chem Lab Med* (2009) 47(4):471–7. doi: 10.1515/ccm.2009.110
31. Fazzini F, Peri G, Doni A, Dell'Antonio G, Dal Cin E, Bozzolo E, et al. PTX3 in small-vessel vasculitides: an independent indicator of disease activity produced at sites of inflammation. *Arthritis Rheum* (2001) 44(12):2841–50. doi: 10.1002/1529-0131(200112)44:12<2841::AID-ART472>3.0.CO;2-6
32. Ristagno G, Fumagalli F, Bottazzi B, Mantovani A, Olivari D, Novelli D, et al. Pentraxin 3 in Cardiovascular Disease. *Front Immunol* (2019) 10(823):1–12. doi: 10.3389/fimmu.2019.00823
33. Muller B, Peri G, Doni A, Torri V, Landmann R, Bottazzi B, et al. Circulating levels of the long pentraxin PTX3 correlate with severity of infection in critically ill patients. *Crit Care Med* (2001) 29(7):1404–7. doi: 10.1097/00003246-200107000-00017
34. Bastrup-Birk S, Skjoedt M-O, Munthe-Fog L, Strom JJ, Ma YJ, Garred P. Pentraxin-3 serum levels are associated with disease severity and mortality in patients with systemic inflammatory response syndrome. *PLoS One* (2013) 8(9):e73119–9. doi: 10.1371/journal.pone.0073119
35. Ramirez GA, Rovere-Querini P, Blasi M, Sartorelli S, Di Chio MC, Baldini M, et al. PTX3 Intercepts Vascular Inflammation in Systemic Immune-Mediated Diseases. *Front Immunol* (2019) 10(1135):1125–34. doi: 10.3389/fimmu.2019.01135
36. Katsube Y, Akao M, Watanabe M, Abe M, Kamisago M, Fukazawa R, et al. PTX3, A New Biomarker for Vasculitis, Predicts Intravenous Immunoglobulin Unresponsiveness in Patients with Kawasaki Disease. *J Am Coll Cardiol* (2011) 57(14s1):E2038. doi: 10.1016/S0735-1097(11)62038-X
37. Yoshikane Y, Fukazawa R, Hirono K, Kobayashi N, Imanaka-Yoshida K, Katsube Y. Predicting the treatment-resistant Kawasaki disease by biomarker, Tenascin C and Pentraxin 3. In: *The 40th Annual Meeting of the Japanese Society of Kawasaki Disease*, vol. 40. The Japanese Society of Kawasaki Disease (2020). p. 76.
38. Ching LL, Nerurkar VR, Lim E, Shohet RV, Melish ME, Bratincsak A. Elevated Levels of Pentraxin 3 Correlate With Neutrophilia and Coronary Artery Dilation During Acute Kawasaki Disease. *Front Pediatr* (2020) 8:295. doi: 10.3389/fped.2020.00295

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Kitoh, Ohara, Muto, Okumura, Baba, Koizumi, Yamagishi, Mikamo, Daigo and Hamakubo. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



PTX3 Regulation of Inflammation, Hemostatic Response, Tissue Repair, and Resolution of Fibrosis Favors a Role in Limiting Idiopathic Pulmonary Fibrosis

OPEN ACCESS

Edited by:

Janos G. Filep,
Université de Montréal, Canada

Reviewed by:

Viswanathan Natarajan,
University of Illinois at Chicago,
United States
Amiram Ariel,
University of Haifa, Israel

*Correspondence:

Remo Castro Russo
remo@ufmg.br
Andrea Doni
andrea.doni@humanitasresearch.it

[†]These authors contributed
equally to this work

Specialty section:

This article was submitted to
Molecular Innate Immunity,
a section of the journal
Frontiers in Immunology

Received: 05 March 2021

Accepted: 02 June 2021

Published: 21 June 2021

Citation:

Doni A, Mantovani A, Bottazzi B and
Russo RC (2021) PTX3 Regulation of
Inflammation, Hemostatic Response,
Tissue Repair, and Resolution of
Fibrosis Favors a Role in Limiting
Idiopathic Pulmonary Fibrosis.
Front. Immunol. 12:676702.
doi: 10.3389/fimmu.2021.676702

Andrea Doni^{1*†}, Alberto Mantovani^{1,2,3†}, Barbara Bottazzi^{1†} and Remo Castro Russo^{4*†}

¹ Unit of Advanced Optical Microscopy, Department of Immunology and Inflammation, Humanitas Clinical and Research Center IRCCS, Milan, Italy, ² Department of Biomedical Sciences, Humanitas University of Milan, Milan, Italy, ³ The William Harvey Research Institute, Queen Mary University of London, London, United Kingdom, ⁴ Laboratory of Pulmonary Immunology and Mechanics, Department of Physiology and Biophysics, Institute of Biological Sciences, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil

PTX3 is a soluble pattern recognition molecule (PRM) belonging to the humoral innate immune system, rapidly produced at inflammatory sites by phagocytes and stromal cells in response to infection or tissue injury. PTX3 interacts with microbial moieties and selected pathogens, with molecules of the complement and hemostatic systems, and with extracellular matrix (ECM) components. In wound sites, PTX3 interacts with fibrin and plasminogen and favors a timely removal of fibrin-rich ECM for an efficient tissue repair. Idiopathic Pulmonary Fibrosis (IPF) is a chronic and progressive interstitial lung disease of unknown origin, associated with excessive ECM deposition affecting tissue architecture, with irreversible loss of lung function and impact on the patient's life quality. Maccarinelli et al. recently demonstrated a protective role of PTX3 using the bleomycin (BLM)-induced experimental model of lung fibrosis, in line with the reported role of PTX3 in tissue repair. However, the mechanisms and therapeutic potential of PTX3 in IPF remained to be investigated. Herein, we provide new insights on the possible role of PTX3 in the development of IPF and BLM-induced lung fibrosis. In mice, PTX3-deficiency was associated with worsening of the disease and with impaired fibrin removal and subsequently increased collagen deposition. In IPF patients, microarray data indicated a down-regulation of PTX3 expression, thus suggesting a potential rational underlying the development of disease. Therefore, we provide new insights for considering PTX3 as a possible target molecule underlying therapeutic intervention in IPF.

Keywords: PTX3, inflammation, hemostasis, resolution, fibrosis, IPF, humoral immunity

INTRODUCTION

Role of PTX3 in Humoral Innate Immunity

The pentraxin family is an ancient group of evolutionarily conserved proteins belonging to humoral innate immunity that act as pattern recognition molecules (PRM). PTX3, the prototype of the long pentraxins arm, differs from the short pentraxins C reactive protein (CRP) and serum amyloid P component (SAP/PTX2) in molecular structure, gene organization, cellular source, and recognized ligands. PTX3 is rapidly produced by mononuclear phagocytes or stromal cells, including mesenchymal, smooth muscle, and endothelial cells (ECs) (1–5), in response to primary proinflammatory cytokines (IL-1 β and TNF- α), TLR agonists, microbial components (LPS or Outer membrane protein-A) and microbes. The molecule acts as an opsonin during infections, facilitating phagocytosis and activating the complement cascade (6). Genetic evidence in mice (7) and humans (8–15) suggests that PTX3 plays an essential role in resistance against selected pathogens, in particular *A. fumigatus*. In addition, PTX3 is induced in response to tissue injury and, through the interaction with the complement system and ECM components, plays non-redundant roles in tissue repair and cancer (1, 16). The relevance of PTX3 in the assembly of the *cumulus oophorus* was the first evidence of its role in ECM. Female subfertility associated with PTX3-deficiency (2, 6) also emphasizes the importance of this protein in ECM assembly and remodeling.

Inflammation activates various tissue response cascades that lead to ECM re-organization, removal of ECM debris, and clearance of apoptotic cells, thus favoring tissue healing. In this context, PTX3 is involved in the turnover of fibrin-rich deposits at wound sites after tissue injury, and consequent collagen deposition (16). Furthermore, when the stimuli persist or the resolution program is broken or stumbled, the inflammatory response may become chronic, impacting tissue remodeling and PTX3 expression. Macrophages are susceptible to the inflammatory environment and are key cells to modulate this system through PRMs. During the early inflammatory phase, M1-polarized macrophages accumulate and orchestrate the inflammatory response. The subsequent switch to an M2-phenotype is crucial for resolving inflammation and tissue repair (17–19). M2-macrophages contribute to tissue homeostasis, dampening inflammation, scavenging ECM debris, and participating in tissue remodeling and repair (19, 20). On the other hand, apoptotic cells generated during chronic inflammation trigger the resolution, with significant changes in macrophage functions. Opsonization of apoptotic cells by PTX3 promotes their recognition by macrophages and subsequent efferocytosis (21), contributing to diverse M2-phenotypes switching (6) and regulating IL-10 and TGF- β 1 production (22). Thus, PTX3 may play a homeostatic role in orchestrating tissue adaptation by coordinating leukocyte migration, resolution, and tissue healing.

PTX3 has been considered an essential regulator of airway mucosal surface homeostasis (23) and is useful as a disease or prognostic marker. In addition, the protein exerts a role in lung immunity against immunological dangers such as respiratory infections, allergy, tissue damage, and malfunction (24). As a

humoral mediator of innate immunity, PTX3 opsonizes pulmonary pathogens promoting the clearance by phagocytosis and triggers the mucosal immune response to fungal or bacterial infections and respiratory viruses. Furthermore, PTX3 also has relevant roles in non-infectious pulmonary diseases. Altogether, PTX3 exerts multiple roles in respiratory diseases. However, its involvement in the development of IPF remains poorly explored.

PTX3 AND LUNG FIBROSIS: LESSONS FROM EXPERIMENTAL MODELS OF PULMONARY FIBROSIS

Bleomycin (BLM) is vastly used to investigate the mechanisms involved in lung fibrosis in mice, and also in the selection of therapeutic drugs for IPF, including Pirfenidone and Nintedanib (25, 26). Several studies have addressed the relevance of PTX3 in different models of pulmonary fibrosis (27–29). Despite the observation that BLM induces PTX3 expression in murine models of lung fibrosis (27, 29), a paucity of information exists on the functional role of the protein in this model. It has been shown that PTX3 promotes murine fibrocyte differentiation dependent on Fc γ RI *in vitro* (28). *In vivo*, PTX3 is localized in fibrotic areas, and its distribution is associated with collagen deposition in lung parenchyma (28) and with macrophage infiltration at sites of fibrogenesis (29), revealing an interplay with macrophages during BLM-induced tissue fibrogenesis. Taking advantage of transgenic mice overexpressing PTX3 (Tie2-PTX3), it was recently shown that the protein could limit lung fibrosis, reducing collagen deposition and fibroblast activation and decreasing leukocyte recruitment (29). Using the BLM-induced fibrosis model (3.75 mg/Kg, *i.n.*), we confirmed macrophage (Ly6C⁺CD115⁺CD11b⁺) accumulation concomitantly with a progressive increase of PTX3 lung levels (**Figure 1A**), as previously described (30). Soon after BLM (2–4 days), RT-PCR showed an increased expression of M1-macrophage genes, followed later on (8–16 days) by an increased expression of M2-macrophage genes (**Figure 1B**). Therefore, increased PTX3 lung content is temporarily associated with a macrophage M2-polarization preceding the pulmonary fibrosis.

In the same model, *Ptx3*^{-/-} mice showed reduced survival (**Figure 1C**) and accentuated weight loss (**Figure 1C**). Corroborating the results by Maccarinelli et al. (29), lungs from *Ptx3*^{-/-} mice showed hemorrhagic areas (day-14)(**Figure 1C**) and increased fibrosis, as assessed by total collagen content (day-22) (**Figure 1D**). In *Ptx3*^{-/-} mice lung homogenates (**Figure 1E**), fibrosis was not associated with differences in IL-4, IL-10, IL-6, and CCL2, thus indicating the independence of PTX3 in the regulation of inflammation (1, 2, 31), while we found increased TGF- β 1 in *Ptx3*^{-/-} mice (**Figure 1E**)(day-14). As described in several models of vascular pathology or tissue repair (16, 32), PTX3 controls the thrombotic response by influencing platelet activation and degranulation. Therefore, it is tempting to speculate that a specific increase in lung TGF- β 1 may be due to a local release derived by platelet degranulation. PTX3 was found localized in the damaged alveolar epithelium and interstitial ECM associated with PDGFR α ⁺

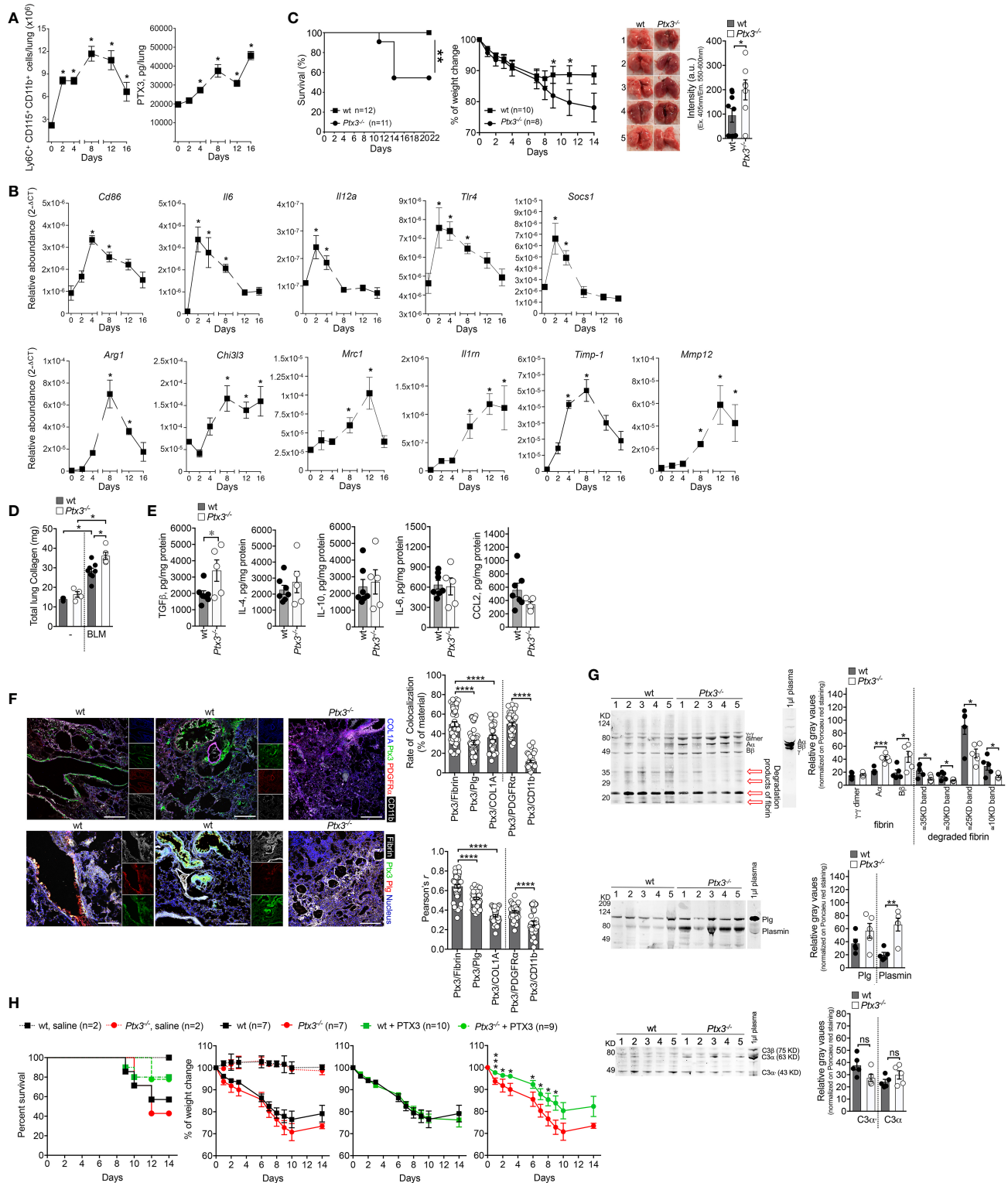


FIGURE 1 | Continued

FIGURE 1 | PTX3 protects mice from BLM-induced pulmonary fibrosis. A model of lung fibrosis was induced by BLM instillation (3.75 mg/Kg, *i.n.*) in Wild Type (WT) and *Ptx3*^{-/-} mice (**A-E**). (**A**, left), kinetic of macrophage influx in the lungs of WT mice after BLM by FACS analysis. (**A**, right), kinetic of PTX3 lung content assessed by ELISA (PTX3 DuoSet® Kit ELISA DY2166, R&D Systems) in tissue homogenates. (**B**), Transcription analysis of M1 genes (*Cd86-Mm00444543_m1*, *Il6-Mm99999064_m1*, *Il12a-Mm99999066_m1*, *Tlr4-Mm00445274_m1*, *Socs1-Mm00782550_s1*) and M2 genes (*Arg1-Mm00475988_m1*, *Chi3l3-Mm00657889_mH*, *Mrc1-Mm01329362_m1*, *Il1rn-Mm00446185_m1*, *Timp1-Mm00441818_m1*, *Mmp12-Mm00500554_m1*) markers of macrophage polarization by TaqMan probes (Applied Biosystems) at different days after BLM treatment. (**C**, **D**), Susceptibility of *Ptx3*^{-/-} mice to lung fibrosis induced by BLM instillation. (**C**, left) survival curve of mice (100% of WT and 54.5% of *Ptx3*^{-/-} mice), as defined by humane end-points (e.g., weight loss of more than 25% of initial body weight, anorexia, excessive decrease in activity, shaggy hair, diarrhea, urinary retention, breathing difficulties). ***P* = 0.01; Log-rank test. (**C**, middle) monitoring of weight loss. **P* < 0.05; unpaired t-test. (**C**, right) representative photographs showing the appearance of lung parenchyma and quantification of autofluorescence intensity (excitation 405nm; emission collection at 550-60nm; CLARIOstar Microplate Reader, BMG Labtech) typically associated to hemoglobin in lung lysates of wt (n=9) and *Ptx3*^{-/-} (n=7) mice (day 14). **P* = 0.05, unpaired t-test. (**D**) lung collagen content after 21 days assessed by Sircol assay. (**E**), measurement of TGF-β₁, IL-4, IL-10, IL-6, and CCL2 in lung lysates of wt (n=7) and *Ptx3*^{-/-} (n=5) mice (day 14 after BLM treatment) by ELISA (R&D Systems). **P* = 0.05, unpaired t-test. (**F**), confocal microscopy analysis on lung specimens (10μm) from WT mice (n=7) 14 days after BLM treatment. (**F**, upper panels) localization of PTX3 (green), Collagen I (blue), PDGFRα⁺ (red) mesenchymal cells and CD11b⁺ (white) immune cells. Representative localization of PTX3 around blood vessels (**F**, upper, left) or associated with fibrotic ECM and damaged epithelium (**F**, upper, middle). (**F**, lower panels) colocalization of PTX3 (green) with fibrin (white) and plasminogen (red) in fibrotic lung associated with blood vessels (**F**, lower, left) or ECM and damaged epithelium (**F**, lower, middle). Blue, nuclei. Lungs obtained from *Ptx3*^{-/-} mice were used as control (Upper and lower panels, right). Bar, 100μm. The following antibodies were used: collagen I, rabbit polyclonal (5μg/ml; AbCam); PTX3, goat polyclonal (0.5μg/ml; R&D Systems); PDGFRα, BV421 rat (APAs, 1.5μg/ml; BD Horizon); CD11b, APC-Cy7™ rat (M1/70, 2μg/ml; BD Pharmingen); plasminogen, rat monoclonal (1μg/ml; Cell Sciences); fibrinogen, rabbit polyclonal (4μg/ml; Dako); species-specific Alexa Fluor 488/568/647- conjugated secondary antibodies were used. (**F**, right) Rate of colocalization (% of material; Fiji software) of PTX3 signal with fibrin, plasminogen, collagen I (COL1A), PDGFRα, and CD11b and relative Pearson's correlation coefficient. Mean ± SEM of 5-8 images acquired for each mouse (n=7). **P* < 0.0001, unpaired t-test. (**G**, upper), Western blot analysis of fibrin in lung lysates (10μg total proteins per lane on 10% SDS-PAGE) of WT (n=5) and *Ptx3*^{-/-} (n=5) mice at day 7. A polyclonal rabbit anti-fibrinogen was used (3μg/ml; Agilent/DAKO). 1μl of basal mouse plasma in ACD-A (Anticoagulant Citrate Dextrose Solution) was used as a control for fibrinogen; 1μl of mouse plasma-ACD incubated with thrombin (1U/ml; 1h) was used as a control for fibrin. A typical band pattern of fibrin (Aα; Bβ, γ-γ dimer) is indicated in the fibrin control and lung lysates. Red arrows, lower molecular weight bands corresponding to degraded fibrin. (**G**, upper, right), quantification of fibrin bands as relative gray values (Fiji software) on Ponceau red staining. ****P* < 0.005, **P* < 0.5; unpaired t-test. (**G**, middle), Western blot analysis of plasminogen and relative band quantification as gray values (Fiji software) in same lysates (50μg total proteins per lane on 10% SDS-PAGE, **G**, middle, right). A polyclonal goat anti-plasminogen was used (0.5μg/ml; R&D Systems). The molecular weight of plasminogen and plasmin activation bands are indicated. (**G**, lower), Western blot analysis of the complement component C3 in the same lysates (10μg total proteins per lane on 10% SDS-PAGE) and relative band quantification as gray values (Fiji software, **G**, lower, right). A polyclonal goat anti-human/mouse C3 and activation fragments (1:3000; Merck-Millipore) was used. (**H**) Effect of PTX3 administration in BLM-induced lung fibrosis. One experiment was performed. Human recombinant PTX3 (50μg/mouse) was injected *i.p.* one day after BLM (5 mg/Kg, *i.n.*) treatment in WT mice. Survival (**H**, left) (*Ptx3*^{-/-} mice from 42.8% to 77.8%, and WT mice from 57.1 to 80% of survival) and body weight (**H**, 3 panels right) were recorded until day 14. Curves referring to weight loss are shown compared to untreated WT and *Ptx3*^{-/-} mice (**H**, first left) or separated by genotype and compared with the correspondent treated group (**H**, right). **P* < 0.05; unpaired t-test. All results were expressed as mean ± SEM. Normalized data were analyzed by One-Way ANOVA with Tukey post-test, using the software GraphPad Prism 8.0. Differences were considered significant at *P* < 0.05.

mesenchymal cells, and *Ptx3*^{-/-} mice showed increased interstitial fibrin deposition and subsequent fibrotic scarring in ALI (acute lung injury) model (5). Similarly, in the BLM-induced fibrotic lungs (day-14), PTX3 is localized in damaged epithelium and areas of ECM rich in collagen-I, preferentially associated with PDGFRα⁺ mesenchymal cells rather than recruited CD11b⁺ cells (day-7) (Figure 1F). Moreover, PTX3 is localized around the blood vessels (Figure 1F). Thus, in line with evidence obtained in different experimental models of lung injury and repair (16, 33, 34), PTX3 plays a non-redundant and protective role during BLM-induced pulmonary fibrosis in mice.

PTX3 promotes arterial thrombosis (32) at wound sites during injury-induced thrombotic response and promotes healing by interacting with plasminogen, favoring timely fibrin removal in acidic microenvironments (5, 16, 33, 35). As presented in Figure 1F, in the BLM-induced fibrotic lungs PTX3 colocalized with fibrin deposits in ECM and damaged epithelium closely associated with plasminogen. A similar colocalization was observed in the endothelium of blood vessels possibly associated with coagulation sites (day-7) (Figure 1F). Coagulation proteases are recognized to exert profibrotic cellular effects *via* activation of protease-activated receptors (PARs) (36). Fibrinolysis is also an essential prerequisite for subsequent tissue remodeling processes leading to efficient repair (37–40). Therefore, analysis of fibrin and

plasminogen content in lung lysates would address whether a defective turnover in fibrin removal was present in *Ptx3*^{-/-} mice. As assessed by Western blot, lungs of *Ptx3*^{-/-} mice showed increased fibrin deposition and decreased fragments of fibrin degradation at the inflammatory phase (day-7) (Figure 1G). Differences in plasminogen deposition and plasmin formation (day-7) (Figure 1G) observed in the same lung homogenates suggest an impairment in ECM turnover of fibrin, possibly at the bases of a subsequent increased lung fibrosis. No evidence of PTX3 regulation on complement activation was observed in this model, as no differences were found in C3 deposition in the lung (Figure 1G). Several reports showed that coagulation cascade elements are involved in lung fibrosis (41). The deficiency of components of the fibrinolytic system caused exacerbated lung injury associated with defective clearance of necrotic tissue and augmented fibrin deposition and fibrosis (42). Lung fibrosis was reverted by overexpression of plasminogen activator genes (38, 39). Therefore, the disruption of fibrin removal and altered ECM turnover with collagen deposition appeared as the central mechanism underlying the phenotypes associated with *Ptx3*^{-/-} mice in response to BLM, corroborating the observations from other models of organ damage (5, 33, 35). TGF-β1-mediated elevated PAI-1 levels and defective fibrinolysis have significant fibrotic consequences for tissue repair (43, 44). Interestingly, TGF-β1 was found to down-regulate PTX3 at mRNA and

protein levels in granuloma (45) and mesenchymal cells (our results, data not shown). TGF- β 1 increased ECM deposition promoting transcription of collagen and protease inhibitors, including tissue inhibitors of metalloproteases (TIMPs) and PAI-1 (46). Concomitantly, TGF- β 1 decreases the secretion of proteases responsible for ECM degradation, including activators of plasminogen (43, 44), thus increasing the overall production of ECM proteins (47). Therefore, PTX3 down-regulation by TGF- β 1 could inhibit the removal of the fibrin matrix and increases ECM deposition during experimental fibrosis.

The evidence obtained from the BLM-induced lung fibrosis model prompted us to evaluate the possible therapeutic effects of PTX3 treatment in the disease. In a first preliminary experiment, shown in **Figure 1H**, a single *i.p.* injection of recombinant PTX3 (50 μ g/mouse) one day after BLM (5 mg/Kg, *i.n.*) was sufficient to increase survival of *Ptx3*^{-/-} mice, with a weak effect even in WT mice survival (**Figure 1H**). PTX3 treatment also attenuated the weight loss in *Ptx3*^{-/-} mice but not in WT mice challenged with BLM (**Figure 1G**), suggesting a potential therapeutic effect of PTX3 in pulmonary fibrosis. However, the actual evaluation of potential PTX3 treatment requires more detailed pharmacological studies on the doses and administration routes.

MECHANISMS OF PTX3 IN IDIOPATHIC PULMONARY FIBROSIS: INTERPRETING DATA FROM IPF

IPF is a chronic and lethal Interstitial Lung Disease characterized by fibroproliferation of unknown origin and insensitive to therapy, associated with excessive ECM deposition in the pulmonary parenchyma (48–50). Clinical signs include progressive loss of lung volume, increased respiratory effort, and abnormal gas exchange, leading to respiratory collapse (48–50). Currently, some hypotheses have been proposed about its controversial origin. Among them, clinical data have revealed a close correlation between pulmonary fibrosis and the profile of inflammatory mediators released by immune cells (19, 51, 52). Data supports that pulmonary fibrosis is the final result of previous alveolitis with excessive scarring (19, 52, 53). Current knowledge about IPF has been derived from detailed pathological analysis of human lung samples that elucidated its unique morphological characteristics, together with observations derived from animal models of disease (26, 52, 54, 55). New insights raised from genetic and transcriptomic studies on IPF samples have given a better comprehension of the molecular and cellular mechanisms determining the lung phenotype of IPF and patient therapeutics (25, 54, 56).

The physiological role of PTX3 in IPF remains to be elucidated. However, present results and previous evidence suggest a role of PTX3 in lung repair in experimental models of lung injury (16), an association of PTX3 and Primary Graft Dysfunction (PGD) in IPF recipients after lung transplant (57), or a potential role of PTX3 produced by fibroblasts and bronchial epithelial cells in fibrocyte differentiation *in vitro* (28). In the lung tissue samples obtained from IPF patients, PTX3 was found

associated with fibrotic areas of ECM, epithelium, and alveolar leukocytes (28). In order to gain a deeper insight into this association, we analyzed microarray data of lung samples from IPF (GEO database: GSE32537) using *Phantasus* (58), a web application for visual and interactive gene expression analysis (<https://genome.ifmo.ru/phantasus>). Besides upregulated genes related to fibrosis (COL1A1, FGFR2, TIMP2, and TGF- β 2/3; **Figures 2A, C**), increased expression of M2-macrophage and inhibition of M1-macrophage related genes were found in IPF samples (**Figures 2A, C**), similarly to WT mice exposed to BLM (**Figure 1B**). Moreover, IL-10 and PTX3 expression were down-regulated in IPF in this set of lung microarray (**Figure 2A**), as shown by normalized data (**Figure 2C**). PLAUR and SERPINE-1, genes belonging to the fibrinolytic system and related to tissue repair, were also down-regulated (**Figures 2B, C**). Thus, PTX3 gene down-regulation in lung samples may be related to active IPF pathology. This recapitulates the mouse phenotype observed *in vivo*, with aberrant collagen deposition in *Ptx3*^{-/-} mice [**Figure 1** (29)], supporting the possibility that endogenous PTX3 exerts a protective role and may be involved in IPF disease. Therefore, PTX3 down-regulation could be part of a TGF- β 1 regulatory program leading to fibrinolysis inhibition and ECM increased deposition, both determinants of IPF pathogenesis and progression.

THERAPEUTIC OPPORTUNITIES OF PTX3 IN THE CONTEXT OF IPF: WHAT MECHANISMS WOULD BE INVOLVED?

Data reported by Maccarinelli et al. (29) and our results suggest that IPF may be related to a low expression of PTX3 in lung tissue. Therefore, we could consider restoring PTX3 levels exogenously (as summarized in **Figure 1G**) as a possible therapeutic approach for IPF. Different roles of PTX3 could be considered:

PTX3 as a Negative Modulator of Chronic Airway Inflammation in IPF

Chronic inflammation can lead to an imbalance in soluble factors production and leukocyte recruitment, turning the healing response into a pathological fibrotic response (52). Chronic neutrophilic airway inflammation occurs in IPF and airway neutrophilia, mainly due to CXCL8 produced by alveolar macrophages (59), and predicts mortality of IPF patient (60). PTX3 regulates neutrophils influx through interaction with P-selectin expressed on the surface of ECs (31, 61), thus, exogenous PTX3 may dampen neutrophil influx into IPF airways (**Figure 2D**). Moreover, soluble PTX3 derived from Human Umbilical Cord Blood-Derived Mesenchymal Stem Cells (UCB-MSCs) has anti-inflammatory effects in ALI, as shown in a model of neonatal hyperoxia-induced lung injury in rats. Similarly, adoptive transfer of MSC from WT but not from *Ptx3*^{-/-} mice improved oxygenation with reduced lung collapse and neutrophils (33), shaping the differentiation of anti-inflammatory macrophages (62). On the other hand, Dendritic cells (DCs), activated by the phagocytosis of apoptotic cells, are described to sustain chronic lung inflammation in IPF (63). Nevertheless, in the presence of PTX3, DCs failed to internalize apoptotic cells (21), thus suggesting that

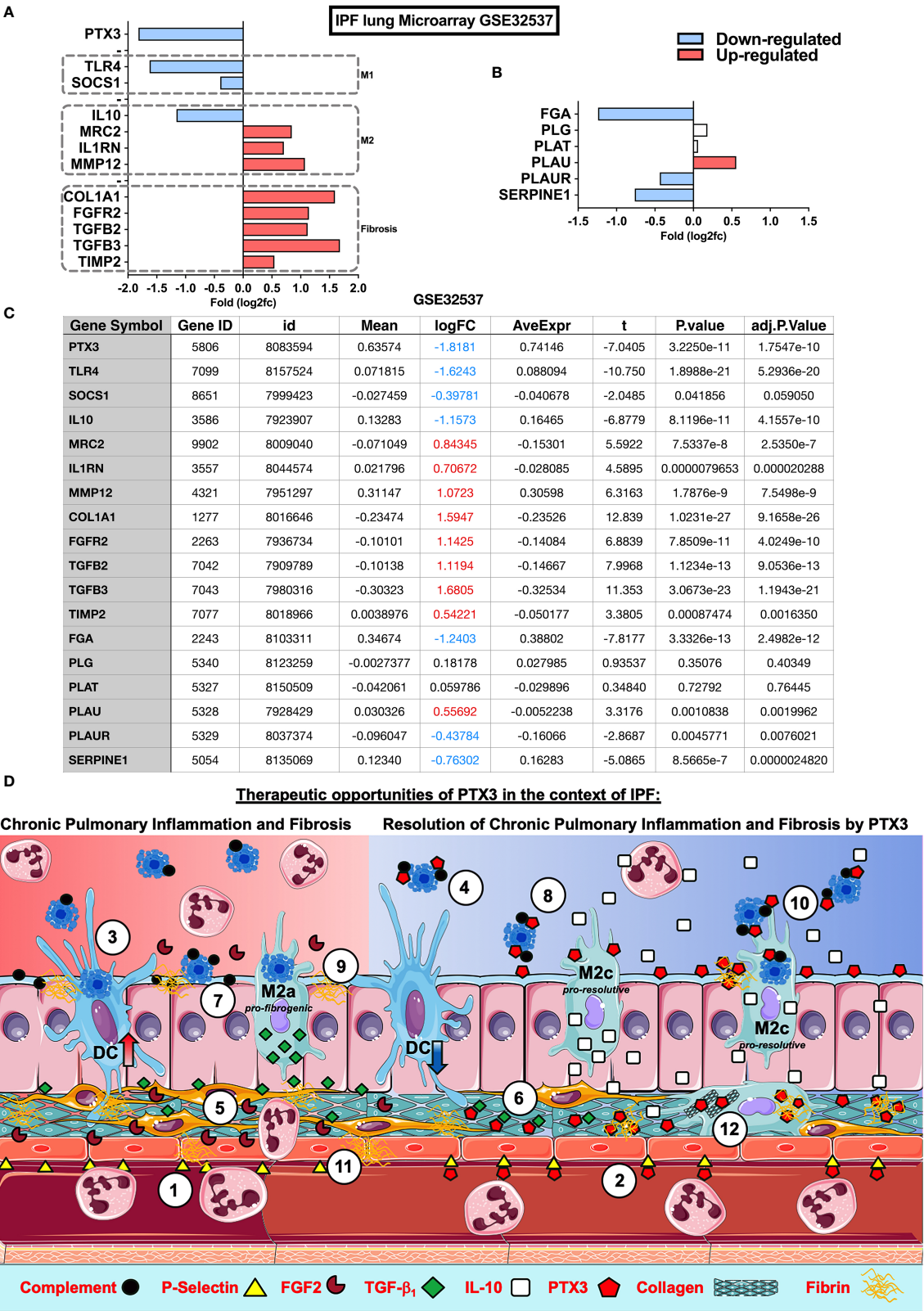


FIGURE 2 | Continued

FIGURE 2 | Impact of PTX3 in Idiopathic Pulmonary Fibrosis and therapeutic opportunities. Microarray analysis of lung samples from IPF (n=119) and healthy (n=50) individuals from GEO database: GSE32537 were analyzed by *Phantasus* (58) (<https://genome.ifmo.ru/phantasus>). **(A)** Lung expression of PTX3, fibrogenic markers as COL1A1, FGFR2, TGF β 2, TGF β 3 and TIMP-2, M1 (SOCS1 and TLR4) and M2 (IL-10, MRC2, IL1RN, and MMP-12) macrophage polarization markers **(B)** Expression of coagulation cascade FGA (Fibrinogen alpha-chain precursor), PLG (Plasminogen precursor), PLAT (Tissue-type Plasminogen Activator precursor), PLAU (Urokinase-type Plasminogen Activator precursor), PLAUR (Urokinase Plasminogen Activator Receptor) and SERPINE1 (PAI-1 Plasminogen Activator Inhibitor-1) in IPF and healthy samples. **(C)** Data table with analysis from GEO database GSE32537 analyzed by *Phantasus* according to the instructions for the use of the application. Differences were considered significant at *P* value <0.05. **(D)** Possible mechanisms and therapeutic opportunities of PTX3 in the context of IPF. IPF is characterized by a reduced PTX3 production (red background), however PTX3 may act as an anti-inflammatory as well as a pro-resolutive modulator of chronic pulmonary inflammation and fibrosis in IPF (blue background) at different levels: (1) Neutrophil influx is facilitated through the interaction with P-selectin expressed on the surface of ECs, (2) PTX3 could antagonize endothelial P-selectin, dampening neutrophil influx during chronic pulmonary inflammation; (3) the abundance of apoptotic cells in airways from IPF is related to DC phagocytosis and activation that sustain chronic lung inflammation, (4) PTX3 may block apoptotic cell internalization and consequent inflammation; (5) FGF2 activates fibroblasts and ECs, (6) however PTX3 interacts with FGF2 reducing its availability for binding to FGFR2 on fibroblasts and consequent fibrosis; (7) complement and apoptotic cell deposition in the lungs lead to chronic inflammation, (8) on the other side PTX3 may act as a scavenger preventing the excessive deposition of both complement components and apoptotic cells in lungs and consequent attenuation of tissue damage and inflammation; (9) Alveolar macrophages from IPF display defective efferocytosis and increased TGF- β 1 production, contributing to tissue fibrogenesis, (10) while PTX3 may enhance macrophage efferocytosis and M2 polarization and resolution of inflammation by IL-10; (11) Finally, defective PTX3 production in IPF may increase fibrin deposition and fibrosis, (12) but PTX3 could contribute to the resolution of fibrosis, interacting with fibrin-clots and disorganized collagen fibers in the lung parenchyma, supporting fibrinolysis and clearance of ECM debris by macrophage phagocytosis, promoting lung tissue healing and repair.

PTX3 may prevent chronic pulmonary inflammation in IPF (Figure 2D). However, whether endogenous or exogenous PTX3 acts as a tissue-protective or resilience factor stimulating lung tissue reepithelization remains unexplored.

PTX3 Involvement in Resolution of Inflammation in IPF

Efficient resolution of inflammation is crucial for the restoration of tissue integrity. PTX3 has been reported to induce the polarization of macrophages into anti-inflammatory M2 phenotype and to stimulate them to secrete the resolutive cytokine IL-10 (62, 64). Moreover, as mentioned above, PTX3 production by MSC reinforced M2 macrophage markers, inducing Dectin-1 and IL-10, and protecting mice from neonatal hyperoxia-induced lung injury (62). Therefore, PTX3 may contribute to the resolution of chronic inflammation in IPF *via* M2-macrophages enhancing IL-10-dependent anti-inflammatory and resolutive functions, such as neutrophil apoptosis (Figure 2D). Nevertheless, impaired efferocytosis can result in inflammation-associated pathologies (65). Indeed, efferocytosis by alveolar macrophages is impaired in IPF samples compared with other interstitial pneumonia (66), and a dysregulated or defective efferocytosis may contribute to the pathogenesis of IPF (65, 66). Notably, post-efferocytotic, satiated macrophages [also termed Mres (67)] produce high levels of TGF- β 1 (68). However, this production seems to be functionally antagonized by the production of IFN- β in these macrophages (69), that directs their anti-fibrotic phenotype (70, 71). Along these lines, apoptotic cell instillation after BLM attenuates lung injury (72) and induces PPAR γ , promoting lung fibrosis resolution *via* regulation of efferocytosis and IL-10 production (73). In this way, PTX3 recognizes apoptotic cells and may facilitate the clearance of dead or dying cells (1). Therefore, the capacity of PTX3 to affect apoptotic cell recognition and efferocytosis could represent an additional mechanism of negative regulation of chronic inflammation in IPF (Figure 2D) (66). PTX3 enhanced complement-mediated clearance of apoptotic debris. The protein is recruited by C4 binding protein (C4BP) on apoptotic cells reducing the deposition of the lytic C5b-9 terminal complex at sites of tissue injury (74), limiting the complement-mediated tissue damage and inflammation (1), and possibly tissue fibrosis (Figure 2D).

PTX3 as a Resolutive Modulator of Tissue Fibrosis in IPF

PTX3 interaction with plasminogen ensures the timely removal of fibrin deposits in the inflammatory ECM of the lung, allowing for the proper sequence of processes leading to efficient tissue repair. TGF- β 1, the major fibrogenic molecule involved in the mechanisms of excessive ECM deposition in pulmonary fibrosis, negatively regulates PTX3 and ECM-degrading molecules (e.g., MMPs, uPA) and up-regulates TIMPs and PAI-1 (45, 75). Thus, defective PTX3-mediated fibrinolysis may represent a key mechanism underlying the development of the disease. Therefore, TGF- β 1 induces suppression of PTX3-mediated fibrinolysis and may represent another mechanism underlying the development of IPF. In other contexts of tissue repair (16, 33, 35), PTX3 administration reversed the defective fibrinolysis associated with PTX3-deficiency and this may represent an important activity underlying PTX3 role in regulating the evolution towards fibrotic scarring of the lung (Figure 2D). PTX3 interacts with FGF2 and modulates the FGFR2-dependent vascularization of tumors and FGFR2-mediated smooth muscle cell proliferation and artery restenosis (76, 77). The expression of FGF2/FGFR2 axis is elevated in IPF samples (78), and FGFR2-dependent signaling is involved in pulmonary fibrosis (76, 79). Therefore, a down-regulation of PTX3 in IPF may represent a possible failure to antagonize these fibrotic pathways (Figure 2D). Finally, PTX3 also interacts with collagens and our own preliminary results indicate a possible involvement of collagen remodeling by mesenchymal cells, thus suggesting an effect in removing the excess of ECM from tissue parenchyma (Figure 2D) and hence in promoting tissue healing.

CONCLUDING AND REMARKS

Failure to control the overlapping events leading to the healing process is the cause of the functional tissue replacement by fibrous scar. Growing evidences indicate that abnormalities in pathways involving fibroblast activation and coagulation cascade drive abnormal fibroproliferation and progressive replacement of lung parenchyma by collagen (37, 80). Understanding the molecules

involved in these pathways during aberrant wound repair may predict new targets and therapeutic intervention strategies. PTX3, besides being an essential fluid-phase PRM of the innate immune system, is involved in the healing at wound sites, favoring timely fibrinolysis through the interaction with fibrin and plasminogen. Results reported by Maccarinelli and colleagues and our observations indicate a protective and regulatory role of PTX3 in BLM-induced lung fibrosis models of lung injury. Besides IPF, other pathological conditions can result in pulmonary fibrosis, last but not least the persistent post-COVID syndrome (81, 82). Many questions remain open, starting from the therapeutic effect of PTX3 to the mechanisms involved in the protective role of the protein and the relationships of PTX3 with the homeostasis of the airway epithelium and with the collagen fibers of the ECM. Little is known about the effect of IPF therapy with Pirfenidone/Nintedanib on PTX3. Further clinical studies will be necessary to answer these and many other questions. Thus, in summary, we assessed the involvement of PTX3 in pulmonary fibrosis. Based on the literature and recent data, we propose that PTX3 may have a physiological and protective role during IPF, interacting with various circuits and representing a potential therapeutic target, acting as a pro-resolutive molecule in the context of pulmonary fibrosis.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: <https://www.ncbi.nlm.nih.gov/geo/>, GSE32537.

REFERENCES

- Garlanda C, Bottazzi B, Magrini E, Inforzato A, Mantovani A. PTX3, a Humoral Pattern Recognition Molecule, in Innate Immunity, Tissue Repair, and Cancer. *Physiol Rev* (2018) 98:623–39. doi: 10.1152/physrev.00016.2017
- Magrini E, Mantovani A, Garlanda C. The Dual Complexity of PTX3 in Health and Disease: A Balancing Act? *Trends Mol Med* (2016) 22:497–510. doi: 10.1016/j.molmed.2016.04.007
- Doni A, Stravalaci M, Inforzato A, Magrini E, Mantovani A, Garlanda C, et al. The Long Pentraxin PTX3 as a Link Between Innate Immunity, Tissue Remodeling, and Cancer. *Front Immunol* (2019) 10:712. doi: 10.3389/fimmu.2019.00712
- Manfredi AA, Rovere-Querini P, Bottazzi B, Garlanda C, Mantovani A. Pentraxins, Humoral Innate Immunity and Tissue Injury. *Curr Opin Immunol* (2008) 20:538–44. doi: 10.1016/j.coi.2008.05.004
- Doni A, Garlanda C, Mantovani A. Innate Immunity, Hemostasis and Matrix Remodeling: PTX3 as a Link. *Semin Immunol* (2016) 28:570–7. doi: 10.1016/j.smim.2016.10.012
- Erreni M, Manfredi AA, Garlanda C, Mantovani A, Rovere-Querini P. The Long Pentraxin PTX3: A Prototypical Sensor of Tissue Injury and a Regulator of Homeostasis. *Immunol Rev* (2017) 280:112–25. doi: 10.1111/imr.12570
- Garlanda C, Hirsch E, Bozza S, Salustri A, De Acetis M, Nota R, et al. Non-Redundant Role of the Long Pentraxin PTX3 in Anti-Fungal Innate Immune Response. *Nature* (2002) 420:182–6. doi: 10.1038/nature01195
- Olesen R, Wejse C, Velez DR, Bisseye C, Sodemann M, Aaby P, et al. DC-SIGN (CD209), Pentraxin 3 and Vitamin D Receptor Gene Variants Associate With Pulmonary Tuberculosis Risk in West Africans. *Genes Immun* (2007) 8:456–67. doi: 10.1038/sj.gene.6364410
- Cunha C, Aversa F, Lacerda JF, Busca A, Kurzai O, Grube M, et al. Genetic PTX3 Deficiency and Aspergillosis in Stem-Cell Transplantation. *N Engl J Med* (2014) 370:421–32. doi: 10.1056/NEJMoa1211161

ETHICS STATEMENT

The animal study was reviewed and approved by by the Italian Ministry of Health (protocol approval n. 803/2015-PR).

AUTHOR CONTRIBUTIONS

AD, AM, BB, and RCR designed the discussion, conclusions and wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

RCR is grateful to the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil) under Grant Agreement No. 312839/2020-0. AD, BB, and AM are grateful to the Italian Association for Cancer Research (AIRC; grant number IG-23465) and the Italian Space Agency (ASI MARS-PRE Project, grant number DC-VUM 2017-006) for financial support.

ACKNOWLEDGMENTS

We would like to thank the professors Dr. Flavio Almeida Amaral and Dr. Jerome Baron from UFMG, for the helpful give-a-take. Schematic Figure (**Figure 2D**) used images from Servier Medical Art, licensed under a Creative Common Attribution 3.0 Generic License. (<http://smart.servier.com/>).

- Cunha C, Monteiro AA, Oliveira-Coelho A, Kuhne J, Rodrigues F, Sasaki SD, et al. Ptx3-Based Genetic Testing for Risk of Aspergillosis After Lung Transplant. *Clin Infect Dis* (2015) 61:1893–4. doi: 10.1093/cid/civ679
- Wojtowicz A, Lecompte TD, Bibert S, Manuel O, Rueger S, Berger C, et al. PTX3 Polymorphisms and Invasive Mold Infections After Solid Organ Transplant. *Clin Infect Dis* (2015) 61:619–22. doi: 10.1093/cid/civ386
- Fisher CE, Hohl TM, Fan W, Storer BE, Levine DM, Zhao LP, et al. Validation of Single Nucleotide Polymorphisms in Invasive Aspergillosis Following Hematopoietic Cell Transplantation. *Blood* (2017) 129:2693–701. doi: 10.1182/blood-2016-10-743294
- Herrero-Sanchez MC, Angomas EB, de Ramon C, Telleria JJ, Corchete LA, Alonso S, et al. Polymorphisms in Receptors Involved in Opsonic and Nonopsonic Phagocytosis, and Correlation With Risk of Infection in Oncohematology Patients. *Infect Immun* (2018) 86(12):e00709-18. doi: 10.1128/IAI.00709-18
- Campos CF, Leite L, Pereira P, Vaz CP, Branca R, Campilho F, et al. PTX3 Polymorphisms Influence Cytomegalovirus Reactivation After Stem-Cell Transplantation. *Front Immunol* (2019) 10:88. doi: 10.3389/fimmu.2019.00088
- Chiarini M, Sabelli C, Melotti P, Garlanda C, Savoldi G, Mazza C, et al. PTX3 Genetic Variations Affect the Risk of Pseudomonas Aeruginosa Airway Colonization in Cystic Fibrosis Patients. *Genes Immun* (2010) 11:665–70. doi: 10.1038/gene.2010.41
- Doni A, Musso T, Morone D, Bastone A, Zambelli V, Sironi M, et al. An Acidic Microenvironment Sets the Humoral Pattern Recognition Molecule PTX3 in a Tissue Repair Mode. *J Exp Med* (2015) 212:905–25. doi: 10.1084/jem.20141268
- Watanabe S, Alexander M, Misharin AV, Budinger GRS. The Role of Macrophages in the Resolution of Inflammation. *J Clin Invest* (2019) 129:2619–28. doi: 10.1172/JCI124615
- Mantovani A, Biswas SK, Galdiero MR, Sica A, Locati M. Macrophage Plasticity and Polarization in Tissue Repair and Remodelling. *J Pathol* (2013) 229:176–85. doi: 10.1002/path.4133

19. Vannella KM, Wynn TA. Mechanisms of Organ Injury and Repair by Macrophages. *Annu Rev Physiol* (2017) 79:593–617. doi: 10.1146/annurev-physiol-022516-034356
20. Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The Chemokine System in Diverse Forms of Macrophage Activation and Polarization. *Trends Immunol* (2004) 25:677–86. doi: 10.1016/j.it.2004.09.015
21. Rovere P, Peri G, Fazzini F, Bottazzi B, Doni A, Bondanza A, et al. The Long Pentraxin PTX3 Binds to Apoptotic Cells and Regulates Their Clearance by Antigen-Presenting Dendritic Cells. *Blood* (2000) 96:4300–6. doi: 10.1182/blood.V96.13.4300
22. Baruah P, Propato A, Dumitriu IE, Rovere-Querini P, Russo V, Fontana R, et al. The Pattern Recognition Receptor PTX3 is Recruited at the Synapse Between Dying and Dendritic Cells, and Edits the Cross-Presentation of Self, Viral, and Tumor Antigens. *Blood* (2006) 107:151–8. doi: 10.1182/blood-2005-03-1112
23. Smole U, Kratzer B, Pickl WF. Soluble Pattern Recognition Molecules: Guardians and Regulators of Homeostasis at Airway Mucosal Surfaces. *Eur J Immunol* (2020) 50:624–42. doi: 10.1002/eji.201847811
24. Balhara J, Koussih L, Zhang J, Gounni AS. Pentraxin 3: An Immuno-Regulator in the Lungs. *Front Immunol* (2013) 4:127. doi: 10.3389/fimmu.2013.00127
25. Tashiro J, Rubio GA, Limper AH, Williams K, Elliot SJ, Ninou I, et al. Exploring Animal Models That Resemble Idiopathic Pulmonary Fibrosis. *Front Med (Lausanne)* (2017) 4:118. doi: 10.3389/fmed.2017.00118
26. Miles T, Hoyne GF, Knight DA, Fear MW, Mutsaers SE, Prele CM. The Contribution of Animal Models to Understanding the Role of the Immune System in Human Idiopathic Pulmonary Fibrosis. *Clin Transl Immunol* (2020) 9:e1153. doi: 10.1002/cti2.1153
27. Della Latta V, Cabiati M, Burchielli S, Frenzilli G, Bernardeschi M, Cecchetti A, et al. Lung Inflammation After Bleomycin Treatment in Mice: Selection of an Accurate Normalization Strategy for Gene Expression Analysis in an *Ex-Vivo* and *in-Vitro* Model. *Int J Biochem Cell Biol* (2017) 88:145–54. doi: 10.1016/j.biocel.2017.05.016
28. Pilling D, Cox N, Vakil V, Verbeek JS, Gomer RH. The Long Pentraxin PTX3 Promotes Fibrocyte Differentiation. *PLoS One* (2015) 10:e0119709. doi: 10.1371/journal.pone.0119709
29. Maccarinelli F, Bugatti M, Schiud AC, Ganzerla S, Vermi W, Presta M, et al. Endogenous Long Pentraxin 3 Exerts a Protective Role in a Murine Model of Pulmonary Fibrosis. *Front Immunol* (2021) 12:617671. doi: 10.3389/fimmu.2021.617671
30. Russo RC, Savino B, Mirolo M, Buracchi C, Germano G, Anselmo A, et al. The Atypical Chemokine Receptor ACKR2 Drives Pulmonary Fibrosis by Tuning Influx of CCR2(+) and CCR5(+) IFN- γ -Producing Gammadelta Cells in Mice. *Am J Physiol Lung Cell Mol Physiol* (2018) 314:L1010–25. doi: 10.1152/ajplung.00233.2017
31. Deban L, Russo RC, Sironi M, Moalli F, Scanziani M, Zambelli V, et al. Regulation of Leukocyte Recruitment by the Long Pentraxin PTX3. *Nat Immunol* (2010) 11:328–34. doi: 10.1038/ni.1854
32. Bonacina F, Barbieri SS, Cutuli L, Amadio P, Doni A, Sironi M, et al. Vascular Pentraxin 3 Controls Arterial Thrombosis by Targeting Collagen and Fibrinogen Induced Platelets Aggregation. *Biochim Biophys Acta* (2016) 1862:1182–90. doi: 10.1016/j.bbdis.2016.03.007
33. Mauri T, Zambelli V, Cappuzzello C, Bellani G, Dander E, Sironi M, et al. Intraperitoneal Adoptive Transfer of Mesenchymal Stem Cells Enhances Recovery From Acid Aspiration Acute Lung Injury in Mice. *Intensive Care Med Exp* (2017) 5:13. doi: 10.1186/s40635-017-0126-5
34. Han B, Haitisma JJ, Zhang Y, Bai X, Rubacha M, Keshavjee S, et al. Long Pentraxin PTX3 Deficiency Worsens LPS-Induced Acute Lung Injury. *Intensive Care Med* (2011) 37:334–42. doi: 10.1007/s00134-010-2067-2
35. Cappuzzello C, Doni A, Dander E, Pasqualini F, Nebuloni M, Bottazzi B, et al. Mesenchymal Stromal Cell-Derived PTX3 Promotes Wound Healing via Fibrin Remodeling. *J Invest Dermatol* (2016) 136:293–300. doi: 10.1038/JID.2015.346
36. Wygrecka M, Kwapiszewska G, Jablonska E, von Gerlach S, Henneke I, Zakrzewicz D, et al. Role of Protease-Activated Receptor-2 in Idiopathic Pulmonary Fibrosis. *Am J Respir Crit Care Med* (2011) 183:1703–14. doi: 10.1164/rccm.201009-1479OC
37. Idell S. Coagulation, Fibrinolysis, and Fibrin Deposition in Acute Lung Injury. *Crit Care Med* (2003) 31:S213–20. doi: 10.1097/01.CCM.0000057846.21303.AB
38. Eitzman DT, McCoy RD, Zheng X, Fay WP, Shen T, Ginsburg D, et al. Bleomycin-Induced Pulmonary Fibrosis in Transgenic Mice That Either Lack or Overexpress the Murine Plasminogen Activator Inhibitor-1 Gene. *J Clin Invest* (1996) 97:232–7. doi: 10.1172/JCI118396
39. Sisson TH, Hattori N, Xu Y, Simon RH. Treatment of Bleomycin-Induced Pulmonary Fibrosis by Transfer of Urokinase-Type Plasminogen Activator Genes. *Hum Gene Ther* (1999) 10:2315–23. doi: 10.1089/10430349950016960
40. Bdeir K, Murciano JC, Tomaszewski J, Koniaris L, Martinez J, Cines DB, et al. Urokinase Mediates Fibrinolysis in the Pulmonary Microvasculature. *Blood* (2000) 96:1820–6. doi: 10.1182/blood.V96.5.1820.h8001820_1820_1826
41. Gunther A, Lubke N, Ermer M, Schermuly RT, Weissmann N, Breitecker A, et al. Prevention of Bleomycin-Induced Lung Fibrosis by Aerosolization of Heparin or Urokinase in Rabbits. *Am J Respir Crit Care Med* (2003) 168:1358–65. doi: 10.1164/rccm.2201082
42. Swaisgood CM, French EL, Noga C, Simon RH, Ploplis VA. The Development of Bleomycin-Induced Pulmonary Fibrosis in Mice Deficient for Components of the Fibrinolytic System. *Am J Pathol* (2000) 157:177–87. doi: 10.1016/S0002-9440(10)64529-4
43. de Giorgio-Miller A, Bottoms S, Laurent G, Carmeliet P, Herrick S. Fibrin-Induced Skin Fibrosis in Mice Deficient in Tissue Plasminogen Activator. *Am J Pathol* (2005) 167:721–32. doi: 10.1016/S0002-9440(10)62046-9
44. Ghosh AK, Vaughan DE. PAI-1 in Tissue Fibrosis. *J Cell Physiol* (2012) 227:493–507. doi: 10.1002/jcp.22783
45. Li H, Chang HM, Shi Z, Leung PCK. SNAIL Mediates TGF- β 1-Induced Downregulation of Pentraxin 3 Expression in Human Granulosa Cells. *Endocrinology* (2018) 159:1644–57. doi: 10.1210/en.2017-03127
46. Massague J, Wotton D. Transcriptional Control by the TGF- β /Smad Signaling System. *EMBO J* (2000) 19:1745–54. doi: 10.1093/emboj/19.8.1745
47. Hall MC, Young DA, Waters JG, Rowan AD, Chantry A, Edwards DR, et al. The Comparative Role of Activator Protein 1 and Smad Factors in the Regulation of Timp-1 and MMP-1 Gene Expression by Transforming Growth Factor- β 1. *J Biol Chem* (2003) 278:10304–13. doi: 10.1074/jbc.M212334200
48. Somogyi V, Chaudhuri N, Torrisi SE, Kahn N, Muller V, Kreuter M. The Therapy of Idiopathic Pulmonary Fibrosis: What is Next? *Eur Respir Rev* (2019) 28(153). doi: 10.1183/16000617.0021-2019
49. Kropski JA, Blackwell TS. Progress in Understanding and Treating Idiopathic Pulmonary Fibrosis. *Annu Rev Med* (2019) 70:211–24. doi: 10.1146/annurev-med-041317-102715
50. Antoniou KM, Wuyts W, Wijsenbeek M, Wells AU. Medical Therapy in Idiopathic Pulmonary Fibrosis. *Semin Respir Crit Care Med* (2016) 37:368–77. doi: 10.1055/s-0036-1582010
51. Heukels P, Moor CC, von der Thusen JH, Wijsenbeek MS, Kool M. Inflammation and Immunity in IPF Pathogenesis and Treatment. *Respir Med* (2019) 147:79–91. doi: 10.1016/j.rmed.2018.12.015
52. Wilson MS, Wynn TA. Pulmonary Fibrosis: Pathogenesis, Etiology and Regulation. *Mucosal Immunol* (2009) 2:103–21. doi: 10.1038/mi.2008.85
53. Chambers RC, Mercer PF. Mechanisms of Alveolar Epithelial Injury, Repair, and Fibrosis. *Ann Am Thorac Soc* (2015) 12 Suppl 1:S16–20. doi: 10.1513/AnnalsATS.201410-448MG
54. Yu G, Ibarra GH, Kaminski N. Fibrosis: Lessons From OMICS Analyses of the Human Lung. *Matrix Biol* (2018) 68–69:422–34. doi: 10.1016/j.matbio.2018.03.014
55. Carrington R, Jordan S, Pitchford SC, Page CP. Use of Animal Models in IPF Research. *Pulm Pharmacol Ther* (2018) 51:73–8. doi: 10.1016/j.pupt.2018.07.002
56. Neumark N, Cosme CJr., Rose KA, Kaminski N. The Idiopathic Pulmonary Fibrosis Cell Atlas. *Am J Physiol Lung Cell Mol Physiol* (2020) 319:L887–93. doi: 10.1152/ajplung.00451.2020
57. Diamond JM, Lederer DJ, Kawut SM, Lee J, Ahya VN, Bellamy S, et al. Elevated Plasma Long Pentraxin-3 Levels and Primary Graft Dysfunction After Lung Transplantation for Idiopathic Pulmonary Fibrosis. *Am J Transplant* (2011) 11:2517–22. doi: 10.1111/j.1600-6143.2011.03702.x
58. Zenkova D, Kamenev V, Sablina R, Artyomov M, Phantasus SA. Visual and Interactive Gene Expression Analysis. doi: 10.18129/B9.bioc.phantasus
59. Carre PC, Mortenson RL, King TE Jr., Noble PW, Sable CL, Riches DW. Increased Expression of the Interleukin-8 Gene by Alveolar Macrophages in Idiopathic Pulmonary Fibrosis. A Potential Mechanism for the Recruitment and Activation of Neutrophils in Lung Fibrosis. *J Clin Invest* (1991) 88:1802–10. doi: 10.1172/JCI115501

60. Kinder BW, Brown KK, Schwarz MI, Ix JH, Kervitsky A, King TE Jr. Baseline BAL Neutrophilia Predicts Early Mortality in Idiopathic Pulmonary Fibrosis. *Chest* (2008) 133:226–32. doi: 10.1378/chest.07-1948
61. Lech M, Rommele C, Grobmayr R, Eka Susanti H, Kulkarni OP, Wang S, et al. Endogenous and Exogenous Pentraxin-3 Limits Postischemic Acute and Chronic Kidney Injury. *Kidney Int* (2013) 83:647–61. doi: 10.1038/ki.2012.463
62. Kim M, Kwon JH, Bae YK, Kim GH, Um S, Ha J, et al. Soluble PTX3 of Human Umbilical Cord Blood-Derived Mesenchymal Stem Cells Attenuates Hyperoxic Lung Injury by Activating Macrophage Polarization in Neonatal Rat Model. *Stem Cells Int* (2020) 2020:1802976. doi: 10.1155/2020/1802976
63. Marchal-Somme J, Uzunhan Y, Marchand-Adam S, Kambouchner M, Valeyre D, Crestani B, et al. Dendritic Cells Accumulate in Human Fibrotic Interstitial Lung Disease. *Am J Respir Crit Care Med* (2007) 176:1007–14. doi: 10.1164/rccm.200609-1347OC
64. Zhang S, Zhu YT, Chen SY, He H, Tseng SC. Constitutive Expression of Pentraxin 3 (PTX3) Protein by Human Amniotic Membrane Cells Leads to Formation of the Heavy Chain (HC)-Hyaluronan (HA)-PTX3 Complex. *J Biol Chem* (2014) 289:13531–42. doi: 10.1074/jbc.M113.525287
65. Kourtzelis I, Hajishengallis G, Chavakis T. Phagocytosis of Apoptotic Cells in Resolution of Inflammation. *Front Immunol* (2020) 11:553. doi: 10.3389/fimmu.2020.00553
66. Morimoto K, Janssen WJ, Terada M. Defective Efferocytosis by Alveolar Macrophages in IPF Patients. *Respir Med* (2012) 106:1800–3. doi: 10.1016/j.rmed.2012.08.020
67. Ariel A, Serhan CN. New Lives Given by Cell Death: Macrophage Differentiation Following Their Encounter With Apoptotic Leukocytes During the Resolution of Inflammation. *Front Immunol* (2012) 3:4. doi: 10.3389/fimmu.2012.00004
68. Schif-Zuck S, Gross N, Assi S, Rostoker R, Serhan CN, Ariel A. Saturated-Efferocytosis Generates Pro-Resolving CD11b Low Macrophages: Modulation by Resolvins and Glucocorticoids. *Eur J Immunol* (2011) 41:366–79. doi: 10.1002/eji.201040801
69. Kumaran Satyanarayanan S, El Kebir D, Soboh S, Butenko S, Sekheri M, Saadi J, et al. IFN-Beta is a Macrophage-Derived Effector Cytokine Facilitating the Resolution of Bacterial Inflammation. *Nat Commun* (2019) 10:3471. doi: 10.1038/s41467-019-10903-9
70. Azuma A, Li YJ, Abe S, Usuki J, Matsuda K, Henmi S, et al. Interferon- β Inhibits Bleomycin-Induced Lung Fibrosis by Decreasing Transforming Growth Factor- β and Thrombospondin. *Am J Respir Cell Mol Biol* (2005) 32:93–8. doi: 10.1165/rcmb.2003-0374OC
71. Butenko S, Satyanarayanan SK, Assi S, Schif-Zuck S, Sher N, Ariel A. Transcriptomic Analysis of Monocyte-Derived Non-Phagocytic Macrophages Favors a Role in Limiting Tissue Repair and Fibrosis. *Front Immunol* (2020) 11:405. doi: 10.3389/fimmu.2020.01003
72. Lee YJ, Moon C, Lee SH, Park HJ, Seoh JY, Cho MS, et al. Apoptotic Cell Instillation After Bleomycin Attenuates Lung Injury Through Hepatocyte Growth Factor Induction. *Eur Respir J* (2012) 40:424–35. doi: 10.1183/09031936.00096711
73. Yoon YS, Kim SY, Kim MJ, Lim JH, Cho MS, Kang JL. Ppargamma Activation Following Apoptotic Cell Instillation Promotes Resolution of Lung Inflammation and Fibrosis via Regulation of Efferocytosis and Proresolving Cytokines. *Mucosal Immunol* (2015) 8:1031–46. doi: 10.1038/mi.2014.130
74. Braunschweig A, Jozsi M. Human Pentraxin 3 Binds to the Complement Regulator c4b-binding Protein. *PLoS One* (2011) 6:e23991. doi: 10.1371/journal.pone.0023991
75. Vaughan DE. PAI-1 and TGF-beta: Unmasking the Real Driver of TGF-Beta-Induced Vascular Pathology. *Arterioscler Thromb Vasc Biol* (2006) 26:679–80. doi: 10.1161/01.ATV.0000209949.86606.c2
76. Guzy RD, Li L, Smith C, Dorry SJ, Koo HY, Chen L, et al. Pulmonary Fibrosis Requires Cell-Autonomous Mesenchymal Fibroblast Growth Factor (FGF) Signaling. *J Biol Chem* (2017) 292:10364–78. doi: 10.1074/jbc.M117.791764
77. Rusnati M, Camozzi M, Moroni E, Bottazzi B, Peri G, Indraccolo S, et al. Selective Recognition of Fibroblast Growth Factor-2 by the Long Pentraxin PTX3 Inhibits Angiogenesis. *Blood* (2004) 104:92–9. doi: 10.1182/blood-2003-10-3433
78. Li L, Zhang S, Wei L, Wang Z, Ma W, Liu F, et al. FGF2 and FGFR2 in Patients With Idiopathic Pulmonary Fibrosis and Lung Cancer. *Oncol Lett* (2018) 16:2490–4. doi: 10.3892/ol.2018.8903
79. Kim MH, Jung SY, Song KH, Park JI, Ahn J, Kim EH, et al. A New FGFR Inhibitor Disrupts the TGF-beta1-induced Fibrotic Process. *J Cell Mol Med* (2020) 24:830–40. doi: 10.1111/jcmm.14793
80. Marshall RP, Bellingan G, Webb S, Puddicombe A, Goldsack N, McNulty RJ, et al. Fibroproliferation Occurs Early in the Acute Respiratory Distress Syndrome and Impacts on Outcome. *Am J Respir Crit Care Med* (2000) 162:1783–8. doi: 10.1164/ajrccm.162.5.2001061
81. George PM, Wells AU, Jenkins RG. Pulmonary Fibrosis and COVID-19: The Potential Role for Antifibrotic Therapy. *Lancet Respir Med* (2020) 8:807–15. doi: 10.1016/S2213-2600(20)30225-3
82. Oronsky B, Larson C, Hammond TC, Oronsky A, Kesari S, Lybeck M, et al. A Review of Persistent Post-Covid Syndrome (Ppcs). *Clin Rev Allergy Immunol* (2021) 1–9. doi: 10.1007/s12016-021-08848-3

Conflict of Interest: AM and BB are inventors of patents on pentraxin-3 and obtain royalties on related reagents.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Doni, Mantovani, Bottazzi and Russo. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



PTX3 Deficiency Promotes Enhanced Accumulation and Function of CD11c⁺CD11b⁺ DCs in a Murine Model of Allergic Inflammation

Jyoti Balhara¹, Latifa Koussih^{1,2}, Ashfaque Mohammed¹, Lianyu Shan¹, Bouchaib Lamkhioued³ and Abdelilah S. Gounni^{1*}

¹ Department of Immunology, Max-Rady College of Medicine, Rady Faculty of Health Sciences, University of Manitoba, Winnipeg, MB, Canada, ² Department des Sciences Experimentales, Université de Saint-Boniface, Winnipeg, MB, Canada, ³ Laboratoire d'Immunologie et de Biotechnologies, EA7509-IRMAIC, Pôle-Santé, Université de Reims Champagne-Ardenne, Reims, France

OPEN ACCESS

Edited by:

Kenji Daigo,
Nippon Medical School, Japan

Reviewed by:

Christophe Combadiere,
INSERM U1135 Centre
d'Immunologie et de Maladies
Infectieuses, France
Jau-Ling Suen,
Kaohsiung Medical University,
Taiwan

*Correspondence:

Abdelilah S. Gounni
abdel.gounni@umanitoba.ca

Specialty section:

This article was submitted to
Molecular Innate Immunity,
a section of the journal
Frontiers in Immunology

Received: 14 December 2020

Accepted: 21 June 2021

Published: 09 July 2021

Citation:

Balhara J, Koussih L, Mohammed A, Shan L, Lamkhioued B and Gounni AS (2021) PTX3 Deficiency Promotes Enhanced Accumulation and Function of CD11c⁺CD11b⁺ DCs in a Murine Model of Allergic Inflammation. *Front. Immunol.* 12:641311. doi: 10.3389/fimmu.2021.641311

PTX3 is a unique member of the long pentraxins family and plays an indispensable role in regulating the immune system. We previously showed that PTX3 deletion aggravates allergic inflammation via a Th17 -dominant phenotype and enhanced CD4 T cell survival using a murine model of ovalbumin (OVA) induced allergic inflammation. In this study, we identified that upon OVA exposure, increased infiltration of CD11c⁺CD11b⁺ dendritic cells (DCs) was observed in the lungs of *PTX3*^{-/-} mice compared to wild type littermate. Further analysis showed that a short-term OVA exposure led to an increased number of bone marrow common myeloid progenitors (CMP) population concomitantly with increased Ly6C^{high} CCR2^{high} monocytes and CD11c⁺CD11b⁺ DCs in the lungs. Also, pulmonary CD11c⁺CD11b⁺ DCs from OVA-exposed *PTX3*^{-/-} mice exhibited enhanced expression of maturation markers, chemokines receptors CCR2, and increased OVA uptake and processing compared to wild type controls. Taken together, our data suggest that PTX3 deficiency heightened lung CD11c⁺CD11b⁺DC numbers and function, hence exacerbating airway inflammatory response.

Keywords: dendritic cells, allergic inflammation, asthma, monocytes, chemokines, allergen uptake, PTX3 protein

INTRODUCTION

Allergic asthma is a chronic disease of the airways that is characterized by a maladaptive T helper immune response. Several previously published reports have elaborated on dendritic cells' crucial role (DCs) in regulating specific helper T cell phenotype and functions in the lungs in response to allergens. Upon allergen/antigen exposure, bone marrow releases CCR2^{high} monocytes that circulate through the blood, reach the pulmonary tissue, and contribute to the generation of inflammatory monocytes derived CD11c⁺CD11b⁺DCs (1).

Airway DCs constitute a network with extended dendrites in the epithelium to capture inhaled antigen or allergen then migrate to the T cell zone in draining lymph nodes (2). During this process, immature DCs undergo maturation characterized by upregulation of cell surface expression of

MHCII, CD40, CD86 and CD80. DCs process aeroallergens and present allergen peptides to the naïve CD4 T cells that initiate differentiation of naïve CD4 T cells to specific Th cells (3). Upon repeated exposures of allergen, differentiated Th cells return to the effector site in the lungs and initiate inflammation (4). During this stage, mature DCs co-localize with effector T cells in inflammatory areas (1). Deletion of DCs at this stage resulted in ablation of Th2-dependent inflammatory responses in mice, suggesting a critical role of such DCs in regulating effector helper T cell-mediated responses (1).

Pentraxin 3 is a member of a long pentraxin family of pattern recognition receptors that plays a critical role in tuning inflammation in several pulmonary diseases (5). Several reports have shown the production of PTX3 by structural and inflammatory cells in mice and humans (6). Dendritic cells, particularly myeloid DCs but not plasmacytoid DCs, are among the primary producer of PTX3 (5, 6). Furthermore, stimulation with TLR ligands, CD40L, IL-10 and IL-1 β induce PTX3 production by DCs (7, 8). PTX3 binds to the late apoptotic T cells and inhibits their internalization by human dendritic cells (9).

Exogenous administration of PTX3 during the early stage of aspergillosis in *p47phox*^{-/-} mice restored anti-aspergillus resistance (10). PTX3 treatment of DCs down-regulated production of IL-23 that resulted in a restricted expansion of IL-23-dependent IL-17A producing $\gamma\delta$ T cells (10). We recently showed that PTX3 deficiency resulted in an IL-17A dominant inflammation in the mouse model of allergic inflammation (11). CD4 T cells exhibited enhanced IL-17A production in ovalbumin (OVA)-exposed *PTX3*^{-/-} mice than their wild-type controls. CD11c⁺CD11b⁺ DCs from *PTX3*^{-/-} mice displayed increased production of Th17 polarizing cytokines, including IL-6 and IL-23, favoring Th17 CD4 T cells (11). However, the mechanism governing the PTX3 effect on regulating dendritic cells in allergic inflammation is not fully understood.

In light of our previous findings (11), we sought to understand the role of PTX3 deletion in shaping CD11c⁺CD11b⁺ DCs function in the context of allergic inflammation. Our study identified that OVA exposure induced increased infiltration of CD11c⁺CD11b⁺ DCs in the lungs in *PTX3*^{-/-} mice compared to wild-type littermate. Upon OVA challenge, *PTX3*^{-/-} bone marrow showed increased frequency of common myeloid progenitors (CMP) population that corresponded with increased Ly6C CCR2^{high} monocytes and CD11c⁺CD11b⁺ DCs in the lungs. In addition, pulmonary CD11c⁺CD11b⁺ DCs exhibited enhanced expression of maturation markers upon OVA-exposed *PTX3*^{-/-} mice than their wild-type controls. These cells also exhibited increased OVA uptake and processing. Together, our data suggest a differential phenotype and function of CD11c⁺CD11b⁺ DCs in the absence of PTX3.

MATERIAL AND METHODS

Mice

PTX3^{-/-} and littermate *PTX3*^{+/+} mice (129SvEv/Bl/6 background) were kindly provided by Dr. M. Matzuk (Baylor

College of Medicine, Texas, USA). Animals were housed in individualized ventilated cages with wooden chip bedding in enriched environment conditions in the animal care facility, the University of Manitoba. Animals were maintained in 12 hours dark-light cycle and supplied with standard chow and water *ad libitum*. Animal usage was strictly according to instructed guidelines by the Canadian Council for Animal Care and the University of Manitoba Animal Ethics Board (Protocol Number 15802).

OVA Sensitization and Challenge, and OVA Uptake and Processing

Six to eight weeks old females *PTX3*^{-/-} and *PTX3*^{+/+} mice were sensitized by intraperitoneal (IP) injection of OVA (Grade V, Sigma Aldrich Canada) and alum (Imject Alum, Biotechnology). In brief, on day 1, mice were sensitized with IP injection of OVA/alum mixture, 250 μ l of 8mg/ml alum mixed with 250 μ l of 4 μ g/ml OVA dissolved in PBS. On day 11, mice were boosted with IP injection of OVA/alum mixture, as in day 1, and 50 μ l of OVA (1mg/ml) administered intranasally (IN). On days 19 and 20, *PTX3*^{-/-} and *PTX3*^{+/+} mice were challenged with IN administration of 50 μ g of OVA in 50 μ l PBS solution. 48 hrs after the last challenge, mice were sacrificed, and tissues were harvested. Naïve mice that received PBS only according to the same protocol were used as controls.

To examine *in vivo* OVA uptake, OVA-sensitized mice, as described above, then were challenged intranasally with 50 μ g OVA-DQ (Life technology-Invitrogen, Carlsbad, CA) in 50 μ l of PBS at day 19. At the indicated time, the lung tissue and mediastinal lymph nodes (MLN), were harvested to examine the uptake and processing of DQ-OVA by DCs using flow cytometry.

In another protocol, mice were IN challenged with 50 μ l of OVA (2mg/ml). Mice were then sacrificed at 12 or 24hrs, and lung tissue, MLN, blood and bone marrow cells were harvested. Again, naïve mice challenged with PBS were used as controls.

Bone Marrow Single-Cell Preparation

Naïve and OVA exposed *PTX3*^{+/+} and *PTX3*^{-/-} mice were sacrificed, and tibia and femur bones were collected as described previously (12). Bones were flushed with 10 ml of complete media, bone marrow cells were filtered using a cell strainer, collected cells were centrifuged at 1200 rpm, and the pellet was resuspended in ACK buffer for 5 minutes to lyse red blood cells. ACK was neutralized with complete media, and cells were then centrifuged and resuspended in fresh complete cell culture media before FACS staining.

Flow Cytometric Analysis of Pulmonary Dendritic Cells

Pulmonary conventional DC subsets were analyzed by FACS from *PTX3*^{-/-} or *PTX3*^{+/+} mice after OVA exposure. Briefly, lungs were removed from mice, and enzymatically digested using 1 mg/ml collagenase IV and 0.5 mg/ml DNase in RPMI 1640 medium, as described previously (13, 14). After Fc blocking, single-cell suspension was incubated with appropriate antibodies (see **Table 1**) followed by acquisition using a BD FACS Canto-II (BD, San Diego, CA) and analyzed using FlowJo software.

TABLE 1 | Antibodies used in flow cytometry.

Antibody	Clone	Company
Lin - PE	mix	Biologend, Inc
SCA-1 - FITC	D7	Biologend, Inc
CD16/32 - PE-Cy7	93	eBiosciences Inc
CD34 - PB	RAM34	eBiosciences Inc
cKit - APC	2B8	eBiosciences Inc
Ly6C - FITC	HK1.4	eBiosciences Inc
CCR2 - APC	SA203G11	Biologend, Inc
CCR5 - APC	HM-CCR5	eBiosciences Inc
CCR6 - PE-Cy7	29-2L17	Biologend, Inc
CD40 - APC	1C10	eBiosciences Inc
MHC II - PB	M5/114.15.2	eBiosciences Inc
CD80 - PE	16-10A1	eBiosciences Inc
CD86 -APC or PE-Cy7	GL-1	eBiosciences Inc
CD45 - PB	30-F11	eBiosciences Inc
B220 - PB	RA3-6B2	eBiosciences Inc
CX3CR1 - BV711	SA011F11	Biologend, Inc
CD11b - PE-Cy7 or BV711	M1/70	eBiosciences Inc
CD11c - FITC	N418	eBiosciences Inc
Gr-1 - PE	RB6-8C5	Biologend, Inc
F4/80 - PE	BM8	eBiosciences Inc
Siglec-F - PE	E50-2440	BD Biosciences.

Similarly, MLN, bone marrow or blood cells were blocked with Fc blocker and stained with respective antibodies (**Table 1**).

For detecting CMP, bone marrow single cells preparation was labeled with anti-mouse lineage Abs (lin-PE: anti-mouse CD3; anti-mouse Ly-6G/Ly-6C; anti-mouse CD11b; anti-mouse CD45R/B220; anti-mouse TER-119), anti-Ly-6A/E- (Sca1) FITC, anti c-kit-APC, anti-CD16/32 PE-Cy7 and anti-CD34 pacific blue. Labeled cells were then acquired on FACS Canto II (BD Biosciences) and analyzed using FlowJo software.

Survival Assay

Apoptosis/survival of lung DCs were determined by staining lung cell suspension with annexin V and DAPI using Apoptosis Detection kit FITC according to manufacturer's instructions (eBioscience Inc.) as we described previously (11). Briefly, cells were first stained with specific fluorochrome-conjugated antibodies, washed with flow buffer once and then in 1X annexin V binding buffer. Resuspended cells were then centrifuged at 1200rpm and stained with FITC labelled annexin V. These cells were incubated at room temperature for 10-15 minutes, followed by washing in 1X binding buffer. After washing, cells were resuspended in 100-200 μ l 1X binding buffer, to which 1 μ l of DAPI was added, and incubated at room temperature for at least 5 minutes. Cells were then acquired on FACS Canto II (BD Biosciences) and analyzed using FlowJo software.

Statistical Analysis

Results are shown as mean $\pm$ SEM, analyzed using two-way ANOVA (Graph Pad prism 8) and *post hoc* Tukey's multiple comparisons, and the Mann-Whitney test. Unless otherwise stated, data are collected from at least three experiments; each experiment contained at least three-five mice in each experimental group. p-value <0.05 was considered significant in all quantitative experiments.

RESULTS

PTX3^{-/-} Mice Exhibited Enhanced Accumulation of CD11c⁺CD11b⁺ DCs in the Lungs Upon OVA Exposure

Recently we showed that the deletion of PTX3 resulted in excessive airway inflammation upon OVA exposure, mainly characterized by enhanced airway and lung tissue infiltration by eosinophils and neutrophils (11). OVA-induced IL-17-dominant inflammation in *PTX3^{-/-}* mice compared to their *PTX3^{+/+}* counterparts was attributed partly to a preponderance of IL-6 and IL-23 producing DCs in *PTX3^{-/-}* mice (11). However, whether PTX3 deletion leads to enhanced recruitment of particular DC subsets in the lung upon OVA challenge is unknown. Using the OVA protocol described in **Figure 1A**, we found that *PTX3^{-/-}* mice showed enhanced accumulation of CD11c⁺ DCs in the lungs compared to *PTX3^{+/+}* mice upon OVA sensitization and challenge (**Figures 1B–D**). CD11c⁺ DCs are further characterized as proinflammatory CD11b^{high} pulmonary DCs known to transport antigen from lungs to the lymphoid organs (15, 16). In *PTX3^{-/-}* mice, we observed a concomitant increase in lung CD11c⁺CD11b⁺ DCs upon OVA exposure compared to their WT littermate (**Figures 1E, F**).

Next, we were interested in understanding the plausible mechanism that supported an increased abundance of lung DCs in *PTX3^{-/-}* mice. *PTX3^{-/-}* CD4 T cells showed enhanced survival compared to CD4 T cells from *PTX3^{+/+}* littermate (11). Thus, we next examined the survival of *PTX3^{-/-}* and *PTX3^{+/+}* lung DCs *ex vivo* by staining cells with annexin V and DAPI to assess whether a similar phenomenon existed for DCs in *PTX3^{-/-}* mice. Interestingly, OVA-exposed *PTX3^{+/+}* and *PTX3^{-/-}* lung DCs showed comparable survival (**Supplementary Figure 1**), suggesting that the survival pathway has no role in increased abundance of DCs in the lungs *PTX3^{-/-}* mice.

PTX3^{-/-} Mice Showed Enhanced Common Myeloid Progenitors Generation in Bone Marrow

Previously published reports indicated that common myeloid progenitors (CMP) in the bone marrow efficiently give rise to Ly-6C^{high}CD11b⁺ inflammatory monocytes (17). Inflammatory monocytes emigrate from the bone marrow, circulate through the blood and rapidly differentiate into CD11c⁺CD11b⁺ DCs in the peripheral tissue under inflammatory conditions (18). Therefore, we aimed to understand whether augmented pulmonary accumulation of CD11b⁺DCs in *PTX3^{-/-}* mice was due to enhanced generation of CMPs, increased recruitment of Ly-6C^{high}CD11b⁺ monocytes, or differentiation of the latter into inflammatory DCs in the lungs. *PTX3^{+/+}* and *PTX3^{-/-}* mice were challenged intranasally with 100 μ g OVA (**Figure 2A**), and the frequency of CD11b⁺CD11c⁺ DCs in the lungs 12 hrs and 24 hrs post-OVA challenge were assessed. As observed in the three weeks OVA sensitization and challenge protocol, short-term OVA challenge increased accumulation of lung CD11c⁺CD11b⁺DCs in *PTX3^{-/-}* mice compared to *PTX3^{+/+}* mice (**Figures 2B, C**, n=6-8, P< 0.01).

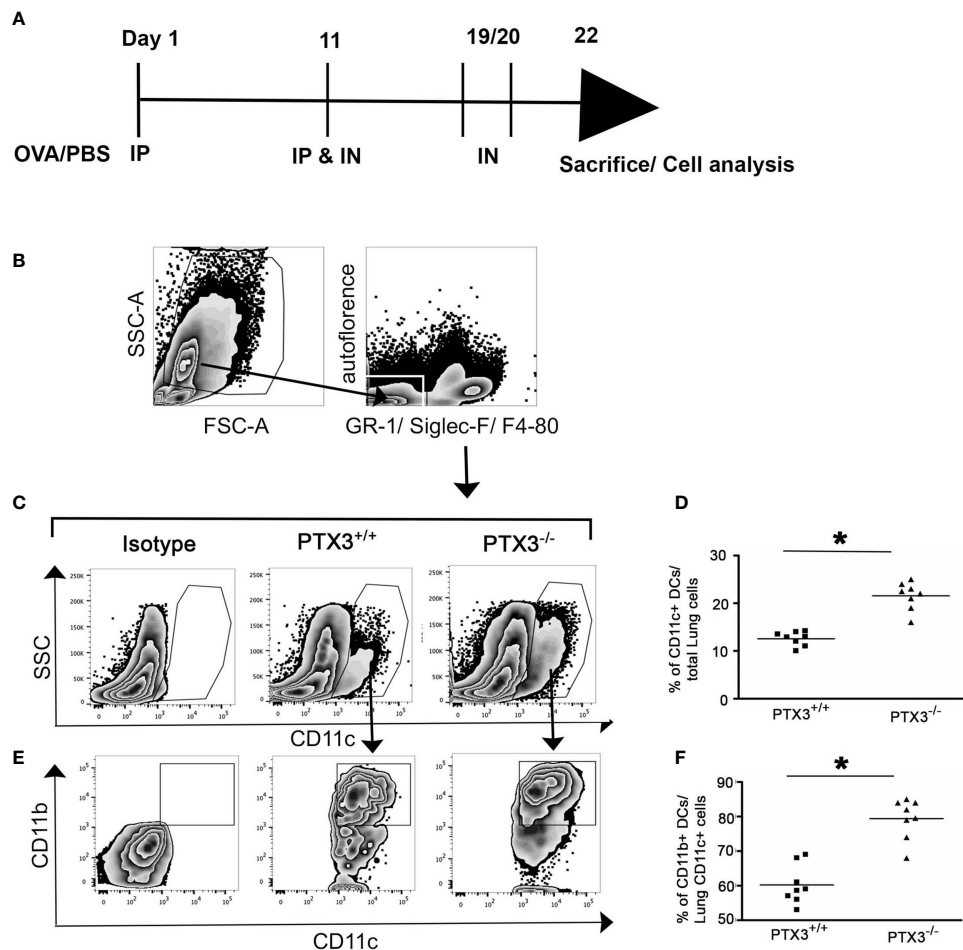


FIGURE 1 | PTX3 deletion results in increased accumulation of CD11c+CD11b+ DCs in the lungs in response to OVA sensitization and challenge. **(A)** Schematic diagram showing the protocol of OVA sensitization and challenge. **(B)** Lung Cells were gated for autofluorescence- Gr-1⁺Siglec-F⁺F4/80⁺ CD11c⁺ cells **(C, D)** that were then gated for CD11b+ cells **(E, F)**. Quantification and statistical analysis of FACS data are shown as graphs. n = 6-8/group, *p < 0.01 using Mann-Whitney test.

We then determined how the OVA challenge affected monocytic lineage progenitors in bone marrow in the presence and absence of PTX3. In contrast to PTX3^{+/+}, CMP cell count in the bone marrow of PTX3^{-/-} mice, gated on Lin⁻ cKit+Sca1⁺ (**Figure 3A**), showed a significant increase upon OVA exposure (**Figures 3B, C**). Conversely, we observed a slight reduction of bone marrow monocytes frequency, gated on GR-F4/80-Siglec- (**Figure 3D**), in PTX3^{-/-} mice 12 hrs post OVA challenge (**Figures 3E, F**). After 24 hrs of OVA exposure, monocytes frequency in PTX3^{-/-} mice come back to the baseline level; however, monocytes accumulated in the BM in PTX3^{+/+} mice (**Figure 3F**).

To investigate the possibility of BM increased exit to the blood and attraction toward lung tissue in PTX3^{-/-} mice compared to WT mice, we analyzed their frequency within the blood and lung (gated on CD11b+ and Ly6C levels, **Figure 4A**). At 12hrs upon OVA challenge, the Ly6C^{int} CD11b+ blood monocytes frequency increased compared to the baseline in PTX3^{-/-} mice but was significantly lower than WT mice (**Figure 4C**). Furthermore, the frequency of blood and lung CD11b+ Ly6C^{low} cells was similar

between both strains (**Figures 4B, E**). Conversely, at 24h post OVA challenge, the Ly6C^{int} CD11b+lung monocytes frequency was higher in PTX3^{-/-} compared to WT mice, but no difference was observed in WT mice (**Figure 4F**). Interestingly, in contrast to WT mice, no difference on the frequency of Ly6C^{high} CD11b+ blood monocytes was detected in the blood of PTX3^{-/-} mice compared to the baseline at 12 and 24hrs post OVA challenge (**Figure 4D**). On the other hand, compared to the baseline level, the frequency of Ly6C^{high} CD11b+ lung monocytes decreased in both PTX3^{-/-} and WT mice (**Figure 4G**).

PTX3^{-/-} Lung DCs Exhibited Increased Expression of CCR2, CCR5 and CCR6

CCR2, a chemokine receptor, plays a key role in directing DC precursors to egress from bone marrow to blood, enabling their migration to the tissue sites upon antigen exposure (19). Furthermore, CCR2 is highly expressed by Ly-6C^{high}CD11b⁺ inflammatory monocytes compared to Ly-6C^{low/int}CD11b⁺ patrolling monocytes (20), and is associated with increased

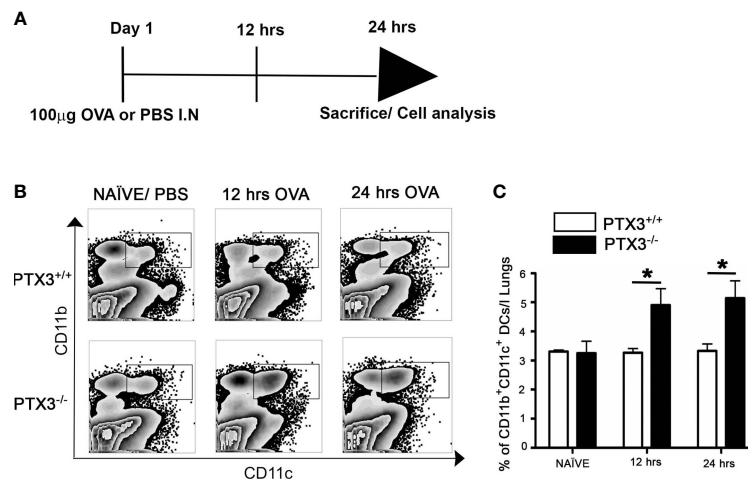


FIGURE 2 | PTX3^{-/-} mice showed increased accumulation of DCs in lungs after short term OVA exposure. **(A)** Schematic diagram showing the protocol of OVA exposure and collection of samples at 12 hrs and 24 hrs. **(B)** Lung Cells were gated for autofluorescence- Gr-1⁺Siglec F F4/80⁺ CD11c⁺ cells. **(C)** Accumulation of lung CD11c⁺ CD11b⁺ DCs 12 hrs and 24 hrs post i.n OVA exposure was compared with naïve lung DCs. **(C)** Quantification and statistical analysis of Flow cytometry data is shown as graphs. n = 6-8/group, *p < 0.01 using two-way ANOVA.

differentiation of Ly-6C^{high}CD11b⁺ monocytes into pulmonary CD11c⁺CD11b⁺ DCs (21). Therefore, we investigated the regulation of CCR2 expression in OVA- challenged PTX3^{-/-} mice. Lung DCs from PTX3^{-/-} showed increased expression of CCR2 at naïve state and 12 hrs post OVA exposure in comparison with WT mice (Figures 5A, B) but not at 24 hrs post OVA challenge.

CCR5 is expressed by immature DCs that facilitate migration later to the tissue sites. Immature DCs also express CCR6 that performs a similar function as CCR5. We evaluated the expression of both chemokine receptors on lung DCs and found that the expression of CCR5 (Figures 5C, D, P<0.01) and CCR6 (Figures 5E, F, P<0.01) was more significant on PTX3^{-/-} DCs at naïve state as compared to PTX3^{+/+} DCs. PTX3^{-/-} lung DCs, although it exhibited increased surface expression of both CCR5 and CCR6 24 hrs post OVA challenge compared to PTX3^{+/+} lung DCs (Figures 5D, F), this expression was not different compared to the baseline expression for CCR5 or even decrease for CCR6 (Figures 5C–F and Supplementary Figure 2). No apparent difference in their expression was found on monocytes from BM, blood and the lungs (data not shown). Altogether our data demonstrated an inherent increased expression of CCR2, CCR5 and CCR6 on lung DCs PTX3^{-/-} mice at the baseline and enhanced CCR2 expression upon OVA challenge compared to WT mice.

PTX3^{-/-} Lung DCs Showed Reduced MHCII but Enhanced CD86 Surface Expression

In OVA-exposed PTX3^{-/-} mice, CD4 T cells exhibited enhanced activation and IL-17A dominant cytokine production (11). Given that DCs process and present antigens to T cells and regulate T cell-dependent inflammatory response through the expression of costimulatory molecules, such as MHCII, CD80, CD86 and CD40, we next assessed whether PTX3 deletion

resulted in differential maturation status of DCs, which may explain the phenotype and functions of CD4 T cells. PTX3^{-/-} lung DCs showed lower surface expression of MHC II (Figures 6A, B) but an enhanced expression of CD86 (Figures 6A, D) upon OVA sensitization/challenge (Figure 1A). Surface expression of CD40 (Figures 6A, C) and CD80 (Figures 6A, E) remained similar in both genotypes at this time point.

We also determined the surface expression of MHCII, CD80, CD86 and CD40 at naïve state and after short-term OVA exposure (Figure 2A). Their surface expression was comparable on naïve lung CD11c⁺CD11b⁺ DCs in both PTX3^{-/-} and WT mice (Supplementary Figures 3A–D). Interestingly, lung DCs from PTX3^{-/-} mice showed an increase in the expression of MHCII, CD80 and CD86 24 hrs OVA challenge compared to WT mice (Supplementary Figures 3A, B, D). CD40 surface expression was similar in DCs isolated from OVA challenged (12 hrs and 24 hrs) mice of both genotypes (Supplementary Figure 3C).

PTX3^{+/+} Lung CD11c⁺CD11b⁺ DCs Exhibit Enhanced OVA Uptake and Processing as Compared to PTX3^{-/-} DCs

DCs take up allergen/antigen and process them into immunogenic peptides for subsequent T cells presentation (1). To study whether PTX3 deletion affected OVA uptake and presentation by lung DCs, we used DQ-OVA *in vivo*. DQ-OVA is digested proteolytically to release photostable and pH-insensitive green fluorescent peptides by antigen-presenting cells (22). The frequency of DCs, which processed OVA, was higher in PTX3^{-/-} mice lungs compared to WT mice (Figures 7A, D). Similar to total lung DCs, a subset of CD11c⁺CD11b⁺ DCs that were actively processing OVA showed reduced MHC II (Figures 7C, F) and increased CD86 (Figures 7B, E) surface expression in PTX3^{-/-} mice.

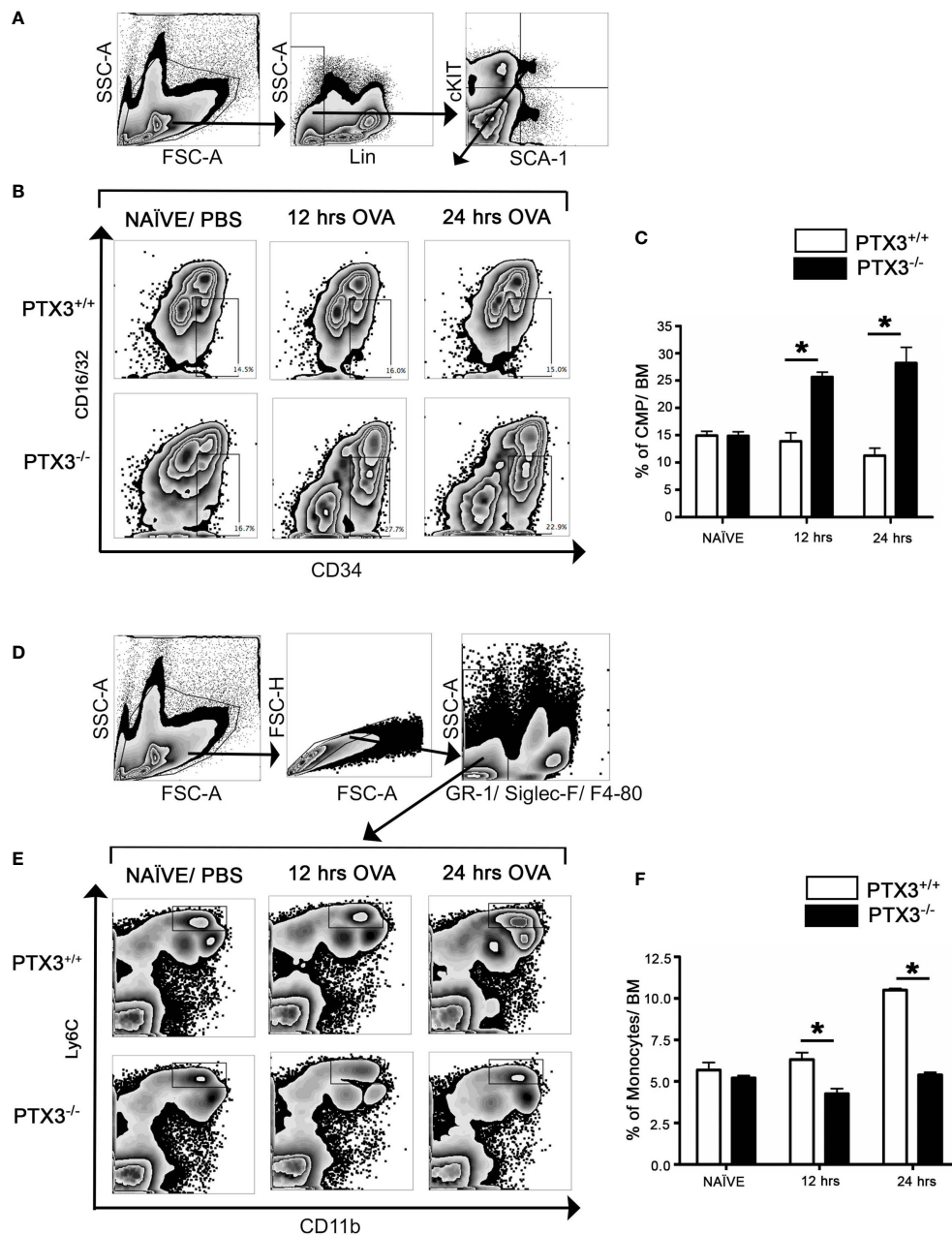


FIGURE 3 | OVA exposure results in increased CMP progenitors and in increased egress of monocytes from the bone marrow in PTX3^{-/-} mice. Lin⁺ckit⁺Sca-1⁻ cells were gated for CMP as (A). (B, C) CMP cells from naïve, 12 hrs and 24 hrs post i.n OVA exposed PTX3^{-/-} mice were compared with CMP cells from PTX3^{+/+} mice. (D) Gating strategy for Ly6C⁺ CD11b⁺ monocytes in bone marrow. (E, F) BM monocytes from naïve, 12 hrs and 24 hrs post i.n OVA exposed PTX3^{-/-} mice were compared with monocytes from PTX3^{+/+} mice. Quantification and statistical analysis of Flow cytometry data is shown as graphs. n = 6/group, *p < 0.01 using two-way ANOVA.

Lung DCs transport allergen to mediastinal lymph nodes and present allergen peptides to T cells to generate a specific effector T cell response (23). Therefore we explored the migration of DQ-OVA containing lung DCs to mesenteric lymph node (MLN). PTX3^{-/-} mice showed greater infiltration of processed DQ-OVA containing DCs into the MLN (Supplementary Figure 4A). DCs in MLN also showed reduced MHCII (Supplementary Figure 4B); however, CD86 expression remained similar (Supplementary Figure 4C).

DISCUSSION

DCs are strategically located in the epithelium to capture antigens/allergens and orchestrate immune response according to the antigen type (1). They are equipped to process allergens and present them to naïve CD4 T cells (4). Recently, we showed that deletion of PTX3 resulted in enhanced infiltration of inflammatory cells in the airways and the lungs, increased

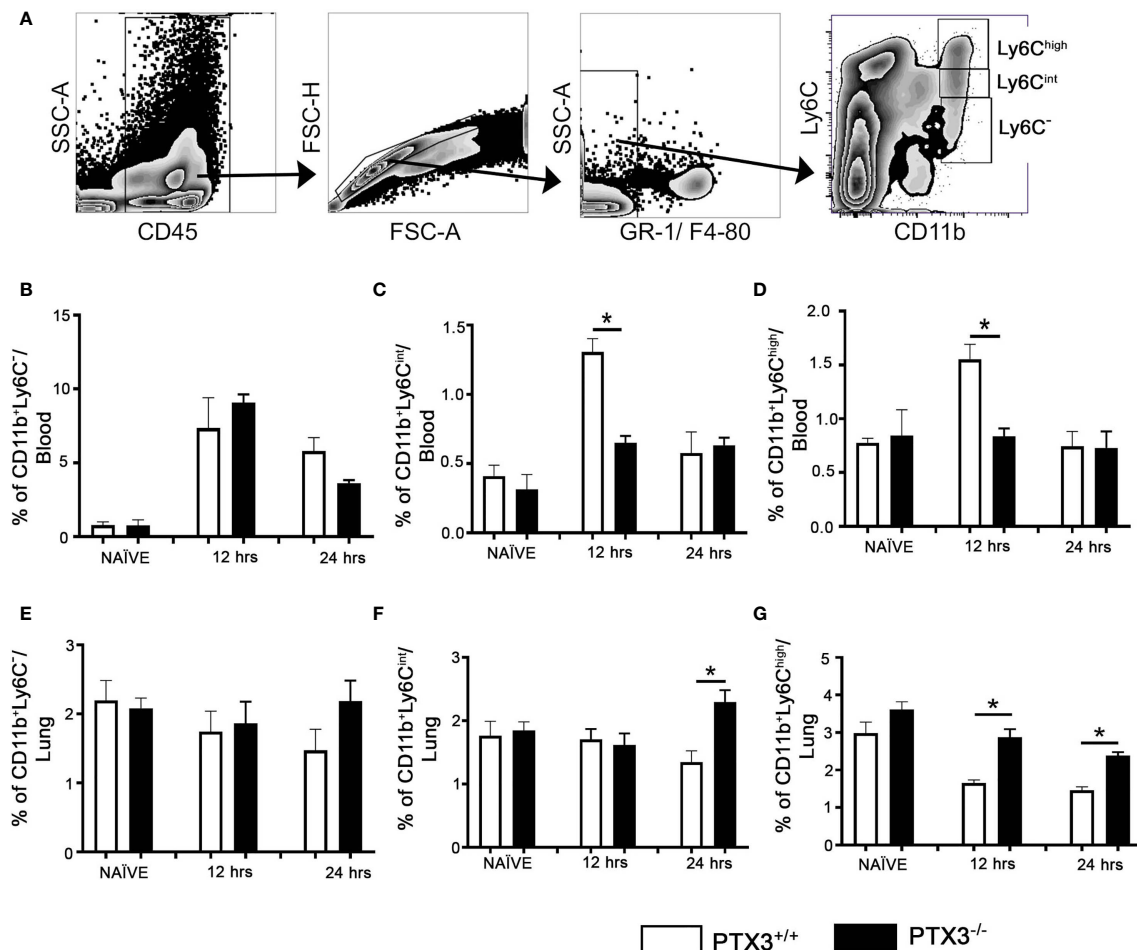


FIGURE 4 | PTX3^{-/-} mice showed increased egress of monocytes from the Blood and increased accumulation of monocytes in the lungs upon OVA exposure. **(A)** Blood monocytes were gated on CD45⁺ Gr1⁻ F4/80⁻. Monocytes from blood **(B–D)** and the lungs **(E–G)** at naïve, 12 hrs and 24 hrs post i.n OVA exposed PTX3^{-/-} mice were compared with monocytes from PTX3^{+/+} mice. Quantification and statistical analysis of Flow cytometry data is shown as graphs. n = 6/group, *p < 0.01 using two-way ANOVA.

production of IgE and enhanced airway hyperreactivity in response to OVA challenge. Also, we identified that PTX3 deletion favored IL-17A-dominant inflammation in the lungs and the draining lymph nodes that is at least in part due to Th17 CD4 T cells (11). In this study, we identified that OVA exposure induced increased infiltration of CD11c⁺CD11b⁺ DCs in the lungs of PTX3^{-/-} mice compared to wild-type littermate. This effect is associated with an increased number and frequency of common myeloid progenitors (CMP) population that may explain increased Ly6C CCR2^{high} monocytes in the blood and subsequently CD11c⁺CD11b⁺ DCs in the lungs. Significantly, pulmonary CD11c⁺CD11b⁺ DCs exhibited enhanced expression of maturation markers upon OVA-exposed PTX3^{-/-} mice compared to their wild-type controls and exhibited increased OVA uptake and processing. Taken together, our data suggest that PTX3 deficiency mediated increased inflammatory response

in the context of allergic asthma is in part due to enhanced DCs function.

In this study, we first showed an increase in CD11b⁺CD11c⁺ DCs infiltration into the lungs of PTX3^{-/-} mice compared to PTX3^{+/+} mice upon OVA exposure. Unlike PTX3^{-/-} CD4 T cells (11), PTX3^{-/-} DCs did not show any apparent difference in survival compared to PTX3^{+/+} DCs. A plausible explanation of such an increase in inflammatory CD11c⁺CD11b⁺ DCs could be enhanced generation of CMP in the bone marrow, which was observed in PTX3 depleted mice compared to their wild-type counterparts. Manz et al. (17) reported that CMP in bone marrow gives rise to granulocyte-monocyte progenitors (GMP), giving rise to functional DCs *in vitro* and *in vivo*. Although in our study, we did not find any difference in GMP population while CMP count was significantly increased in PTX3^{-/-} compared to PTX3^{+/+} mice. Monocytes, an intermediate stage between GMP and tissue

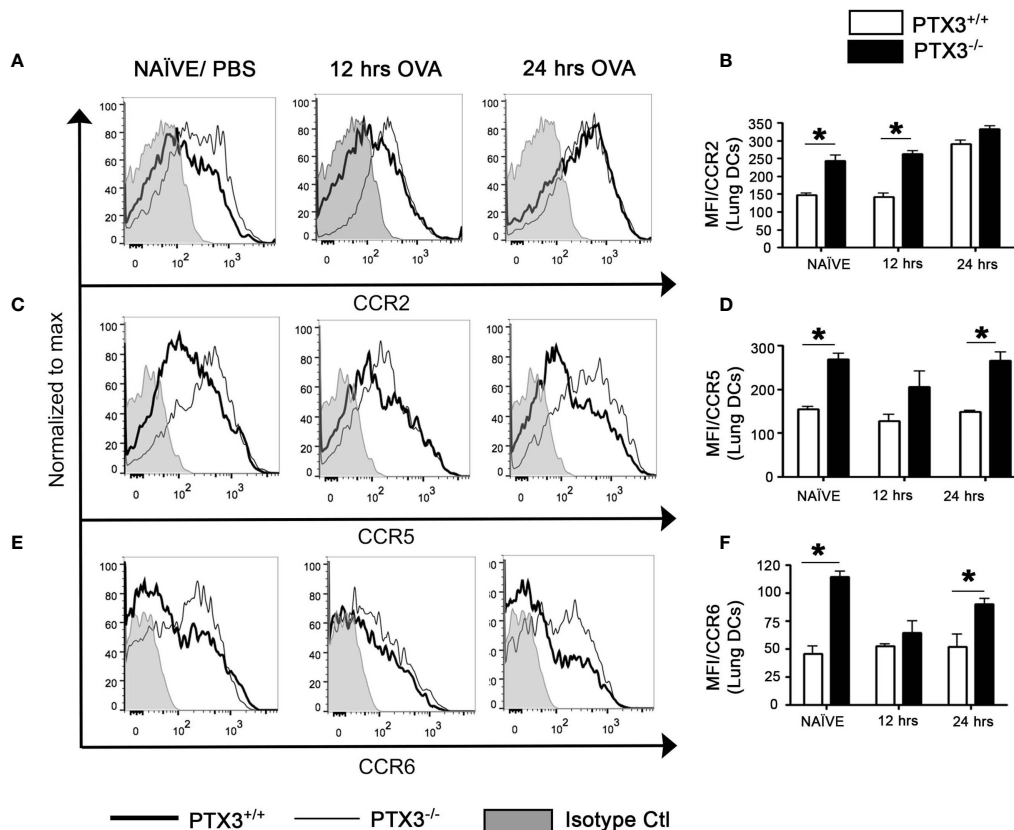


FIGURE 5 | PTX3 deletion affect CCR2, CCR5 and CCR6 expression on lungs DCs. Expression of CCR2 (A, B), CCR5 (C, D) and CCR6 (E, F) were assessed by flow cytometry on CD11c+CD11b+ DCs from the lungs. Gating strategy as shown in Figure 1B. White bars represent PTX3^{+/+} and black bars represent PTX3^{-/-}. Quantification and statistical analysis of Flow cytometry data is shown as graphs. n = 6/group, *p < 0.01 using two-way ANOVA.

DCs, give rise to DCs that infiltrate the tissue upon inflammation. To understand whether the increased accumulation of lungs DCs depended on monocytes' differentiation into DCs, we monitored monocytes in the bone marrow, blood, and the lungs. Reduction of monocytes in the bone marrow and blood but increase in their count in the lungs of PTX3^{-/-} mice upon OVA exposure partly explains the increased accumulation of lung CD11c+CD11b+ DCs. Such monocytes, which can differentiate to functional DCs upon inflammation, are known as inflammatory monocytes. These are characterized by high expression of CCR2 in contrast to Ly6C^{low/int} CCR2^{low} patrolling monocytes (24).

CCR2 plays a critical role in facilitating the egress of inflammatory monocytes from the bone marrow to blood and from blood to the lungs in response to allergen challenge (19). In our study, we observed that inflammatory monocytes were CCR2^{high} in both genotypes. As CCR2 expression on monocytes at basal level was comparable in PTX3^{-/-} and PTX3^{+/+} mice, it rules out the plausibility of an effect of PTX3 deletion on CCR2 expression in naïve state. Although we did not find a significant difference in the CCR2 expression on BM, blood and lung monocytes, lung DCs showed upregulation of CCR2 expression

upon OVA exposure. Such observation could stem from two possibilities. PTX3^{-/-} lung DCs with increased CCR2 represent monocytes that had increased CCR2, which upon antigen encounter differentiated into DCs; and/or the increase in CCR2 in PTX3^{-/-} DCs regulates their accumulation in the lungs independent of its role on monocytes. Previously, diesel particles induced inflammation was shown to upregulate the expression of CCR2 in the lungs (25). Recruitment of inflammatory monocytes, monocyte-derived DCs and induction of subsequent pulmonary inflammation was also CCR2 dependent (25). Similarly, CCR2-dependent recruitment of monocytes-derived DCs has also been reported upon influenza infection, exposure to LPS and particulate antigens (19, 26). Loss of CCR2 could not be compensated by other chemokines- chemokine receptor systems (19).

Other chemokine receptors, including CCR5 and CCR6, have also been involved in directing monocytes to the tissue sites. These chemokine receptors are expressed on immature DCs and regulate their immigration to the tissue sites (27, 28). Previously Norata et al. (8) also showed enhanced CCR5 expression in the vascular wall of PTX3^{-/-} apoE^{-/-} mice compared to their PTX3^{+/+} counterpart. In our study, we observed an increased expression

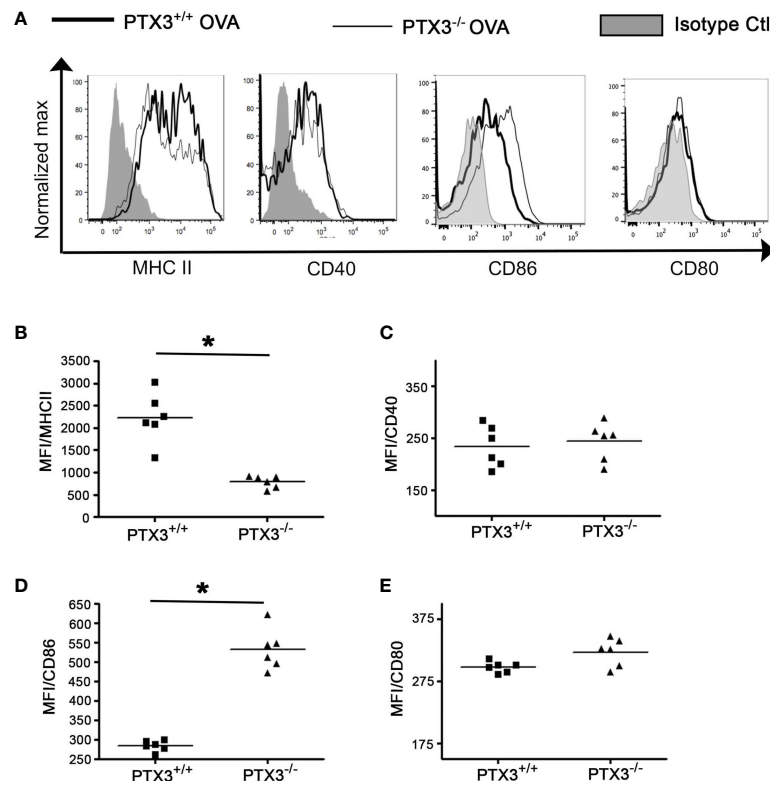


FIGURE 6 | PTX3 deletion results in reduced surface expression of MHCII but an enhanced surface expression of CD80 and CD86 in response to OVA. After 3 weeks of OVA sensitization and challenge regimen, lung CD11b⁺CD11c⁺ DCs were analyzed for the expression of MHCII (A, B), CD40 (A, C), CD86 (A, D) and CD80 (A, E) by flow cytometry. Quantification and statistical analysis of Flow cytometry data is shown as graphs. n = 6 mice group, *p < 0.01 using Mann-Whitney test.

of CCR5 and CCR6 on naïve supposedly immature lung DCs in *PTX3*^{-/-} mice at steady state. Surface expression of CCR5 and CCR6 downregulate as DCs undergo maturation. Similarly, we found a reduction in CCR6 but not CCR5 expression 12 hrs post OVA challenge for *PTX3*^{-/-} mice, suggesting the possibility of acquiring naïve like phenotype by lung DCs 24 hrs after the challenge. Altogether it seems likely that increased CCR6 on naïve *PTX3*^{-/-} lung DCs renders them more responsive to chemokines, which are released immediately after exposure to OVA. CCR6, on the other hand, regulates the migration of Tregs and Th17 cells into the inflammatory region (29). CCR5 expression, known to be associated with Th1 cells (30), was not different in both *PTX3* and WT mice lung DCs. Whether the altered expression of these chemokine receptors on lung DCs is also involved in the regulation of Th17 promoting cytokines needs further studies.

PTX3 interacts with P-selectin and inhibits the migration of neutrophils to the peripheral tissues (31). This phenomenon was reversed upon disruption of PTX3 and P-selectin interaction either through PTX3 deletion or antibody-mediated inhibition (32). Furthermore, increased expression of P-selectin has been observed in *PTX3*^{-/-} mice, further supporting the likelihood of P-selectin-dependent migration of inflammatory cells in the

absence of PTX3. As monocytes are also dependent on P-selectin for migration, it is tempting to hypothesize this pathway's involvement in enhanced monocytes and CD11c⁺CD11b⁺ DCs accumulation in the lungs.

In the lungs, DCs exist in an immature state and initiate their maturation upon allergen exposure process. Mature DCs process allergen, present allergen epitopes through MHCII along with the expression of an array of costimulatory molecules including CD40, CD80, and CD86, which are well known as maturation markers of DCs. *PTX3*^{-/-} CD11c⁺CD11b⁺ DCs showed a greater ability to uptake and process OVA compared to *PTX3*^{+/+} DCs. Surprisingly, *PTX3*^{-/-} DCs expressed a reduced level of MHCII. Whether the lower expression of MHCII by *PTX3*^{-/-} DCs was due to increased recycling of MHCII or reduced biosynthesis or transport to the membrane is unknown (33). Engagement of CD80/86 by CD28 induces the production of IL-6 by DCs (34). In our work, we observed enhanced expression of CD80 and CD86 by *PTX3*^{-/-} CD11c⁺CD11b⁺ DCs in contrast to *PTX3*^{+/+} CD11c⁺CD11b⁺ DCs, which is also concurrent with increased production of IL-6 by *PTX3*^{-/-} DCs (11). Consistent with increased maturation, antigen uptake, and processing, we also observed more significant migration of OVA peptide containing CD11c⁺CD11b⁺ DCs from lungs to the draining lymph nodes in

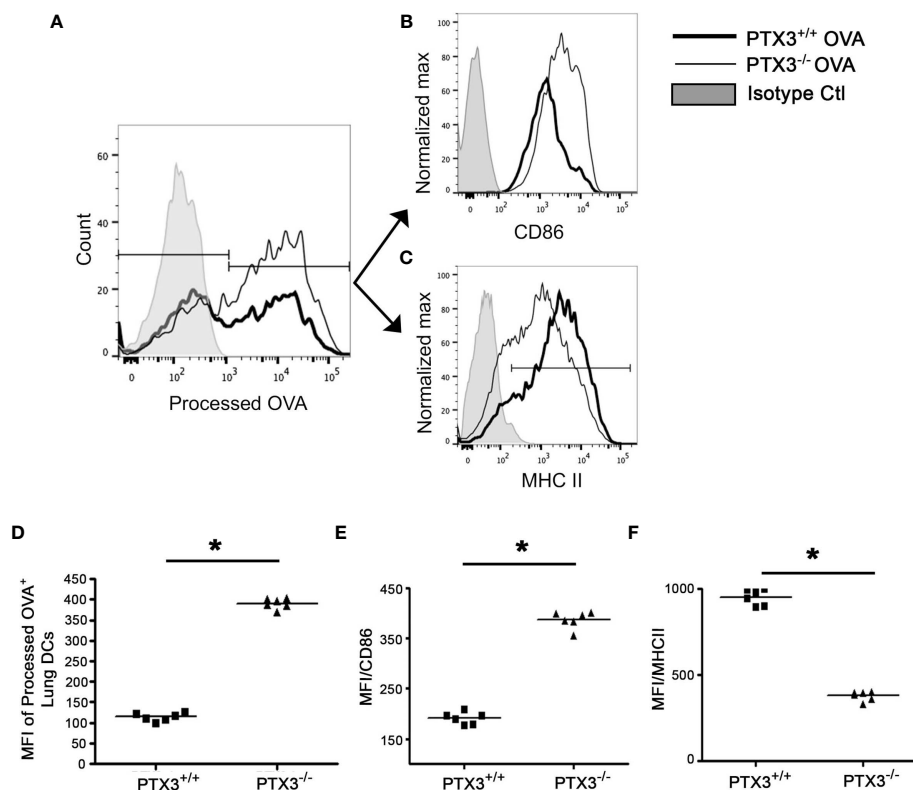


FIGURE 7 | PTX3^{-/-} lung DCs showed enhanced OVA uptake and processing. DQ-OVA was administered intranasally to PTX3^{-/-} and PTX3^{+/+} mice and lung DCs were assessed for processed OVA signal (FITC) 16 hrs post administration (**A**, **D**). Surface expression of CD86 (**B**, **E**) and MHCII (**C**, **F**) was determined on processed OVA+ lung DCs (gated on CD11b+CD11c+). Quantification and statistical analysis of Flow cytometry data is shown as graphs (**D–F**). *n* = 6 mice per group, **p* < 0.01 using Mann-Whitney test.

PTX3^{-/-} mice as compared to their wild type controls. Altogether our data provide plausible insight into how deletion of PTX3 has rendered pulmonary DCs hyper mature upon OVA exposure and affected their function.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Animal usage was strictly according to instructed guidelines by the Canadian Council for Animal Care and the University of Manitoba Animal Ethics Board (Protocol Number 15802).

AUTHOR CONTRIBUTIONS

AG supervised the study, provided scientific insight, reviewed and edited the manuscript. JB performed the animal study and analyzed the data, and drafted the manuscript. LK analyzed the data, drafted and revised the manuscript. LS performed the

animal study and analyzed the data. AM helped in the animal study and the chemokines experiments. LB provided insight into the study design and manuscript writing. All authors contributed to the article and approved the submitted version.

FUNDING

This study was funded by a Canadian Institute of Health Research bridge fund (Project#149062).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2021.641311/full#supplementary-material>

Supplementary Figure 1 | PTX3^{+/+} and PTX3^{-/-} lung DCs show comparable survival. **(A)** Survival of lung DCs (CD11c+CD11b+) was assessed by annexin V and DAPI staining. % of gated cells is presented as mean ± SEM in graph **(B)**, *n* = 8–10/group. **P* < 0.01.

Supplementary Figure 2 | PTX3 deletion affect CCR2 expression on lungs DCs. Expression of CCR2 **(A)**, CCR5 **(B)** and CCR6 **(C)** were assessed by flow cytometry

on CD11c+CD11b+ DCs from the lungs. Quantification and statistical analysis of Flow cytometry data was done using two way ANOVA. $n = 6/\text{group}$, p values are indicated above the graph bars.

Supplementary Figure 3 | Quantification and statistical analysis of the expression of MHCII (A), CD86 (B), CD40 (C) and CD80 (D) at naïve state, 12 and 24 hrs after single i.n OVA challenge. $n = 6/\text{group}$, * $p < 0.01$.

REFERENCES

- Lambrecht BN, Hammad H. The Immunology of Asthma. *Nat Immunol* (2015) 16:45–56. doi: 10.1038/ni.3049
- Tibbitt CA, Stark JM, Martens L, Ma J, Mold JE, Deswarte K, et al. Single-Cell RNA Sequencing of the T Helper Cell Response to House Dust Mites Defines a Distinct Gene Expression Signature in Airway Th2 Cells. *Immunity* (2019) 51:169–84.e165. doi: 10.1016/j.immuni.2019.05.014
- Butcher MJ, Zhu J. Recent Advances in Understanding the Th1/Th2 Effector Choice. *Fac Rev* (2021) 10:30. doi: 10.12703/r/10-30
- Schuijs MJ, Hammad H, Lambrecht BN. Professional and ‘Amateur’ Antigen-Presenting Cells In Type 2 Immunity. *Trends Immunol* (2019) 40:22–34. doi: 10.1016/j.it.2018.11.001
- Balhara J, Koussih L, Zhang J, Gounni AS. Pentraxin 3: An Immuno-Regulator in the Lungs. *Front Immunol* (2013) 4:127. doi: 10.3389/fimmu.2013.00127
- Bottazzi B, Inforzato A, Messa M, Barbagallo M, Magrini E, Garlanda C, et al. The Pentraxins PTX3 and SAP in Innate Immunity, Regulation of Inflammation and Tissue Remodelling. *J Hepatol* (2016) 64:1416–27. doi: 10.1016/j.jhep.2016.02.029
- Introna M, Alles VV, Castellano M, Picardi G, De Gioia L, Bottazzai B, et al. Cloning of Mouse Ptx3, A New Member of the Pentraxin Gene Family Expressed at Extrahepatic Sites. *Blood* (1996) 87:1862–72. doi: 10.1182/blood.V87.5.1862.1862
- Doni A, Michela M, Bottazzi B, Peri G, Valentino S, Polentarutti N, et al. Regulation of PTX3, a Key Component of Humoral Innate Immunity in Human Dendritic Cells: Stimulation by IL-10 and Inhibition by IFN- γ . *J Leukoc Biol* (2006) 79:797–802. doi: 10.1189/jlb.0905493
- Rovere P, Peri G, Fazzini F, Bottazzi B, Doni A, Bondanza A, et al. The Long Pentraxin PTX3 Binds to Apoptotic Cells and Regulates Their Clearance by Antigen-Presenting Dendritic Cells. *Blood* (2000) 96:4300–6. doi: 10.1182/blood.V96.13.4300.h8004300_4300_4306
- D’Angelo C, De Luca A, Zelante T, Bonifazi P, Moretti S, Giovannini G, et al. Exogenous Pentraxin 3 Restores Antifungal Resistance and Restrains Inflammation in Murine Chronic Granulomatous Disease. *J Immunol* (2009) 183:4609–18. doi: 10.4049/jimmunol.0900345
- Balhara J, Shan L, Zhang J, Muhuri A, Halayko AJ, Almiski MS, et al. Pentraxin 3 Deletion Aggravates Allergic Inflammation Through a TH17-Dominant Phenotype and Enhanced CD4 T-Cell Survival. *J Allergy Clin Immunol* (2017) 139:950–63.e959. doi: 10.1016/j.jaci.2016.04.063
- Gupta G, Mou Z, Jia P, Sharma R, Zayats R, Viana SM, et al. The Long Pentraxin 3 (PTX3) Suppresses Immunity to Cutaneous Leishmaniasis by Regulating CD4(+) T Helper Cell Response. *Cell Rep* (2020) 33:108513. doi: 10.1016/j.celrep.2020.108513
- Movassagh H, Shan L, Mohammed A, Halayko AJ, Gounni AS. Semaphorin 3E Deficiency Exacerbates Airway Inflammation, Hyperresponsiveness, and Remodeling in a Mouse Model of Allergic Asthma. *J Immunol* (2017) 198:1805–14. doi: 10.4049/jimmunol.1601514
- Plantinga M, Guillems M, Vanheerswynghels M, Deswarte K, Branco-Madeira F, Toussaint W, et al. Conventional and Monocyte-Derived CD11b(+) Dendritic Cells Initiate and Maintain T Helper 2 Cell-Mediated Immunity to House Dust Mite Allergen. *Immunity* (2013) 38:322–35. doi: 10.1016/j.immuni.2012.10.016
- Shi C, Pamer EG. Monocyte Recruitment During Infection and Inflammation. *Nat Rev Immunol* (2011) 11:762–74. doi: 10.1038/nri3070
- Chen K, Liu M, Liu Y, Wang C, Yoshimura T, Gong W, et al. Signal Relay by CC Chemokine Receptor 2 (CCR2) and Formylpeptide Receptor 2 (Fpr2) in the Recruitment of Monocyte-Derived Dendritic Cells in Allergic Airway Inflammation. *J Biol Chem* (2013) 288:16262–73. doi: 10.1074/jbc.M113.450635
- Manz MG, Traver D, Miyamoto T, Weissman IL, Akashi K. Dendritic Cell Potentials of Early Lymphoid and Myeloid Progenitors. *Blood* (2001) 97:3333–41. doi: 10.1182/blood.V97.11.3333
- GeurtsvanKessel CH, Lambrecht BN. Division of Labor Between Dendritic Cell Subsets of the Lung. *Mucosal Immunol* (2008) 1:442–50. doi: 10.1038/mi.2008.39
- Robays LJ, Maes T, Lebecque S, Lira SA, Kuziel WA, Brusselle GG, et al. Chemokine Receptor CCR2 But Not CCR5 or CCR6 Mediates the Increase in Pulmonary Dendritic Cells During Allergic Airway Inflammation. *J Immunol* (2007) 178:5305–11. doi: 10.4049/jimmunol.178.8.5305
- Palframan RT, Jung S, Cheng G, Weninger W, Luo Y, Dorf M, et al. Inflammatory Chemokine Transport and Presentation in HEV: A Remote Control Mechanism for Monocyte Recruitment to Lymph Nodes in Inflamed Tissues. *J Exp Med* (2001) 194:1361–73. doi: 10.1084/jem.194.9.1361
- Osterholzer JJ, Chen GH, Olszewski MA, Curtis JL, Huffnagle GB, Toews GB. Accumulation of CD11b+ Lung Dendritic Cells in Response to Fungal Infection Results From the CCR2-Mediated Recruitment and Differentiation of Ly-6Chigh Monocytes. *J Immunol* (2009) 183:8044–53. doi: 10.4049/jimmunol.0902823
- Schmudde I, Strover HA, Vollbrandt T, König P, Karsten CM, Laumonnier Y, et al. C5a Receptor Signalling in Dendritic Cells Controls the Development of Maladaptive Th2 and Th17 Immunity in Experimental Allergic Asthma. *Mucosal Immunol* (2013) 6:807–25. doi: 10.1038/mi.2012.119
- Trombetta ES, Mellman I. Cell Biology of Antigen Processing *In Vitro* and *In Vivo*. *Annu Rev Immunol* (2005) 23:975–1028. doi: 10.1146/annurev.immunol.22.012703.104538
- Menezes S, Melandri D, Anselmi G, Perchet T, Loschko J, Dubrot J, et al. The Heterogeneity of Ly6C(hi) Monocytes Controls Their Differentiation Into iNOS(+) Macrophages or Monocyte-Derived Dendritic Cells. *Immunity* (2016) 45:1205–18. doi: 10.1016/j.immuni.2016.12.001
- Provoost S, Maes T, Joos GF, Tournoy KG. Monocyte-Derived Dendritic Cell Recruitment and Allergic T(H)2 Responses After Exposure to Diesel Particles are CCR2 Dependent. *J Allergy Clin Immunol* (2012) 129:483–91. doi: 10.1016/j.jaci.2011.07.051
- Maus UA, Wellmann S, Hampl C, Kuziel WA, Srivastava M, Mack M, et al. CCR2-Positive Monocytes Recruited to Inflamed Lungs Downregulate Local CCL2 Chemokine Levels. *Am J Physiol Lung Cell Mol Physiol* (2005) 288: L350–358. doi: 10.1152/ajplung.00061.2004
- Bracke KR, D’Hulst A I, Maes T, Demedts IK, Moerloose KB, Kuziel WA, et al. Cigarette Smoke-Induced Pulmonary Inflammation, But Not Airway Remodelling, Is Attenuated in Chemokine Receptor 5-Deficient Mice. *Clin Exp Allergy* (2007) 37:1467–79. doi: 10.1111/j.1365-2222.2007.02808.x
- Bracke KR, D’Hulst A I, Maes T, Moerloose KB, Demedts IK, Lebecque S, et al. Cigarette Smoke-Induced Pulmonary Inflammation and Emphysema Are Attenuated in CCR6-Deficient Mice. *J Immunol* (2006) 177:4350–9. doi: 10.4049/jimmunol.177.7.4350
- Yamazaki T, Yang XO, Chung Y, Fukunaga A, Nurieva R, Pappu B, et al. CCR6 Regulates the Migration of Inflammatory and Regulatory T Cells. *J Immunol* (2008) 181:8391–401. doi: 10.4049/jimmunol.181.12.8391
- Odum N, Bregenholt S, Eriksen KW, Skov S, Ryder LP, Bendtsen K, et al. The CC-Chemokine Receptor 5 (CCR5) Is a Marker of, But Not Essential for the Development of Human Th1 Cells. *Tissue Antigens* (1999) 54:572–7. doi: 10.1034/j.1399-0039.1999.540606.x
- Deban L, Russo RC, Sironi M, Moalli F, Scanziani M, Zambelli V, et al. Regulation of Leukocyte Recruitment by the Long Pentraxin PTX3. *Nat Immunol* (2010) 11:328–34. doi: 10.1038/ni.1854

32. Chen J, Matzuk MM, Zhou XJ, Lu CY. Endothelial Pentraxin 3 Contributes to Murine Ischemic Acute Kidney Injury. *Kidney Int* (2012) 82:1195–207. doi: 10.1038/ki.2012.268
33. Roche PA, Furuta K. The Ins and Outs of MHC Class II-Mediated Antigen Processing and Presentation. *Nat Rev Immunol* (2015) 15:203–16. doi: 10.1038/nri3818
34. Koorella C, Nair JR, Murray ME, Carlson LM, Watkins SK, Lee KP. Novel Regulation of CD80/CD86-Induced Phosphatidylinositol 3-Kinase Signaling by NOTCH1 Protein in Interleukin-6 and Indoleamine 2,3-Dioxygenase Production by Dendritic Cells. *J Biol Chem* (2014) 289:7747–62. doi: 10.1074/jbc.M113.519686

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Balhara, Koussih, Mohammed, Shan, Lamkhioed and Gounni. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



A Novel Model Based on Serum Biomarkers to Predict Primary Non-Response to Infliximab in Crohn's Disease

OPEN ACCESS

Edited by:

Silvano Sozzani,
Sapienza University of Rome, Italy

Reviewed by:

Gerard Dijkstra,
University Medical Center Groningen,
Netherlands

Barbara Bottazzi,
University of Milan, Italy

*Correspondence:

Shenghong Zhang
shenghongzhang@163.com;
zhshh3@mail.sysu.edu.cn

[†]These authors have contributed
equally to this work and
share first authorship

Specialty section:

This article was submitted to
Cytokines and Soluble
Mediators in Immunity,
a section of the journal
Frontiers in Immunology

Received: 27 December 2020

Accepted: 07 July 2021

Published: 22 July 2021

Citation:

Li L, Chen R, Zhang Y,
Zhou G, Chen B, Zeng Z,
Chen M and Zhang S (2021)
A Novel Model Based on
Serum Biomarkers to Predict
Primary Non-Response to
Infliximab in Crohn's Disease.
Front. Immunol. 12:646673.
doi: 10.3389/fimmu.2021.646673

Li Li[†], Rirong Chen[†], Yingfan Zhang, Gaoshi Zhou, Baili Chen, Zhirong Zeng,
Minhu Chen and Shenghong Zhang*

Division of Gastroenterology, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China

Background: Infliximab is effective in inducing and maintaining remission in patients with Crohn's disease (CD), but primary non-response (PNR) occurs in 10-30% of cases. We investigated whether serum biomarkers are effective in predicting PNR in patients with CD.

Methods: From January 2016 to April 2020, a total of 260 patients were recruited to this prospective and retrospective cohort study. Serum samples were collected at baseline and week 2 of infliximab treatment. Serum levels of 35 cytokines were assessed in 18 patients from the discovery cohort and were further evaluated in the 60-patient cohort 1. Then, candidate cytokines and other serological biomarkers were used to construct a predictive model by logistic regression in a 182-patient cohort 2. PNR was defined based on the change of CD activity index or clinical symptoms.

Results: Among the 35 cytokines, matrix metalloproteinase 3(MMP3) and C-C motif ligand 2 (CCL2) were two effective serum biomarkers associated with PNR in both the discovery cohort and cohort 1. In cohort 2, serum level of MMP3, CCL2 and C-reactive protein (CRP) at 2 weeks after infliximab injection were independent predictors of PNR, with odds ratios (95% confidence interval) of 1.108(1.059-1.159), 0.940(0.920-0.965) and 1.102(1.031-1.117), respectively. A PNR classifier combining these three indicators had a large area under the curve [0.896(95% CI:0.895-0.897)] and negative predictive value [0.918(95%CI:0.917-0.919)] to predict PNR to infliximab.

Conclusions: MMP3, CCL2, and CRP are promising biomarkers in prediction of PNR to infliximab, and PNR classifier could accurately predict PNR and may be useful in clinical practice for therapy selection.

Keywords: Crohn's disease, primary non-response, infliximab, serum biomarkers, prediction

INTRODUCTION

The introduction of infliximab (IFX), a chimeric monoclonal antibody against tumour necrosis factor (TNF)- α , has significantly improved therapy to induce and maintain remission in Crohn's disease (CD) (1). However, 10–30% of patients receiving IFX are non-responsive during induction therapy (primary non-response, PNR) (2). Furthermore, IFX therapy is expensive and may give rise to some adverse events, such as infusion reactions and infections (1). Incorrect use of IFX in PNR patients would delay treatment as well as lead to disease progression (3). Thus, it is important to explore a method for precisely predicting PNR in patients of CD.

Previous studies have demonstrated that clinical characteristics (4, 5), including disease duration, age at diagnosis, concomitant use of immunosuppressant drugs, and disease location or behaviour, were associated with the efficacy of IFX. Besides, genetic polymorphisms (6, 7), microbiome (8) and serum biomarkers (5, 9) have also been studied with regard to response to IFX. Although some risk factors of PNR were confirmed, the mechanisms underlying PNR have not been broadly defined. It has been widely proposed that non-TNF- α mediated inflammation may result in PNR to IFX and some pro-inflammatory pathways could even be regulated by TNF α blockade (10). Therefore, we believe that the inflammatory state of patients could affect the IFX response, and alterations of inflammatory cytokines in serum might be an appropriate biomarker to predict PNR.

Previous studies have shown that inflammatory cytokines, including TNF- α , interleukin (IL)-6, IL-1 β , IL-17A, IL-23, and IL-12, may be predictive factors of PNR to IFX (11–13). However, these studies only found the discrepant expression levels of the cytokines between the respondent and non-respondent groups, and none of these cytokines has been widely used in clinical practise. Thus, identifying effective serum cytokines that affect therapeutic failure could help select the most appropriate patients for IFX treatment. In this prospective and retrospective cohort study, we assessed circulating inflammatory cytokines levels before and after the administration of IFX to identify potential serum biomarkers, and then constructed a model in prediction of PNR to IFX in CD patients.

PATIENTS AND METHODS

Study Design and Patient Population

This was a prospective and retrospective, single-centre cohort study, approved by the institutional ethics committee (IEC) for Clinical Research and Animal Trials of the First Affiliated Hospital of Sun Yat-sen University (No. 2019-383). Informed consent was obtained from all patients. Patients with a definite diagnosis of CD and receiving IFX therapy in the First Affiliated Hospital of Sun Yat-sen University were consecutively included in the study from January 2016 to April 2020. Other inclusion criteria were as follows: (1) treatment with 5 mg/kg IFX intravenously at week 0, 2, and 6; (2) naïve to anti-TNF and other biologicals therapy; and (3) CD activity index (CAI) of

≥ 150 before IFX treatment. Exclusion criteria included the following: (1) lack of evaluation for disease activity at baseline or at week 14 after the first treatment with IFX; (2) unavailable blood samples at baseline and week 2; (3) delayed infusion; (4) pregnancy; and (5) lost to follow-up.

The Montreal system was used to classify the location and behaviour of CD patients (14). The CDAI was calculated for all patients at baseline and week 14 after IFX therapy. At baseline, clinical characteristics including sex, age at first IFX therapy, age at diagnosis, disease duration, body mass index, disease location and behaviour, presence of extraintestinal manifestations, history of smoking and surgery, concomitant azathioprine, and CDAI score were recorded. The endoscopic activity of patients was assessed using the Simple Endoscopic Score for Crohn's Disease (SES-CD). C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), albumin, haemoglobin, white blood cell, neutrophil, lymphocytes, and platelets counts were assessed at baseline and week 2 after IFX therapy.

PNR of IFX was defined as a failure of the CDAI to drop more than 70 points or to <150 at week 14 after IFX administration. Situations in which patients received an alternative therapy schedule such as an escalation of corticosteroid therapy, switching to other agents or having the surgery before week 14 were also defined as PNR.

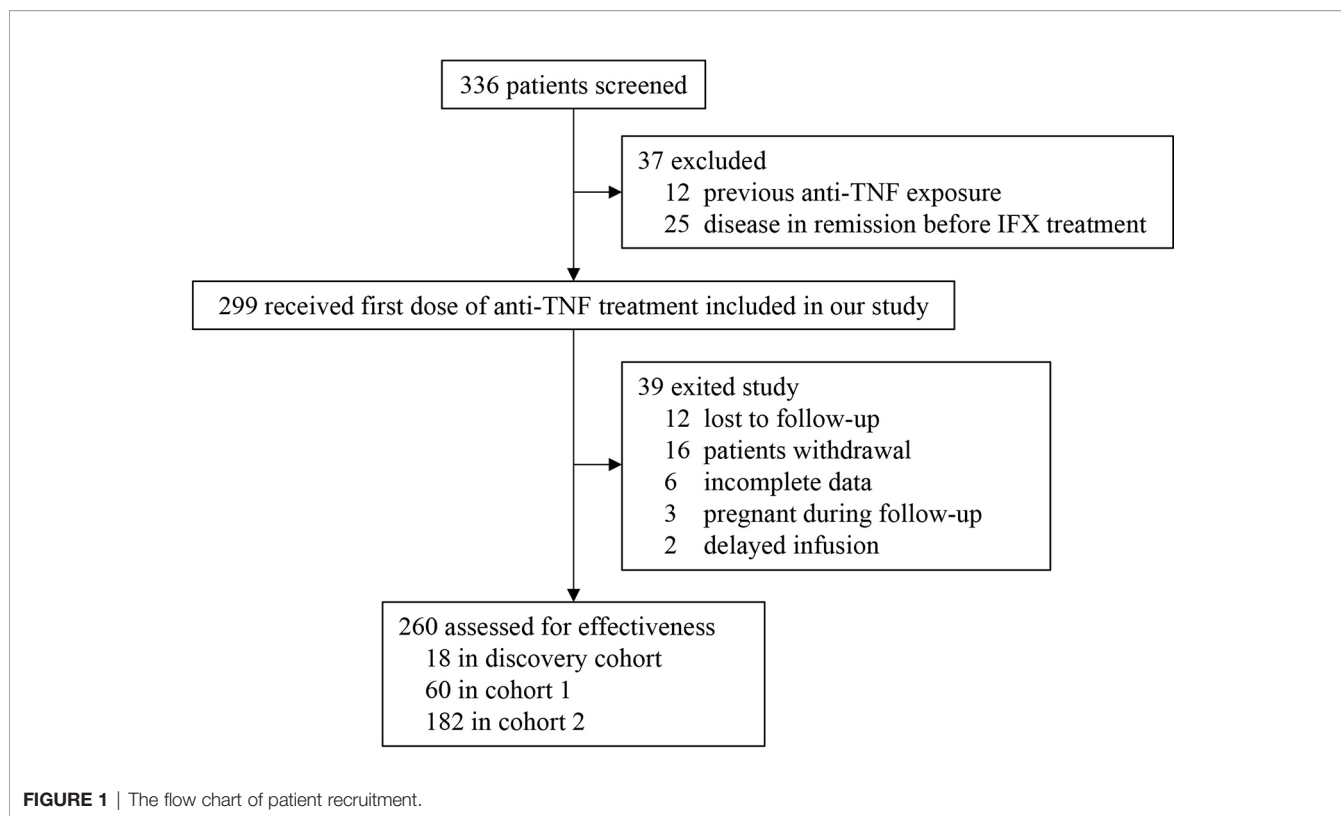
In this prospective and retrospective study, a total of 260 patients were eligible (**Figure 1**). Retrospective single-centre cohorts were designed to screen (discovery cohort, $n=18$) and validate (Cohort 1, $n=60$) the candidate cytokines. The first 9 primary responders and 9 non-responders constituted a discovery cohort to identify potential biomarkers associated with PNR. Serum levels of 35 cytokines at baseline and week 2 after IFX therapy were examined by the Luminex cytokine multiplex assay in 18 patients from the discovery cohort. The latter 60 patients were recruited into cohort 1 to further verify and screen the candidate cytokines selected from the discovery cohort. Thereafter, a prospective single-centre cohort (cohort 2, $n=182$) was recruited from May 2018 to April 2020 in the First Affiliated Hospital of Sun Yat-sen University, which were used to construct a predictive model based on the findings from cohort 1 and do internal validation through the bootstrap validation approach.

Sample Collection

The blood samples were collected into serum separator tubes (BD Biosciences, Franklin Lakes, NJ, USA) before intravenous injection of Infliximab at baseline and week 2. The tubes were centrifuged at $1900\times g$ for 15 minutes, then the serum samples were aliquoted with 0.5ml tubes (Ambion, Foster City, CA) and stored at -80°C until analyses.

Assay for Serum Biomarkers

Serum levels of 35 cytokines, including C-C motif ligand (CCL)2, CCL4, CCL11, CCL20, CCL25, CCL26, CX3CL1, CXCL8, CXCL11, ADAMTS13, adiponectin, α -2-macroglobulin, matrix metalloproteinase (MMP)3, insulin-like growth factor binding protein-1, TNF- α , IL-1 β , IL-2, IL-4, IL-6, IL-7, IL-10, IL-11, IL-12 p70, IL-13, IL-17, IL-18, IL-23, IL-27, IL-28A, IL-28B, IL-31,



IL-33, IL-34, and IL-36 β , at baseline and week 2 in discovery cohort were measured using a Luminex cytokine assay kit (LXSAHM-35) and a Luminex X-200 instrument according to the manufacturer's protocol (R&D Systems, Minneapolis, MN, USA).

Enzyme-linked immunosorbent assay (ELISA) was used to measure levels of the molecules selected after the analysis of Luminex results obtained in the discovery cohort. In cohort 1 and 2, serum MMP3 levels were measured using a quantitative sandwich enzyme immunoassay (R&D Systems) at baseline and week 2. The serum CCL2 concentration was measured at week 2 using an ELISA kit (Abcam, Cambridge, UK, Ab179886). Serum IL-7 levels were measured using an ELISA kit (R&D Systems). Other indicators, such as CRP, ESR, albumin and so on, were tested with standard institutional protocols by the laboratory physicians at the First Affiliated Hospital of Sun Yat-sen University.

Statistical Analyses

Continuous and categorical variables were described as medians with interquartile ranges and frequency with percentages, respectively. The non-parametric Wilcoxon and chi-square tests for continuous and categorical variables, respectively, were used to assess differences between responders and non-responders. The False Discovery Rate (Benjaminiand-Hochberg method) was performed to adjust the p-value in the comparison of 35 serum biomarkers between primary responders and non-responders in the discovery cohort. A p-value of <0.05 denoted statistical significance. Multivariate logistic regression (enter)

was used to evaluate the relationship between the clinical or serological variables with the significant differences in the univariate analysis and the outcome of PNR among all patients in cohort 2, and also in patients with determined response status by CDAI in cohort 2. The variables with a p-value of <0.05 in the multivariate analyses were defined as independent predictive indicators, and were selected to construct a logistic regression model, the PNR classifier. Multivariate logistic regression was also performed to calculate the odds ratio (OR) of the independent predictive indicators and PNR classifier in predicting PNR. The area under the receiver operating characteristics curve (AUC), sensitivity, specificity, positive predictive value and negative predictive value were used to assess the predictive ability of the model, and their respective mean [95% confidence interval (CI)] were calculated by bootstrapping with 2,000 replications. Moreover, the bootstrap validation approach was also used to assess the internal validation of the independent predictive indicators and the PNR classifier for predicting PNR in cohort 2. R software, version 4.0.0, was used for statistical analyses.

RESULTS

Patients Characteristics

Between January 2016 and April 2020, 260 patients were included in this prospective and retrospective cohort study (Figure 1). Baseline characteristics for discovery cohort, and cohorts 1 and 2 are documented in **Supplement Table 1** and

Table 1, respectively. Only perianal disease ($p<0.001$) and serum albumin concentration ($p=0.019$) were significantly different between the two cohorts. In PNR patients, only ESR ($p=0.015$) was significantly different between cohort 1 and cohort 2 (**Supplement Table 2**). To evaluate the disease activity at week 14 more objectively, we performed a comparison of CRP, ESR and SESCD between primary responders and primary non-responders (**Supplement Table 3**). The results showed that primary non-responders had higher CRP, ESR and SESCD levels than primary responders at week 14 in both cohort 1 (CRP: $p<0.001$; ESR: $p<0.001$; SESCD: $p<0.001$) and cohort 2 (CRP: $p<0.001$; ESR: $p<0.001$; SESCD: $p<0.001$).

Distinct Serum Cytokine Profiles in CD Patients Receiving IFX Treatment

In the discovery cohort, Luminex multiplex assay was performed using serum samples collected before IFX treatment and at week 2. Through non-parametric Wilcoxon test, we found that the serum CCL2 level at week 2 ($p=0.013$), MMP3 level at baseline ($p=0.050$), MMP3 level at week 2 ($p=0.008$), and the change of IL-7 from baseline to week 2 (Δ IL-7) ($p=0.050$) were significantly different between the primary responders and non-responders (**Supplement Table 4**). However, after adjusting the p-value by False Discovery Rate, none of the indicators was significantly different.

To verify the discrepant levels of these four serum indicators, we performed further analyses by ELISA in cohort 1. MMP3 level at baseline ($p=0.040$), MMP3 level at week 2 ($p=0.004$) and CCL2 level at week 2 ($p=0.001$) showed significant difference between primary non-responders and responders, while Δ IL-7 showed no significant change ($p=0.094$) (**Table 2**). Furthermore, we observed that CCL2 and MMP3 levels at week 2 were significantly altered in the multivariate analysis; the odds ratios (ORs) (95% CI) were 0.903 (0.877-0.986, $p=0.010$) and 1.203 (1.039-1.394, $p=0.008$), respectively. The receiver operating characteristics analysis revealed that CCL2 [AUC (95% CI): 0.790 (0.786-0.793)] and MMP3 [AUC (95% CI): 0.764 (0.760-0.767)] levels at week 2 could better predict PNR than MMP3 at baseline [AUC (95% CI): 0.683 (0.678-0.687)] and Δ IL-7 [AUC (95% CI): 0.632 (0.627-0.637)] (**Figure 2**).

CCL2, MMP3, and CRP at Week 2 Are Independently Predictive of PNR

Considering that CCL2 and MMP3 at week 2 were the most important predictors of PNR to IFX, we further analysed these two indicators, as well as other clinical and serological markers, in cohort 2.

Univariate analyses showed that three clinical characteristics and eight serological indicators, including age at first IFX therapy

TABLE 1 | Baseline characteristics of Cohort 1 and 2.

	Cohort 1 (n=60)		Cohort 2 (n=182)		P value*
	All patients	PNR patients	All patients	PNR patients	
Primary non-responders	12 (20)	12 (100)	45 (24.7)	45 (100)	0.489
Failure of CDAl reduction		10 (83.3)		38 (84.4)	
Therapy alteration		2 (16.7)		7 (15.6)	
Male	47 (78.3)	9 (75.0)	128 (70.3)	32 (71.1)	0.249
Age at 1 st IFX therapy (years)	24.5 (18.5-29.0)	24.38 (19.2-34.7)	24.9 (18.0-34.0)	28.8 (22.6-36.5)	0.242
Body-mass index (kg/m ²)	17.1 (15.8-19.4)	17.7 (16.0-21.3)	17.7 (16.1-19.2)	17.4 (15.8-18.8)	0.371
Age at diagnosis (years)	21.8 (17.4-26.2)	22.6 (18.3-27.0)	22.0 (16.7-29.0)	23.0 (18.3-29.3)	0.775
Disease duration (years)	1.0 (0.5-3.9)	1.3 (0.8-8.4)	1.6 (0.7-4.9)	4.6 (1.0-6.9)	0.100
Disease location					0.373
L1 (ileal disease)	6 (10.0)	2 (16.7)	30 (16.5)	11 (24.4)	
L2 (colonic disease)	4 (6.7)	0 (0)	8 (4.4)	1 (2.2)	
L3 (ileocolonic disease)	50 (83.3)	10 (83.8)	144 (79.1)	33 (73.3)	
Presence of upper GI disease	11 (18.3)	2 (16.7)	42 (23.1)	9 (20.2)	0.478
Disease behavior					0.382
B1 (non stricturing, non-penetrating)	37 (61.7)	6 (50.0)	95 (52.2)	13 (28.9)	
B2 (stricturing)	18 (30.0)	4 (33.3)	62 (34.1)	25 (55.6)	
B3 (penetrating)	5 (8.3)	2 (16.7)	25 (13.7)	7 (15.6)	
Perianal disease	16 (26.7)	4 (33.3)	70 (38.5)	14 (31.1)	<0.001
Presence of extraintestinal manifestations	6 (10)	2 (16.7)	34 (18.7)	11 (24.4)	0.160
Previous surgery	8 (13.3)	2 (16.7)	40 (22.0)	13 (28.9)	0.191
History of smoking	3 (5)	0 (0)	15 (8.2)	3 (6.7)	0.573
Concomitant Azathioprine	32 (53.3)	8 (66.7)	79 (43.4)	19 (42.2)	0.181
CDAl score	251 (199-296)	268 (194-296)	234 (198-282)	232 (198-245)	0.519
C-reactive protein (mg/L)	29.4 (9.7-49.0)	33.0 (19.3-58.6)	21.7 (8.5-39.5)	19.4 (2.3-35.0)	0.154
Erythrocyte sedimentation rate (mm/h)	52.0 (31.3-70.0)	69.0 (47.5-85.8)	42.0 (24.0-72.0)	38.0 (19.5-60.5)	0.253
Albumin (g/L)	32.9 (30.0-36.3)	34.0 (30.8-36.5)	35.0 (31.2-38.9)	35.4 (31.2-38.2)	0.019
Haemoglobin (g/L)	111 (92-124)	113 (100-127)	113 (96-128)	114 (95-135)	0.319
Platelet count ($\times 10^9/L$)	348 (259-461)	364 (301-442)	350 (280-450)	343 (267-459)	0.910

*The p value was from the non-parametric Wilcoxon test or chi-square test in comparison of baseline characteristics between all patients in cohort 1 and cohort 2. Continuous variables and categorical variables are described as median (IQR) and n (%), respectively. IQR, interquartile range; GI, gastrointestinal; CDAl, Crohn's disease activity index.

TABLE 2 | Comparison of serum biomarkers detected by ELISA between primary non-responders and responders in cohort 1.

	Univariate analysis			Multivariate analysis	
	Primary response (n=48)	PNR (n=12)	P value	Odds Ratio (95% CI)	P value
Δ IL-7 (pg/ml)	-5.5 (-10.1- -3.2)	-4.5 (-6.0- -2.1)	0.095	1.261 (0.948-1.677)	0.111
MMP3 at baseline (ng/mL)	21.4 (13.6-27.0)	26.4 (21.2-35.7)	0.040	0.943 (0.869-1.024)	0.161
MMP3 at week 2 (ng/ml)	13.5 (8.2-18.5)	19.7 (14.2-38.3)	0.004	1.203 (1.039-1.394)	0.013
CCL2 at week 2 (pg/ml)	93.7 (77.3-128.5)	63.6 (59.3-86.7)	0.001	0.903 (0.877-0.986)	0.015

ELISA, Enzyme-linked immunosorbent assay; Δ IL-7, change of IL-7 from baseline to week 2; CCL, C-C motif ligand; MMP, matrix metalloproteinase.

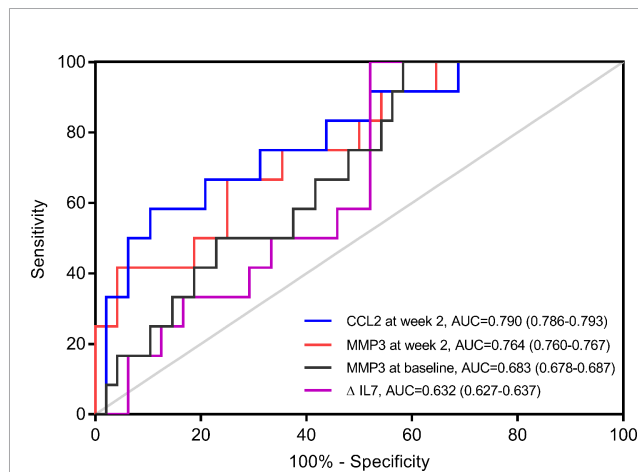


FIGURE 2 | Receiver operating characteristics (ROC) analyses of potential predictors in cohort 1. ROC Curve of change of IL-7 from baseline to week 2 (Δ IL-7), matrix metalloproteinase (MMP) 3 at baseline, MMP3 at week 2 and C-C motif ligand (CCL) 2 at week 2 for predicting primary non-response (n=12) in cohort 1 (n=60).

($p=0.044$), disease duration ($p=0.002$), CD behaviour ($p=0.001$), neutrophil percentage ($p=0.010$), lymphocytes counts($p=0.042$), lymphocyte percentage ($p=0.007$), platelet-to-lymphocyte ratio

($p=0.028$), neutrophil-to-lymphocyte ratio ($p=0.008$), CRP ($p<0.001$), MMP3 ($p<0.001$) and CCL2 levels ($p<0.001$) at week 2, were significantly different between primary non-responders and responders (**Table 3** and **Supplemental Table 5**).

Multivariate analyses of all patients in cohort 2 revealed that only CRP [OR 1.102 (95%CI: 1.031-1.117), $p=0.004$], MMP3 [OR 1.108 (95%CI: 1.059-1.159), $p<0.001$], and CCL2 levels at week 2 [OR 0.940 (95%CI: 0.920-0.965), $p<0.001$] were independently associated with PNR (**Table 3**). Another multivariate analysis was performed in patients with determined response status by CDAI, and the result was similar to that obtained while including all patients (**Supplement Table 6**).

A Novel Model for Predicting PNR

The predictive values of CRP, CCL2, and MMP3 at week 2 are shown in **Table 4**. Both CRP and MMP3 had large specificity [(95% CI): 0.768 (0.765-0.770) and 0.799 (0.796-0.802), respectively], whereas CCL2 had high sensitivity [0.783 (0.778-0.788)], but low specificity [0.687 (0.681-0.692)].

To better predict PNR, we built a PNR classifier combining serum levels of CRP, CCL2, and MMP3 at week 2 through logistic regression, and the variance inflation factors of CRP, MMP3 and CCL2 was equal to 1.004, 1.008 and 1.012,

TABLE 3 | Significant univariate association of clinical characteristics and serological markers for primary non-response in cohort 2.

	Univariate analysis			Multivariate analysis	
	Primary response (n=137)	PNR (n=45)	P value	Odds Ratio (95% CI)	P value
Clinical characteristics					
Age at 1 st IFX therapy (years)	23.6 (18.3-32.6)	28.8 (22.6-36.5)	0.044	1.012 (0.953-1.076)	0.692
Disease duration (years)	1.2 (0.6-4.0)	4.6 (1.0-6.9)	0.002	1.069 (0.964-1.185)	0.205
CD behaviour			0.001	1.540 (0.765-3.099)	0.226
B1 (non stricturing, non-penetrating)	82 (59.9)	13 (28.9)			
B2 (stricturing)	37 (27.0)	25 (55.6)			
B3 (penetrating)	18 (13.1)	7 (15.6)			
Level of serological markers at weeks 2					
Neutrophil percentage (%)	58.6 (51.8-64.1)	63.6 (55.8-71.7)	0.010	1.010 (0.902-1.131)	0.866
Lymphocytes ($\times 10^9/L$)	1.6 (1.3-2.0)	1.4 (1.1-1.9)	0.042	1.658 (0.468-6.064)	0.425
Lymphocyte percentage (%)	30.6 (24.5-35.1)	24.9 (17.8-32.2)	0.007	0.974 (0.838-1.132)	0.727
PLR	180 (142-230)	217 (152-291)	0.028	1.005 (0.997-1.013)	0.243
NLR	2.0 (1.5-2.6)	2.5 (1.7-3.8)	0.008	0.980 (0.301-3.191)	0.947
CRP (mg/L)	1.5 (0.8-3.6)	4.0 (1.0-8.7)	<0.001	1.102 (1.031-1.117)	0.004
CCL2 (pg/ml)	98.9 (80.0-120.5)	72.0 (55.7-88.0)	<0.001	0.940 (0.920-0.965)	<0.001
MMP3 (ng/ml)	12.7 (8.1-17.4)	24.7 (16.0-33.6)	<0.001	1.108 (1.059-1.159)	<0.001

PLR, platelet-to-lymphocyte ratio; NLR, neutrophil-to-lymphocyte ratio; CRP, C-reactive protein; CCL, C-C motif ligand; MMP, matrix metalloproteinase.

TABLE 4 | The ability of PNR classifier in predicting primary non-response to IFX.

	AUC (95% CI)	Cut-off value (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
PNR classifier	0.896 (0.895-0.897)	0.331 (0.327-0.335)	0.758 (0.755-0.762)	0.888 (0.885-0.890)	0.703 (0.699-0.708)	0.918 (0.917-0.919)
CRP at week 2	0.679 (0.677-0.681)	3.79 (3.76-3.82)	0.614 (0.610-0.617)	0.768 (0.765-0.770)	0.468 (0.465-0.471)	0.859 (0.857-0.860)
CCL2 at week 2	0.781 (0.779-0.782)	84.84 (84.44-85.23)	0.783 (0.778-0.788)	0.687 (0.681-0.692)	0.468 (0.465-0.472)	0.912 (0.910-0.913)
MMP3 at week 2	0.779 (0.777-0.781)	19.15 (19.05-19.25)	0.712 (0.709-0.716)	0.799 (0.796-0.802)	0.548 (0.544-0.551)	0.895 (0.894-0.897)

PPV, Positive predictive value; NPV, Negative predictive value; PNR, Primary non-response; PLR, platelet-to-lymphocyte ratio; CRP, C-reactive protein; CCL, C-C motif ligand; MMP, matrix metalloproteinase.

respectively. PNR classifier had a large AUC of 0.896 (95% CI: 0.895-0.897) to predict PNR (**Table 4**). PNR classifier also had the largest specificity [0.888 (95%CI: 0.885-0.890)], negative predictive value [0.918 (95%CI: 0.917-0.919)] and positive predictive value [0.703 (95%CI: 0.699-0.708)] in the prediction of PNR among these 4 indicators. The OR for getting PNR in patients with a value of PNR classifier > 0.331 was 6.467 (95% CI: 1.928-21.687; $p=0.002$), after adjustment for covariates (**Supplement Table 7**). Furthermore, patients with serum concentration of MMP3 > 19.15pg/ml [OR (95% CI): 8.478 (2.307-31.160); $p=0.001$] or CRP > 3.79 mg/L [OR (95% CI): 6.314 (1.956-20.377); $p=0.002$] also had the high risk to getting PNR.

DISCUSSION

IFX therapy is currently an important choice to induce and maintain remission in CD patients, but approximately 30% of patients exhibit PNR to IFX (1, 2). To achieve the goal of treating each patient at the right time with the optimal drug, it is necessary to detect which group of patients are unlikely to benefit from IFX treatment. In this prospective and retrospective cohort study of 260 CD patients, we found that clinical characteristics such as older age at first IFX therapy, longer disease duration, and stricturing behaviour were significantly associated with PNR to IFX. Furthermore, serum CRP, MMP3, and CCL2 levels at week 2 after IFX injection were the three independent predictors for PNR. Additionally, to better monitor CD, we further established a PNR classifier incorporating these three inflammation mediators, which was demonstrated to have a larger AUC (0.898 [95% CI: 0.837-0.947]) in discriminating primary non-responders from primary responders than using CRP, MMP3 or CCL2 independently. Thus, it can effectively identify patients who are suitable for IFX induction therapy.

MMP3 is a pattern of the large family of zinc dependent matrix metalloproteinases (MMPs), which plays an active role in the pathogenesis of inflammation in inflammatory bowel disease (IBD). The expression and secretion of MMPs are sensitive to the condition of intestinal inflammation, and are either augmented or moderated by a series of inflammatory cytokines, especially TNF- α (15). MMP3 is regarded as a crucial effector molecule of inflammatory cells, while it can also modify cytokines and chemokines (15). Thus, the up-regulated expression of MMPs prompts an initiation phase of acute inflammation. In our study,

a high level of serum MMP3 at week 2 from baseline manifested as an effective predictor of PNR to IFX. Similarly, previous study had found that a high serum MMP3 concentration at week 14 was associated with a loss of response after 52 weeks of IFX treatment (16). Biancheri et al. showed that MMP3 was involved in cleaving anti-TNF agents, such as infliximab, adalimumab, and etanercept, in the colon mucosa of inflammatory bowel disease patients (17). This may explain the predictive function of MMP3 in PNR to IFX. However, whether MMP3 can predict a loss of response to other anti-TNF drugs has not been studied.

CCL2, namely monocyte chemotactic protein 1, is a CC-type chemokine that plays an important role in regulating migration and infiltration of monocytes, lymphocytes, or other immunocytes to inflammation sites in the pathogenesis of chronic inflammatory diseases such as CD (18–20). Previous studies have shown that the expression of CCL2 in intestinal epithelial cells, mucosa, circulating monocytes, or plasma was significantly increased in CD patients, especially those with active CD, compared to healthy individuals (21–23). Thus, CCL2 may be a pro-inflammatory factor in CD. Swedish patients with ulcerative colitis responding to infliximab therapy have been shown to express decreased levels of CCL2 in the serum at Week 2 (24). However, it is confusing that CD patients with PNR had lower serum CCL2 concentrations at week 2 after IFX treatment than responders in our study. A hypothesis that might explain this phenomenon is that the high serum concentration of CCL2 in primary responders is attributable to the decrease in CCR2 expression. A recent study showed that serum CCL2 was removed in a CCR2-dependent but G-protein independent manner, and when CCR2 expression decreased, serum CCL2 concentrations increased (25). Furthermore, anti-TNF non-responders with inflammatory bowel disease had upregulated CCR2 expression (26), which might cause lower serum CCL2 levels than those seen in the responders. Although this theoretical explanation makes sense, experimental validation is lacking. Therefore, it is attractive to research the change and function of the CCL2-CCR2 axis in CD patients before and after anti-TNF therapy for further studies.

CRP is one of the most common biomarkers used in clinical practice and is also useful for monitoring clinical or endoscopic activity in CD patients (27, 28). Post-injection CRP levels are a potential predictor for loss of response to anti-TNF therapy (29, 30). However, it remains controversial whether the baseline CRP level can predict loss of response to anti-TNF therapy in CD (31, 32). Our study found that serum CRP at week 2, but not baseline, was an independent predictor for PNR. However, CRP reflects

the status of generic inflammation, not specific inflammatory pathways. In this study, we combined CRP with MMP3 and CCL2 levels, which are closely correlated with the pathogenesis of CD, and developed a well-founded PNR classifier.

Previous studies have demonstrated that the mechanisms underlying PNR are multifactorial. These mechanisms include disease characteristics, drug and treatment strategy-related factors, and inflammation and immune status of the patients (5). Disease duration, phenotype, and location may contribute to primary nonresponse. However, the role of patient-related characteristics was unclear because of controversial outcomes of different studies (5). Drug related factors includes through drug levels and the accumulation of anti-TNF antibodies. A recent study demonstrated that serum trough levels of IFX at week 2 could predict short-term clinical efficacy of IFX (33). Moreover, low IFX levels and high anti-TNF antibody levels to IFX at week 2 could predict PNR to IFX, with AUCs of 0.68 and 0.78, respectively (34). Nevertheless, the response status could be altered by changing the IFX dose or the combination of immunosuppressive drugs, and the effect of through drug levels and the anti-TNF antibodies could not count as primary mechanisms in the process of PNR. Emerging evidence suggest that primary nonresponse occurs in the patients with a disease course of non-TNF-driven inflammatory process. It has been thought that non-TNF-driven inflammatory processes were mainly involved in the mechanisms underlying PNR. Our research mainly focused on the non-TNF-driven inflammatory processes. We assumed that the changes of inflammatory states, which is presented by the alterations of the cytokines, chemokines and other inflammatory related factors, were significantly different between patients with TNF-driven inflammatory pathway and those were not. In the end, we explored MMP3 and CCL2 which were correlated with PNR.

There are some limitations to our study. From an analysis perspective, the reference cytokine subset we used as the discovery cohort to identify potential biomarkers is not a complete compendium of all of the immune related factors in the CD patients. Nevertheless, these potential factors have all been previously described in CD, which could compensate for the selection bias to some extent. In addition, the initial screening of cytokines in the discovery cohort was based on only 18 patients, which might be insubstantial to yield conclusive data. However, we screened potential biomarkers through analyses based not only on these 18 patients but also the patients in Cohort 1. we verified these potential cytokines selected from the discovery cohort in cohort 1 by univariate and multivariate analyses, which could reduce the incidence of false positives to a certain extent. Moreover, we did not detect IFX trough levels or anti-TNF antibody concentrations in the patients. These two indicators were thought to be able to predict loss of response of IFX (35, 36). However, this study mainly discussed the primary non-response of IFX, while trough serum concentrations of drug and anti-TNF antibodies are probably more relevant for the secondary loss of response (10). Another limitation is that all patients in our study are Chinese, which might make our results with limited generalisability.

In conclusion, our prospective and retrospective cohort study showed that CCL2 and MMP3 at week 2 play important roles in predicting PNR to IFX in CD patients. Furthermore, a PNR classifier was developed and proved effective in predicting PNR. However, further studies need to be performed on other patient populations to further substantiate our findings.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

This study was approved by the IEC for Clinical Research and Animal Trials of the First Affiliated Hospital of Sun Yat-sen University (No. 2019-383). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

All authors were responsible for the study concept and design. LL and RC: drafting of manuscript. LL, RC, YZ, and GZ analyzed the serum samples and interpretation of the data. RC and LL performed the statistical analysis. BC ZZ, MC, and SZ: critical revision of manuscript for important intellectual content. All authors contributed to the article and approved the submitted version.

FUNDING

This project was supported by grants from the National Natural Science Foundation of China (#81630018, #81670498, #81870374, #82070538), Guangzhou Science and Technology Department (#202002030041), Guangdong Science and Technology (#2017A030306021, #2020A1515010249), China Postdoctoral Science Foundation (#2019M653228), and Science and Technology Innovation Young Talents of Guangdong Special Support Plan (#2016TQ03R296).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2021.646673/full#supplementary-material>

REFERENCES

- Hanauer SB, Feagan BG, Lichtenstein GR, Mayer LF, Schreiber S, Colombel JF, et al. Maintenance Infliximab for Crohn's Disease: The ACCENT I Randomised Trial. *Lancet* (2002) 359:1541–9. doi: 10.1016/S0140-6736(02)08512-4
- Gisbert JP, Panes J. Loss of Response and Requirement of Infliximab Dose Intensification in Crohn's Disease: A Review. *Am J Gastroenterol* (2009) 104:760–7. doi: 10.1038/ajg.2008.88
- Rosenblum H, Amital H. Anti-TNF Therapy: Safety Aspects of Taking the Risk. *Autoimmun Rev* (2011) 10:563–8. doi: 10.1016/j.autrev.2011.04.010
- Kennedy NA, Heap GA, Green HD, Hamilton B, Bewshea C, Walker GJ, et al. Predictors of Anti-TNF Treatment Failure in Anti-TNF-Naïve Patients With Active Luminal Crohn's Disease: A Prospective, Multicentre, Cohort Study. *Lancet Gastroenterol Hepatol* (2019) 4:341–53. doi: 10.1016/S2468-1253(19)30012-3
- Gisbert JP, Chaparro M. Predictors of Primary Response to Biologic Treatment [Anti-TNF, Vedolizumab, and Ustekinumab] in Patients With Inflammatory Bowel Disease: From Basic Science to Clinical Practice. *J Crohns Colitis* (2020) 14:694–709. doi: 10.1093/ecco-jcc/jjz195
- Hlavaty T, Pierik M, Henckaerts L, Ferrante M, Joossens S, van Schuerbeek N, et al. Polymorphisms in Apoptosis Genes Predict Response to Infliximab Therapy in Luminal and Fistulizing Crohn's Disease. *Aliment Pharmacol Ther* (2005) 22:613–26. doi: 10.1111/j.1365-2036.2005.02635.x
- Barber GE, Yajnik V, Khalili H, Giallourakis C, Garber J, Xavier R, et al. Genetic Markers Predict Primary Non-Response and Durable Response to Anti-TNF Biologic Therapies in Crohn's Disease. *Am J Gastroenterol* (2016) 111:1816–22. doi: 10.1038/ajg.2016.408
- Zhou Y, Xu ZZ, He Y, Yang Y, Liu L, Lin Q, et al. Gut Microbiota Offers Universal Biomarkers Across Ethnicity in Inflammatory Bowel Disease Diagnosis and Infliximab Response Prediction. *mSystems* (2018) 3. doi: 10.1128/mSystems.00188-17
- Chen P, Zhou G, Lin J, Li L, Zeng Z, Chen M, et al. Serum Biomarkers for Inflammatory Bowel Disease. *Front Med* (2020) 7:123. doi: 10.3389/fmed.2020.00123
- D'Haens GR, Panaccione R, Higgins PD, Vermeire S, Gassull M, Chowers Y, et al. The London Position Statement of the World Congress of Gastroenterology on Biological Therapy for IBD With the European Crohn's and Colitis Organization: When to Start, When to Stop, Which Drug to Choose, and How to Predict Response? *Am J Gastroenterol* (2011) 106(2):199–212. doi: 10.1038/ajg.2010.392
- Ogawa K, Matsumoto T, Esaki M, Torisu T, Iida M. Profiles of Circulating Cytokines in Patients With Crohn's Disease Under Maintenance Therapy With Infliximab. *J Crohns Colitis* (2012) 6:529–35. doi: 10.1016/j.crohns.2011.10.010
- Martinez-Borra J, Lopez-Larrea C, Gonzalez S, Fuentes D, Dieguez A, Deschamps EM, et al. High Serum Tumor Necrosis Factor-Alpha Levels are Associated With Lack of Response to Infliximab in Fistulizing Crohn's Disease. *Am J Gastroenterol* (2002) 97:2350–6. doi: 10.1111/j.1572-0241.2002.05990.x
- Andus T, Herfarth H, Obermeier F, Kuehbachner T, Mascheretti T, Thriene T, et al. Prediction of Response to Treatment With Infliximab (Remicade) in a German Prospective Open-Label Multicenter Trial in Refractory Crohn's Disease. *Gastroenterology* (2001) 120:A621. doi: 10.1016/S0016-5085(08)83089-2
- Gomollón F, Dignass A, Annese V, Tilg H, Van Assche G, Lindsay JO, et al. 3rd European Evidence-Based Consensus on the Diagnosis and Management of Crohn's Disease 2016: Part 1: Diagnosis and Medical Management. *J Crohns Colitis* (2017) 11:3–25. doi: 10.1093/ecco-jcc/jjw168
- O'Shea NR, Smith AM. Matrix Metalloproteinases Role in Bowel Inflammation and Inflammatory Bowel Disease: An Up to Date Review. *Inflammation Bowel Dis* (2014) 20:2379–93. doi: 10.1097/MIB.0000000000000163
- Barberio B, D'Inca R, Facchin S, Gasperina M, Fohom Tagne CA, Cardin R, et al. Matrix Metalloproteinase 3 Predicts Therapeutic Response in Inflammatory Bowel Disease Patients Treated With Infliximab. *Inflamm Bowel Dis* (2020) 26:756–63. doi: 10.1093/ibd/izz195
- Biancheri P, Brezski RJ, Di Sabatino A, Greenplate AR, Soring KL, Corazza GR, et al. Proteolytic Cleavage and Loss of Function of Biologic Agents That Neutralize Tumor Necrosis Factor in the Mucosa of Patients With Inflammatory Bowel Disease. *Gastroenterology* (2015) 149:1564–74.e3. doi: 10.1053/j.gastro.2015.07.002
- Melgarejo E, Medina MÁ, Sánchez-Jiménez F, Urdiales JL. Monocyte Chemoattractant Protein-1: A Key Mediator in Inflammatory Processes. *Int J Biochem Cell Biol* (2009) 41:998–1001. doi: 10.1016/j.biocel.2008.07.018
- Moser B, Loetscher P. Lymphocyte Traffic Control by Chemokines. *Nat Immunol* (2001) 2:123–8. doi: 10.1038/84219
- He J, Song Y, Li G, Xiao P, Liu Y, Xue Y, et al. Fbxw7 Increases CCL2/7 in CX3CR1hi Macrophages to Promote Intestinal Inflammation. *J Clin Invest* (2019) 129:3877–93. doi: 10.1172/JCI123374
- Grip O, Janciauskiene S, Lindgren S. Circulating Monocytes and Plasma Inflammatory Biomarkers in Active Crohn's Disease: Elevated Oxidized Low-Density Lipoprotein and the Anti-Inflammatory Effect of Atorvastatin. *Inflamm Bowel Dis* (2004) 10:193–200. doi: 10.1097/00054725-200405000-00003
- Reinecker HC, Loh EY, Ringler DJ, Mehta A, Rombeau JL, MacDermott RP. Monocyte-Chemoattractant Protein 1 Gene Expression in Intestinal Epithelial Cells and Inflammatory Bowel Disease Mucosa. *Gastroenterology* (1995) 108:40–50. doi: 10.1016/0016-5085(95)90006-3
- Christophi GP, Rong R, Holtzapfel PG, Massa PT, Landas SK. Immune Markers and Differential Signaling Networks in Ulcerative Colitis and Crohn's Disease. *Inflammation Bowel Dis* (2012) 18:2342–56. doi: 10.1002/ibd.22957
- Magnusson MK, Strid H, Isaksson S, Bajor A, Lasso A, Ung K, et al. Response to Infliximab Therapy in Ulcerative Colitis is Associated With Decreased Monocyte Activation, Reduced CCL2 Expression and Downregulation of Tenascin C. *J Crohn's Colitis* (2015) 9:56–65. doi: 10.1093/ecco-jcc/jju008
- Kaushansky N, Bakos E, Becker-Herman S, Shachar I, Ben-Nun A. Circulating Picomolar Levels of CCL2 Downregulate Ongoing Chronic Experimental Autoimmune Encephalomyelitis by Induction of Regulatory Mechanisms. *J Immunol* (2019) 203:1857–66. doi: 10.4049/jimmunol.1900424
- Gaujoux R, Starosvetsky E, Maimon N, Vallania F, Bar-Yoseph H, Pressman S, et al. Cell-Centred Meta-Analysis Reveals Baseline Predictors of Anti-TNFα non-Response in Biopsy and Blood of Patients With IBD. *Gut* (2019) 68:604–14. doi: 10.1136/gutjnl-2017-315494
- Ma C, Battat R, Khanna R, Parker CE, Feagan BG, Jairath V. What is the Role of C-Reactive Protein and Fecal Calprotectin in Evaluating Crohn's Disease Activity? *Best Pract Res Clin Gastroenterol* (2019) 38–39:101602. doi: 10.1016/j.bpg.2019.02.004
- Mosli MH, Zou G, Garg SK, Feagan SG, MacDonald JK, Chande N, et al. C-Reactive Protein, Fecal Calprotectin, and Stool Lactoferrin for Detection of Endoscopic Activity in Symptomatic Inflammatory Bowel Disease Patients: A Systematic Review and Meta-Analysis. *Am J Gastroenterol* (2015) 110:802–19; quiz 820. doi: 10.1038/ajg.2015.120
- Rodrigues-Pinto E, Magro F, Santos-Antunes J, Vilas-Boas F, Lopes S, Nunes A, et al. High C-Reactive Protein in Crohn's Disease Patients Predicts Nonresponse to Infliximab Treatment. *J Crohns Colitis* (2013) 7:S208. doi: 10.1016/S1873-9946(13)60516-4
- Reinisch W, Wang Y, Oddens BJ, Link R. C-Reactive Protein, an Indicator for Maintained Response or Remission to Infliximab in Patients With Crohn's Disease: A Post-Hoc Analysis From ACCENT I. *Aliment Pharmacol Ther* (2012) 35:568–76. doi: 10.1111/j.1365-2036.2011.04987.x
- Colombel JF, Sandborn WJ, Reinisch W, Mantzaris GJ, Kornbluth A, Rachmilewitz D, et al. Infliximab, Azathioprine, or Combination Therapy for Crohn's Disease. *N Engl J Med* (2010) 362:1383–95. doi: 10.1056/NEJMoa0904492
- Sprakes MB, Ford AC, Warren L, Greer D, Hamlin J. Efficacy, Tolerability, and Predictors of Response to Infliximab Therapy for Crohn's Disease: A Large Single Centre Experience. *J Crohns Colitis* (2012) 6:143–53. doi: 10.1016/j.crohns.2011.07.011
- Goncz L, Vegh Z, Golovics PA, Rutka M, Gecse KB, Bor R, et al. Prediction of Short- and Medium-Term Efficacy of Biosimilar Infliximab Therapy. Do Trough Levels and Antidrug Antibody Levels or Clinical And Biochemical Markers Play the More Important Role? *J Crohns Colitis* (2017) 11:697–705. doi: 10.1093/ecco-jcc/jjw203
- Bar-Yoseph H, Levhar N, Selinger L, Manor U, Yavzori M, Picard O, et al. Early Drug and Anti-Infliximab Antibody Levels for Prediction of Primary

- Nonresponse to Infliximab Therapy. *Aliment Pharmacol Ther* (2018) 47:212–8. doi: 10.1111/apt.14410
35. Ding NS, Hart A, De Cruz P, Levesque BG, Vermeire S, Sandborn WJ. Systematic Review: Predicting and Optimising Response to Anti-TNF Therapy in Crohn's Disease - Algorithm for Practical Management. *Aliment Pharmacol Ther* (2016) 43:30–51. doi: 10.1111/apt.13445
 36. Papamichael K, Gils A, Rutgeerts P, Levesque BG, Vermeire S, Sandborn WJ, et al. Role for Therapeutic Drug Monitoring During Induction Therapy With TNF Antagonists in IBD: Evolution in the Definition and Management of Primary Nonresponse. *Inflamm Bowel Dis* (2015) 21:182–97. doi: 10.1097/MIB.0000000000000202

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Li, Chen, Zhang, Zhou, Chen, Zeng, Chen and Zhang. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Advantages of publishing in Frontiers



OPEN ACCESS

Articles are free to read
for greatest visibility
and readership



FAST PUBLICATION

Around 90 days
from submission
to decision



HIGH QUALITY PEER-REVIEW

Rigorous, collaborative,
and constructive
peer-review



TRANSPARENT PEER-REVIEW

Editors and reviewers
acknowledged by name
on published articles

Frontiers

Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org

Contact us: frontiersin.org/about/contact



REPRODUCIBILITY OF RESEARCH

Support open data
and methods to enhance
research reproducibility



DIGITAL PUBLISHING

Articles designed
for optimal readership
across devices



FOLLOW US

@frontiersin



IMPACT METRICS

Advanced article metrics
track visibility across
digital media



EXTENSIVE PROMOTION

Marketing
and promotion
of impactful research



LOOP RESEARCH NETWORK

Our network
increases your
article's readership