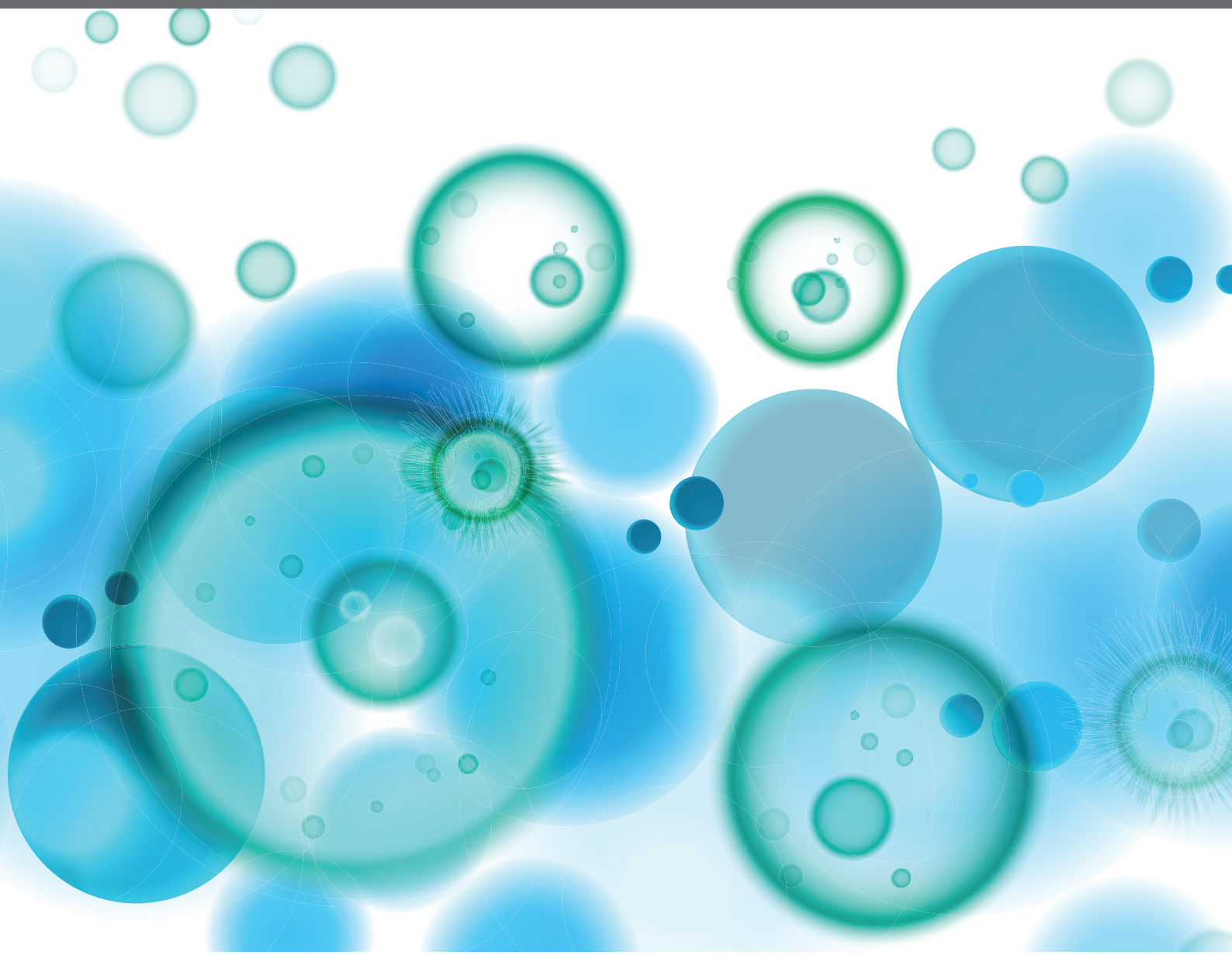
TOWARDS PRECISION MEDICINE IN VASCULITIS

EDITED BY: Federico Alberici, Andreas Kronbichler and Giacomo Emmi
PUBLISHED IN: Frontiers in Immunology



frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88966-349-1
DOI 10.3389/978-2-88966-349-1

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: researchtopics@frontiersin.org

TOWARDS PRECISION MEDICINE IN VASCULITIS

Topic Editors:
Federico Alberici, University of Milan, Italy
Andreas Kronbichler, Innsbruck Medical University, Austria
Giacomo Emmi, University of Florence, Italy

Citation: Alberici, F., Kronbichler, A., Emmi, G., eds. (2021). Towards Precision Medicine in Vasculitis. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-88966-349-1

Table of Contents

frontiers in Immunology

EDITORIAL
published: 17 November 2020
doi: 10.3389/fimmu.2020.616221

Editorial: "Toward Precision Medicine in Vasculitis"

Federico Alberici[1,2]*, *Giacomo Emmi*[3,4] and *Andreas Kronbichler*[5]

[1] Department of Medical and Surgical Specialties, Radiological Sciences and Public Health, University of Brescia, Brescia, Italy, [2] Nephrology Unit, Spedali Civili Hospital, ASST Spedali Civili di Brescia, Brescia, Italy, [3] Department of Experimental and Clinical Medicine, University of Firenze, Firenze, Italy, [4] Internal Interdisciplinary Medicine Unit, Careggi University Hospital of Firenze, Firenze, Italy, [5] Department of Internal Medicine IV (Nephrology and Hypertension), Medical University Innsbruck, Innsbruck, Austria

Keywords: antineutrophil cytoplasmic antibodies (ANCA)-associated vasculitis, biomarkers, Behçet's syndrome, uveitis, treatment

Vasculitis is a group of rare systemic autoimmune diseases that may be classified according to the size of the vessels involved (1). The prognosis of these diseases has been dramatically improved by immunosuppression although toxicity of such therapies is not negligible and the response to treatment may vary exposing subgroups of patients to the risk of relapsing and refractory disease (2). Of note, the therapeutic arrays are rapidly expanding and new treatment protocols combining different target therapies are being proposed urging the identification of clinical characteristics as well as biomarkers able to identify subgroups of patients more likely to benefit from specific approaches (3). Furthermore, the deepening of the understanding of the mechanisms of action of the drugs employed poses also the rationale for patients' monitoring that may in some cases guide re-treatment. For example, in the field of antineutrophil cytoplasmic antibodies (ANCA) associated vasculitis (AAV), the kinetics of CD20+ B-cells repopulation or increase of the ANCA titer in patients treated with the chimeric monoclonal anti-CD20 antibody rituximab, may be associated to an increased relapse risk (4). Moreover, these biomarkers have been explored as potentially able to guide patients' re-treatment (5). In this perspective, the research is also proposing biomarkers able to identify subgroups of patients less likely to respond to a specific therapy as well as posing the rationale for combining different biological drugs (6).

The aim of this special issue is to describe the state of the art of precision medicine in vasculitis. This collection contains 14 articles including eight original research publications and six reviews. The nice balance between original manuscripts and literature reviews supports the idea of this topic as an evolving concept.

Two reviews approached the broad topic of how genetic association and pharmacogenetics studies as well as studies of single-gene high-penetrance mutations, epigenetics factors, metabolomics and proteomics are contributing to the precision medicine in this field. These approaches are providing the rationale for advanced diseases classification, patients stratification while improving the understanding of the pathogenesis as well as identifying new therapeutic targets (Acosta-Herrera et al.; Demirkaya et al.).

OPEN ACCESS

Edited and reviewed by:
Pietro Ghezzi,
Brighton and Sussex Medical School, United Kingdom

***Correspondence:**
Federico Alberici
federico.alberici@unibs.it

Specialty section:
This article was submitted to Inflammation, a section of the journal Frontiers in Immunology

Received: *11 October 2020*
Accepted: *20 October 2020*
Published: *17 November 2020*

Citation:
Alberici F, Emmi G and Kronbichler A (2020) Editorial: "Toward Precision Medicine in Vasculitis". Front. Immunol. 11:616221. doi: 10.3389/fimmu.2020.616221

Of note, the vast majority of the remaining manuscripts of this issue focused on small vessels vasculitis: six original papers and three reviews.

In the three reviews of the literature, key opinion leaders described the state of the art in the field of precision medicine in AAV. Wallace et al. focused on the role of ANCA specificity in defining a personalized approach to patients' management further supporting the superiority for an ANCA-based classification of AAV compared to a classification based on clinical characteristics (Wallace and Stone). Shochet et al. focused on the role of animal models of AAV in the field of translational research addressing the intriguing issue of difficulties in the generation of animal models for PR3-ANCA AAV, leaving uncertainties on the pathogenetic role of PR3-ANCA compared to the well-established pathogenetic role of MPO-ANCA. Segelmark et al. reviewed the rationale and the data supporting the potential role of IdeS and EndoS, two enzymes produced by *Streptococcus pyogenes* capable of degrading IgG, and their potential as innovative therapeutic strategies in antibody mediated diseases such as small vessels vasculitis.

Original articles on AAV published in this issue focused on insights into disease pathogenesis, potential biomarkers, improvement in phenotypic characterization, and identification of potential therapeutic targets. Sun et al. demonstrated that thrombin could enhance MPO-ANCA induced activation of glomerular endothelial cells and that the protein sphingosine-1-phospahte (S1P) may act as link of the hyper-activation of the coagulation and inflammation system. This paper therefore provides a further rationale to the well-known tight link between inflammation and thrombosis as well as new potential therapeutic targets.

Two works focused on potential new biomarkers. Mhaonaigh et al. showed that low-density granulocytes may be associated to active vasculitis (Ui Mhaonaigh et al.), while Van Daalen et al. focused on the study of podocytes in patients with kidney involvement of AAV. In this report, of interest, proteinuria ten weeks after diagnosis correlated with podocytes foot process width; moreover, this characteristic was associated to different histological features at light microscopy. This study would be in support for a thorough assessment of podocytes at the moment of kidney biopsy providing important prognostic information further supporting the central role of kidney biopsies in patients with AAV (van Daalen et al.).

In everyday clinical practice, relying on clear and validated phenotypic classification of patients is key in order to have prognostic information especially in the field of rare diseases. Marques et al. described a big multicenter French cohort of patients with anti-glomerular basement membrane (anti-GBM) disease identifying several factors associated to poor prognosis in terms of overall survival as well as risk of end-stage kidney disease contributing significantly to the improvement of patients stratification (Marques et al.).

Eventually, the aim of focusing on precision medicine is improving patients' management, which also includes the identification of new potential therapeutic targets: two of the articles on AAV focused on this aspect. Pang et al., *via* the study of recombinant PR3 antigens and their interaction with the monoclonal antibody moANCA518, hypothesizes that the interaction between PR3 and PR3-ANCA may represent a potential target of interest (Pang et al.). On the other hand, Werner et al. showed that the negative co-stimulator B- and T-lymphocyte attenuator (BTLA) was diminished on double negative T-cells in remission samples of AAV patients and correlated with disease activity and relapse rate. The same study showed that T-cell inhibition *via* BTLA during T-cell receptor-mediated stimulation led to suppression of T-cell proliferation, inhibition of interleukin (IL)-17 and interferon (IFN)-gamma, suggesting that this may also represent a therapeutic target.

Behçet syndrome (BS) is a systemic vasculitis frequently posing diagnostic challenges and for which classification uncertainties do exist; a review by Bettiol et al. report the phenotypic rationale for sub-classifying BS in three subgroups (mucocutaneous and articular; extra-parenchymal neurological and peripheral vascular phenotype; the parenchymal neurological and ocular phenotype), further supporting the clinical observation that this condition may indeed be a complex spectrum of diseases ranging from mild phenotypes to life/organ threatening forms. Of note, the same article recommends a different therapeutic management for these phenotypes with a huge impact on patients' management.

In an original manuscript on BS, Emmi et al. focused on circulating hematopoietic progenitor cells (CPC), identifying a reduction of this stem cells in cases compared to healthy controls; moreover, the data would support the hypothesis that oxidative stress may contribute to CPC apoptosis suggesting a possible role for these mechanisms in counteracting the vascular repair actions of these cells (19).

Typically, BS is characterized by ocular involvement (i.e posterior uveitis/panuveitis). Bonacini et al. profiled cytokines in aqueous humor of patients with non-infectious uveitis secondary to BS, Vogt Koyanagi Harada disease and healthy controls. The authors found a different intra-ocular cytokine profile in patients with auto-inflammatory uveitis. At the same time, the profile of cytokines identified as potential therapeutic targets are already part of the therapeutic armamentarium and their impact should be better clarified in future.

In conclusion, this special issue collects reports highlighting current state of the art in the field of precision medicine in vasculitides as well as original manuscripts identifying new biological as well as phenotypic markers that may contribute to further progress in this field. We believe that the reader will benefit from a broad overview that will contribute deeply to the general the understanding on this topic.

AUTHOR CONTRIBUTIONS

FA drafted a version. GE and AK reviewed and completed it. All authors contributed to the article and approved the submitted version.

REFERENCES

1. Jennette JC, Falk RJ, Bacon PA, Basu N, Cid MC, Ferrario F, et al. Revised International Chapel Hill Consensus Conference Nomenclature of Vasculitides. *Arthritis Rheumatol* (2012) 2013:65(1):1–11. doi: 10.1002/art.37715
2. Alberici F, Jayne DRW. Impact of rituximab trials on the treatment of ANCA-associated vasculitis. *Nephrol Dial Transplant Off Publ Eur Dial Transpl Assoc Eur Ren Assoc* (2014) 29(6):1151–9. doi: 10.1093/ndt/gft318
3. Tedesco M, Gallieni M, Pellegata F, Cozzolino M, Alberici F. Update on ANCA-associated vasculitis: from biomarkers to therapy. *J Nephrol* (2019) 32 (6):871–82. doi: 10.1007/s40620-019-00628-9
4. Alberici F, Smith RM, Jones RB, Roberts DM, Willcocks LC, Chaudhry A, et al. Long-term follow-up of patients who received repeat-dose rituximab as maintenance therapy for ANCA-associated vasculitis. *Rheumatol Oxf Engl* (2015) 54(7):1153–60. doi: 10.1093/rheumatology/keu452
5. Charles P, Terrier B, Perrodeau É, Cohen P, Faguer S, Huart A, et al. Comparison of individually tailored versus fixed-schedule rituximab regimen to maintain ANCA-associated vasculitis remission: results of a multicentre, randomised controlled, phase III trial (MAINRITSAN2). *Ann Rheum Dis* (2018) 77(8):1143–9. doi: 10.1136/annrheumdis-2017-212878
6. Alberici F, Smith RM, Fonseca M, Willcocks LC, Jones RB, Holle JU, et al. Association of a TNFSF13B (BAFF) regulatory region single nucleotide polymorphism with response to rituximab in antineutrophil cytoplasmic antibody-associated vasculitis. *J Allergy Clin Immunol* (2017) 139(5):1684–7.e10. doi: 10.1016/j.jaci.2016.08.051

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.

frontiers in Immunology

ORIGINAL RESEARCH
published: 15 February 2019
doi: 10.3389/fimmu.2019.00237

Thrombin Contributes to Anti-myeloperoxidase Antibody Positive IgG-Mediated Glomerular Endothelial Cells Activation Through SphK1-S1P-S1PR3 Signaling

Xiao-Jing Sun[1], *Min Chen*[1,2*] *and Ming-Hui Zhao*[1,2,3]

[1] *Renal Division, Department of Medicine, Peking University, First Hospital, Peking University Institute of Nephrology, Beijing, China,* [2] *Key Laboratory of Renal Disease, Ministry of Health of China, Beijing, China,* [3] *Key Laboratory of Chronic Kidney Disease Prevention and Treatment, Ministry of Education, Peking-Tsinghua Center for Life Sciences, Peking University, Beijing, China*

Background: Activation of coagulation system plays an important role in antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) pathogenesis. Thrombin, generated during coagulation could disrupt endothelial barrier integrity through protease-activated receptor 1 (PAR1). Our previous study found that sphingosine-1-phosphate (S1P) contributed to myeloperoxidase (MPO)-ANCA-positive IgG-induced glomerular endothelial cell (GEnC) activation through a S1P receptor (S1PR)-dependent route. In recent years, S1P signaling was reported to be involved in thrombin effects on endothelial cells. This current study investigated whether the interaction between thrombin-PAR and S1P-S1PR signaling contributed to MPO-ANCA-positive IgG-induced GEnC dysfunction.

Methods: The effect of thrombin on GEnC activation was analyzed from three aspects. First, morphological alteration of GEnCs was observed. Second, permeability assay was performed to determine GEnC monolayer activation quantitatively. Third, endothelin-1 (ET-1) levels were measured. Expression levels of sphingosine kinases (SphKs) and S1PRs were detected. In addition, antagonists of PAR1 and S1PR3 were employed to determine their roles. Eventually, PAR1 and tissue factor (TF) expression levels as well as TF procoagulant activity were analyzed.

Results: Thrombin induced further damage of tight junction, increase in endothelial monolayer permeability as well as upregulation of ET-1 levels in GEnCs stimulated with MPO-ANCA-positive IgG. Blocking PAR1 downregulated ET-1 levels in the supernatants of GEnCs treated by thrombin plus MPO-ANCA-positive IgG. Expression levels of SphK1, S1PR3 increased significantly in GEnCs treated with thrombin plus MPO-ANCA-positive IgG. S1P upregulated PAR1 and TF expression, and enhanced procoagulant activity of TF in MPO-ANCA-positive IgG-stimulated GEnCs.

Conclusion: Thrombin synergized with SphK1-S1P-S1PR3 signaling pathway to enhance MPO-ANCA-positive IgG-mediated GEnC activation.

Keywords: ANCA, vasculitis, thrombin, sphingosine-1-phosphate, endothelium

OPEN ACCESS

Edited by:
Andreas Kronbichler, Innsbruck Medical University, Austria

Reviewed by:
Matija Crnogorac, Clinical Hospital Dubrava, Croatia
Sina Maren Coldewey, Universitätsklinikum Jena, Germany

***Correspondence:**
Min Chen
chenmin74@sina.com

Specialty section:
This article was submitted to Inflammation, a section of the journal Frontiers in Immunology

Received: *24 October 2018*
Accepted: *28 January 2019*
Published: *15 February 2019*

Citation:
Sun X-J, Chen M and Zhao M-H (2019) Thrombin Contributes to Anti-myeloperoxidase Antibody Positive IgG-Mediated Glomerular Endothelial Cells Activation Through SphK1-S1P-S1PR3 Signaling. Front. Immunol. 10:237. doi: 10.3389/fimmu.2019.00237

INTRODUCTION

Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) consists of eosinophilic granulomatosis with polyangiitis (EGPA), granulomatosis with polyangiitis (GPA) and microscopic polyangiitis (MPA) (1). AAV is characterized by necrotizing inflammation of the small blood vessels, which involves glomerular endothelial cell (GEnC) injury in particular. The serological hallmarks for AAV are ANCAs against either proteinase 3 (PR3) or myeloperoxidase (MPO) (2, 3). The majority of Chinese AAV patients are MPO-ANCA-positive, as reported in our previous studies (4, 5). In addition, cumulating evidences suggest that MPO-ANCAs cause GEnC activation and injury directly in AAV (6, 7).

Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid metabolite and produced by phosphorylation of sphingosine by sphingosine kinases (SphKs). S1P is the ligand for five G-protein-coupled receptors (GPCRs) named S1PR1-5 (8). S1P and S1PRs participate in the pathogenesis of a variety of vascular inflammatory conditions including ischemia-reperfusion injury, atherosclerosis and sepsis (9–11). In recent years, clinical trials that targeted S1PRs for autoimmune diseases have attracted wide interest. Of note, FTY720 (Fingolimod, Gilenya, Novartis), a functional antagonist of S1PR1, 3, 4, and 5, has already been approved and used in treating multiple sclerosis (12–14). Moreover, cumulating evidences supported a vital role of FTY720 in endothelial barrier enhancement both *in vivo* and *in vitro* (15–17). In our previous studies, we found that the circulating levels of S1P and the renal expression of S1PRs correlated with renal involvement and disease activity of AAV. In addition, it was found that S1P enhanced MPO-ANCA-positive IgG-induced GEnC activation through S1PR2-5 and RhoA signaling pathway (18–20). All these studies indicated a pathogenic role of S1P in AAV.

Although the pathogenesis of AAV is not yet fully clear, the interaction among ANCA, neutrophils and complement activation is of vital importance in the development of this disease [reviewed by Chen et al. (21)]. In recent years, more and more evidence has suggested that activation of coagulation system may also play an important role. Patients with AAV are in a hypercoagulable state, with an increased risk of developing venous thromboembolic events (22, 23). Moreover, the interaction between coagulation and complement system also contributes to the pathogenesis of glomerular capillary tuft infarction and to the increased frequency of thromboembolic events in AAV. Some serine proteases from the coagulation cascade, in particular plasmin and thrombin, can directly activate C3 and C5, independent of the traditional C3/C5 convertase (24, 25). C5a-primed neutrophils produce tissue-factor-expressing microparticles and neutrophil extracellular traps (NETs) after stimulation with ANCAs, which subsequently activate the coagulation system (26). Platelets are activated *via* thrombin-PARs pathway and can activate the alternative complement pathway in AAV (27).

The coagulation system is initiated in two distinct mechanisms: the contact pathway and the tissue factor (TF) pathway. Both pathways result in the generation of thrombin, the best-characterized activator of protease-activated receptors (PARs) (28). PARs are a family of G protein-coupled receptors including 4 members named PAR1-4. PAR1 is the major effector of thrombin signaling in most cell types including endothelial cells. Thrombin activates PAR1 by catalyzing the cleavage of the Arg41-Ser42 peptide bond on the N-terminal extracellular domain of the receptor (29). It was reported that thrombin-activated PAR1 could induce disruption of endothelial barrier integrity (30).

Thrombin effects in endothelial cells involve S1P signaling. According to Tauseef et al. SphK1-S1P-S1PR1 signaling could counteract the detrimental effect of thrombin-PAR1 signaling on endothelial barrier function. On the one hand, thrombin-activated-PAR1 interrupts endothelial barrier integrity *via* Rho signaling pathway; on the other hand, thrombin also induces expression of SphK1 and increases S1P generation, which in turn transactivates S1PR1 leading to the activation of Rac1 signaling pathway. This effect improves endothelial integrity to counteract and limit thrombin-induced endothelial damage and vascular leakage (31). However, some other studies revealed a synergistic effect of S1P on thrombin-induced endothelial dysfunction, including enhanced NF-κB binding activity and TF expression in endothelial cells (32, 33). Given the potential effect of thrombin-PAR and SphK-S1P-S1PR signaling on regulating endothelial barrier function, our current study aimed to investigate whether the interaction between thrombin-PAR and SphK-S1P-S1PR signaling participated in MPO-ANCA-positive IgG-induced GEnC dysfunction.

MATERIALS AND METHODS

Cell Culture

Primary human glomerular endothelial cells (GEnC; ScienCell, San Diego, CA, USA) were cultured in endothelial cell basal medium (ECM) (ScienCell San Diego, CA, USA) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% endothelial cell growth factor. Cultures were grown in an atmosphere of 5% CO_2 at 37°C. After starving in ECM with additional 0.5% FBS for 8 h, GEnC in selected wells were washed with phosphate buffered saline (PBS) and then stimulated with thrombin (Sigma, Darmstadt, Germany), MPO-ANCA-positive IgG, normal IgG or 2 μmol/L S1P (Sigma, Darmstadt, Germany), which was comparable to the levels of circulating S1P in AAV patients at active stage, as demonstrated by our previous study (18).

Preparation of Immunoglobulin (Ig)Gs

Preparation of IgGs was performed according to the methods described previously (34). MPO-ANCA-positive IgGs and normal IgGs and were prepared from plasma exchange liquid of eight patients with active MPO-ANCA-positive primary small vessel vasculitis and plasma of six healthy donors, respectively. Then we further screened the prepared IgGs for the presence of anti-endothelial cell antibody (AECA) through an ELISA method described previously (35), and AECA-positive IgGs were excluded in our following experiments. Eventually, normal IgGs from plasma of five healthy donors and MPO-ANCA-positive

IgGs from plasma exchange liquid of five AAV patients were included, respectively. Our research was in compliance with the Declaration of Helsinki and approved by the clinical research ethics committee of the Peking University First Hospital.

Measurements of GEnC Activation and Injury

Immunofluorescence Staining of Zonula Occludens-1 (ZO-1) and Vascular Endothelial (VE)-Cadherin

As important markers for endothelial barrier function, the distribution of the tight junction scaffolding protein ZO-1 and adherens junction protein VE-cadherin were observed (36). After relevant treatment, GEnCs were washed in PBS and fixed with 4% formaldehyde for 30 min. Next, the GEnCs were permeabilized with 0.5% Triton X-100, washed and blocked with 5% BSA for 1 h at room temperature. After incubation with primary antibodies (ZO-1, dilution 1/100, Life, Carlsbad, CA, USA; VE-cadherin, dilution 1/200, Abcam, Cambridge, MA, USA) at 4°C overnight and a thorough wash in PBS, the GEnCs were incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (for the detection of ZO-1, dilution 1/200, Jackson ImmunoResearch, West Grove, PA, USA; for the detection of VE-cadherin, dilution 1/500, Abcam, Cambridge, MA, USA) at 37°C for 1 h. Eventually, the specimens were stained with 10 μg/ml 4',6-diamidino-2-phenylindole (DAPI) and mounted with Mowiol. The immunofluorescence staining was photographed by a fluorescence microscope (Nikon Eclipse 90i, Nikon Instruments Inc., Tokyo, Japan). At least 10 visual fields per slide of GEnCs at ×400 were observed blindly. Image J software (National Institutes of Health, Bethesda, MD, USA) was used to evaluate the immunofluorescence staining of ZO-1 and VE-cadherin. Positive signals were quantified as signal intensity.

Permeability Assay

The permeability of GEnC monolayers was determined using Costar Transwell plate with 0.5-μm porous filters and FITC-labeled BSA (Sigma-Aldrich, Darmstadt, Hessen, Germany), as described previously (37). GEnCs were grown on the upper chamber of Costar Transwell until confluent. The tracer protein FITC-albumin was added to the upper chamber after relevant stimulation. After incubation at 37°C for 30 min, samples from both the upper and lower chambers were collected for fluorometric analysis. Fluorescent intensity (FI) was measured using a microplate fluorescence reader (Tristar™ LB941, Berthold, Germany) with filter settings of 485 nm (excitation) and 538 nm (emission). Eventually, these fluorescence readings were used for calculation of the permeability coefficient, which is indicative of vascular barrier disruption. The permeability coefficient was calculated according to the following formula:

Permeability coefficient = FI (lower chamber) × 100% / (FI (upper chamber) + FI (lower chamber)).

Evaluation of Endothelium Activation by Endothelin-1 (ET-1) Quantification

As a biomarker of endothelial cell activation and injury (38), ET-1 levels in GEnC supernatants were measured using commercial ELISA kits (R&D, Minneapolis, MN, USA).

TABLE 1 | Sequences of PCR primers used.

Gene	Forward primer 5'-3'	Reverse primer 5'-3'
SphK1	AAACCCCTGTGTAGCCTCCC	AGCAGGTTCATGGGT GACAG
SphK2	GCACAGCAACAGTGAGCA-3'	GAGCCTGAG TGAGTG GGA
S1PR1	CACTCTGACCAACAAGGAGATG	GATGATGGGTCGCTTGAATTTG
S1PR2	AAGTTCCACTCGGCAATGTA	AGCCAGAGAGCAAGGTATTG
S1PR3	TCTCCGAAGGTCAAGGAAGA	TCAGTTGCAGAAGATCCCATTC
S1PR4	CTGAAGACGGTGCTGATGAT	CAGAGGTTGGAGCCAAAGA
S1PR5	GGTCATCGTCCTGCATTACA	CTAGATTCTCTAGCACGATGA AGG
PAR1	CAGGCACTACAAATACTGTGG	TGTAGACTTGATTGACGGGTT
TF	GCCAGGAGAAAGGGGAAT	CAGTGCAATATAGCATTTGCA GTAGC
β-actin	GGACCTGACTGACTACCTCAT	CGTAGCACAGCTTCTCCTTAAT
GAPDH	GAGTCAACGGATTTGGTCGT	GACAAGCTTCCCGTTCTCAG

Measurement of SphKs, S1PRs, PARs, and TF

SphK1 and 2, S1PR1-5, PAR1, and TF expression levels were determined by quantitative real-time polymerase chain reaction (qRT-PCR). GEnCs were washed in Dulbecco's phosphate-buffered saline (D-PBS) and total RNA was extracted using a commercial RNA purification kit (Thermo scientific, Waltham, MA, USA). Concentration and purity of RNA samples were determined by reading absorbance at 260 and 280 nm with a spectrophotometer (Nanodrop, Thermo fisher scientific, Wilmington, DE, USA). After cDNA synthesis using GoScript™Reverse Transcriptase (Promega, Madison, WI, USA), mRNA levels were determined by quantitative polymerase chain reaction (q-PCR) on an Applied Biosystems system (ViiA7) using Power SYBR® Green PCR Master Mix (Applied Biosystems, Austin, TX, USA). Amplifications were pre-incubation at 95°C for 10 min, followed by 40 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s. Values were expressed as $2^{-\Delta\Delta CT}$. β-actin and GAPDH were used as endogenous controls. Primers used are listed in **Table 1**.

Detection of SphK1 by Western Blot

Samples were incubated for 10 min at 95°C in loading buffer. Samples were then subjected to electrophoresis on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated with primary antibodies (for the detection of SphK1, dilution 1/1000, Abcam, Cambridge, MA, USA; for the detection of β-actin, dilution 1/1000, Santa Cruz, Dallas, TX, USA) followed by horseradish peroxidase-conjugated secondary antibodies (each diluted 1:2000; both from Proteintech, Chicago, IL, USA). Proteins were visualized on autoradiographic film using an ECL Plus Western blot detection system (GE Healthcare).

Inhibition of PAR1 and S1PR3

RWJ 56110 (RWJ; Tocris, Louis, MO, USA) is a selective PAR1 antagonist (39). TY52156 (TY; Tocris, Louis, MO, USA) is a specific antagonist for S1PR3 (40). In thrombin-induced ET-1 expression assay, GEnCs were incubated with RWJ and TY for

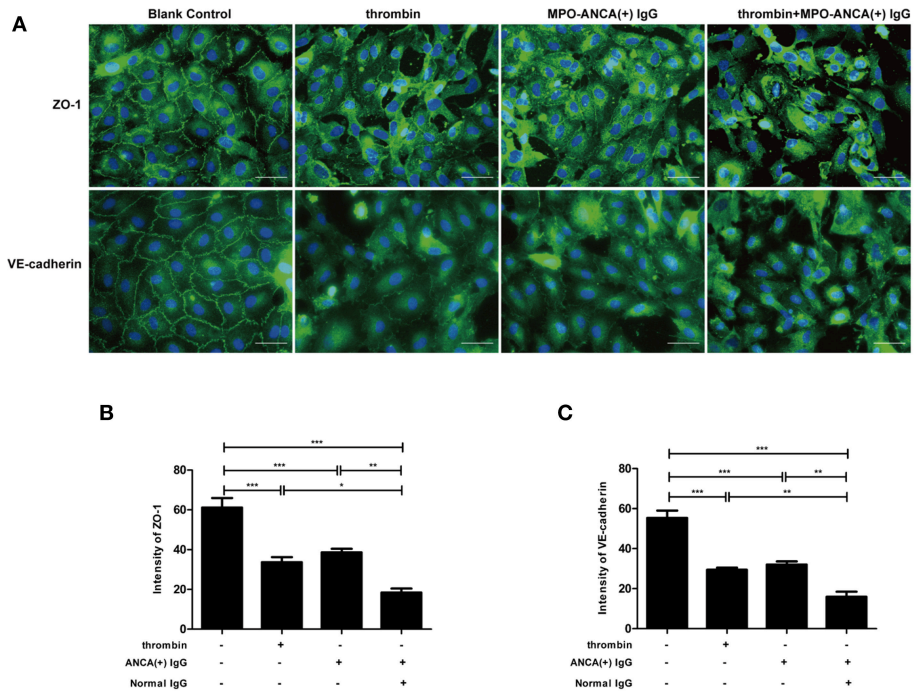


FIGURE 1 | Thrombin could enhance MPO-ANCA-positive IgG-mediated GEnC activation. **(A)** Thrombin could induce alterations in cellular morphology of GEnCs in the presence of MPO-ANCA-positive IgG. **(B)** Quantitive assessment of ZO-1 in GEnCs upon stimulation by thrombin and MPO-ANCA-positive IgG. **(C)** Quantitive assessment of VE-cadherin in GEnCs upon stimulation by thrombin and MPO-ANCA-positive IgG. Bars represent mean ± SD of repeated measurements of five independent experiments or donors. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

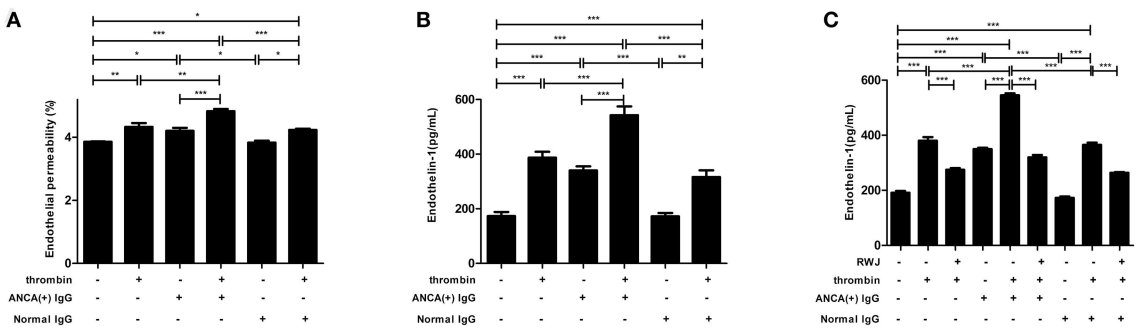


FIGURE 2 | (A) Thrombin could induce increased endothelial permeability of GEnC monolayers in the presence of MPO-ANCA-positive IgG. **(B)** Thrombin could upregulate ET-1 levels in the supernatant of GEnCs in the presence of MPO-ANCA-positive IgG. **(C)** PAR1 mediated the thrombin-induced ET-1 upregulation in GEnCs in the presence of MPO-ANCA-positive IgG. Bars represent mean ± SD of repeated measurements of five independent experiments or donors. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

different doses and time points. Eventually, 1 μM RWJ at 15 min and 1 μM TY at 15 min were selected for the experiments due to the highest inhibition rates.

TF Procoagulant Activity Assay

To analyze TF procoagulant activity, a Cell Tissue Factor Assay Kit (Genmed Scientifics Inc, Wilmington, DE, USA) was used following manufacturer's instructions. GEnCs were lysed and 50 μg proteins and $\sim 2 \times 10^6$ cells of each sample were used. Samples were incubated with prothrombin complex (including Factor II, VII, IX, X) and $CaCl_2$. Reaction was terminated by adding EDTA buffer. Eventually, we added a chromogenic substrate (Spectrozyme factor Xa) and measured the absorbance at 405 nm.

Statistical Analysis

SPSS version 13.0 (SPSS Inc., Chicago, IL, USA) was used to perform data analysis. Normality of the data was evaluated by kurtosis and skewness (both the absolute values were <3). Data was generally presented as mean ± standard deviation (SD) and compared by ANOVA followed by Bonferroni correction for multiple testing. $P < 0.05$ were considered statistically significant.

RESULTS

Thrombin Amplifies MPO-ANCA-Positive IgG-Mediated GEnC Dysfunction *via* PAR1

Thrombin Induces GEnC Morphological Alteration of GEnC Monolayers

Immunofluorescence staining of ZO-1 and VE-cadherin were performed to observe the structure of the tight junction and adherens junction in GEnCs, respectively. We found that compared with untreated cells, the application of thrombin or MPO-ANCA-positive IgG alone could disrupt tight junction and adherens junction structures (33.71 ± 5.65 vs. 61.14 ± 10.83, $P < 0.001$; 38.60 ± 4.05 vs. 61.14 ± 10.83, $P < 0.001$; 32.04 ± 3.63 vs. 55.39 ± 8.11, $P < 0.001$; 29.44 ± 2.41 vs. 55.39 ± 8.11, $P < 0.001$, respectively). Moreover, combined application of thrombin and MPO-ANCA-positive IgG induced further damage of tight junction and adherens junction compared with all the above-mentioned cell groups (18.43 ± 4.46 vs. 61.14 ± 10.83, $P < 0.001$; 18.43 ± 4.46 vs. 33.71 ± 5.65, $P < 0.05$; 18.43 ± 4.46 vs. 38.60 ± 4.05, $P < 0.01$; 15.98 ± 5.57 vs. 55.39 ± 8.11, $P < 0.001$; 15.98 ± 5.57 vs. 32.04 ± 3.63, $P < 0.01$; 15.98 ± 5.57 vs. 29.44 ± 2.41, $P < 0.01$, respectively) (**Figure 1**). These data revealed that thrombin synergized with MPO-ANCA-positive IgG to exert damage effects on endothelial barrier integrity.

Thrombin Induces Increased Endothelial Permeability in GEnC Monolayers

We used a transwell system and a FITC-labeled BSA to investigate the effect of S1P on monolayer permeability in GEnCs. The results revealed that compared with untreated cells, monolayer permeability increased in GEnCs stimulated with thrombin or MPO-ANCA positive IgG alone (4.33 ± 0.27% vs. 3.86 ± 0.03%, $P < 0.01$; 4.21 ± 0.21% vs. 3.86 ± 0.03%, $P < 0.05$, respectively). Furthermore, compared with the above cells, monolayer permeability still increased significantly in GEnCs stimulated by thrombin plus MPO-ANCA-positive IgG (4.83 ± 0.15% vs. 3.86 ± 0.03%, $P < 0.001$; 4.83 ± 0.15% vs. 4.33 ± 0.27%, $P < 0.01$; 4.83 ± 0.15% vs. 4.21 ± 0.21%, $P < 0.001$, respectively) (**Figure 2A**). These data suggested that thrombin enhanced MPO-ANCA-positive IgG-mediated increasing of GEnC permeability.

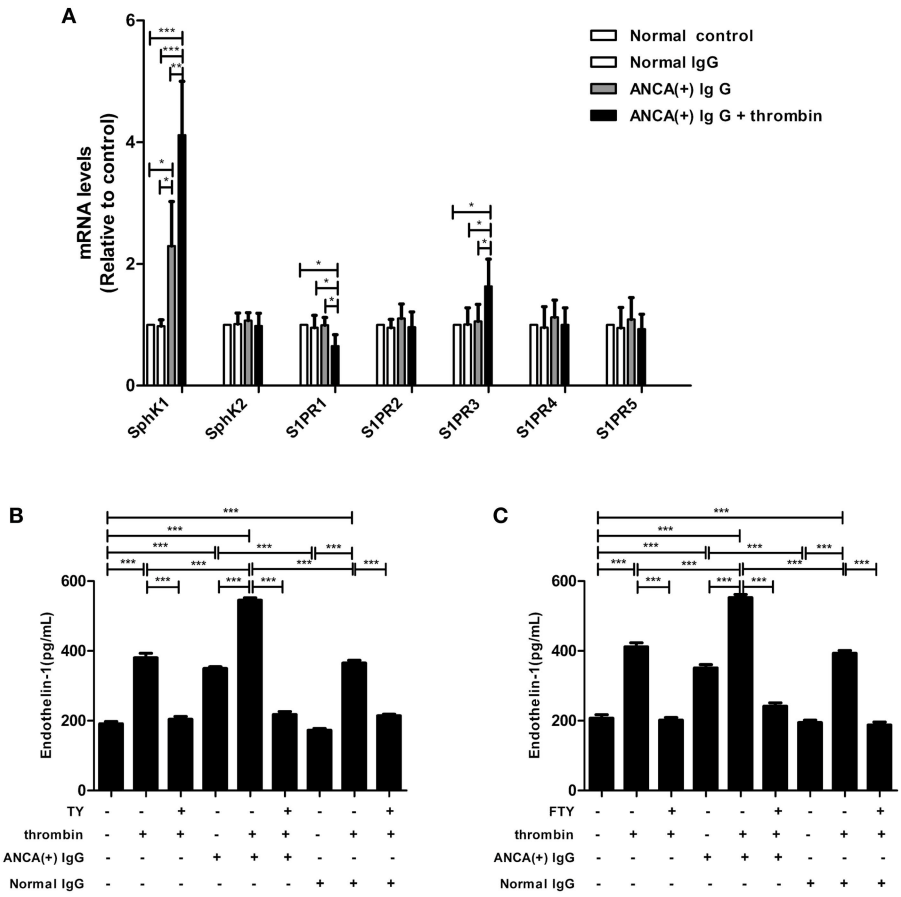


FIGURE 3 | SphK1–S1P-S1PR3 signaling was involved in thrombin -induced MPO-ANCA-positive IgG-mediated GEnC activation. **(A)** SphK1 and S1PR3 expression levels were elevated in MPO-ANCA-positive IgG-treated GEnCs upon thrombin stimulation. **(B)** S1PR3 mediated the thrombin-induced ET-1 upregulation in MPO-ANCA-positive IgG-treated GEnCs. **(C)** FTY720 significantly downregulated ET-1 levels in the supernatants of GEnCs stimulated by thrombin and MPO-ANCA-positive IgG. Bars represent mean ± SD of repeated measurements of five independent experiments or donors. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Thrombin Increases ET-1 Levels in GEnC Supernatants

As a biomarker of endothelial cell activation and injury, ET-1 levels in the supernatants of GEnCs were measured. It was found that compared with unstimulated cells, cells stimulated by thrombin or MPO-ANCA-positive IgG alone, the levels of ET-1 increased significantly in GEnCs treated with thrombin and MPO-ANCA-positive IgG (542.82 ± 71.58 pg/ml vs. 173.10 ± 33.48 pg/ml, $P < 0.001$; 542.82 ± 71.58 pg/ml vs. 387.33 ± 47.89 pg/ml, $P < 0.001$; 542.82 ± 71.58 pg/ml vs. 340.47 ± 32.77 pg/ml, $P < 0.001$, respectively) (**Figure 2B**). Collectively, these data illustrated that thrombin synergized with MPO-ANCA-positive IgG to upregulate the levels of ET-1 in the GEnC supernatants.

PAR1 Mediates the Thrombin-Induced Endothelial Dysfunction

GEnCs were pre-incubated with PAR1 antagonist RWJ for 15 min before stimulation with thrombin and MPO-ANCA-positive IgG, and the ET-1 levels in the supernatants were measured. We found that the ET-1 levels reduced from 545.39 ± 15.06 pg/ml in the supernatants of GEnCs stimulated by thrombin and MPO-ANCA-positive IgG to 319.86 ± 19.07 pg/ml, upon pre-incubation with PAR1 antagonist RWJ (compared with that without the antagonist, $P < 0.001$, with the inhibition rate of 41.35 ± 3.50%) (**Figure 2C**). These data revealed that PAR1 mediated ET-1 upregulation in thrombin and MPO-ANCA-positive IgG-treated GEnCs.

Thrombin Amplifies MPO-ANCA-Positive IgG-Mediated GEnC Dysfunction Through SphK-S1P-S1PR Signaling Crosstalk

SphK1 and S1PR3 Expression Levels Are Elevated in MPO-ANCA-Positive IgG-Treated GEnCs Upon Thrombin Stimulation

SphK1,2 and S1PR1-5 expression levels in GEnCs were measured by qRT-PCR. It was found that compared with GEnCs stimulated by MPO-ANCA-positive IgG alone, the expression levels of SphK1 and S1PR3 in GEnCs treated with thrombin plus MPO-ANCA-positive IgG increased significantly (4.12 ± 0.88 vs. 2.30 ± 0.73, $P < 0.01$; 1.63 ± 0.45 vs. 1.06 ± 0.28, $P < 0.05$, respectively), whereas S1PR1 expression level decreased significantly in GEnCs treated with thrombin plus MPO-ANCA-positive IgG (0.65 ± 0.19 vs. 0.99 ± 0.13, $P < 0.05$) (**Figure 3A**). The protein expression levels of SphK1 were also detected with Western blot. Consistent with the results of PCR, the expression levels of SphK1 in GEnCs stimulated with thrombin plus MPO-ANCA-positive IgG were higher than those in the other groups (**Figure S1**).

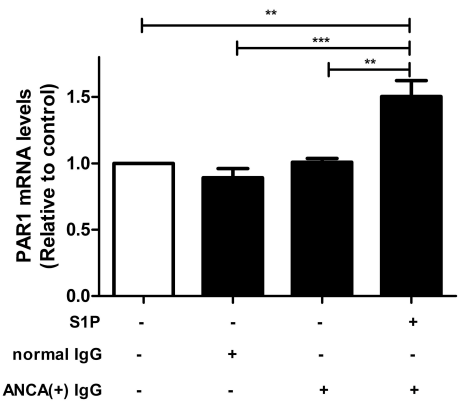


FIGURE 4 | PAR1 expression levels were elevated in MPO-ANCA-positive IgG-treated GEnCs upon stimulation by S1P. Bars represent mean ± SD of repeated measurements of five independent experiments or donors. $^{**}P < 0.01$, $^{***}P < 0.001$.

S1PR3 Mediates the Thrombin-Induced Endothelial Dysfunction

GEnCs were pre-incubated with S1PR3 antagonist TY for 15 min before stimulation with thrombin and MPO-ANCA-positive IgG, and the ET-1 levels in the supernatants were measured. We found that pre-incubation of GEnCs with TY significantly decreased ET-1 level in the supernatants of GEnCs stimulated by thrombin and MPO-ANCA-positive IgG (545.39 ± 15.06 pg/ml vs. 217.52 ± 18.99 pg/ml, $P < 0.001$, with the inhibition rate of 60.12 ± 3.48%) (**Figure 3B**). These data revealed that S1PR3 activation was involved in thrombin-induced ET-1 upregulation in GEnCs in the presence of MPO-ANCA-positive IgG.

We also pre-incubated GEnCs with FTY720 before stimulated with thrombin and MPO-ANCA-positive IgG, and the ET-1 levels in the supernatants were measured. We found that the ET-1 levels reduced from 552.69 ± 20.46 pg/ml in the supernatants of GEnCs stimulated by thrombin and MPO-ANCA-positive IgG to 241.53 ± 21.22pg/ml, upon pre-incubation with FTY720 (compared with those without FTY720, $P < 0.001$, with the inhibition rate of 43.70 ± 3.84%) (**Figure 3C**).

PAR1 Expression Levels Are Elevated in GEnCs Upon Stimulation by S1P

GEnCs were stimulated with MPO-ANCA-positive IgG plus 2 μmol/L S1P, which was comparable to the levels of circulating S1P in AAV patients at active stage, as demonstrated by our previous study (12), and PAR1 expression levels in GEnCs were measured by qRT-PCR. It was found that compared with GEnCs stimulated by MPO-ANCA-positive IgG alone, the expression levels of PAR1 in GEnCs treated with S1P plus MPO-ANCA-positive IgG increased significantly (1.50 ± 0.27 vs. 1.01 ± 0.06, $P < 0.01$) (**Figure 4**).

S1P Enhances the Expression and Activity of TF in GEnCs in the Presence of MPO-ANCA-Positive IgG

TF expression levels were detected using qRT-PCR, and it was found that compared with untreated cells, cells stimulated by S1P or MPO-ANCA-positive IgG alone, the TF levels increased significantly in GEnCs stimulated by S1P and MPO-ANCA-positive IgG (3.03 ± 0.66 vs. 1.00, $P < 0.001$; 3.03 ± 0.66 vs. 2.08 ± 0.39, $P < 0.05$; 3.03 ± 0.66 vs. 2.00 ± 0.68, $P < 0.05$, respectively) (**Figure 5A**). TF procoagulant activity was also measured using a commercial kit. The results demonstrated

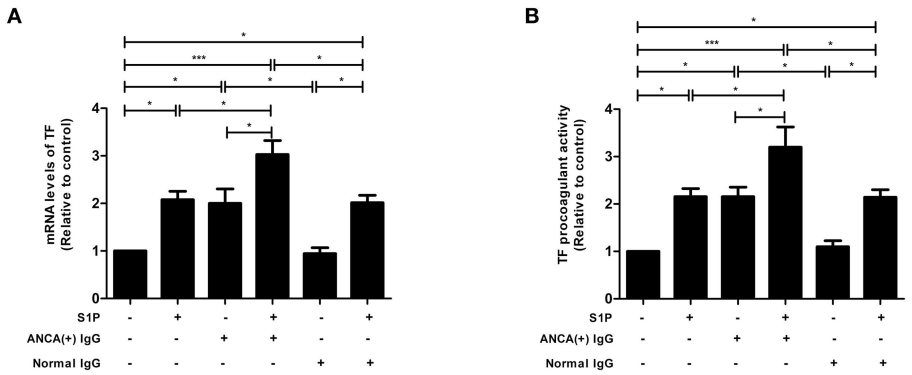


FIGURE 5 | S1P enhanced the expression and activity of TF in GEnCs in the presence of MPO-ANCA-positive IgG. **(A)** S1P enhanced the expression levels of TF in GEnCs in the presence of MPO-ANCA-positive IgG. **(B)** S1P enhanced the procoagulant activity of TF in GEnCs in the presence of MPO-ANCA-positive IgG. Bars represent mean ± SD of repeated measurements of five independent experiments or donors. $^{*}P < 0.05$, $^{***}P < 0.001$.

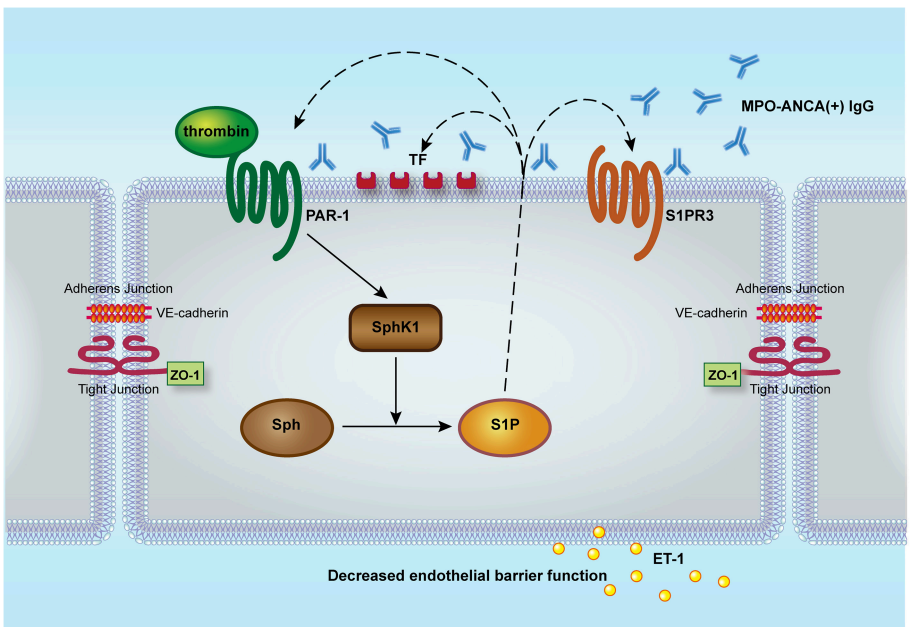


FIGURE 6 | Proposed working model for the role of SphK1-S1P-S1PR3 in thrombin-induced GEnC activation in the presence of MPO-ANCA-positive IgG. Thrombin could enhance MPO-ANCA-positive IgG-induced GEnC activation and injury *via* PAR1. At the same time, thrombin might activate SphK1-S1P-S1PR3 axis in GEnCs in the presence of MPO-ANCA-positive IgG. Furthermore, S1P of pathophysiological concentration in active AAV patients might induce PAR1 expression as well as enhance both expression level and activity of tissue factor in MPO-ANCA-positive IgG-treated endothelial cells, which might further activate the coagulation system, thus forming a vicious loop. S1P, sphingosine-1-phosphate; S1PR, sphingosine-1-phosphate receptor; Sph, sphingosine; SphK, sphingosine kinases; PAR, protease-activated receptor; ET-1, endothelin-1; ZO-1, zonula occludens-1.

that compared with untreated cells, cells stimulated by S1P or MPO-ANCA-positive IgG alone, the activity of TF increased significantly in GEnCs stimulated by S1P and MPO-ANCA-positive IgG (3.20 ± 0.95 vs. 1.00, $P < 0.001$; 3.20 ± 0.95 vs. 2.16 ± 0.38, $P < 0.05$; 3.20 ± 0.95 vs. 2.15 ± 0.45, $P < 0.05$, respectively) (**Figure 5B**). Collectively, these data illustrated that S1P, with pathophysiological concentration of active AAV patients, synergized with MPO-ANCA-positive IgG to promote both the expression and activity of TF in GEnCs.

DISCUSSION

In our current study, we demonstrated that thrombin could enhance MPO-ANCA-positive IgG-induced GEnC activation *via* PAR1, and thrombin could activate SphK1-S1P-S1PR3 axis in GEnCs in the presence of MPO-ANCA-positive IgG. At the same time, S1P, at pathophysiological concentration in active AAV patients, could induce PAR1 expression as well as enhance both expression level and procoagulant activity of TF in MPO-ANCA-positive IgG-treated GEnCs, which may

further activate the coagulation system, thus forming a vicious loop (**Figure 6**).

Anti-MPO antibody could cause activation of GEnCs by recognizing moesin even though MPO is not expressed in endothelial cells (41). Moesin, whose full name is membrane-organizing extension spike protein, shares certain similar sequences with those on the N-terminal region of the MPO heavy chain (7). Binding of anti-MPO antibody to moesin was able to increase permeability and to up-regulate adhesion molecules of human GEnCs (42). Recently, it was reported that thrombin was able to induce phosphorylation of moesin within seconds (43). Likewise, S1P could also cause acute and potent moesin activation (44, 45). Therefore, we speculate that moesin recognized by MPO-ANCA could be further activated by thrombin or S1P, which might cause enhanced GEnC activation *in vitro*. However, anti-PR3 antibody might induce endothelial cells dysfunction through different mechanisms. According to the study by Le Roux S et al., anti-PR3 antibodies could induce a potent inhibitor of vascular endothelial growth factor named soluble Flt1 to release from monocytes rather than endothelial cells, therefore leading to an anti-angiogenic state that hinders endothelial repair in AAV (46).

In our current study, we found that thrombin could activate SphK1-S1P-S1PR3 axis, thrombin induced upregulation of SphK1 expression levels in GEnCs in the presence of MPO-ANCA-positive IgG (confirmed by both Western blot and PCR), therefore promoting the generation of S1P. However, the exact involvement of PAR1 during this process remains to be determined. According to the study by Parker et al. thrombin could cause activation of the small GTPase RhoA *in vivo* (47). This is of particular interest, because small GTPases are confirmed to play critical roles in mediating signaling responses of the S1PR (48), and our previous work also demonstrated that RhoA activated by S1PR2-5 dominated the S1P-induced MPO-ANCA-positive IgG-mediated GEnC activation (20). Activation of RhoA signaling induces endothelial barrier disruption by remodeling cytoskeleton and enhancing the formation of contractile stress fibers which are connected to junctions and generate pulling forces within neighboring cells, thus destabilizing cell contact and forming "discontinuous" adherens junctions and tight junctions (49). All these evidences not only suggest a mutual interaction of S1PR-initiated signaling and regulation of S1P synthesis, but also provide clues to the synergistic effect of thrombin and SphK-S1P-S1PR3 signaling on endothelial barrier dysfunction.

Under homeostatic conditions, high levels of S1P in circulation ($\sim$1 μM) are a result of its release from endothelial cells and red blood cells, while platelets may only release large amounts of S1P upon platelet activation when endothelial cells are damaged. Thromboxane plays a crucial role in S1P release from human platelets. The coagulation factors thrombin and FXa interact with local S1P availability and its cellular effects at multiple levels (8). A recent study by Campos et al. demonstrated that in rodent models of stroke, the functional S1P receptor antagonist fingolimod could enhance blood-brain barrier integrity and reduce infarct size, indicating S1P as a potential link between coagulation and inflammation system (50). Our previous studies illustrated that the renal expression of S1PRs correlated with both inflammatory and coagulation parameters among AAV patients, and S1P contributed to MPO-ANCA-positive IgG induced GEnC activation through S1PR2-5 and RhoA signaling pathway (18–20). In our current study, we found that thrombin-PAR could interact with SphK-S1P-S1PR signaling pathway to induce GEnC dysfunction in the presence of MPO-ANCA-positive IgG, and S1P could enhance both expression level and activity of TF in MPO-ANCA-positive IgG-treated GEnCs, thus further activating the coagulation system. Therefore, we speculate that in AAV, S1P might act as a mutual link between inflammation and coagulation system. Blockade of this Sphk1-S1P-S1PR3 signaling pathway may be critical for attenuating the pathological processes associated with over-activation of both coagulation system and inflammation system in AAV.

CONCLUSIONS

In conclusion, thrombin is able to enhance MPO-ANCA-positive IgG-mediated GEnC activation *via* Sphk1-S1P-S1PR3 signaling pathway. These findings are helpful to figure out the linking role of S1P between coagulation and inflammation in AAV, thus provide potential clues for intervention strategies.

ETHICS STATEMENT

Informed consent from each participant was obtained. The study was conducted in line with the Declaration of Helsinki and was approved by the ethics committees of Peking University First Hospital.

AUTHOR CONTRIBUTIONS

X-JS conducted the experiments, analyzed the data, and drafted the manuscript. M-HZ involved in its design, assisted with interpretation of data, and provided suggestion for revising the manuscript. MC conceived of the study, participated in the revision of the manuscript, and provided final approval of the version of the submitted manuscript. All authors read and approved the manuscript.

FUNDING

This study was supported by three grants from the National Natural Science Foundation of China (No. 81870478, 81425008, and 81621092), the grant from the National Key Research and Development Program (No. 2016YFC0906102), and the grant of University of Michigan Health System and Peking University Health Sciences Center Joint Institute for Translational and Clinical Research.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00237/full#supplementary-material

Figure S1 | Detection of SphK1 expression with Western blot.

REFERENCES

1. Jennette JC, Falk RJ, Bacon PA, Basu N, Cid MC, Ferrario F, et al. Revised International Chapel Hill consensus conference Nomenclature of Vasculitis. *Arthritis Rheum.* (2013) 65:1–11. doi: 10.1002/art.37715
2. Segelmark M, Wieslander J. IgG subclasses of antineutrophil cytoplasm autoantibodies (ANCA). *Nephrol Dial Transplant* (1993) 8:696–702. doi: 10.1093/ndt/8.8.696
3. Falk RJ, Terrell RS, Charles LA, Jennette JC. Anti-neutrophil cytoplasmic autoantibodies induce neutrophils to degranulate and produce oxygen radicals in vitro. *Proc Natl Acad Sci USA.* (1990) 87:4115–9. doi: 10.1073/pnas.87.11.4115
4. Chen M, Yu F, Zhang Y, Zou WZ, Zhao MH, Wang HY. Characteristics of Chinese patients with Wegener's granulomatosis with anti-myeloperoxidase autoantibodies. *Kidney Int.* (2005) 68:2225–9. doi: 10.1111/j.1523-1755.2005.00679.x
5. Li ZY, Chang DY, Zhao MH, Chen M. Predictors of treatment resistance and relapse in antineutrophil cytoplasmic antibody-associated vasculitis: a study of 439 cases in a single Chinese center. *Arthritis Rheumatol.* (2014) 66:1920–6. doi: 10.1002/art.38621
6. Nagao T, Matsumura M, Mabuchi A, Ishida-Okawara A, Koshio O, Nakayama T, et al. Up-regulation of adhesion molecule expression in glomerular endothelial cells by anti-myeloperoxidase antibody. *Nephrol Dial Transplant* (2007) 22:77–87. doi: 10.1093/ndt/gfl555
7. Nagao T, Suzuki K, Utsunomiya K, Matsumura M, Saiga K, Wang PC, et al. Direct activation of glomerular endothelial cells by anti-moesin activity of anti-myeloperoxidase antibody. *Nephrol Dial Transplant* (2011) 26:2752–60. doi: 10.1093/ndt/gfr032
8. Proia RL, Hla T. Emerging biology of sphingosine-1-phosphate: its role in pathogenesis and therapy. *J Clin Invest.* (2015) 125:1379–87. doi: 10.1172/JCI76369
9. Stone ML, Sharma AK, Zhao Y, Charles EJ, Huerter ME, Johnston WF, et al. Sphingosine-1-phosphate receptor 1 agonism attenuates lung ischemia-reperfusion injury. *Am J Physiol Lung Cell Mol Physiol.* (2015) 308:L1245–52. doi: 10.1152/ajplung.00302.2014
10. Potì F, Ceglarek U, Burkhardt R, Simoni M, Nofer JR. SKI-II–a sphingosine kinase 1 inhibitor–exacerbates atherosclerosis in low-density lipoprotein receptor-deficient (LDL-R-/-) mice on high cholesterol diet. *Atherosclerosis* (2015) 240:212–5. doi: 10.1016/j.atherosclerosis.2015.03.020
11. Winkler MS, Nierhaus A, Poppe A, Greiwe G, Gräler MH, Daum G. Sphingosine-1-phosphate (S1P): a Potential biomarker and therapeutic target for endothelial Dysfunction and sepsis? *Shock* (2017) 47:666–72. doi: 10.1097/SHK.0000000000000814
12. Pelletier D, Hafler DA. Fingolimod for multiple sclerosis. *N Engl J Med.* (2012) 366:339–47. doi: 10.1056/NEJMct1101691
13. Brinkmann V, Billich A, Baumruker T, Heining P, Schmouder R, Francis G, et al. Fingolimod (FTY720): discovery and development of an oral drug to treat multiple sclerosis. *Nat Rev Drug Discov.* (2010) 9:883–97. doi: 10.1038/nrd3248
14. Huwiler A, Zangemeister-Wittke U. The sphingosine 1-phosphate receptor modulator fingolimod as a therapeutic agent: recent findings and new perspectives. *Pharmacol Ther.* (2018) 185:34–49. doi: 10.1016/j.pharmthera.2017.11.001
15. Hemdan NY, Weigel C, Reimann CM, Gräler MH. Modulating sphingosine 1-phosphate signaling with DOP or FTY720 alleviates vascular and immune defects in mouse sepsis. *Eur J Immunol.* (2016) 46:2767–77. doi: 10.1002/eji.201646417
16. Camp SM, Chiang ET, Sun C, Usatyuk PV, Bittman R, Natarajan V, et al. Pulmonary endothelial cell barrier enhancement by novel FTY720 analogs: methoxy-FTY720, fluoro-FTY720, and β-glucuronide-FTY720. *Chem Phys Lipids* (2015) 191:16–24. doi: 10.1016/j.chemphyslip.2015.08.004
17. Imeri F, Blanchard O, Jenni A, Schwalm S, Wünsche C, Zivkovic A, et al. FTY720 and two novel butterfly derivatives exert a general anti-inflammatory potential by reducing immune cell adhesion to endothelial cells through activation of S1P(3) and phosphoinositide 3-kinase. *Naunyn Schmiedebergs Arch Pharmacol.* (2015) 388:1283–92. doi: 10.1007/s00210-015-1159-5
18. Sun XJ, Wang C, Zhang LX, Yu F, Chen M, Zhao MH. Sphingosine-1-phosphate and its receptors in anti-neutrophil cytoplasmic antibody–associated vasculitis. *Nephrol Dial Transplant* (2017) 32:1313–22. doi: 10.1093/ndt/gfw427
19. Sun XJ, Chen M, Zhao MH. Sphingosine-1-phosphate (S1P) enhances glomerular endothelial cells activation mediated by anti-myeloperoxidase antibody-positive IgG. *J Cell Mol Med.* (2018) 22:1769–77. doi: 10.1111/jcmm.13458
20. Sun XJ, Chen M, Zhao MH. Rho GTPases are involved in S1P-enhanced glomerular endothelial cells activation with anti-myeloperoxidase antibody positive IgG. *J Cell Mol Med.* (2018) 22:4550–4. doi: 10.1111/jcmm.13736
21. Chen M, Jayne DRW, Zhao MH. Complement in ANCA-associated vasculitis: mechanisms and implications for management. *Nat Rev Nephrol.* (2017) 13:359–67. doi: 10.1038/nrneph.2017.37
22. Salmela A, Ekstrand A, Joutsi-Korhonen L, Räisänen-Sokolowski A, Lassila R. Activation of endothelium, coagulation and fibrinolysis is enhanced and associates with renal anti-neutrophil cytoplasmic antibody-associated vasculitis. *Nephrol Dial Transplant* (2015) 30 (Suppl.1):i53–9. doi: 10.1093/ndt/gfu379
23. Stassen PM, Derks RP, Kallenberg CG, Stegeman CA. Venous thromboembolism in ANCA-associated vasculitis—incidence and risk factors. *Rheumatology* (2008) 47:530–4. doi: 10.1093/rheumatology/ken035
24. Amara U, Flierl MA, Rittirsch D, Klos A, Chen H, Acker B, et al. Molecular intercommunication between the complement and coagulation systems. *J Immunol.* (2010) 185:5628–36. doi: 10.4049/jimmunol.0903678
25. Conway EM. Reincarnation of ancient links between coagulation and complement. *J Thromb Haemost.* (2015) 13 (Suppl. 1):S121–32. doi: 10.1111/jth.12950
26. Huang YM, Wang H, Wang C, Chen M, Zhao MH. Promotion of hypercoagulability in antineutrophil cytoplasmic antibody-associated vasculitis by C5a-induced tissue factor-expressing microparticles and neutrophil extracellular traps. *Arthritis Rheumatol.* (2015) 67:2780–90. doi: 10.1002/art.39239
27. Miao D, Li DY, Chen M, Zhao MH. Platelets are activated in ANCA-associated vasculitis via thrombin-PARs pathway and can activate the alternative complement pathway. *Arthritis Res Ther.* (2017) 19:252. doi: 10.1186/s13075-017-1458-y
28. Swieringa F, Spronk HMH, Heemskerk JWM, van der Meijden PEJ. Integrating platelet and coagulation activation in fibrin clot formation. *Res Pract Thromb Haemost.* (2018) 2:450–60. doi: 10.1002/rth2.12107
29. Nieman MT. Protease-activated receptors in hemostasis. *Blood* (2016) 128:169–177. doi: 10.1182/blood-2015-11-636472
30. Grimsey NJ, Trejo J. Integration of Endothelial Protease-activated Receptor-1 Inflammatory Signaling by Ubiquitin. *Curr Opin Hematol.* (2016) 23:274–9. doi: 10.1097/MOH.0000000000000232
31. Tauseef M, Kini V, Knezevic N, Brannan M, Ramchandaran R, Fyrst H, et al. Activation of sphingosine kinase-1 reverses the increase in lung vascular permeability through sphingosine-1-phosphate receptor signaling in endothelial cells. *Circ Res.* (2008) 103:1164–72. doi: 10.1161/01.RES.0000338501.84810.51
32. Takeya H, Gabazza EC, Aoki S, Ueno H, Suzuki K. Synergistic effect of sphingosine 1-phosphate on thrombin-induced tissue factor expression in endothelial cells. *Blood* (2003) 102:1693–700. doi: 10.1182/blood-2002-11-3607
33. Mahajan-Thakur S, Böhm A, Jedlitschky G, Schrör K, Rauch BH. Sphingosine-1-phosphate and its receptors: a mutual link between blood coagulation and inflammation. *Mediators Inflamm.* (2015) 2015:831059. doi: 10.1155/2015/831059
34. Schreiber A, Rolle S, Peripelittchenko L, Rademann J, Schneider W, Luft FC, et al. Phosphoinositol 3-kinase-gamma mediates antineutrophil cytoplasmic autoantibody-induced glomerulonephritis. *Kidney Int.* (2010) 77:118–28. doi: 10.1038/ki.2009.420
35. Carvalho D, Savage CO, Black CM, Pearson JD. IgG antiendothelial cell autoantibodies from scleroderma patients induce leukocyte adhesion to human vascular endothelial cells *in vitro*. *J Clin Invest.* (1996) 97:111–9.
36. Hartsock A, Nelson WJ. Adherens and tight junctions: structure, function and connections to the actin cytoskeleton. *Biochim Biophys Acta* (2008) 1778:660–9.

37. Tinsley JH, Wu MH, Ma W, Taulman AC, Yuan SY. Activated neutrophils induce hyperpermeability and phosphorylation of adherens junction proteins in coronary venular endothelial cells. *J Biol Chem.* (1999) 274:24930–4. doi: 10.1074/jbc.274.35.24930
38. Szmitko PE, Wang CH, Weisel RD, de Almeida JR, Anderson TJ, Verma S. New markers of inflammation and endothelial cell activation: part I. *Circulation* (2003) 108:1917–23. doi: 10.1161/01.CIR.0000089190.95415.9F
39. Andrade-Gordon P, Maryanoff BE, Derian CK, Zhang HC, Addo MF, Darrow AL, et al. Design, synthesis, and biological characterization of a peptide-mimetic antagonist for a tethered-ligand receptor. *Proc Natl Acad Sci USA.* (1999) 96:12257–62. doi: 10.1073/pnas.96.22.12257
40. Hirata N, Yamada S, Shoda T, Kurihara M, Sekino Y, Kanda Y. Sphingosine-1-phosphate promotes expansion of cancer stem cells via S1PR3 by a ligand-independent Notch activation. *Nat Commun.* (2014) 5:4806. doi: 10.1038/ncomms5806
41. Pendergraft WF, Alcorta DA, Segelmark M, Yang JJ, Tuttle R, Jennette JC, et al. ANCA antigens, proteinase 3 and myeloperoxidase, are not expressed in endothelial cells. *Kidney Int.* (2000) 57:1981–90. doi: 10.1046/j.1523-1755.2000.00048.x
42. Deng H, Wang C, Chang DY, Hu N, Chen M, Zhao MH. High mobility group box-1 contributes to anti-myeloperoxidase antibody-induced glomerular endothelial cell injury through a moesin-dependent route. *Arthritis Res Ther.* (2017) 19:125. doi: 10.1186/s13075-017-1339-4
43. Nakamura F, Amieva MR, Furthmayr H. Phosphorylation of threonine 558 in the carboxyl-terminal actin-binding domain of moesin by thrombin activation of human platelets. *J Biol Chem.* (1995) 270:31377–85. doi: 10.1074/jbc.270.52.31377
44. Adada M, Canals D, Hannun YA, Obeid LM. Sphingolipid regulation of ezrin, radixin, and moesin proteins family: implications for cell dynamics. *Biochim Biophys Acta* (2014) 1841:727–37. doi: 10.1016/j.bbalip.2013.07.002
45. Adada MM, Canals D, Jeong N, Kelkar AD, Hernandez-Corbacho M, Pulkoski-Gross MJ, et al. Intracellular sphingosine kinase 2-derived sphingosine-1-phosphate mediates epidermal growth factor-induced ezrin-radixin-moesinphosphorylation and cancer cell invasion. *FASEB J.* (2015) 29:4654–69. doi: 10.1096/fj.15-274340
46. Le Roux S, Pepper RJ, Dufay A, Néel M, Meffray E, Lamandé N. Elevated soluble Flt1 inhibits endothelial repair in PR3-ANCA-associated vasculitis. *J Am Soc Nephrol.* (2012) 23:155–64. doi: 10.1681/ASN.2010080858
47. Parker WH, Qu ZC, May JM. Intracellular ascorbate prevents endothelial barrier permeabilization by thrombin. *J Biol Chem.* (2015) 290:21486–97. doi: 10.1074/jbc.M115.662098
48. Singleton PA, Dudek SM, Ma SF, Garcia JG. Transactivation of sphingosine 1-phosphate receptors is essential for vascular barrier regulation. Novel role for hyaluronan and CD44 receptor family. *J Biol Chem.* (2006) 281:34381–93. doi: 10.1074/jbc.M603680200
49. Rho SS, Ando K, Fukuhara S. Dynamic regulation of vascular permeability by vascular endothelial cadherin-mediated endothelial cell-cell junctions. *J Nippon Med Sch.* (2017) 84:148–59. doi: 10.1272/jnms.84.148
50. Campos F, Qin T, Castillo J, Seo JH, Arai K, Lo EH, et al. Fingolimod reduces hemorrhagic transformation associated with delayed tissue plasminogen activator treatment in a mouse thromboembolic model. *Stroke* (2013) 44:505–11. doi: 10.1161/STROKEAHA.112.679043

Conflict of Interest Statement: 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.

frontiers in Immunology

ORIGINAL RESEARCH
published: 26 June 2019
doi: 10.3389/fimmu.2019.01405

Podocytes and Proteinuria in ANCA-Associated Glomerulonephritis: A Case-Control Study

Emma E. van Daalen[1], Peter Neeskens[1], Malu Zandbergen[1], Lorraine Harper[2], Alexandre Karras[3], Augusto Vaglio[4], Janak de Zoysa[5], Jan A. Bruijn[1] and Ingeborg M. Bajema[1]*

[1] Department of Pathology, Leiden University Medical Center, Leiden, Netherlands, [2] School of Immunity and Infection, College of Medical and Dental Sciences, University of Birmingham, Birmingham, United Kingdom, [3] Nephrology Department, HEGP Hospital, Assistance Publique Hôpitaux de Paris, Paris, France, [4] Nephrology Unit, Meyer Children's Hospital and Department of Biomedical Experimental and Clinical Sciences "Mario Serio", University of Firenze, Firenze, Italy, [5] Renal Services, North Shore Hospital, Auckland, New Zealand

Proteinuria has been identified as prognosticator of renal outcome in patients with ANCA-associated glomerulonephritis, but whether proteinuria is related to podocyte abnormalities in these patients is largely unknown. We here investigate podocyte foot process width and number of podocytes positive for the podocyte marker WT-1 in diagnostic renal biopsies of 25 Caucasian patients with ANCA-associated glomerulonephritis in relation to proteinuria. Control tissue was used from pre-transplantation donor kidney biopsies. Proteinuria at 10 weeks follow-up correlated significantly with foot process width ($P = 0.04$). Biopsies with foot process width ≥600 nm belonged more often to the crescentic or mixed class, whereas biopsies with a foot process width <600 nm were most often categorized as focal class ($P = 0.03$). The mean number of podocytes based upon expression of WT-1 was significantly lower in patients compared to controls (15 vs. 34 podocytes per glomerulus; $P < 0.0001$). The significant decrease in expression of the podocyte WT-1 marker in ANCA-associated glomerulonephritis is considered indicative of actual podocyte loss or at least, of a loss of functionality. Furthermore, our study indicates that podocyte foot process width at baseline could be indicative for proteinuria at short term follow up. For prognostic purposes, we therefore suggest to include a description of the foot process width in the diagnostic report of a biopsy with ANCA-associated glomerulonephritis.

Keywords: podocyte, proteinuria, ANCA, vasculitis, renal biopsy

OPEN ACCESS

Edited by:
Andreas Kronbichler, Innsbruck Medical University, Austria

Reviewed by:
Matija Crnogorac, Clinical Hospital Dubrava, Croatia
Seerapani Gopaluni, University of Cambridge, United Kingdom

****Correspondence:***
Emma E. van Daalen
E.E.van_Daalen@lumc.nl

Specialty section:
This article was submitted to Autoimmune and Autoinflammatory Disorders, a section of the journal Frontiers in Immunology

Received: *17 April 2019*
Accepted: *04 June 2019*
Published: *26 June 2019*

Citation:
van Daalen EE, Neeskens P, Zandbergen M, Harper L, Karras A, Vaglio A, de Zoysa J, Bruijn JA and Bajema IM (2019) Podocytes and Proteinuria in ANCA-Associated Glomerulonephritis: A Case-Control Study. Front. Immunol. 10:1405. doi: 10.3389/fimmu.2019.01405

INTRODUCTION

In the patient care and research of anti-neutrophil cytoplasmic antibody (ANCA-) associated glomerulonephritis (AAGN), proteinuria is a subject matter which so far received relatively little attention. Studies on AAGN have mainly focused on renal function deterioration in combination with findings in the urine sediment. However, there are some data indicating that the degree of proteinuria at diagnosis is associated with renal outcome in patients with AAGN (1–3). Also, preliminary data combined from three European Vasculitis Society (EUVAS) clinical trials show

that the level of proteinuria during follow-up is a prognostic marker of chronic kidney disease progression (4). At disease presentation, the majority of patients with AAGN have proteinuria, the amount of which is quite variable (5).

In general, the presence of proteinuria in kidney diseases is associated with changes in podocyte morphology (6, 7). Podocytes are highly specialized epithelial cells that, together with the glomerular basement membrane (GBM) and glomerular endothelial cells, constitute the filtration barrier of the glomerular capillary wall. The notion that podocytes react to injury by effacement is generally accepted, but exactly how this reactive change relates to the level of proteinuria, remains a matter of debate (8). Two recent studies investigating foot process effacement in different human glomerulopathies suggested that the amount of foot process effacement is related to the type of glomerulopathy rather than to the amount of proteinuria; for example, patients with IgA nephropathy and minimal change nephrotic syndrome had similar proteinuria levels at diagnosis, but foot processes were significantly more effaced in minimal change nephrotic syndrome (9, 10).

To study podocyte morphology, images at high magnification with electron microscopy (EM) of the podocytes are required. In most centers, EM is not routinely performed in AAGN, because the characteristic findings by light microscopy (LM) and the pauci-immune pattern by immunofluorescence are usually diagnostic. A number of studies investigated EM samples from patients with AAGN (11–15), but only one described the podocyte morphology in detail (16). This was a recent study from China showing that foot process width (FPW) was significantly higher and that podocyte density was significantly lower in an Asian group of patients with AAGN compared to healthy controls. In the current study, we investigate the podocyte morphology and number in renal biopsies of a Caucasian population of patients with AAGN. We analyzed whether and how these parameters were related to proteinuria at baseline and during follow-up.

METHODS

Study Population

Patients with histopathologically proven AAGN were retrieved from the Pathology database at Leiden University Medical Center, the Netherlands. Patients had to fulfill the criteria for ANCA-associated vasculitis as specified in the 2012 Revised International Chapel Hill Consensus Conference Nomenclature of Vasculitides (17). Only patients with available samples for EM could be included. Samples were either retrieved from tissue obtained by renal biopsy that had previously been stored in glutaraldehyde, or from the paraffin blocks in case of which the quality for EM had to be sufficient for the evaluation of podocyte morphology. Control human renal tissue was used from five pre-transplantation donor kidney biopsies, which showed no abnormalities by LM and from which it was known that the donors were non-proteinuric at time of donation. This study was conducted in accordance with the ethical principles stated in the Declaration of Helsinki.

Clinical Data

Medical records were used to retrieve data on sex, age, diagnosis (granulomatosis with polyangiitis or microscopic polyangiitis), serology (proteinase 3-[PR3-] or myeloperoxidase-[MPO-]ANCA), and laboratory results (serum creatinine and proteinuria). The estimated glomerular filtration rate (eGFR) at time of biopsy and during follow-up was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation (18). Proteinuria was expressed as total protein excretion in 24-h urine. In case this value was missing, proteinuria by dipstick measurement (scale from negative to $+++$) was used. All patients were classified as having either moderately or severely increased proteinuria according to the Kidney Disease: Improving Global Outcomes (KDIGO) clinical guidelines: moderately increased proteinuria was defined as a protein excretion rate of 0.15–0.50 g/day, or as trace or $+$ on protein dipstick test; severely increased proteinuria was defined as total protein excretion over 0.50 g/day, or as $+$ or more on protein dipstick (19). Proteinuria levels were assessed at least twice during follow-up: at 10 weeks and 1 year, which were regular moments of outpatient visits for all patients.

Histopathological Parameters

Renal biopsies were re-evaluated and classified as either focal, crescentic, mixed, or sclerotic class, following the Berden classification (20). Moreover, inflammatory infiltrate (<10%, 10–25%, 26–50%, or >50% of unscarred parenchyma), interstitial infiltrate and tubular atrophy (IFTA [0%, <25%, 26–50%, or >50% of cortical area]), and tubulitis (no mononuclear cells in tubules, foci with 1–4 cells/tubular cross section, foci with 5–10 cells/tubular cross section, or foci with >10 cells/tubular cross section) were determined for each case, according to the Banff classification for allograft pathology (21).

Measurement of Foot Process Effacement

Renal specimens were fixed in 1.5% GA/1.0% PF fixative or formalin, post-fixed in osmium tetroxide, and embedded in epon (LADD Research Industries Inc., USA). EM sections were stained with uranyl acetate and lead citrate. For each patient and control, 15 pictures were taken with a JEM-1011 electron microscope (JEOL USA, Inc.) at 10.000-fold magnification. As a measure of foot process effacement, FPW was calculated using the formula

$$\frac{\pi}{4} * \frac{\Sigma \text{GBM length}}{\Sigma \text{foot processes}},$$

where $\sum$foot processes is the total number of foot processes, $\sum$GBM length is the total length of GBM, and $\frac{\pi}{4}$ is a correction factor for random variation in the angle of section relative to the long axis of the podocyte (9). The total length of GBM in each picture was measured by ImageJ 1.46r software (National Institutes of Health, rsb.info.nih.gov/ij). The number of foot processes was manually counted.

Measurement of Podocyte Number

We used immunohistochemistry to identify and count podocytes based on staining for WT-1, a podocyte-specific transcription factor (22). Paraffin sections (4-μm thickness) were stained with

rabbit anti-human WT-1 (sc-192, Santa Cruz Biotechnology, Dallas, TX, USA), followed by goat anti-rabbit EnVision-HRP conjugate (Dako, Glostrup, Denmark) with diaminobenzidine as the chromogen. The sections were counterstained with hematoxylin. The number of WT-1 positive nuclei per glomerular tuft (referred to as number of podocytes) was counted in three glomeruli unaffected by light microscopic lesions per patient. In the control group, six glomeruli per biopsy were analyzed. The number of podocytes was expressed as number of WT-1 positive nuclei per glomerulus. In the same glomeruli, all nuclei and the surface area of the glomerular tuft were quantified. The software used to count podocytes and nuclei and to measure glomerular surface areas was IMS viewer (Philips Digital Pathology Solution).

Statistical Analysis

Means were compared between groups by using the student's *t*-test or one-way analysis of variance. Categorical data were compared by using the chi-square test or Fisher's exact test. FPW was correlated to demographic and clinical parameters with Pearson correlation coefficients. All analyses were performed with SPSS statistical software, version 23 (IBM Corp., Armonk, NY, USA). $P < 0.05$ were considered significant.

RESULTS

Patient Characteristics

A total of 25 patients were included in this study. The mean ± SD age at biopsy was 55.4 ± 13.5 years, which was similar to the mean age in the control group (47.2 ± 17.3; $P = 0.24$). The 24-hour proteinuria at baseline ($proteinuria_0$) was available in 23 patients; the mean was 1.6 ± 1.9 g/day (**Table 1**). The two patients whose 24-h $proteinuria_0$ was unavailable had a positive dipstick (+ and ++ respectively). The mean eGFR at baseline ($eGFR_0$) was 42.3 ± 28.6 ml/min/1.73 m^2. The level of $proteinuria_0$ and $eGFR_0$ did not correlate ($r = 0.07$; $P = 0.75$), similar to the level of $proteinuria_0$ and eGFR at 1 year ($eGFR_{1year}$) ($r = 0.17$; $P = 0.48$). Treatment regimens were as follows: all patients were treated with prednisone; 24 patients received cyclophosphamide, which was switched to maintenance therapy with azathioprine in 17 patients. Six patients received angiotensin converting enzyme—inhibitor (ACE-I) therapy before or after the diagnosis of AAGN; their level of $proteinuria_0$ was non-significantly higher than the level in patients who did not receive ACE-I therapy (2.3 ± 2.9 vs. 1.3 ± 1.5 g/day; $P = 0.45$). After 10 weeks of follow-up, the level of proteinuria ($proteinuria_{10weeks}$) was similar in patients receiving ACE-I therapy and patients not receiving ACE-I therapy (1.6 ± 0.9 vs. 1.4 ± 1.6; $P = 0.76$). The levels of proteinuria at 1-year follow-up ($proteinuria_{1year}$) were lower in patients treated with ACE-I compared to patients who did not receive this treatment (0.9 ± 0.8 vs. 0.6 ± 0.9; $P = 0.58$).

TABLE 1 | Characteristics of the study cohort and according to FPW.

	All patients ($n = 25$)	Patients with FPW <600 nm ($n = 11$)[a]	Patients with FPW ≥600 nm ($n = 10$)[a]	*P*-value[b]
Male	15 (60)	6 (55)	6 (60)	1.00
Age, yr	55.4 ± 13.5	51.3 ± 14.4	60.4 ± 13.1	0.15
Diagnosis				0.39
GPA	16 (64)	8 (73)	5 (50)	
MPA	9 (36)	3 (27)	5 (50)	
ANCA serotype				0.43
PR3-ANCA	13 (52)	7 (64)	4 (40)	
MPO-ANCA	9 (36)	4 (36)	4 (40)	
Double positive	2 (8)	0 (0)	1 (10)	
Negative	1 (4)	0 (0)	1 (10)	
Histopathological class				0.03
Focal	13 (54)	9 (82)	3 (33)	
Crescentic/mixed	11 (46)	2 (18)	6 (67)	
Podocytes/glomerulus	15.0 ± 6.5	15.8 ± 6.6	13.4 ± 6.4	0.49
$eGFR_0$, mL/min/1.73 m^2	42.3 ± 28.6	49.4 ± 33.9	38.1 ± 21.4	0.38
$eGFR_{1year}$, mL/min/1.73 m^2	59.1 ± 23.4	68.4 ± 19.1	57.3 ± 22.5	0.31
$Proteinuria_0$, g/day	1.6 ± 1.9	0.9 ± 0.5	2.4 ± 2.7	0.14
$Proteinuria_{10weeks}$, g/day	1.4 ± 1.4	1.0 ± 1.1	2.0 ± 2.0	0.21
$Proteinuria_{1year}$, g/day	0.7 ± 0.9	0.7 ± 1.0	1.0 ± 0.9	0.58
ESRD[c]	3 (12.0)	0 (0.0)	1 (12.5)	0.44

Values are reported as number (%) or mean ± SD.

[a] *FPW could not be measured in four patients, because of insufficient EM material.*

[b] *Indicating differences between patients with FPW<600 nm and ≥600 nm.*

[c] *Missing data for two patients due to limited follow-up. eGFR, estimated glomerular filtration rate; ESRD, end-stage renal disease; FPW, foot process width; GPA, granulomatosis with polyangiitis; MPA, microscopic polyangiitis; PR3-ANCA, proteinase 3 ANCA; MPO-ANCA, myeloperoxidase ANCA.*

Glomerular and Tubulointerstitial Parameters

Thirteen biopsies were scored as focal, five as crescentic, six as mixed, and one could not be classified due to insufficient number of glomeruli (i.e., <7). Patients with a biopsy categorized as focal class had the lowest level of $proteinuria_0$ (0.9 ± 0.5 g/day), followed by mixed class (1.2 ± 1.1 g/day), and crescentic class (3.4 ± 3.1 g/day; $P = 0.02$). $Proteinuria_{10weeks}$ did not differ between classes ($P = 0.39$), similar to the level of $proteinuria_{1year}$ ($P = 0.35$). Inflammatory infiltrate, IFTA, and tubulitis were not associated to the level of proteinuria at baseline or during follow-up.

Foot Process Width

Figure 1 shows examples of EM pictures from the patient and control group. EM material turned out to be insufficient in four patients. The mean FPW in renal biopsies of 21 patients with AAGN was 603 ± 66 nm. In the control group (biopsies from five living donors), mean FPW was 571 ± 35 nm, which is in accordance with the normal range of FPW as reported in previous studies (7, 9, 10, 16). The mean FPW in patients was not significantly different from the FPW in controls ($P = 0.31$), but the three patients presenting with nephrotic range proteinuria (i.e., >3 g/day) did have a higher FPW compared to controls (657 ± 35 nm; $P = 0.02$). Because the highest FPW in the normal control group was 602 nm, characteristics were compared between patients with FPW <600 and ≥600 nm. Biopsies from patients with a FPW <600 nm were most often

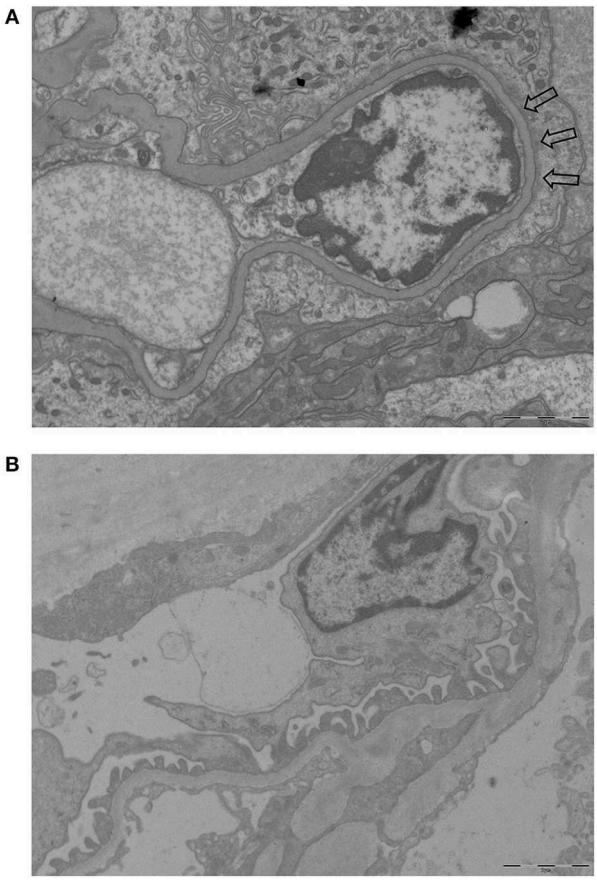


FIGURE 1 | Examples of EM pictures used to calculate FPW (magnification 10.000-fold). **(A)** EM picture of a patient with AAGN showing foot process effacement (part of the effacement is pointed by arrows). **(B)** EM picture of a control with normal foot processes.

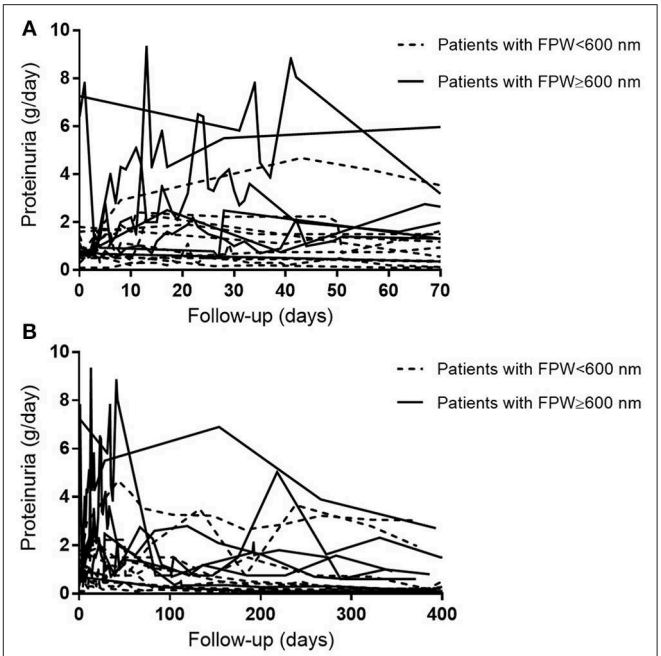


FIGURE 2 | Course of patients' individual 24-h proteinuria levels during follow-up. **(A)** Proteinuria levels during 10 weeks of follow-up. **(B)** Proteinuria levels during 400 days of follow-up.

categorized as focal class, whereas biopsies with FPW ≥600 nm belonged more often to the crescentic or mixed class (P = 0.03; **Table 1**). Tubulointerstitial parameters were not different between the two groups of FPW. The mean level of proteinuria$_0$ was not significantly higher in patients with FPW ≥600 nm compared to patients with FPW <600 nm (2.4 ± 2.7 vs. 0.9 ± 0.5 g/day; P = 0.14; **Table 1**). **Figure 2** shows proteinuria levels during follow-up of individual patients according to FPW subgroups. Proteinuria$_{10weeks}$ correlated significantly with FPW ($r = 0.50$; $P = 0.04$). At 1-year follow-up, the correlation between proteinuria and the FPW at biopsy was lost (r = 0.22; P = 0.40). A correlation of borderline significance was found between FPW and age at biopsy (r = 0.43; P = 0.05). No significant correlation was observed between FPW and eGFR at baseline and during follow-up.

Number of Podocytes

Material for immunohistochemistry was available in 19 patients, of which four were excluded due to the absence of at least 3 glomeruli without light microscopic lesions. The remaining 15 patients had a mean of 15 ± 7 podocytes per glomerulus. The mean number of podocytes was 34 ± 4 per glomerulus in the control group, which was significantly higher compared to the patients with AAGN (**Figure 3**; $P < 0.0001$). The mean surface area of the glomerular tuft was not significantly different in patients vs. controls (0.019 ± 0.006 mm^2 and 0.025 ± 0.012 mm^2 respectively; P = 0.12); also the total number of nuclei per glomerulus was not significantly different between patients and controls (84 ± 24 and 98 ± 12 respectively; P = 0.30). The percentage nuclei positive for WT-1 of the total number of nuclei was significantly lower in patients compared to controls (19.4 ± 9.0% vs. 34.3 ± 1.1%; $P < 0.001$). The number of podocytes per glomerulus in patients with AAGN did not correlate with FPW ($r = -0.190$; $P = 0.52$) or any of the clinical parameters. No significant differences were observed between patients with less and more than the median of 18 podocytes per glomerulus (**Table 2**).

DISCUSSION

Previous studies have underlined the importance of proteinuria as a prognostic marker in patients with AAGN (1–4). Since proteinuria has been associated with podocyte abnormalities, we here investigated the structural changes in podocytes in Caucasian patients presenting with AAGN. Although the FPW in patients was not statistically different from the mean FPW in healthy controls, we did identify an interesting association with clinical data as FPW correlated with the level of proteinuria 10 weeks after diagnosis. During these 10 weeks, the level of proteinuria increased in particular in patients whose FPW ≥600 nm (**Figure 2B**). Therefore, studying podocyte morphology

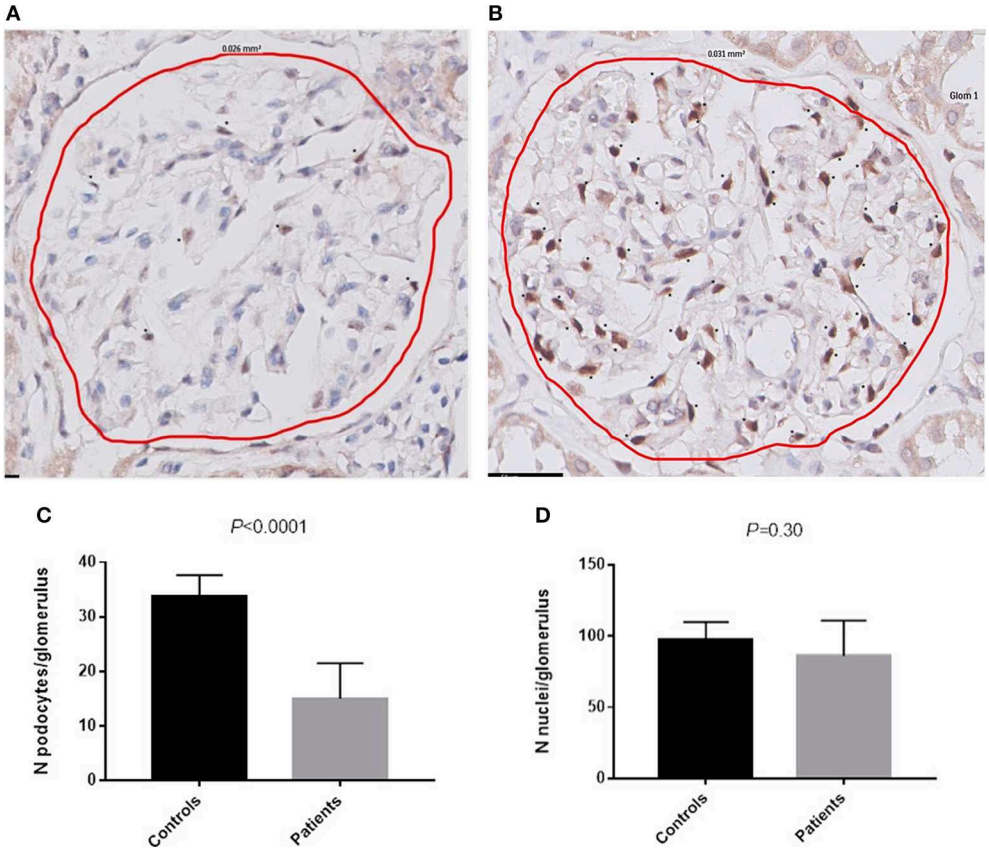


FIGURE 3 | Podocytes positive for WT-1. **(A)** WT-1 staining in a glomerulus of a patient with AAGN. **(B)** WT-1 staining in a glomerulus of a control. Asterisks (*) indicate a podocyte positive for WT-1. **(C)** Number of podocytes per glomerulus in controls and in patients ($P < 0.0001$). **(D)** Number of nuclei per glomerulus in controls and in patients.

in patients with AAGN may be indicative of whether or not patients will have an increase of proteinuria at short-term follow-up. At 1 year, the correlation between FPW and proteinuria was lost. The anti-inflammatory effect of immunosuppressive therapy may reduce the altered permeability of the glomerular capillary wall, thereby reducing the leak of proteins (23). Moreover, *in vitro* experiments have demonstrated a direct effect of corticosteroids on podocytes, enhancing their survival and promoting their repair (24, 25). Therefore, in addition to reducing inflammation, it could be hypothesized that corticosteroids cause podocytes to regain their normal morphology, leading to the observed decrease in level of proteinuria during 1-year follow-up in our patients with AAGN (**Figure 2A**). Only by performing EM on repeat protocolized biopsies, which were unavailable in the current study, more insights in this process could be obtained.

The exact relationship between foot process effacement and level of proteinuria is a topic of debate; some studies on glomerular diseases found a correlation between the degree of foot process effacement and amount of proteinuria (6, 7), whereas others did not (9, 10). In our study, FPW did not correlate with the amount of proteinuria at baseline, but we observed severely increased levels of proteinuria at baseline in all patients with a FPW $\geq$600 nm. Moreover, the three patients presenting with nephrotic range proteinuria had a FPW of 627, 648, and 696 nm; all higher than the highest reported value of 602 nm in controls. These data suggest that foot process effacement and proteinuria are related in patients with AAGN; however, a firm association could not be established.

In contrast to our results, the study by Zou et al. reported a mean FPW of 1269 nm in patients with AAGN, which was significantly higher than the mean FPW of 586 nm they measured in controls (16). In their study, the FPW was higher in patients with elevated serum creatinine (>133 μmol/L). However, they did not find a correlation between FPW and proteinuria at baseline, and did not report on proteinuria during follow-up. Values for FPW in normal controls were similar in the study by Zou et al. and our study, but the mean FPW in our patients with AAGN was much lower than in the Zou study. FPW in our study did correlate to proteinuria levels at 10 weeks. The different results in the study by Zou et al. and ours could have arisen from some differences in study cohorts: 96% of patients from the Zou study were positive for ANCA directed against MPO-ANCA, vs. 36% in our study; and the mean level of proteinuria was higher in their study (2.6 vs. 1.6 g/day in our study). In particular the difference between MPO-ANCA and PR3-ANCA distribution in our study and the study by Zou et al. underlines the differences

TABLE 2 | Characteristics according to number of podocytes.

	Patients with <18 podocytes/ glomerulus ($n = 9$)[a]	Patients with ≥18 podocytes/ glomerulus ($n = 6$)[a]	*P*-value
Male	5 (55.6)	4 (66.7)	1.00
Age, yr	54.2 ± 19.4	58.0 ± 8.4	0.62
Diagnosis			1.00
GPA	5 (55.6)	4 (66.7)	
MPA	4 (44.4)	2 (33.3)	
ANCA serotype			0.61
PR3-ANCA	4 (44.4)	4 (66.7)	
MPO-ANCA	5 (55.6)	2 (33.3)	
Histopathological class			0.59
Focal	3 (37.5)	4 (66.7)	
Crescentic/mixed	5 (62.5)	2 (33.3)	
$eGFR_0$, mL/min/1.73 m^2	34.4 ± 18.7	48.6 ± 14.8	0.14
$eGFR_{1year}$, mL/min/1.73 m^2	56.3 ± 18.9	59.6 ± 7.2	0.75
$Proteinuria_0$, g/day	2.4 ± 2.7	1.6 ± 1.7	0.59
$Proteinuria_{10weeks}$, g/day	1.7 ± 1.9	0.9 ± 0.9	0.46
$Proteinuria_{1year}$, g/day	0.9 ± 0.9	0.3 ± 0.1	0.21
ESRD[b]	0 (0.0)	1 (16.7)	0.46

Values are reported as number (%) or mean ± SD.

[a]Material for immunohistochemistry was available in 19 patients, of which four were excluded due to the absence of glomeruli without light microscopic lesions.

[b]Missing data for two patients due to limited follow-up. eGFR, estimated glomerular filtration rate; ESRD, end-stage renal disease; GPA, granulomatosis with polyangiitis; MPA, microscopic polyangiitis; PR3-ANCA, proteinase 3 ANCA; MPO-ANCA, myeloperoxidase ANCA.

between Asian and Caucasian patients with AAGN (26, 27). Whether FPW in AAGN varies between populations should be the focus of future studies.

In the current study, we found that biopsies containing a relatively high amount of lesions characteristic for AAGN (i.e., crescentic or mixed class) more often had a FPW ≥600 nm than biopsies with a small number of lesions (i.e., focal class). This is in line with the findings by Zou et al., showing a correlation between FPW and percentage of crescents (16). It has been suggested that podocytes have an active role in crescent formation; in the early stages before crescent formation, they form bridges between the tuft and Bowman's capsule (28). In a later stage, they constitute a component of the crescent, and during the transformation to crescentic cells, they lose podocyte-specific antigens, such as WT-1 (29, 30). In line with this hypothesis, it is telling that we found a 50% decrease in podocytes positive for WT-1 compared to healthy controls, probably reflecting either loss of podocytes or changes in functionality of the podocyte. Our finding of similar numbers of nuclei in glomeruli of patients and controls is suggestive for the latter explanation, and given the diminishment of proteinuria during follow-up this change may be reversible.

The current study has limitations, of which sample size is the major issue. However, EM material of patients with AAGN is scarce, and data on proteinuria are often not routinely documented. We acknowledge that larger studies are required to study podocyte morphology in AAGN into more detail, especially in different populations. Our study included both PR3- and MPO-positive patients, however, perhaps due to limited power, differences in podocyte morphology were not found between different serological phenotypes. Another limitation is that we could not investigate changes in podocyte morphology during follow-up, since repeated biopsy sampling is not part of the standard protocol in AAGN. Moreover, data on factors influencing proteinuria, such as blood pressure, were not available.

In conclusion, we here firstly describe the details of podocyte morphology in Caucasian patients with AAGN. In renal biopsies with AAGN a significant decrease of the podocyte WT-1 marker was found that could be indicative of actual podocyte loss or at least, of a loss of functionality. Patients had variable amounts of FPW, and in particular biopsies with a crescentic or mixed class had the highest FPW. These findings together merit further studies into the morphology and functionality of the podocyte in AAGN. In the meantime, our study indicates that podocyte FPW at baseline could be indicative for proteinuria at short term follow up. Therefore, it would be valuable for prognostic purposes to include a description of the FPW in the diagnostic report of a biopsy with AAGN.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

Following the Dutch legislation, this study was not presented to an ethical committee.

AUTHOR CONTRIBUTIONS

EvD, AK, and IB designed the study. EvD, LH, AV, and JdZ collected material. EvD and PN performed the FPW measurement with EM and analyzed the results. EvD and MZ performed the WT-1 staining and analyzed these results. All authors contributed to the data interpretation. EvD and IB wrote the manuscript, and all authors reviewed, and approved the manuscript. EvD takes responsibility that this study has been reported honestly, accurately, and transparently, that no important aspects of the study have been omitted and that any discrepancies from the study as planned and registered have been explained.

FUNDING

EvD was supported by the Kolff Student Researcher Grant (number 16OKK53) from the Dutch Kidney Foundation.

ACKNOWLEDGMENTS

The authors would like to thank Daniel Blockmans, Terry Cook, Chen Peh, and Giles Walters for their contribution to this study.

REFERENCES

1. Cordova-Sanchez BM, Mejia-Vilet JM, Morales-Buenrostro LE, Loyola-Rodriguez G, Uribe-Uribe NO, Correa-Rotter R. Clinical presentation and outcome prediction of clinical, serological, and histopathological classification schemes in ANCA-associated vasculitis with renal involvement. *Clin Rheumatol.* (2016) 35:1805–16. doi: 10.1007/s10067–016-3195-z
2. Neumann I, Kain R, Regele H, Soleiman A, Kandutsch S, Meisl FT. Histological and clinical predictors of early and late renal outcome in ANCA-associated vasculitis. *Nephrol Dial Transplant.* (2005) 20:96–104. doi: 10.1093/ndt/gfh563
3. Franssen CF, Stegeman CA, Oost-Kort WW, Kallenberg CG, Limburg PC, Tiebosch A, et al. Determinants of renal outcome in anti-myeloperoxidase-associated necrotizing crescentic glomerulonephritis. *J Am Soc Nephrol.* (1998) 9:1915–23.
4. Karras A, Hiemstra TF, Jones RB, Jayne D. Prognostic value of persistent heamaturia and proteinuria in ANCA-Associated Vasculitis: Data from the European Vasculitis Study Group (EUVAS) therapeutic trials. *J Am Soc Nephrol.* (2015) 26:447A. doi: 10.1038/kisup.2012.73
5. Hilhorst M, Wilde B, van Breda Vriesman P, van Paassen P, and Cohen Tervaert JW. Estimating renal survival using the ANCA-associated GN classification. *J Am Soc Nephrol.* (2013) 24:1371–5. doi: 10.1681/ASN.2012090912
6. Powell HR. Relationship between proteinuria and epithelial cell changes in minimal lesion glomerulopathy. *Nephron.* (1976) 16:310–7. doi: 10.1159/000180616
7. Koop K, Eikmans M, Baelde HJ, Kawachi H, De Heer E, Paul LC, et al. Expression of podocyte-associated molecules in acquired human kidney diseases. *J Am Soc Nephrol.* (2003) 14:2063–71. doi: 10.1097/01.ASN.0000078803.53165.C9
8. Shankland SJ. The podocyte's response to injury: role in proteinuria and glomerulosclerosis. *Kidney Int.* (2006) 69:2131–47. doi: 10.1038/sj.ki.5000410
9. van den Berg JG, van den Bergh Weerman MA, Assmann KJ, Weening JJ, Florquin S. Podocyte foot process effacement is not correlated with the level of proteinuria in human glomerulopathies. *Kidney Int.* (2004) 66:1901–6. doi: 10.1111/j.1523–1755.2004.00964.x
10. Deegens JK, Dijkman HB, Borm GF, Steenbergen EJ, van den Berg JG, Weening JJ, et al. Podocyte foot process effacement as a diagnostic tool in focal segmental glomerulosclerosis. *Kidney Int.* (2008) 74:1568–76. doi: 10.1038/ki.2008.413
11. Haas M, and Eustace JA. Immune complex deposits in ANCA-associated crescentic glomerulonephritis: a study of 126 cases. *Kidney Int.* (2004) 65:2145–52. doi: 10.1111/j.1523–1755.2004.00632.x
12. Neumann I, Regele H, Kain R, Birck R, and Meisl FT. Glomerular immune deposits are associated with increased proteinuria in patients with ANCA-associated crescentic nephritis. *Nephrol Dial Transplant.* (2003) 18:524–31. doi: 10.1093/ndt/18.3.524
13. Horn RG, Fauci AS, Rosenthal AS, Wolff SM. Renal biopsy pathology in Wegener's granulomatosis. *Am J Pathol.* (1974) 74:423–40.
14. Yu F, Chen M, Wang SX, Zou WZ, Zhao MH, Wang HY. Clinical and pathological characteristics and outcomes of Chinese patients with primary anti-neutrophil cytoplasmic antibodies-associated systemic vasculitis with immune complex deposition in kidney. *Nephrology.* (2007) 12:74–80. doi: 10.1111/j.1440–1797.2006.00713.x
15. Zhao L, David MZ, Hyjek E, Chang A, Meehan SM. M2 macrophage infiltrates in the early stages of ANCA-associated pauci-immune necrotizing GN. *Clin J Am Soc Nephrol.* (2015) 10:54–62. doi: 10.2215/CJN.03230314
16. Zou R, Wang SX, Liu G, Yu F, Chen M, Zhao MH. Podocyte Detachment Is Associated with Renal Prognosis in ANCA-Associated Glomerulonephritis: a retrospective cohort study. *Medicine.* (2016) 95:e3294. doi: 10.1097/MD.0000000000003294
17. Jennette JC, Falk RJ, Bacon PA, Basu N, Cid MC, Ferrario F, et al. 2012 revised international chapel hill consensus conference nomenclature of vasculitides. *Arthritis Rheum.* (2013) 65:1–11. doi: 10.1002/art.37715
18. Levey AS, Stevens LA, Schmid CH, Zhang YL, Castro AF, Feldman HI, et al. A new equation to estimate glomerular filtration rate. *Ann Intern Med.* (2009) 150:604–12. doi: 10.7326/0003–4819-150–9-200905050–00006
19. National Kidney Foundation: KDIGO 2012 Clinical Practice Guideline for the Evaluation and Management of Chronic Kidney Disease. *Kidney Int Suppl.* (2013) 3:1–150.
20. Berden AE, Ferrario F, Hagen EC, Jayne DR, Jennette JC, Joh K, et al. Histopathologic classification of ANCA-associated glomerulonephritis. *J Am Soc Nephrol.* (2010) 21:1628–36. doi: 10.1681/ASN.2010050477
21. Racusen LC, Solez K, Colvin RB, Bonsib SM, Castro MC, Cavallo T, et al. The Banff 97 working classification of renal allograft pathology. *Kidney Int.* (1999) 55:713–23. doi: 10.1046/j.1523–1755.1999.00299.x
22. Mundlos S, Pelletier J, Darveau A, Bachmann M, Winterpacht A, Zabel B. Nuclear localization of the protein encoded by the Wilms' tumor gene WT1 in embryonic and adult tissues. *Development.* (1993) 119:1329–41.
23. Mathieson PW. Proteinuria and immunity–an overstated relationship? *N Engl J Med.* (2008) 359:2492–4. doi: 10.1056/NEJMcibr0806881
24. Xing CY, Saleem MA, Coward RJ, Ni L, Witherden IR, Mathieson PW. Direct effects of dexamethasone on human podocytes. *Kidney Int.* (2006) 70:1038–45. doi: 10.1038/sj.ki.5001655
25. Ransom RF, Lam NG, Hallett MA, Atkinson SJ, Smoyer WE. Glucocorticoids protect and enhance recovery of cultured murine podocytes via actin filament stabilization. *Kidney Int.* (2005) 68:2473–83. doi: 10.1111/j.1523–1755.2005.00723.x
26. Fujimoto S, Watts RA, Kobayashi S, Suzuki K, Jayne DR, Scott DG, et al. Comparison of the epidemiology of anti-neutrophil cytoplasmic antibody-associated vasculitis between Japan and the U.K. *Rheumatology.* (2011) 50:1916–20. doi: 10.1093/rheumatology/ker205
27. Watts RA, Scott DG. L32. ANCA vasculitis over the world. What do we learn from country differences? *Presse Med.* (2013) 42:591–3. doi: 10.1016/j.lpm.2013.01.032
28. Le Hir M, Keller C, Eschmann V, Hahnel B, Hosser H, Kriz W. Podocyte bridges between the tuft and Bowman's capsule: an early event in experimental crescentic glomerulonephritis. *J Am Soc Nephrol.* (2001) 12:2060–71.
29. Thorner PS, Ho M, Eremina V, Sado Y, Quaggin S. Podocytes contribute to the formation of glomerular crescents. *J Am Soc Nephrol.* (2008) 19:495–502. doi: 10.1681/ASN.2006101115
30. Bariety J, Bruneval P, Meyrier A, Mandet C, Hill G, Jacquot C. Podocyte involvement in human immune crescentic glomerulonephritis. *Kidney Int.* (2005) 68:1109–19. doi: 10.1111/j.1523–1755.2005.00503.x

Conflict of Interest Statement: 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.

frontiers in Immunology

ORIGINAL RESEARCH
published: 18 July 2019
doi: 10.3389/fimmu.2019.01665

Prognostic Factors in Anti-glomerular Basement Membrane Disease: A Multicenter Study of 119 Patients

Cindy Marques[1,2,3,4,5†], *Julien Carvelli*[6†], *Lucie Biard*[7], *Stanislas Faguer*[8], *François Provôt*[9], *Marie Matignon*[10], *Jean-Jacques Boffa*[11], *Emmanuelle Plaisier*[11], *Alexandre Hertig*[11], *Maxime Touzot*[12], *Olivier Moranne*[13], *Xavier Belenfant*[14], *Djillali Annane*[15], *Thomas Quéméneur*[16], *Jacques Cadranel*[17], *Hassan Izzedine*[18], *Nicolas Bréchot*[19], *Patrice Cacoub*[1,2,3,4,5], *Alexis Piedrafita*[8], *Noémie Jourde-Chiche*[6‡] *and David Saadoun*[1,2,3,4,5*‡]

[1] Inflammation-Immunopathology-Biotherapy Department (DHU i2B), Sorbonne Université, UPMC Univ Paris 06, UMR 7211, Paris, France, [2] INSERM, UMR_S 959, Paris, France, [3] CNRS, FRE3632, Paris, France, [4] AP-HP, Groupe Hospitalier Pitié-Salpêtrière, Department of Internal Medicine and Clinical Immunology, Paris, France, [5] Centre de Référence des Maladies Auto-Immunes et Systémiques Rares, Centre de Référence des Maladies Auto-Inflammatoires et de l'Amylose, Paris, France, [6] Aix-Marseille Univ, APHM, C2VN, INRA 1260, INSERM 1263, CHU de la Conception, Centre de Néphrologie et Transplantation Rénale, Marseille, France, [7] Department of Biostatistics and Medical Information, INSERM UMR1153 ECSTRRA Team, Hôpital Saint Louis, AP-HP, Paris, France, [8] Département de Néphrologie et Transplantation d'organes, Centre de référence des maladies rénales rares, Hôpital Rangueil, CHU de Toulouse, Toulouse, France, [9] Department of Nephrology, Centre Hospitalier Régional Universitaire de Lille, Lille, France, [10] Department of Nephrology and Renal Transplantation, Groupe Hospitalier Henri-Mondor, AP-HP, Créteil, France, [11] Sorbonne Université, UPMC Université Paris 06, Hôpital Tenon, Urgences Néphrologiques et Transplantation Rénale, Paris, France, [12] AURA Paris Plaisance, Paris, France, [13] Service Néphrologie-Dialyses-Aphérèse, Hôpital Caremeau, CHU Nîmes, et Faculté de Médecine Université de Montpellier-nimes, Nîmes, France, [14] Nephrology and Dialysis, Centre Hospitalier Intercommunal André Grégoire, Montreuil, France, [15] General ICU, Hôpital Raymond Poincaré, AP-HP, Garches, France, [16] Department of Internal Medicine, Centre Hospitalier, Valenciennes, France, [17] Chest Department and Constitutive Center for Rare Pulmonary Disease, Hôpital Tenon, AP-HP; Inflammation-Immunopathology-Biotherapy Department (DHU i2B) and Sorbonne Université, Paris, France, [18] Department of Nephrology, Peupliers Private Hospital, Ramsay Générale de Santé, Paris, France, [19] Medical-Surgical Intensive Care Unit, AP-HP, Groupe Hospitalier Pitié-Salpêtrière, Paris, France

OPEN ACCESS

Edited by:
Andreas Kronbichler, Innsbruck Medical University, Austria

Reviewed by:
Mårten Segeelmark, Linköping University, Sweden
Stephen McAdoo, Imperial College London, United Kingdom

***Correspondence:**
David Saadoun
david.saadoun@aphp.fr

[†]*Co first authors*

[‡]*Co-senior authors*

Specialty section:
This article was submitted to Autoimmune and Autoinflammatory Disorders, a section of the journal Frontiers in Immunology

Received: *19 December 2018*
Accepted: *03 July 2019*
Published: *18 July 2019*

Citation:
Marques C, Carvelli J, Biard L, Faguer S, Provôt F, Matignon M, Boffa J-J, Plaisier E, Hertig A, Touzot M, Moranne O, Belenfant X, Annane D, Quéméneur T, Cadranel J, Izzedine H, Bréchot N, Cacoub P, Piedrafita A, Jourde-Chiche N and Saadoun D (2019) Prognostic Factors in Anti-glomerular Basement Membrane Disease: A Multicenter Study of 119 Patients. Front. Immunol. 10:1665. doi: 10.3389/fimmu.2019.01665

We report the overall and renal outcome in a French nationwide multicenter cohort of 119 patients with anti-glomerular basement membrane (anti-GBM) disease. Sixty-four patients (54%) had an exclusive renal involvement, 7 (6%) an isolated alveolar hemorrhage and 48 (40%) a combined renal and pulmonary involvement. Initial renal replacement therapy (RRT) was required in 78% of patients; 82% received plasmapheresis, 82% cyclophosphamide, and 9% rituximab. ANCA positive (28%) patients were older (70 vs. 47 years, $p < 0.0001$), less frequently smokers (26 vs. 54%, $p = 0.03$), and had less pulmonary involvement than ANCA- patients. The 5 years overall survival was 92%. Risk factors of death ($n = 11$, 9.2%) were age at onset [HR 4.10 per decade (1.89–8.88) $p = 0.003$], hypertension [HR 19.9 (2.52–157 0.2) $p = 0.005$], dyslipidemia [HR 11.1 (2.72–45) $p = 0.0008$], and need for mechanical ventilation [HR 5.20 (1.02–26.4) $p = 0.047$]. The use of plasmapheresis was associated with better survival [HR 0.29 (0.08–0.98) $p = 0.046$]. At 3 months, 55 (46%) patients had end-stage renal disease (ESRD) vs. 37 (31%) ESRD-free and 27 (23%) unevaluable with follow-up < 3 months. ESRD patients were older, more frequently female and had

a higher serum creatinine level at presentation than those without ESRD. ESRD-free survival was evaluated in patients alive without ESRD at 3 months ($n = 37$) using a landmark approach. In conclusion, this large French nationwide study identifies prognosis factors of renal and overall survival in anti-GBM patients.

Keywords: anti-glomerular basement membrane disease, Goodpasture's disease, glomerulonephritis, vasculitis, outcome, mortality

INTRODUCTION

Anti-glomerular basement membrane (anti-GBM) disease is a rare small vessel vasculitis that affects the capillary beds of the kidneys and lungs (1). It is an organ-specific autoimmune disease mediated by circulating autoantibodies directed against the non-collagenous domain of the α3 chain of type IV collagen [α3(IV)NC1] (2–5). Clinical presentation, related to the involvement of both glomerular and alveolar membranes, includes rapidly progressive glomerulonephritis and pulmonary hemorrhage. A majority of patients with anti-GBM have both pulmonary and renal involvement, but 20–40% and <10% of patients have kidney or pulmonary involvement only, respectively. Twenty-one to 47% of patients also have antineutrophil cytoplasmic antibodies (ANCA) (6–10). They mostly display anti-myeloperoxidase (MPO) specificity (11, 12) and could be older than those with anti-GBM positivity alone (13), with a male preponderance (9).

The standard treatment for anti-GBM relies on plasma exchanges to rapidly remove pathogenic autoantibodies, combined with glucocorticoids and cyclophosphamide (CYC) (14). CYC is most often administered orally but some protocols include intravenous administration. Despite the lack of randomized controlled studies given the rarity and severity of the disease, the use of combination therapy has been the gold standard since the 1970s. According to the severity of the clinical course, some patients will require prolonged treatment with immunosuppressive drugs for as long as 6–12 months. Moreover, the addition of anti-CD20 rituximab monoclonal antibody therapy (375 mg/m^2/week for 4 weeks) has been proposed for patients with severe and/or refractory anti-GBM disease (15). Similarly, the use of mycophenolate mofetil and cyclosporine has been reported in individual cases or small series (16–18).

Given the small number of large and homogeneous cohorts, few data are available on prognostic factors for renal and overall long-term evolution. A large Chinese study of 221 patients confirmed that the combination of plasmapheresis and corticosteroids correlated with overall and renal survival (19). A British study from 2015 showed that short-term renal survival was determined by the severity of initial renal impairment (oliguria and percentage of histological crescents); and that age, ANCA positivity, oliguria, and the presence of comorbidities were predictive of overall survival (OS) (13). In a recent study from the French Society of Hemapheresis, renal survival was only predicted by the severity of the renal presentation (20).

The present study was undertaken to report the outcome of anti-GBM. We compared anti-GBM patients according to ANCA status, and analyzed prognostic factors of overall and renal survival in a French nationwide cohort of 119 patients with anti-GBM disease.

METHODS

Patients

We retrospectively reviewed the data of patients with anti-GBM disease diagnosed in 16 French centers between 1981 and 2017. Diagnosis of anti-GBM was based on the presence of circulating anti-GBM antibodies detected by ELISA or immunofluorescence and/or linear IgG fluorescence along the GBM on renal biopsy, which is the gold-standard for diagnosis of anti-GBM disease (21). A diagnosis of pulmonary hemorrhage was retained in patients with overt hemoptysis and/or pulmonary interstitial opacities on chest computed tomography (CT) and/or proven alveolar hemorrhage on bronchoalveolar lavage. Relapses were defined as pulmonary (i.e., recurrence of hemoptysis) and/or renal worsening (i.e., increase in serum creatinine level and proteinuria) more than 3 months after diagnosis elevation of anti-GBM autoantibodies and/or compatible renal biopsy. Before 3 months, we considered that it was a worsening of the disease. Included patients did not belong to the cohort-based study from the French Society of Hemapheresis (20). The study was approved by the ethical committee of Pitié-Salpêtrière University Hospital.

Data Collection

Demographic data, medical history, clinical, biological, radiological, and histological data at presentation were collected. Intensive care stays, number of plasma exchanges as well as number and dose of different treatments regimen, were also reported. End-stage renal disease (ESRD) was defined as the persistence of renal failure with anuria or estimated glomerular filtration rate <15 ml/min/1.73 m^2 after 3 months of evolution. Finally, overall and renal survival data up to 60 months of follow-up, adverse events, kidney or pulmonary transplants and relapses were also collected.

Statistical Analyses

For description according to ANCA status and to renal status at M3, quantitative variables were compared with the Wilcoxon test or Kruskal-Wallis test when appropriate. Qualitative variables were compared with the Fisher test or the χ^2 test when appropriate. Overall survival (OS) was defined as the time from the date of diagnosis to the date of death or last follow-up. Renal

TABLE 1 | Characteristics of 119 anti-GBM patients at presentation.

Clinical features	
Age (years, median [IQR])	54 [29; 72]
Female (%)	59 (50)
Ethnic group*	
Caucasian (%)	94 (83)
Other (%)	19 (17)
Toxics	
Tobacco (%)*	50 (46)
Cannabis (%)*	6 (6)
Other (%)	12 (10)
Comorbidities	
Hypertension (%)*	40 (34)
Diabetes (%)*	9 (8)
Dyslipidemia (%)*	14 (12)
Time between onset and diagnosis (months, median [IQR])	0.4 [0.1; 0.9]
Symptom leading to the medical consultation*	
Fatigue (%)	38 (33)
Fever (%)	10 (9)
Dyspnea (%)	11 (10)
Cough (%)	7 (6)
Hemoptysis (%)	15 (13)
Microscopic hematuria (%)	9 (8)
Biological anomaly (%)	25 (22)
Biological features	
ANCA positivity (%)*	30 (28)
Hemoglobin level (g/dl, median [IQR])*	9 [8; 10]
CRP (mg/L, median [IQR])*	93 [38; 164]
Renal involvement	
Acute renal failure (%)*	101 (91)
Serum creatinine (mg/dl, median [IQR])*	7.2 [4.2; 11.4]
Proteinuria (> 0.5 g/dl, %)*	72 (91)
Microscopic hematuria (%)*	81 (98)
Leukocyturia (%)*	42 (93)
Serum albumin (g/l, median [IQR])*	27 [22; 31]
Renal biopsy (%)*	101 (86)
Extracapillary proliferation (%)*	69 (68)
Capsular rupture (%)*	32 (76)
Interstitial fibrosis (%)*	38 (64)
Hyaline thrombi (%)*	11 (15)
Immunofluorescence positivity (%)*	91 (99)
Pulmonary involvement	
Dyspnea (%)*	42 (38)
Cough (%)*	39 (35)
Overt hemoptysis (%)*	31 (27)
Pulmonary interstitial opacities on chest CT (*n*, %)*	40 (57)
Alveolar hemorrhage on bronchoalveolar lavage (*n*, %)*	23 (92)
PaO2 (mmHg, median [IQR])*	77 [60; 86]
Therapeutic regimens	
Admission to intensive care (%)*	36 (31)
Mechanical ventilation (%)	8 (22)
Initial hemodialysis (%)*	91 (78)
Plasmapheresis (%)*	97 (82)
Corticosteroid pulses (%)*	81 (70)
Oral corticosteroids (%)*	115 (97)
Cyclophosphamide (%)*	97 (82)
Intravenous (%)*	67 (73)
Oral (%)*	25 (27)
Cumulative dose (mg, median [IQR])*	4,000 [1,100; 6,112]
Rituximab (%)*	11 (9)
Other immunosuppressive agent (%)*	4 (3)

*Presence of missing values.
IQR, interquartile range; ANCA, antineutrophil cytoplasm antibodies; CRP, C reactive protein; CT, computed tomography.

survival (RS) was examined both in the global population at M3 (3 months after the initial hospitalization), as a categorical endpoint, and in patients without ESRD alive at M3, as a time-to-event endpoint (ESRD-free survival) using a landmark approach (22). ESRD-free survival was defined as the time from M3 (confirmation if ESRD- profile) to the date of first ESRD diagnosis, death or last follow-up, whichever occurred first. Time-to-event outcomes were estimated using the Kaplan-Meier method. Univariate analyses of factors associated with survival outcomes were performed in Cox regression models, or using the LogRank test when appropriate. The proportional hazards assumption and loglinearity assumption for quantitative variables were assessed.

Tests were two-sided and a significance level smaller than 0.05 was considered to indicate a significant association. Analyzes were carried out with the statistical software R, version 3.4.1 (https://cran.r-project.org/).

RESULTS

Characteristics of Anti-GBM Patients

The main clinical, laboratory, pathological, and immunological features are summarized in **Table 1**. We included 119 patients with a male to female ratio of 1 (60:59). The median age at the time of diagnostic was 54 years (range: 5–86) following a bimodal distribution with a first peak during the third decade and a second one around the age of 60. Fifty patients (42%) patients were smokers. Twelve patients (10%) reported a toxic exposure in the weeks preceding the onset of symptoms such as cannabis, ecstasy, pesticides, or cleaner household product. Twelve patients (10%) had a personal history of autoimmune or inflammatory disease including systemic scleroderma or Hashimoto thyroiditis; or vasculitis such as Takayasu arteritis.

The main symptoms at presentation were fatigue, fever, dyspnea, hemoptysis, and microscopic hematuria. One hundred and one (91%) had acute kidney injury at diagnostic with a median serum creatinine level of 7.2 mg/dl. Microscopic hematuria was found in 98% of patients, leukocyturia in 93%, and median proteinuria was 1.76 g/l. Fifty-four patients had alveolar hemorrhage

TABLE 2 | Comparison of anti-GBM patients according to ANCA status.

	ANCA – ($n = 77$)	ANCA + ($n = 30$)	*P*-value
Clinical features			
Age (years, median [IQR])	47 [26; 62]	70 [57; 78]	**< 0.0001**
Female (%)	35 (45)	17 (57)	0.39
Toxics			
Tobacco (%)*	40 (54)	6 (26)	**0.03**
Cannabis (%)*	5 (7)	0 (0)	0.33
Other (%)	9 (12)	3 (10)	1
Comorbidities			
Hypertension (%)*	22 (29)	13 (46)	0.10
Diabetes (%)*	8 (10)	1 (3)	0.44
Dyslipidemia (%)*	7 (9)	7 (24)	0.055
Renal involvement			
Acute renal failure (%)*	64 (86)	30 (100)	0.059
Serum creatinine (mg/dl, median [IQR])*	7.3 [4.2; 10.4]	7.0 [3.8; 11.8]	0.74
Proteinuria (> 0.5 g/d, %)*	48 (89)	19 (100)	0.33
Microscopic hematuria (%)*	56 (98)	21 (100)	1
Leukocyturia (%)*	25 (89)	15 (100)	0.54
Serum albumin (g/l, median [IQR])*	26 [22; 31]	29 [25; 33]	0.40
Renal biopsy (%)	65 (84)	26 (87)	1
Extracapillary proliferation (%)*	48 (94)	14 (93)	1
Capsular rupture (%)*	23 (85)	9 (64)	0.23
Interstitial fibrosis (%)*	24 (60)	11 (69)	0.76
Hyaline thrombi (%)*	7 (15)	3 (15)	1
Immunofluorescence positivity (%)*	60 (100)	23 (96)	0.29
Pulmonary involvement			
Dyspnea (%)*	29 (39)	10 (34)	0.82
Cough (%)*	28 (37)	9 (31)	0.65
Overt hemoptysis (%)*	24 (32)	4 (14)	0.085
Pulmonary interstitial opacities on chest CT (*n*, %)*	27 (55)	12 (63)	0.60
Alveolar hemorrhage on bronchoalveolar lavage (*n*, %)*	15 (88)	7 (100)	1
PaO2 (mmHg, median [IQR])*	77 [60; 82]	81 [75; 93]	0.41
Therapeutic regimens			
Admission to intensive care (%)*	25 (32)	8 (28)	0.81
Mechanical ventilation (%)	5 (20)	3 (38)	0.37
Initial hemodialysis (%)*	57 (74)	24 (83)	0.45
Plasmapheresis (%)	64 (83)	25 (83)	1
Corticosteroid pulses (%)	56 (73)	19 (63)	0.36
Oral corticosteroids (%)	76 (99)	29 (97)	0.48
Cyclophosphamide (%)	63 (82)	25 (83)	1
Rituximab (%)	6 (8)	5 (17)	0.29
Other immunosuppressive agent (%)	3 (4)	1 (3)	1

**Presence of missing values. Significant P-values are represented in bold. IQR, interquartile range; ANCA, antineutrophil cytoplasm antibodies; CRP, C reactive protein; CT, computer scan.*

confirmed by chest CT in 40 patients and bronchoalveolar lavage in 23 patients. Forty-eight individuals (40%) had combined kidney and lung involvement whereas 64 (54%) and 7 (6%) had isolated renal or pulmonary involvement, respectively.

Diagnosis of anti-GBM disease was assessed by the presence of anti-GBM antibodies ($n = 103$, 93%) and/or by renal histology revealing linear glomerular basement IgG deposits ($n = 91$, 99%) when tested.

One third of patients was admitted in an intensive care unit, 8 of them required mechanical ventilation, and 3 needed a vasopressor support. Initial renal replacement therapy was required in 91 patients (78%). Ninety-seven patients (82%) received plasma exchanges. The non-use of plasma exchange was most often decided in cases of advanced renal damage with scarring. Among the 115 patients who received tapering doses of oral prednisone, 81 also received 1 to 3 intravenous pulses of methylprednisolone (70%). A total of 97 (82%)

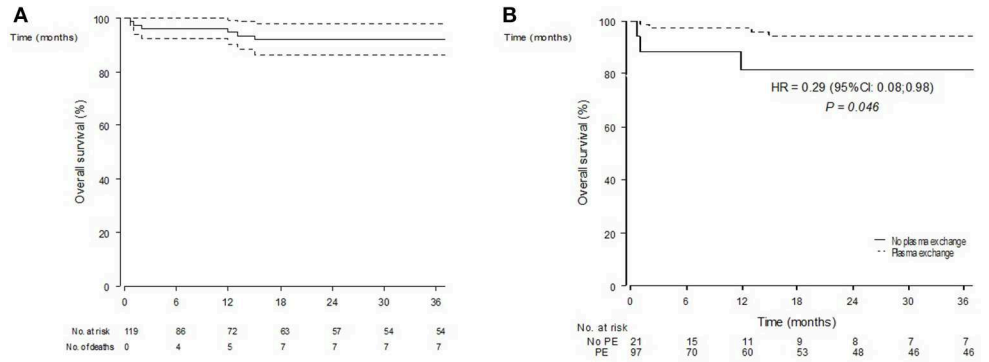


FIGURE 1 | Overall survival estimates (Kaplan-Meier estimator) in $n = 119$ included patients **(A)** and according to the initial use of plasma exchanges **(B)**.

individuals received CYC, intravenously in two-thirds of cases. Rituximab therapy was initiated within 3 months following the diagnosis in 11 (9%) patients. Four patients received other immunosuppressive agents (azathioprine, $n = 3$, mycophenolate mofetil, $n = 1$).

Comparison of Anti-GBM Patients According to ANCA Status

Of the 107 patients tested, 30 were positive for ANCA (ANCA+, 28%), with anti-MPO specificity in the majority of cases (27/30). ANCA positive (ANCA+) patients were significantly older (median age 70 vs. 47 years-old, $p < 0.0001$), were less likely smokers (26 vs. 54%, $p = 0.03$), and cannabis users (0 vs. 7%) compare to ANCA negative (ANCA-) patients (**Table 2**). All of ANCA+ patients had acute renal failure at diagnosis. Conversely, only 4 (14%) of ANCA+ presented hemoptysis compared to 24 (32%) of ANCA- patients.

Both groups had comparable rates of hospitalization in intensive care unit, with a higher rate of mechanical ventilation, vasopressor support, and hemodialysis in the ANCA+ group, although not statistically significant. Therapeutic regimens included plasma exchanges, corticosteroids, and cyclophosphamide in comparable rates. However, rituximab treatment was initiated in 17% of ANCA+ vs. only 8% of ANCA-, although this difference was not statistically significant.

Overall Survival

The OS was 95% (95% CI: 90–99) at 1 year and 92% (95% CI: 86–98) at 3 and 5 years (**Figure 1A**). Median survival was not reached during a median follow-up of 24 months (6–54). Eleven patients died during this follow-up. Among those, the median time from presentation until death was 13 months (1.5–60), 4 patients died during the first 6 months, and 5 during the first year. The serum creatinine levels at presentation were >500 μmol/L for 9 of them. They all required hemodialysis within the first month and 5 had isolated renal involvement. Causes of death were infections in 2 patients, acute congestive heart failure in 1 patient, discontinuation of hemodialysis after cessation of treatment in 1 patient, neoplastic complications in 3 patients (1 pulmonary cancer at 104 months, 2 urothelial bladder cancers at 15 and 168 months, respectively) and bedridden condition in 1 patient. In the other cases, the cause of death was not specified.

OS prognostic factors are summarized in **Table 3**. In univariate analyses, older age at presentation [HR for 10 years: 4.10 (1.89–8.88) $p = 0.0003$], history of hypertension [HR 19.9 (2.52–157.2) $p = 0.005$], or dyslipidemia [HR 11.1 (2.72–45) $p = 0.0008$], and initial mechanical ventilation [HR 5.20 (1.02–26.4) $p = 0.047$] were associated to death. Conversely, plasma exchanges use was associated with a better survival [HR 0.29 (0.08–0.98) $p = 0.046$] (**Figure 1B**). Gender, alveolar hemorrhage, ANCA status or the use of an alternative immunosuppressor was not associated to death.

Renal Survival

We examined the baseline characteristics according to renal status, as diagnosed after 3 months of follow-up: ESRD+ ($n = 55$, 46%), ESRD- patients ($n = 37$, 31%), or not evaluable [follow-up shorter than 3 months, lost-to-follow-up (LFUP), $n = 27$, 23%; **Figure 2A**]. The ESRD+ and ESRD- group data are summarized in **Table 4**. The complete table including the LFUP group data is available in **Supplementary Material**. ESRD+ patients were older than ESRD- patients (57 vs. 37 years, $p = 0.003$). The biological parameters were similar including the positivity of the ANCAs. Serum creatinine level at presentation was significantly higher in ESRD+ patients than in ESRD- [9.1 (6.3; 14.3) vs. 4.0 mg/dl (1.4; 5.9), $p < 0.0001$]. The histological parameters seemed also associated with short-term renal impairment, although not statistically significant at the pre-defined threshold, with greater observed proportions of extracapillary proliferation (73 vs. 60%), capsular rupture (89 vs. 55%), interstitial fibrosis (69 vs. 56%), and hyaline thrombi (19 vs. 10%). Conversely, the initial pulmonary involvement seemed more frequent in ESRD- patients with more cough (58 vs. 23%, $p = 0.004$) and alveolar hemorrhage (71 vs. 36%) than in ESRD+. Concerning initial treatments, ESRD+ had required more frequently renal replacement therapy at the onset (96 vs. 44% in ESRD-, $p < 0.0001$), and tended to receive less CYC (76 vs. 94%, $p = 0.060$) than ESRD-.

The majority of patients presented with severe renal failure at diagnosis. However, of the 50 patients with a serum creatinine

TABLE 3 | Overall survival prognostic factors.

	HR [95% CI]	*P*-value
Clinical features		
Age (HR for 10 years)	4.10 [1.89; 8.88]	**0.0003**
Male	1.02 [0.31; 3.34]	0.98
Toxics		
Tobacco	0.59 [0.17; 2.01]	0.40
Cannabis		0.50*
Other	1.37 [0.17; 11.0]	0.77
Comorbidities		
Hypertension	19.9 [2.52; 157.2]	**0.005**
Diabetes		0.51*
Dyslipidemia	11.1 [2.71; 45.0]	**0.0008**
Time between onset and diagnosis (HR for 1 month)	0.010 [0.000; 1.69]	0.078
Biological features		
ANCA positivity	3.01 [0.78; 11.7]	0.11
Hemoglobin level	0.87 [0.32; 2.36]	0.79
CRP (HR for 10 mg/l)	0.79 [0.46; 1.37]	0.41
Renal involvement		
Serum creatinine (HR for 1 mg/dl)	0.97 [0.86; 1.09]	0.57
Proteinuria (> 0.5 g/dl)		0.41*
Microscopic hematuria		0.67*
Serum albumin	1.22 [0.79; 1.89]	0.38
Renal biopsy		
Extracapillary proliferation	1.91 [0.23; 16.0]	0.55
Immunofluorescence positivity		0.88*
Pulmonary involvement		
Dyspnea	0.73 [0.19; 2.86]	0.66
Cough	0.52 [0.11; 2.45]	0.41
Alveolar hemorrhage	1.13 [0.34; 3.72]	0.84
Therapeutic regimens		
Admission to intensive care	1.67 [0.42; 6.56]	0.46
Mechanical ventilation	5.20 [1.02; 6.56]	**0.047**
Initial hemodialysis		**0.092***
Plasmapheresis	0.29 [0.08; 0.98]	**0.046**
Corticosteroid pulses	0.73 [0.21; 2.50]	0.42
Cyclophosphamide	0.58 [0.15; 2.20]	0.42
Rituximab		0.33*
Other immunosuppressive agent		0.50*

**P-values from Log Rank tests, due to limited number of events across groups defined by the candidate variables. Significant P-values (<0.05) are represented in bold. HR, hazard ratio; CI, confidence interval; ANCA, antineutrophil cytoplasm antibodies; CRP, C reactive protein.*

level of <6.8 mg/dl (i.e., 600 μmol/L) at diagnosis, 26 were nevertheless dialyzed immediately because of a rapid degradation of their renal function. The description of the cohort according to the creatinine level (< or ≥ 6.8 mg/dl) and the Kaplan-Meier curves for overall survival by group are available in **Supplementary Material**.

Ninety-one (78%) patients required dialysis at presentation. Of these, 53 progressed to chronic end stage renal failure (ESDR+), 15 have recovered renal function (ESRD-), and 23 have been lost to follow-up (LFUP) at M3. ESRD- patients at M3 had a lower serum creatinine at presentation [6.1 mg/dl (6.1;12.1) vs. 9.8 (6.5;14.6), $p = 0.006$], were less likely to have hypertension at diagnosis (29 vs. 46%, $p = 0.024$), had more often pulmonary involvement (hemoptysis 47 vs. 18%, $p = 0.019$; alveolar hemorrhage 73 vs. 37%, $p = 0.022$) and have more often required the use of mechanical ventilation (100 vs. 13%, $p = 0.001$) than ESRD+ patients (**Supplementary Material**).

ESRD-free survival in patients without ESRD alive at M3 ($n = 37$) is represented in **Figure 2B**. Starting from M3, the median follow-up was 44 months (9–81). During the follow-up, 10 of the 37 M3-ESRD- patients eventually developed ESRD, following the adverse course of renal function or relapse of the disease; two of them died. In the M3-ESRD-population, ESRD-free survival prognostic factors are presented in **Table 5**. The main predictors of poor renal outcome were: the presence of hyaline thrombi on renal biopsy [HR 17 (95% confidence interval (CI) 1.06; 271.6) $p = 0.045$]; and cannabis use [HR 7.64 (1.80; 32.5) $p = 0.006$].

At the end of the follow-up, among all patients who reached ESRD ($n = 62$, 67%), 29 patients were still in hemodialysis and 33 had received kidney transplant. Five patients (4%) had a relapse during the follow-up with a median of 12 months following diagnosis. Among them, two were renal relapses, one pulmonary relapse, and one affecting both organs. All pulmonary relapses involved patients with isolated lung involvement. Relapsing patients received therapeutic regimen including 4/5 (80%) plasma exchanges, 5/5 (100%) corticosteroids, 4/5 (80%) CYC, and none received rituximab. No relapse was observed after transplantation.

DISCUSSION

Anti-GBM disease is a rare disease with an estimated incidence between 0.5 and 1.6 case per million per year (23) but it represents 1 to 5% of all types of glomerulonephritis and ∼20% of rapidly progressive glomerulonephritis (24–26). The severity of disease imposes an early diagnosis to initiate rapidly plasmapheresis and immunosuppressive treatments. There are still unmet needs to identify prognostic factors prior to complications to target patients needing more aggressive therapy.

In this large French nationwide multicenter cohort, we first analyzed anti-GBM patients according to ANCA status. ANCA positivity was found in 28% of patients. Double-positive patients were older, less frequently smokers, and had less pulmonary involvement. Consistently with previous series (7, 10, 27), we reported a high frequency of ANCA positive anti-GBM disease patients. Olson et al. (28) suggest that ANCA-induced glomerular inflammation may be a trigger for the development of an anti-GBM response, perhaps by modifying or exposing usually sequestered disease epitopes in GBM, since it has been shown that ANCA may be detected before the onset of anti-GBM disease. Our ANCA positive patients experienced severe renal involvement since all of them presented acute renal failure at onset compared to 86% of their ANCA negative counterpart. In contrast, lung involvement was less frequent. In a large European cohort, McAdoo et al showed that double-positive patients

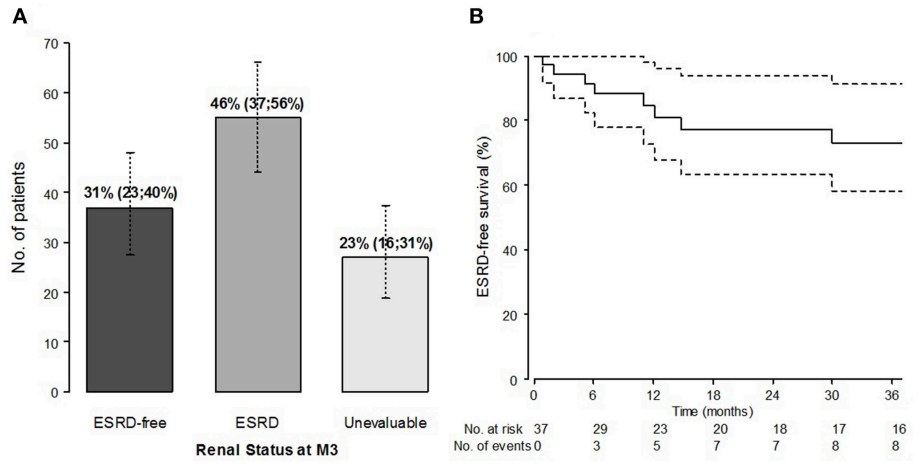


FIGURE 2 | Renal outcome: prevalence of patients with ESRD at M3 (%, 95% confidence interval) **(A)** and ESRD-free survival (Kaplan-Meier estimates) from M3, in patients alive and without ESRD at M3 ($n = 37$) **(B)**.

had severe kidney and lung disease at presentation, requiring aggressive immunosuppressive therapy, and plasma exchange (10). During long-term follow-up, they relapsed at a frequency comparable to a parallel cohort of patients with ANCA-associated vasculitis (AAV), suggesting they warrant more careful long-term follow-up and maintenance immunosuppression, unlike patients with single-positive anti-GBM disease.

The presence of hyaline thrombi on renal biopsy and cannabis use were significantly associated with ESRD in patients without initial ESRD. In our study, ESRD at 3 months was observed in 46% of cases. ESRD positive patients were older, more frequently men, and had higher serum creatinine level at presentation than those without ESRD. These results are consistent with those of previous studies showing that the occurrence of oliguria or anuria, elevated serum creatinine at presentation and the percentage of crescents were shown to be risk factors for ESRD (19, 29).

This large cohort allowed us to identify four prognostic factors of overall survival. We identified age at onset, existence of cardiovascular risk factors, aggressiveness of initial management with mechanical ventilation, and the absence of plasmapheresis as significantly associated with death in anti-GBM patients. Mortality in anti-GBM used to be extremely high, up to 95% in older series (30) and was mainly related to pulmonary hemorrhage, or to end-stage renal failure. New protocols including plasmapheresis, glucocorticoids, and cyclophosphamide (CYC) had dramatically improved patient's outcomes. In our study, the 1 and 5-year survival reached 95 and 92%, respectively. This rate was higher than OS observed in recent other series. Proskey et al. reported 88% survival rate in an English study over 20 years (14). Huart et al reported 86.9% 1-year survival rate (20). This improvement of survival rate could be explained by the relatively low rate of infectious complications (23%), and severe infections accounted only for 2 out of 11 deaths. In contrast, 3 deaths were attributable to cancers (at 15, 108, and 162 months after presentation, respectively), and 2 others occurred after renal transplantation. This underlines the need to take into account the toxicity of immunosuppressive treatments (mainly CYC) used in the acute phase of the disease, and anti-rejection treatments after transplantation. In this respect, induction therapy with rituximab, may reduce the risk of developing secondary cancer.

Surprisingly, pulmonary involvement was not a factor of poor prognosis. Our study confirms the results of other series on the importance of plasma exchanges positively associated with overall survival. Huart et al. showed that a cut-off of 8 plasmapheresis sessions was associated with positive and negative predictive survival rates of 95 and 47%, respectively (20). Given the physiopathological importance of the clearance of autoantibodies in the disease, the number of plasma exchanges could be monitored according to the course of circulating anti-GBM levels. On 111 patients tested, 8 (7%) were antibody-negative anti-GBM disease. Seven of them had acute renal failure and half had alveolar hemorrhage at presentation. These results differ somewhat from those of a recent study reporting 19 cases of negative antibody patients with better renal function at biopsy and less lung involvement than in classic anti-GBM patients (31).

We acknowledge some limitations in our study. Our analysis was performed in a retrospective manner. We were unable to collect complete longitudinal data on patients who were seen only on an intermittent consultation basis. A few initial patients (27/119, 23%) were lost-to follow-up soon after diagnosis, before M3, most often due to a change of medical center for dialysis or pre-transplant assessment. However, to prevent selection biases, these patients, categorized at unevaluable at M3 for renal function, were included in the descriptive analysis and evaluating prognostic factors of ESRD status at M3 (**Supplementary Material**). Furthermore, given that ESRD diagnosis requires a 3-month follow-up time window by definition, we used a landmark approach to examine prognostic factors of ERD-free survival from M3. The sample was therefore restricted to ESRD-free patients alive at M3 ($n = 37$) and limited

TABLE 4 | Comparison of anti-GBM patients according to ESRD status at M3 (ESRD status was categorized in 3 groups: ESRD–, ESRD+, Not evaluable [FUP < 3 months]).

Variables	ESRD– (*n* = 37)	ESRD+ (*n* = 55)	*P*-value†
Clinical features			
Age (years)	37 [25; 56]	57 [38; 74]	**0.003**
Female (%)	15 (41)	31 (56)	0.35
Toxics			
Tobacco (%)*	20 (57)	22 (43)	0.22
Cannabis (%)*	3 (9)	1 (2)	0.34
Other (%)	5 (14)	2 (4)	0.057
Comorbidities			
Hypertension (%)*	11 (31)	19 (35)	0.88
Diabetes (%)*	3 (8)	2 (4)	0.15
Dyslipidemia (%)*	4 (11)	6 (11)	0.87
Time between onset and diagnosis (months, median [IQR])*	0.5 [0.1; 1.0]	0.3 [0.1; 0.8]	0.32
Biological features*			
ANCA positivity (%)	8 (24)	14 (29)	0.70
Hemoglobin level (g/dl)*	9 [8; 10]	9 [8; 10]	0.70
CRP (mg/L)*	84 [28; 142]	128 [86; 239]	0.044
Renal involvement			
Serum creatinine (mg/dl)*	4.0 [1.4; 5.9]	9.1 [6.4; 14.3]	**< 0.0001**
Proteinuria (> 0.5 g/dl, %)*	25 (86)	27 (96)	0.43
Microscopic hematuria (%)*	31 (97)	28 (100)	0.74
Leukocyturia (%)*	16 (89)	12 (100)	0.77
Serum albumin (g/l)*	30 [22; 33]	26 [23; 31]	0.25
Renal biopsy (%)*	30 (83)	50 (93)	
Extracapillary proliferation (%)*	18 (60)	37 (74)	0.41
Capsular rupture (%)*	6 (55)	16 (89)	0.12
Interstitial fibrosis (%)*	6 (56)	20 (69)	0.64
Hyaline thrombi (%)*	2 (10)	7 (19)	0.69
Immunofluorescence positivity (%)*	29 (97)	46 (100)	0.50
Pulmonary involvement			
Dyspnea (%)*	16 (50)	16 (30)	0.20
Cough (%)*	19 (58)	12 (23)	**0.004**
Alveolar hemorrhage (%)*	25 (71)	19 (36)	**0.002**
Therapeutic regimens			
Admission to intensive care (%)*	8 (24)	15 (27)	0.11
Mechanical ventilation (%)	5 (62)	2 (13)	**0.015**
Initial hemodialysis (%)*	15 (44)	53 (96)	**< 0.0001**
Plasmapheresis (%)*	32 (89)	44 (80)	0.45
Corticosteroid pulses (%)*	25 (69)	36 (68)	0.84
Cyclophosphamide (%)*	34 (94)	42 (76)	0.06
Rituximab (%)*	6 (17)	3 (5)	0.23
Other immunosuppressive agent (%)*	2 (6)	2 (4)	0.69

**Presence of missing values. †P-values for Fisher's exact tests or Kruskal-Wallis tests comparing discrete and continuous variables, respectively, across ESRD+, ESRD–, and LFUP (lost-to-follow-up before 3 months) groups. Significant P-values (<0.05) are represented in bold. ANCA, antineutrophil cytoplasm antibodies; CRP, C reactive protein.*

TABLE 5 | ESRD-free survival prognostic factors, in ESRD-free patients alive at M3 (*n* = 37).

	HR [95% CI]	*P*-value
Clinical features		
Age (HR for 10 years)	1.02 [0.67; 15.7]	0.14
Male	3.24 [0.67; 1.45]	0.91
Toxics		
Tobacco	1.00 [0.27; 3.74]	1
Cannabis	7.64 [1.80; 32.5]	**0.006**
Other	0.9 [0.11; 7.32]	0.92
Comorbidities		
Hypertension	0.66 [0.14; 3.17]	0.60
Diabetes		0.29*
Dyslipidemia	1.20 [0.15; 9.59]	0.87
Time between onset and diagnosis (HR for 1 month)	0.76 [0.42; 1.40]	0.38
Biological features		
ANCA positivity	1.15 [0.23; 5.74]	0.86
Hemoglobin level	1.34 [0.68; 2.64]	0.40
CRP (HR for 10 mg/L)	1.01 [0.83; 1.23]	0.92
Renal involvement		
Serum creatinine (HR for 1 mg/dl)	1.01 [0.80; 1.27]	0.95
Proteinuria (> 0.5 g/dl)		0.35*
Microscopic hematuria		0.49*
Leukocyturia		0.35*
Serum albumin	0.91 [0.77; 1.09]	0.31
Renal biopsy		
Extracapillary proliferation	0.76 [0.18; 3.22]	0.71
Capsular rupture	0.82 [0.05; 13.2]	0.89
Interstitial fibrosis	3.11 [0.34; 28.7]	0.32
Hyaline thrombi	17 [1.06; 271.6]	**0.045**
Immunofluorescence positivity		0.73*
Pulmonary involvement		
Dyspnea	1.26 [0.31; 5.08]	0.75
Cough	2.99 [0.60; 14.9]	0.18
Alveolar hemorrhage	3.81 [0.47; 30.5]	0.21
Therapeutic regimens		
Admission to intensive care	1.43 [0.29; 7.16]	0.66
Mechanical ventilation	2.73 [0.55; 13.6]	0.22
Initial hemodialysis	1.72 [0.46; 6.44]	0.42
Plasmapheresis	0.30 [0.06; 1.48]	0.14
Corticosteroid pulses	1.62 [0.34; 7.84]	0.55
Cyclophosphamide		0.45*
Rituximab		0.18*
Other immunosuppressive agent	1.57 [0.20; 12.6]	0.67

**P-values from Log Rank tests, due to limited number of events across groups defined by the candidate variables. Significant P-values (<0.05) are represented in bold. HR, hazard ratio; CI, confidence interval; ANCA, antineutrophil cytoplasm antibodies; CRP, C reactive protein.*

in size; nonetheless, the landmark approach is an adequate approach to prevent immortal time bias (22). The quantification of diuresis and the evolution of urinary sediment would have let us evaluate anuria and proteinuria as potential pejorative prognostic factors of renal evolution. Our study also comes up against the lack of proofreading of anatomopathological features of renal biopsies. The presence of hyaline thrombi remains a non-specific element, especially in acute glomerulonephritis. We

could not specify either their location or their number. Similarly, the presence of acute tubular necrosis lesions and/or vasculitis has not been reported. In a recent study classifying 123 anti-GBM kidney biopsy samples according to ANCA-associated GN, histopathological class, and kidney survival were associated. Low percentage of normal glomeruli and large extent of interstitial infiltrate were associated with poor renal survival (32). Anti-GBM antibodies levels could only very rarely be collected during follow-up. Thus, their rate after plasma exchange was available for only 47% of patients, limiting the interpretation of the impact of treatments on the clearance of autoantibodies. Although we provide univariate analyses of EFS and of OS, due to the limited number of events, we were unable to perform robust multivariate analyzes for these outcomes (33). Prospective enrollment and data collection from the time of diagnosis would have been ideal but are difficult to achieve with such rare diseases.

In conclusion, this French nationwide study shows that older age at diagnosis, female gender, a high serum creatinine level at presentation, and extracapillary proliferation predicted renal survival in patients with anti-GBM disease. We identified age at onset, existence of cardiovascular risk factors, aggressiveness of initial management with mechanical ventilation and the absence of plasmapheresis as significantly associated with death in anti-GBM patients.

AUTHOR CONTRIBUTIONS

CM, JC, NJ-C, and DS contributed conception and design of the study. CM, JC, and AP organized the database. LB performed the statistical analysis. CM wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01665/full#supplementary-material

REFERENCES

1. Jennette JC, Falk RJ, Bacon PA, Basu N, Cid MC, Ferrario F, et al. 2012 Revised International Chapel Hill Consensus Conference Nomenclature of Vasculitides. *Arthritis Rheumat.* (2013) 65:1–11. doi: 10.1002/art.37715
2. Krakower CA, Greenspon SA. Localization of the nephrotoxic antigen within the isolated renal glomerulus. *AMA Arch Pathol.* (1951) 51:629–39.
3. Pusey CD. Anti-glomerular basement membrane disease. *Kidney Int.* (2003) 64:1535–50. doi: 10.1046/j.1523-1755.2003.00241.x
4. Greco A, Rizzo MI, De Virgilio A, Gallo A, Fusconi M, Pagliuca G, et al. Goodpasture's syndrome: a clinical update. *Autoimmun Rev.* (2015) 14:246–53. doi: 10.1016/j.autrev.2014.11.006
5. Segelmark M, Hellmark T. Anti-glomerular basement membrane disease: an update on subgroups, pathogenesis and therapies. *Nephrol Dial Transplant.* (2018) 2018:327. doi: 10.1093/ndt/gfy327
6. DE Zoysa J, Taylor D, Thein H, Yehia M. Incidence and features of dual anti-GBM-positive and ANCA-positive patients. *Nephrology.* (2011) 16:725–9. doi: 10.1111/j.1440-1797.2011.01484.x
7. Jayne DR, Marshall PD, Jones SJ, Lockwood CM. Autoantibodies to GBM and neutrophil cytoplasm in rapidly progressive glomerulonephritis. *Kidney Int.* (1990) 37:965–70. doi: 10.1038/ki.1990.72
8. Verburgh CA, Bruijn JA, Daha MR, van Es LA. Sequential development of anti-GBM nephritis and ANCA-associated Pauci-immune glomerulonephritis. *Am. J. Kidney Dis.* (1999) 34:344–8. doi: 10.1016/S0272-6386(99)70366-5
9. Hellmark T, Niles JL, Collins AB, McCluskey RT, Brunmark C. Comparison of anti-GBM antibodies in sera with or without ANCA. *J Am Soc Nephrol.* (1997) 8:376–85.
10. McAdoo SP, Tanna A, Hrušková Z, Holm L, Weiner M, Arulkumaran N, et al. Patients double-seropositive for ANCA and anti-GBM antibodies have varied renal survival, frequency of relapse, and outcomes compared to single-seropositive patients. *Kidney Int.* (2017) 92:693–702. doi: 10.1016/j.kint.2017.03.014
11. Levy JB, Hammad T, Coulthart A, Dougan T, Pusey CD. Clinical features and outcome of patients with both ANCA and anti-GBM antibodies. *Kidney Int.* (2004) 66:1535–40. doi: 10.1111/j.1523-1755.2004.00917.x
12. Bosch X, Mirapeix E, Font J, Borrellas X, Rodríguez R, López-Soto A, et al. Prognostic implication of anti-neutrophil cytoplasmic autoantibodies with myeloperoxidase specificity in anti-glomerular basement membrane disease. *Clin Nephrol.* (1991) 36:107–13.
13. Alchi B, Griffiths M, Sivalingam M, Jayne D, Farrington K. Predictors of renal and patient outcomes in anti-GBM disease: clinicopathologic analysis of a two-centre cohort. *Nephrol Dial Transplant.* (2015) 30:814–21. doi: 10.1093/ndt/gfu399
14. Proskey AJ, Weatherbee L, Easterling RE, Greene JA Jr, Weller JM. Goodpasture's syndrome. A report of five cases and review of the literature. *Am J Med.* (1970) 48:162–73. doi: 10.1016/0002-9343(70)90112-9
15. Touzot M, Poisson J, Faguer S, Ribes D, Cohen P, Geffray L, et al. Rituximab in anti-GBM disease: a retrospective study of 8 patients. *J Autoimmun.* (2015) 60:74–9. doi: 10.1016/j.jaut.2015.04.003
16. Mori M, Nwaogwugwu U, Akers GR, McGill RL. Anti-glomerular basement membrane disease treated with mycophenolate mofetil, corticosteroids, and plasmapheresis. *Clin Nephrol.* (2013) 80:67–71. doi: 10.5414/CN107333
17. García-Cantón C, Toledo A, Palomar R, Fernandez F, Lopez J, Moreno A, et al. Goodpasture's syndrome treated with mycophenolate mofetil. *Nephrol Dial Transplant.* (2000) 15:920–2. doi: 10.1093/ndt/15.6.920
18. Kiykim AA, Horoz M, Gok E. Successful treatment of resistant antiglomerular basement membrane antibody positivity with mycophenolic acid. *Intern Med.* (2010) 49:577–80. doi: 10.2169/internalmedicine.49.2321
19. Cui Z, Zhao J, Jia XY, Zhu SN, Jin QZ, Cheng XY, et al. Anti-glomerular basement membrane disease: outcomes of different therapeutic regimens in a large single-center Chinese cohort study. *Medicine.* (2011) 90:303–11. doi: 10.1097/MD.0b013e31822f6f68
20. Huart A, Josse AG, Chauveau D, Korach JM, Heshmati F, Bauvin E, et al. Outcomes of patients with Goodpasture syndrome: a nationwide cohort-based study from the French Society of Hemapheresis. *J Autoimmun.* (2016) 73:24–9. doi: 10.1016/j.jaut.2016.05.015
21. McAdoo SP, Pusey CD. Anti-glomerular basement membrane disease. *Clin J Am Soc Nephrol.* (2017) 12:1162–72. doi: 10.2215/CJN.01380217
22. Gleiss A, Oberbauer R, Heinze G. An unjustified benefit: immortal time bias in the analysis of time-dependent events. *Transpl Int.* (2018) 31:125–30. doi: 10.1111/tri.13081
23. Canney M, O'Hara PV, McEvoy CM, Medani S, Connaughton DM, Abdalla AA, et al. Spatial and temporal clustering of anti-glomerular basement membrane disease. *Clin J Am Soc Nephrol.* (2016) 11:1392–9. doi: 10.2215/CJN.13591215
24. Kluth DC, Rees AJ. Anti-glomerular basement membrane disease. *J Am Soc Nephrol.* (1999) 10:2446–53.
25. Andrassy K, Küster S, Waldherr R, Ritz E. Rapidly progressive glomerulonephritis: analysis of prevalence and clinical course. *Nephron.* (1991) 59:206–12. doi: 10.1159/000186552

26. Tang W, McDonald SP, Hawley CM, Badve SV, Boudville NC, Brown FG, et al. Anti-glomerular basement membrane antibody disease is an uncommon cause of end-stage renal disease. *Kidney Int.* (2013) 83:503–10. doi: 10.1038/ki.2012.375
27. Rutgers A, Slot M, van Paassen P, van Breda Vriesman P, Heeringa P, Tervaert JW. Coexistence of anti-glomerular basement membrane antibodies and myeloperoxidase-ANCAs in crescentic glomerulonephritis. *Am J Kidney Dis.* (2005) 46:253–62. doi: 10.1053/j.ajkd.2005.05.003
28. Olson SW, Arbogast CB, Baker TP, Owshalimpur D, Oliver DK, Abbott KC, et al. Asymptomatic autoantibodies associate with future anti-glomerular basement membrane disease. *J Am Soc Nephrol.* (2011) 22:1946–52. doi: 10.1681/ASN.2010090928
29. Levy JB, Turner AN, Rees AJ, Pusey CD. Long-term outcome of anti-glomerular basement membrane antibody disease treated with plasma exchange and immunosuppression. *Ann Intern Med.* (2001) 134:1033–42. doi: 10.7326/0003-4819-134-11-200106050-00009
30. Benoit FL, Rulon DB, Theil GB, Doolan PD, Watten RH. Goodpasture's syndrome: a clinicopathologic entity. *Am J Med.* (1964) 37:424–44. doi: 10.1016/0002-9343(64)90199-8
31. Liang D, Liang S, Xu F, Zhang M, Li X, Tu Y, et al. Clinicopathological features and outcome of antibody-negative anti-glomerular basement membrane disease. *J Clin Pathol.* (2018) 72:31–7. doi: 10.1136/jclinpath-2018-205278
32. van Daalen EE, Jennette JC, McAdoo SP, Pusey CD, Alba MA, Poulton CJ, et al. Predicting Outcome in Patients with Anti-GBM Glomerulonephritis. *Clin J Am Soc Nephrol.* (2018) 13:63–72. doi: 10.2215/CJN.04290417
33. Harrell F. *Regression Modeling Strategies: With Applications to Linear Models, Logistic Regression, and Survival Analysis*. New York, NY: Springer-Verlag (2001).

Conflict of Interest Statement: 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.

frontiers in Immunology

REVIEW
published: 02 August 2019
doi: 10.3389/fimmu.2019.01796

Leveraging Genetic Findings for Precision Medicine in Vasculitis

Marialbert Acosta-Herrera[1], *Miguel A. González-Gay*[2], *Javier Martín*[1] *and Ana Márquez*[1,3]*

[1] *Instituto de Parasitología y Biomedicina "López-Neyra," CSIC, Granada, Spain,* [2] *Division of Rheumatology and Epidemiology, Genetics and Atherosclerosis Research Group on Systemic Inflammatory Diseases, Hospital Universitario Marqués de Valdecilla, IDIVAL, University of Cantabria, Santander, Spain,* [3] *Systemic Autoimmune Disease Unit, Hospital Clínico San Cecilio, Instituto de Investigación Biosanitaria ibs.GRANADA, Granada, Spain*

Vasculitides are a heterogeneous group of low frequent disorders, mainly characterized by the inflammation of blood vessels that narrows or occlude the lumen and limits the blood flow, leading eventually to significant tissue and organ damage. These disorders are classified depending on the size of the affected blood vessels in large, medium, and small vessel vasculitis. Currently, it is known that these syndromes show a complex etiology in which both environmental and genetic factors play a major role in their development. So far, these conditions are not curable and the therapeutic approaches are mainly symptomatic. Moreover, a percentage of the patients do not adequately respond to standard treatments. Over the last years, numerous genetic studies have been carried out to identify susceptibility *loci* and biological pathways involved in vasculitis pathogenesis as well as potential genetic predictors of treatment response. The ultimate goal of these studies is to identify new therapeutic targets and to improve the use of existing drugs to achieve more effective treatments. This review will focus on the main advances made in the field of genetics and pharmacogenetics of vasculitis and their potential application for ameliorating long-term outcomes in patient management and in the development of precision medicine.

Keywords: systemic vasculitis, polymorphism, genome-wide association studies, immunochip, precision medicine

OPEN ACCESS

Edited by:
Giacomo Emmi,
University of Florence, Italy

Reviewed by:
Augusto Vaglio,
University of Parma, Italy
Davide Martorana,
University Hospital of Parma, Italy

***Correspondence:**
Ana Márquez
anamaort@ipb.csic.es

Specialty section:
This article was submitted to Inflammation, a section of the journal Frontiers in Immunology

Received: *20 May 2019*
Accepted: *16 July 2019*
Published: *02 August 2019*

Citation:
Acosta-Herrera M, González-Gay MA, Martín J and Márquez A (2019) Leveraging Genetic Findings for Precision Medicine in Vasculitis. Front. Immunol. 10:1796. doi: 10.3389/fimmu.2019.01796

INTRODUCTION

Systemic vasculitides represent a heterogeneous group of chronic diseases characterized by the inflammation of the blood vessels. These disorders are classified according to the diameter of the affected vessels in large, medium and small vessel vasculitis, and may affect one or several organs and tissues of the body, resulting in different clinical presentations. In the past years, considerable therapeutic advances have been made in the treatment of vasculitis; however, the lack of appropriate therapeutic response and the appearance of side effects remain a major concern (1).

Although the specific mechanisms underlying vasculitis are not fully understood, it is currently known that these conditions show a complex etiology in which both genetic and environmental factors appear to contribute to their pathogenesis (2). In recent years, our knowledge of the genetic landscape of vasculitis has experienced a significant increase, mainly due to the development of large-scale genetic scans, including genome-wide association studies (GWASs) and Immunochip studies, focused on analyzing single-nucleotide polymorphisms (SNPs) in cases and controls

(**Figure 1**). In addition to the human leukocyte antigen (HLA) region, which represents the strongest association in vasculitis, multiple *loci* located outside the HLA have been shown to play a role in the genetic predisposition to these disorders (**Table 1**).

Identification of genes and molecular pathways deregulated in vasculitis is crucial to better understand disease pathogenesis and for the development of more effective therapeutic approaches. In this sense, Nelson et al. (27) demonstrated that a drug with genetic support has twice the possibilities of going from phase I to approval in the different phases of drug development, than those drugs without genetic support. The authors found that genes associated with a broad spectrum of human diseases were significantly enriched in target genes for drugs approved in the United States or the European Union, highlighting the importance of the provided genetic knowledge in different drug mechanisms. In addition, genetic studies not only have the potential to identify molecular targets for new therapies, but they also allow us to determine the best way to administer current treatments. In this regard, several pharmacogenetic studies based on candidate genes have identified a number of genetic variants influencing treatment response in different vasculitides (**Table 2**).

This review aims to provide an update of the main findings obtained from genetic and pharmacogenetic studies as well as their potential application to precision medicine in vasculitis.

CONTRIBUTION OF GENETICS TO NEW THERAPEUTIC APPROACHES IN VASCULITIS

Takayasu Arteritis

Takayasu arteritis (TAK) is a chronic vasculitis characterized by granulomatous inflammation of large vessels, predominantly the aortic arch and its branches, which results in non-specific constitutional symptoms, such as fever and weight loss, and serious complications, including arterial stenosis, occlusion and aneurysm. This disease affects mainly young females with a higher incidence in Asia and Latin America (46).

Genetic studies have shown that the HLA region represents the main genetic risk factor in TAK. Specifically, an association at the genome-wide significance level between the classical allele HLA-B*52:01 and this vasculitis has been reported in TAK patients from Japanese, Turkish and European-American origin (3, 47), and confirmed in Greek, Mexican Mestizo, India, Thai, and Korean populations (48–52). Moreover, independent associations within the HLA class II region, specifically with the DRB1*07 classical allele and DRB1/DQB1 polymorphisms, have also been reported (3, 53, 54); however, additional studies in well-powered populations are required to confirm these findings.

Outside the HLA region, five *loci* have been consistently associated with TAK through three large-scale genetic analyses (3–5), *IL6* (interleukin 6), encoding a cytokine that plays a crucial role in the immune response by regulating the balance between Th17 cells and regulatory T cells (Treg) (55); *LILRB3* (leukocyte immunoglobulin like receptor B3), which encodes a protein that binds to HLA class I molecules to inhibit immune cell stimulation (56); *IL12B*, encoding the p40 subunit of IL-12 and IL-23, two cytokines with a key role in the inflammatory responses mediated by Th1 and Th17 cells, respectively (57); *FCGR2A* (Fc fragment of IgG receptor IIa) that encodes an immunoglobulin Fcg receptor (FcgR), which has a relevant role in humoral immunity by participating in modulation of antibody production by B cells, phagocytosis, and clearing of immune complexes (58); and an intergenic locus on chromosome 21q22 near *PSMG1* (proteasome assembly chaperone 1).

Interestingly, some genes associated with TAK are being explored as therapeutic targets for this vasculitis. On one hand, tocilizumab, a humanized monoclonal antibody against IL-6 receptor (IL-6R), has shown clinical efficacy in TAK patients in several case series studies (59). This efficiency was confirmed in a prospective clinical trial evaluating tocilizumab in refractory TAK, although the primary end-point was not met, probably due to the low number of individuals included in this study (60). A phase III clinical trial evaluating this biological agent as a first-line therapy in TAK patients is currently underway (NCT02101333).

On the other hand, administration of ustekinumab, a monoclonal antibody to the p40 subunit common to IL-12 and IL-23, to patients with active TAK achieved decrease of inflammatory markers but did not improve vascular lesions in a pilot clinical trial (61). In a more recent study, this drug was used to treat a patient with refractory TAK and psoriasis (for which this drug is approved), two diseases that share the genetic risk locus *IL12B*, with satisfactory results (62). Ustekinumab allowed a significant reduction in glucocorticoid dose and full reduction of vessel wall thickness, thus demonstrating the usefulness of drug repositioning based on the existence of a common genetic component.

Moreover, several evidences, including the association observed between *FCGR2A* and TAK, indicate that, in addition to T lymphocytes, B cells are also involved in the pathogenesis of this vasculitis. In this regard, depletion of B cells using rituximab, a chimeric anti-CD20 monoclonal antibody, has been shown to be effective in a case series study, achieving clinical and laboratory remission (63). Nevertheless, a randomized control trial is needed in order to confirm the efficacy of rituximab in patients with TAK.

Giant Cell Arteritis

Giant cell arteritis (GCA) is a vasculitis characterized by chronic inflammation of medium- and large-sized blood vessels, mainly the aorta and external carotid arteries and their branches. A severe complication of this disorder is the occlusion of the ophthalmic artery, which leads to acute and irreversible blindness. GCA represents the most frequent vasculitis in elderly individuals from Western countries affecting predominantly women and people over 50 years of age (64).

In the last years, a high number of candidate gene association studies have been performed in GCA, most of them focused on analyzing genes encoding inflammatory cytokines (65). These studies identified the HLA class II region, specifically the classical allele DRB1*04, as the main genetic risk factor in GCA. However, both the low sample size and the lack of replication cohorts of these studies have been limiting factors in the identification of robust genetic associations outside the HLA region. Nevertheless,

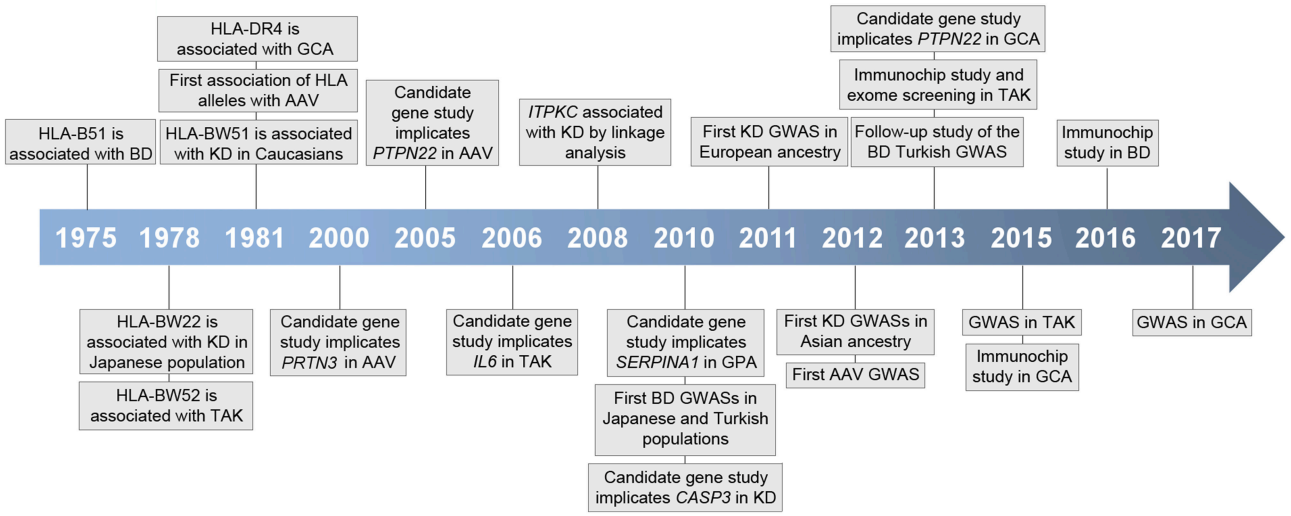


FIGURE 1 | Timeline representing key events in vasculitis genetic research. HLA, human histocompatibility complex; BD, Behçet's disease; GCA, giant cell arteritis; AAV, ANCA-associated vasculitis; KD, Kawasaki's disease; TAK, Takayasu arteritis; GPA, granulomatosis with polyangiitis; GWAS, genome-wide association study.

some of the non-HLA loci associated with this vasculitis using candidate-gene approaches were replicated in different populations (66–71) and, therefore, they represent potential genetic risk factors in GCA, including *IL33*, which encodes a member of the IL-1 family involved in pro-inflammatory cytokines production, angiogenesis and vascular permeability (72, 73); *IL17A*, encoding a pro-inflammatory cytokine with a relevant role in the differentiation of Th17 lymphocytes (74); *VEGF* (vascular endothelial growth factor), encoding a proangiogenic mediator (75); and *NLRP1* (NLR family pyrin domain containing 1), encoding a protein implicated in the formation of the inflammasome, which activates caspases 1 and 5 leading to the activation of pro-inflammatory cytokines such as IL-1β and IL-18 (76).

More recently, the emergence of massive genotyping platforms and the formation of a large consortium focused on the study of the genetic basis of GCA have allowed a significant progress in the identification of this genetic component. Until now, two large-scale genetic studies, a GWAS and an Immunochip, have been performed in GCA (6, 8). Both of them have confirmed the classical allele HLA-DRB1*04 as the most consistent association with this vasculitis. In addition, several non-HLA loci have been also found to play a role in the GCA genetic predisposition, including *PTPN22* (protein tyrosine phosphatase non-receptor type 22), *PLG* (plasminogen), and *P4HA2* (prolyl 4-hydroxylase subunit alpha 2).

The association between *PTPN22* and GCA was initially identified in a candidate-gene association study (7) and subsequently confirmed by using the Immunochip strategy (6). This gene encodes LYP, a tyrosine phosphatase involved in several immune signaling pathways, such as the T cell receptor (TCR) pathway and the humoral activity of B cells. The strongest signal within this *locus* corresponds to a functional variant (rs2476601), previously associated with multiple immune-mediated disorders, that results in a non-synonymous arginine to tryptophan amino acid change (R620W). It has been described that carrying the rs2476601 risk allele results in enhanced B lymphocyte autoreactivity, deregulated TCR signaling, and reduced capacity for TLR-induced type 1 interferon (IFN) production (77). On the other hand, *PLG*, encoding plasminogen, is involved in different processes relevant for GCA, such as angiogenesis, lymphocyte recruitment, and production of inflammatory mediators, including tumor necrosis factor alpha (TNF-α) and IL-6 (78), and *P4HA2*, encoding an isoform of the alpha subunit of the collagen prolyl 4-hydroxylase, is an important hypoxia response gene whose expression is induced by hypoxia-inducible factor-1 (HIF-1), which also induces the expression of other genes involved in GCA such as *IL6*, *MMP9* (matrix metallopeptidase 9), and *VEGF* (79).

These genetic findings, together with other lines of evidence, have contributed to the identification of several molecular pathways implicated in the GCA pathogenesis. Currently, it is known that both Th1 and Th17 cells are relevant player in GCA with two main cytokine clusters contributing to the local inflammation, the IL-6/IL-17 and the IL-12/IFN-γ axes (74). Interestingly, whereas the inflammatory activity of the IL-6/IL-17 cytokine cluster seems to be affected by glucocorticoid treatment, the IL-12/IFN-γ cytokine cluster is resistant to this therapy. This, together with the adverse events associated with long-term glucocorticoids use, has led to the search for new therapeutical agents.

Considering the major role of IL-6 in the pathogenesis of GCA, the potential use of tocilizumab in the treatment of this vasculitis has been explored. IL-6 inhibition has shown clinical efficacy in several randomized controlled trials (80, 81), thus representing a promising therapeutic strategy for

TABLE 1 | Non-HLA loci associated with vasculitis at genome-wide significance level.

Type of vasculitis	Susceptibility locus	Chromosomic region	Population	Approach	References
TAK	*FCGR2A*	1q23.3	Turkish, European-American	Immunochip	(3)
	IL12B	5q33.3	Turkish, European-American, Japanese	GWAS	(3, 4)
	IL6	7p15.3	Turkish, European-American	GWAS	(5)
	LILR3B	19q13.42	Turkish, European-American	GWAS	(5)
	PSMG1	21q22.2	Turkish, European-American	GWAS	(5)
GCA	*PTPN22*	1p13.2	European	Candidate-gene, Immunochip	(6, 7)
	PLG	6q26	European	GWAS	(8)
	P4HA2	5q31.1	European	GWAS	(8)
AAV	*SERPINA1*	14q32.13	European	Candidate-gene, GWAS	(9–11)
	PRTN3	19p13.3	European	Candidate-gene, GWAS	(9, 10, 12)
	PTPN22	1p13.2	European	Candidate-gene, GWAS	(10, 13–15)
	SEMA6A	5q23.1	European	GWAS	(13)
BD	*IL10*	1q32.1	Japanese, Turkish	GWAS	(16, 17)
	IL23R/IL12RB2	1p31.3	Japanese, Turkish, European	GWAS, Immunochip	(16–18)
	CCR1/CCR3	3p21.31	Turkish	GWAS follow-up	(19)
	STAT4	2q32.2-q32.3	Turkish	GWAS follow-up	(19)
	ERAP1	5q15	Turkish	GWAS follow-up	(19)
	KLRC4	12p13.2	Turkish	GWAS follow-up	(19)
	GIMAP4	7q36.1	Korean	GWAS	(20)
	IL12A	3q25.33	European, Middle East and Turkish	GWAS, Immunochip	(18, 21)
	JRKL/CNTN5	11q22.1	European	Immunochip	(18)
KD	*ITPKC*	19q13.2	European, Asian	GWAS	(22)
	FCGR2A	1q23.3	European, Asian	GWAS	(22)
	CASP3	4q35.1	European, Japanese	Candidate-gene	(23)
	BLK	8p23.1	Han Chinese, Japanese, Korean	GWAS	(24–26)
	CD40	20q13.12	Han Chinese, Japanese	GWAS	(25, 26)

TAK, Takayasu arteritis; GCA, giant cell arteritis; AAV, Anti-neutrophil cytoplasmic antibody-associated vasculitis; BD, Behçet's disease; KD, Kawasaki's disease; GWAS, genome-wide association study.

this type of vasculitis. Indeed, tocilizumab has been recently approved to treat GCA by the United States Food and Drug Administration (FDA). Furthermore, other biological agents, such as ustekinumab and abatacept, a fusion protein comprising the Fc region of IgG1 and the extracellular domain of cytotoxic T lymphocyte antigen 4 (CTLA4) that inhibits the co-stimulatory signal required for T cell activation, have also shown encouraging but more moderate results (82, 83). Better powered studies are required in order to evaluate the efficacy of these drugs in GCA.

Finally, the potential therapeutic application of two monoclonal antibodies, anakinra and secukinumab, targeted against IL-1β receptor and IL-17A (one of the genes associated with GCA), respectively, is currently under investigation (NCT02902731 and NCT03765788). Both cytokines are crucial for the differentiation of Th17 cells and, therefore, their inhibition could be a therapeutic option in patients with GCA.

ANCA-Associated Vasculitis

Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is a group of disorders characterized by necrosing inflammation of small vessels, including arterioles, capillaries and venules, that comprises three separate conditions, granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA), and eosinophilic granulomatosis with polyangiitis (EGPA). AAV frequently affects small vessels in the respiratory tract and kidneys and is characterized by the presence of antibodies directed against two proteins, proteinase 3 (PR3) and myeloperoxidase (MPO), located on the membrane of monocytes and neutrophils (84).

Both candidate gene association studies and GWASs published in last years have identified several loci associated with these forms of vasculitis (2). Specifically, three GWASs on AAV have been performed so far (9, 10, 13), one in European patients with GPA and MPA and two in North American patients of European descent (one including patients with GPA and the other one including patients with GPA and MPA). Interestingly, these studies have shown that the genetic background of AAV depends on auto-antibody specificity rather than clinically defined disorders. In this regard, different HLA genes have been associated with the different ANCA subgroups; whereas

TABLE 2 | Genes associated with treatment response in vasculitis.

Vasculitis	Locus	Region	Treatment	Population	Approach	References
KD	*ITPKC*	19q13.2	IVIG	Japanese, Taiwanese	Candidate-gene	(28, 29)
	CASP3	4q35.1	IVIG	Japanese, Taiwanese	Candidate-gene	(28, 29)
	FCGR2C	1q23.3	IVIG	European, Asian, African-American, Hispanic	Candidate-gene	(30)
	FCGR3B	1q23.3	IVIG	European, Asian, African-American, Hispanic	Candidate-gene	(30, 31)
	FCGR2B	1q23.3	IVIG	European, Asian, African-American, Hispanic	Candidate-gene	(32)
	CCL17	16q21	IVIG	Taiwanese	Candidate-gene	(33)
	CCR5	3p21.31	IVIG	Japanese	Candidate-gene	(34)
	CCL3L1	17q21.1	IVIG	Japanese	Candidate-gene	(34)
	IL1B	2q14.1	IVIG	Taiwanese	Candidate-gene	(35)
	IFNG	12q15	IVIG	Taiwanese	Candidate-gene	(36)
	HMGB1	13q12.3	IVIG	Korean	Candidate-gene	(37)
	BCL2L11	2q13	IVIG	Korean	GWAS	(38)
	STX1B	16p11.2	IVIG	European	Immunochip	(39)
	BAZ1A/C14orf19	14q13.1-q13.2	IVIG	European	Immunochip	(39)
	SAMD9L	7q21.2	IVIG	Korean	GWAS	(40)
AAV	*HLA-DRB1*0405*	6p21.32	Remission induction therapy	China	Candidate-gene	(41)
	FCGR2A	1q23.3	Rituximab or cyclophosphamide	–	Candidate-gene	(42)
	TNFSF13B	13q33.3	Rituximab	European	Candidate-gene	(43)
BD	*ABCB1*	7q21.12	Colchicine	Turkish	Candidate-gene	(44)
	MTHFR	1p36.22	Colchicine	Turkish	Candidate-gene	(45)

KD, Kawasaki's disease; AAV, Anti-neutrophil cytoplasmic antibody-associated vasculitis; BD, Behçet's disease; IVIG, intravenous immunoglobulin; GWAS, genome-wide association study.

polymorphisms within the *DPB1* and *DPA1* genes appeared to be associated with PR3-ANCA-positive patients, *DQB1* showed a specific effect in the MPO-ANCA subgroup (10).

Additionally, four non-HLA genetic *loci*, *SERPINA1* (serpin family A member 1), *PRTN3* (proteinase 3), *PTPN22*, and *SEMA6A* (semaphorin 6A) have been associated with AAV at genome-wide significance level (9, 10, 13), the first two showing a specific association with the subgroup of patients positive for PR3-ANCA.

SERPINA1 encodes α1-antitrypsin (α1AT), an inhibitor of the serine proteases, including proteinase 3. The association between this gene and AAV was initially described in a candidate gene study (11), in which a functional genetic variant known to cause a deficient production of α1-AT appeared to be associated with GPA, and subsequently confirmed by GWAS (9, 10). It has been proposed that, since PR3 is a target of α1AT, a decreased production of this inhibitor could result in higher levels of circulating PR3, leading to the synthesis of anti-PR3 ANCA (11).

Regarding *PRTN3*, the role of this gene in AAV was described in a candidate gene association study, in which a genetic variant affecting a putative transcription factor-binding site was associated with GPA (12). Subsequently, two of the GWASs performed in AAV have confirmed this association (9, 10). Interestingly, the most recent GWAS reported that the lead SNP at this gene (rs62132293), which is in almost complete linkage disequilibrium (LD) with that described in the original study, acts as an expression-quantitative trait locus (eQTL) that results in an increased expression of *PRTN3* in neutrophils (10).

The protein encoded by *SEMA6A* has been characterized as a critical regulator of angiogenesis by modulating VEGF signaling (85). Nevertheless, it should be considered that, although polymorphisms within this locus showed genome-wide significance (13), this association was not subsequently validated in a replication study performed in a well-powered cohort of European AAV patients (including GPA, MPA, and EGPA cases) (86) or in the subsequent GWAS carried out by the same group (10). Therefore, further genetic association studies are needed to confirm the role of *SEMA6A* in AAV.

Finally, *PTPN22* has been consistently associated with both GPA and MPA by candidate gene and genome-wide studies (10, 13–15). The highest signal within the *PTPN22* locus lies on the R620W functional variant, the same one associated with GCA, thus pointing to a pleiotropic effect of this polymorphism in both vasculitis.

On the other hand, polymorphisms within the *CTLA4* locus, encoding a protein which transmits an inhibitory signal to T cells by blocking the interaction between CD28 on the T cell and CD80 or CD86 on the antigen-presenting cell, have been implicated in AAV by candidate gene analyses in different populations (87–92) and have shown suggestive association in GWASs (9, 13), thus supporting the idea that this locus represents a genetic risk factor for AAV.

Regarding EGPA, no GWAS has been published in this disease so far and the few associations reported to date have been identified using a candidate-gene strategy. In this regard, an early candidate-gene study reported an association between EGPA

and the HLA-DRB4 gene (93), which highlights the existence of different HLA associations for the different AAV subgroups. In addition, polymorphisms within several non-HLA genes, including *FCGR3B* and *IL10*, have also been associated with this vasculitis (94, 95). On the other hand, a role of eotaxin-3 in the EGPA pathogenesis was proposed. Interestingly, serum levels of this molecule were found to be increased in active patients and correlated with eosinophil counts, total immunoglobulin E (IgE) levels and acute-phase parameters (96, 97). However, a genetic study failed to identify an association between the gene encoding this molecule, *CCL26,* and EGPA, maybe due to a lack of statistical power (97). Therefore, the role of this gene as a susceptibility factor for EGPA remains to be clarified.

Although we are still far from fully understanding the pathogenic mechanisms implicated in AAV, genetic studies are contributing to their elucidation. Insights into these pathogenic pathways have opened new strategies for biological treatment. In this line, the central role that ANCA-mediated neutrophil activation plays in these disorders has led to the therapeutic use of B cell depleting drugs for AAV. Rituximab has proved to be highly effective for both remission induction and maintenance treatment (98), representing one of the major breakthroughs of the last decade in the treatment of these vasculitides. Additionally, the therapeutic potential of other B cell-targeting agents, such as belimumab, is being evaluated (NCT01663623). Belimumab is a humanized monoclonal antibody against BAFF, a potent B cell activator, which represents an interesting target for AAV treatment, since it has been reported to increase the production of PR3-ANCA in GPA patients (99).

On the other hand, B cells require T cell help to differentiate into antigen-specific Ig-producing plasma cells. Therefore, blockade of the co-stimulation signal required for full T cell activation using abatacept (CTLA4-Ig) is also an interesting treatment option that has shown clinical efficacy in an open-label clinical trial (100).

Behçet's Disease

Behçet's disease (BD) is an inflammatory disorder that may affect arteries and veins of all sizes. It is characterized by heterogeneous clinical manifestations, including oral and genital ulcers, which are the hallmark lesions of this vasculitis, as well as vascular, gastrointestinal, articular, and central nervous system manifestations. This condition shows a male preponderance and is more frequent in the Middle East and Asia (101).

BD is the vasculitis that has benefited most from the genome-wide era, with five large-scale genetic studies performed on this disorder so far, four GWASs and an Immunochip (16–18, 20, 21). This has lead to the discovery of a significant number of consistent genetic risk loci, including the HLA region that, as in other vasculitis, is the main susceptibility locus for BD. Specifically, an association between the classical allele HLA-B*51 and this disorder has been consistently identified in different ethnic groups during the last years (102). Moreover, dense genotyping and imputation of this region have evidenced additional independent signals. In this regard, a study published in 2013 reported that the association between HLA-B*51 and BD was explained by a SNP located between the HLA-B and MICA genes (103). They also identified three independent signals within the HLA region, located within *PSORS1C1* (psoriasis susceptibility 1 candidate 1), upstream HLA-F-AS1 (HLA-F antisense RNA 1), and within HLA-Cw*16:02. In addition, a subsequent Immunochip study also described two signals, HLA-B*57 and HLA-A*03, that showed an independent effect to that conferred by HLA-B*51 (18).

Several loci outside the HLA region have also shown robust associations with this vasculitis. The first GWASs on BD, performed in Japanese and Turkish populations and simultaneously published in 2010, evidenced the role of *IL10* and *IL23R*/*IL12RB2* as genetic risk factors in BD (16, 17). The association with the *IL23R*/*IL12RB2* locus was subsequently confirmed in an Immunochip study performed in BD patients from Spain (18). *IL10* encodes a cytokine that has an anti-inflammatory role by suppressing the expression of pro-inflammatory cytokines, such as IL-6, IL-12, and IL-1, but also promotes B cell responses by enhancing B cell survival, proliferation, and antibody production (104). The *IL23R*/*IL12RB2* locus contains two genes with a crucial role in the inflammatory response. *IL23R* encodes a subunit of the IL-23 receptor, whereas *IL12RB2* encodes an IL-12 receptor chain. As previously indicated, IL-12 and IL-23 participate in the immune responses mediated by Th1 and Th17 cells, respectively (57).

In addition, a follow-up study, in which data from the Turkish GWAS were imputed, identified four new loci contributing to the BD susceptibility, *CCR1*/*CCR3* (C-C motif chemokine receptor 1/3), *STAT4* (signal transducer and activator of transcription 4), *KLRC4* (killer cell lectin like receptor C4), and *ERAP1* (endoplasmic reticulum aminopeptidase 1) (19). All these loci play relevant roles in the immune response. The *CCR1* and *CCR3* genes form a chemokine receptor gene cluster, which also includes *CCR2*, *CCRL2*, *CCR5,* and *CCXCR1*, on the chromosomal region 3p21. These genes encode proteins critical for the recruitment of effector immune cells to the site of inflammation (105). The protein encoded by *STAT4* is a member of the STAT family of transcription factors that mediates responses to IL-12, IL-23, and type 1 IFNs, and regulates the differentiation of Th1 and Th17 lymphocytes (106). The signal detected at the *KLRC4* region is located within a haplotype block that contains five natural killer (NK) cell receptor genes (*KLRK1*, *KLRC1*, *KLRC2*, *KLRC3*, and *KLRC4*), some of which act as co-stimulators for CD4+ and CD8+ T cells (107). Finally, *ERAP1* encodes an amino peptidase that is crucial for antigen presentation through HLA class I molecules. Interestingly, *ERAP1* variants conferred risk for BD in HLA-B*51 positive individuals preferentially, thus suggesting the existence of an interaction between both proteins (19).

In 2013, a third GWAS performed on BD patients from Korea reported *GIMAP4* (guanosine-5′-triphosphatase (GTPase) IMAP family member 4) as a new susceptibility locus (20). This gene encodes a protein belonging to the GTP-binding superfamily and to the immuno-associated nucleotide (IAN) subfamily of nucleotide-binding proteins that seems to play a role in regulating T cell apoptosis (108). Functional studies performed by the authors revealed that the minor allele of the most associated SNP within this region correlated with lower

protein activity, and that CD4+ T cells from BD patients have a diminished *GIMAP4* expression (20).

A genome-wide association between *IL12A*, encoding the p35 subunit of IL-12, and BD has also been described in a GWAS performed on an admixed cohort including Western Europeans, Middle Eastern and Turkish cases with BD (21). This association was confirmed in a subsequent Immunochip study, in which the *JRKL*/*CNTN5* (jerky like/contactin 5) locus was also identified as a new genetic risk factor for BD (18). The protein encoded by *JRKL* has an unknown function, whereas *CNTN5* encodes a member of the immunoglobulin superfamily that mediates cell surface interactions during nervous system development (109).

BD treatment has undergone a significant evolution over the years, thanks to the increased knowledge of the pathogenic mechanisms involved in this disease. Genetic findings have evidenced the prominent role of immune responses mediated by Th1 and Th17 cells in BD, with multiple pro-inflammatory molecules contributing to its pathological landscape. This has led to the study of new biological therapies, most of them targeted against cytokines.

In this line, inhibition of IL-1 and IL-6 has shown the most interesting results in both small case series and clinical trials. Specifically, three IL-1 blockers have shown clinical efficacy in BD patients, the IL-1 receptor antagonist anakinra, as well as canakinumab and gevokizumab, targeting the IL-1 molecule directly, which have proved to be effective for all BD manifestations, especially for the most severe ocular involvement (110). In relation to IL-6 inhibition, tocilizumab has proved to be highly effective in treating BD patients with neurological involvement as well as in controlling uveitis, although less promising results were found regarding the treatment of mucocutaneous manifestations (110). Ustekinumab and secukinumab have also shown clinical efficacy in case series studies (111, 112). In addition, the clinical utility of Ustekinumab is currently being evaluated in a phase 2 clinical trial (NCT02648581).

Although there are more clear evidences of T cell involvement in BD, several studies have suggested a possible pathogenic role of B lymphocytes. Indeed, depletion of B cells using rituximab has also emerged as a promising therapy in BD patients (110).

Kawasaki's Disease

Kawasaki's disease (KD) is a systemic vasculitis that affects small and medium size vessels. It mainly affects children younger than 5 years of age, especially of Asian origin. The most serious complication of KD is the development of coronary artery lesions (CALs), representing the main cause of acquired heart disease among children in Japan, Europe and the USA (113).

Although seven GWASs and an Immunochip have been published in both European and Asian KD cohorts in recent years (22, 24–26, 39, 114–116), only a few consistent genetic associations have been described so far, probably due to the lack of statistical power of most of these studies.

As in other vasculitis, the HLA locus seems to be involved in the KD genetic predisposition. However, contradictory results have been found regarding the specific HLA alleles associated with this disease. Whereas early candidate gene studies identified associations with HLA-Bw54 (previously known as Bw22) in Japanese population (117, 118) and with HLA-Bw51 and HLA-B44 in European patients (119–121), a genetic variant located within the HLA class II region (between HLA-DQB2 and HLA-DOB) was identified as the strongest signal in a GWAS performed on KD cases from Japan (26). This association within the HLA class II region was then replicated in an European-American case-parent trio study using the Immunochip platform (39). In addition, a more recent GWAS identified several SNPs within the HLA class I region associated with KD in Korean population, but failed to replicate this association in an independent case/control set from Japan (24). The small sample sizes of these studies and the varying LD patterns observed across different populations are likely explanations of these heterogeneous findings.

Regarding genetic risk factors outside the HLA locus, genome-wide associations within the *ITPKC* and the *FCGR2A* loci were identified in a GWAS performed in European KD patients and replicated in independent cohorts of Asian and European descent (22). The KD-associated SNP within *FCGR2A* is a functional variant encoding a H131R substitution. It has been reported that the presence of arginine instead of histidine at this amino acid position reduces the affinity of the receptor for the IgG2 isotype (122). *ITPKC* (inositol-trisphosphate 3-kinase C), encoding one of the three isoenzymes of ITPK that phosphorylate inositol 1,4,5-trisphosphate (IP3), was initially implicated in KD by linkage analysis using sib-pairs (123). This same study showed for the first time that ITPKC acts as a negative regulator of T cell activation through the Ca2+/nuclear factor of activated T cells (NFAT) signaling pathway. Interestingly, a subsequent study showed that the genetic variant associated with KD has functional consequences, influencing the ITPKC protein levels, which regulates the production of IL-1β and IL-18 (124). Moreover, using a positional candidate gene study for the 4q35 region, previously linked to KD, Onouchi et al. identified several genome-wide associations within the *CASP3* (caspase 3) gene, which encodes a caspase with a crucial role in apoptosis (23). Similarly to the function identified for *ITPKC*, this study also reported that one of the associated SNPs, located within the 5′ untranslated region of the gene, had functional implications, affecting binding of NFATc2 to the DNA sequence surrounding this polymorphism.

In 2012, two subsequent GWASs, published simultaneously, identified two new susceptibility loci for KD, *BLK* (BLK proto-oncogene, Src family tyrosine kinase) and *CD40* (CD40 molecule) (25, 26). *BLK* encodes a non-receptor tyrosine-kinase of the src family of proto-oncogenes with a crucial role in B cell receptor signaling, thus participating in B-cell activation and antibody secretion (125). The *CD40* gene is a member of the TNF-receptor superfamily that encodes a receptor expressed on antigen-presenting cells involved in inflammation through selection of autoreactive T cells and activation of B and T cells (126).

Additionally, large-scale genetic studies have reported suggestive signals in different loci, including *CAMK2D* (calcium/calmodulin-dependent protein kinase II delta), *CSMD1* (CUB and Sushi multiple domains 1), *LNX1* (ligand of numb-protein X1), *NAALADL2* (N-acetylated alpha-linked

acidic dipeptidase-like 2), *TCP1* (t-complex 1), *PELI1* (pellino E3 ubiquitin protein ligase 1), *DAB1* (Dab reelin signal transducer homolog 1), *COPB2* (coatomer protein complex beta-2 subunit), *ERAP1*, *NMNAT2* (nicotinamide nucleotide adenylyltransferase 2), *FUT1* [fucosyltransferase 1 (H blood group)], *RASIP1* (Ras interacting protein 1), and *BRD7P2* (bromodomain containing 7 pseudogene 2) (24, 39, 114–116). However, these associations did not reach genome-wide significance level nor were replicated in later studies and, therefore, they cannot be considered as established susceptibility loci.

As shown by genetic studies, both T and B cells participate in the pathogenic mechanisms implicated in KD. The involvement of the *FCGR2A* gene evidences the relevant role of IgG receptors in the pathogenesis of this vasculitis, providing a biological basis for the use of intravenous immunoglobulin (IVIG), the standard treatment for KD patients. However, approximately up to 20% of patients do not fully respond to this therapy, presenting an increased risk for the development of coronary aneurysms (127). Therefore, several therapeutic options are being tested for treatment of refractory cases.

Considering the role of TNF-α in the pathogenesis of KD, the clinical efficacy of TNF-α inhibitors, such as infliximab and etanercept, has been evaluated. Although treatment with infliximab has shown clinical efficacy in different studies, including reduction of fever duration, markers of inflammation and immunoglobulin reaction rates, its role on the prevention of CALs is still to be determined (128). A phase III trial comparing the efficacy of a second dose of IVIG with infliximab treatment is currently recruiting participants (NCT03065244). In addition, a phase II clinical trial to determine the safety and efficacy of etanercept in reducing the incidence of persistent or recurrent fever in KD patients is currently ongoing (NCT00841789).

On the other hand, studies in mice have demonstrated that both IL-1α and IL-1β are involved in the development of CALs in KD (129, 130). Interestingly, *PELI1*, encoding a protein that acts an intermediate component in the signaling cascade initiated by the IL-1 receptor (131), showed a suggestive association with CALs development in a KD GWAS (115). Thus, genetic findings also support the role of this pathway as a potential drug target in this vasculitis. Considering this, the potential clinical efficacy of blocking IL-1β receptor using anakinra is currently being explored. Three case reports have reported the beneficial clinical use of this biological agent (128). Moreover, two phase II clinical trials exploring the efficacy of anakinra are currently underway (NCT02179853 and NCT02390596).

Polyarteritis Nodosa

Polyarteritis nodosa (PAN) is a systemic, necrotizing medium-sized vessel vasculitis, mainly affecting adults between the ages of 40–60 years, although it can also appear in children. The clinical features of PAN depend on the affected organs and include systemic symptoms and involvement of the gastrointestinal, renal and peripheral nervous systems.

In 2014, two independent studies identified loss-of-function mutations in the *CECR1* (cat eye syndrome chromosome region candidate 1) gene, which encodes the extracellular adenosine deaminase 2 (ADA2), using whole-exome sequencing (132, 133). Interestingly, in many cases, both the clinical manifestations and the histological findings of the deficiency of ADA2 (DADA2) were consistent with the diagnosis of PAN, which suggests that DADA2 contributes to the clinical phenotype of this vasculitis. The ADA2 protein is mainly expressed by myeloid cells and plays a role in the proliferation and differentiation of macrophages. In this regard, its deficiency has been linked to an imbalance in monocytes differentiation toward proinflammatory M1 macrophages (133, 134).

Clinical management of patients with DADA2 is challenging. None of the commonly used immunosuppressive drugs have resulted particularly effective. Anti-TNF agents have shown promise in the management of the inflammatory syndrome and vasculitis; however, this therapy is not able to completely control the disease manifestations in all treated patients. Considering that bone marrow–derived monocytes and macrophages are the main source of secreted ADA2, it was hypothesized that hematopoietic stem cell transplantation (HSCT) could be an effective treatment for this condition. In this regard, two studies have reported that HSCT was able to normalize the plasmatic levels of ADA2 and to control the disease manifestations (135–137), thus suggesting that this therapy could represent a definitive treatment of DADA2. In addition, enzyme-replacement therapies have also been considered as a potential treatment for these conditions. However, the results obtained using this strategy have not been entirely satisfactory (138).

GENETICS DETERMINANTS OF TREATMENT RESPONSE IN VASCULITIS

The genetic basis of treatment response has only been evaluated in three types of vasculitis so far, KD, AAV, and BD, mainly by means of candidate-gene association studies. This has led to the identification of several potential genetic predictors of treatment efficacy.

Most of the pharmacogenetic studies performed in vasculitis have analyzed genetic variants involved in the resistance to IVIG therapy in KD. As it was already mentioned, this treatment is highly effective, but around 10–20% of patients are resistant and have a higher risk for CALs. Therefore, it is essential to elucidate the causes of this resistance in order to predict the responsiveness of patients during the early stages of the disease.

Polymorphisms previously associated with KD have been evaluated in relation to the IVIG response. A study published in 2013 showed that the risk alleles of the *ITPKC* and *CASP3* susceptibility variants (rs28493229 and rs72689236, respectively) were overrepresented in IVIG resistant patients with respect to responding patients (28). These associations were replicated in a subsequent study (29), thus supporting the role of these genes in the response to IVIG treatment. Interestingly, a functional study demonstrated that the poor response observed in patients homozygous for the risk allele of the *ITPKC* locus correlated with increased cellular production of IL-1β and IL-18 (124).

On the other hand, additional studies have evaluated the implication of candidate genes in the clinical efficacy of IVIG based on their functional role. Given that the anti-inflammatory

activity of IVIG is partly mediated through FcgR (139), the role of several genes encoding these proteins have been explored. In this regard, polymorphisms within *FCGR2B*, *FCGR2C,* and *FCGR3B* have been involved in the response to this drug in different studies performed by the same group (30–32). In addition, genetic variants located within genes encoding chemokine receptors and their ligands, including *CCR5* (C-C motif chemokine receptor 5), *CCL3L1* (C-C motif chemokine ligand 3 like 1), and *CCL17* (C-C motif chemokine ligand 17), as well as genes encoding pro-inflammatory cytokines, such as *IL1B* and *IFNG*, have also been implicated in the IVIG treatment resistance (33–36). Moreover, an association between a polymorphism of the *HMGB1* (high mobility group box 1) locus, involved in inflammation and cell differentiation, and the clinical efficacy of this treatment has been recently reported (37). However, all these associations need to be confirmed in independent studies.

The genetic basis of IVIG response in KD has also been explored through comprehensive large-scale genetic analyses (38–40). Both GWAS and Immunochip data have been used to identify genetic variants associated with IVIG resistance by stratifying KD patients according to treatment response. A polymorphism within the *BCL2L11* (BCL2 like 11) gene showed a specific association at the genome-wide significance level with the subgroup of responder patients in an IVIG response-stratified genome-wide association study (38). The protein encoded by this gene, known as Bim, is an important regulator of the negative selection of B lymphocytes in the bone marrow and of T lymphocytes both in the thymus and the periphery (140). In addition, a very recent GWAS performed in Korean KD patients identified the *SAMD9L* (sterile alpha-motif domain-containing 9-like) gene as a susceptibility factor for IVIG resistance (40). This gene encodes a cytoplasmic protein involved in multiple cellular processes, such as cell proliferation and innate immune responses to viral infections (141). Several suggestive signals that could be involved in the response to IVIG therapy were identified using the Immunochip platform, including an intronic SNP of the *STX1B* (syntaxin 1B) gene and a genetic variant located in the intergenic region of *BAZ1A* (bromodomain adjacent to zinc finger domain 1A) and *C14orf19* (39).

A study published in 2017 developed a genetic model to predict IVIG resistance in KD patients (142). In this study, the additive effect of 11 SNPs associated with IVIG response ($p < 1 \times 10^{-05}$) was used to calculate a GWAS-based weighted genetic risk score (wGRS). A significant association between wGRS and the response was found, suggesting that this scoring system can significantly increase the sensitivity and specificity of prediction of IVIG responsiveness.

Regarding AAV, the advances achieved in its therapeutic management in recent years have allowed these forms of vasculitis to go from presenting high mortality to becoming chronic diseases. Currently, the standard treatment for AAV consists of glucocorticoids together with cyclophosphamide or rituximab. However, despite the success of this therapy, a high percentage of patients do not reach complete remission.

Only three pharmacogenetic studies have evaluated the role of genetic variants as predictors of treatment response in AAV so far. One of them, performed in 152 AAV patients from China, was focused on analyzing the possible implication of the HLA locus in the response to remission induction therapy after 6 months (41). Among the 56 HLA-DRB1, HLA-DPB1, HLA-DQB1, and HLA-DQA1 alleles analyzed, HLA-DRB1*0405 appeared to be associated with the clinical efficacy of this treatment; specifically, the proportion of patients showing treatment failure was higher in the subgroup of patients carrying this allele (41.7%) than in the subgroup of patients negative for HLA-DRB1*0405 (12.9%).

On the other hand, the main mechanism through which rituximab achieves B cell depletion is antibody-dependent cell mediated cytotoxicity (ADCC), which is mediated through FcgR. Regarding cyclophosphamide, it requires activation by the hepatic cytochrome P450 (CYP) enzymes. Considering this, a more recent study has explored the role of several polymorphisms, located within three genes encoding FcgR (*FCGR2A*, *FCGR2B*, *FCGR3A*) and two genes encoding different CYP isoforms (*CYP2B6* and *CYP2C19*), in the response to the treatment with rituximab and cyclophosphamide, respectively (42). When both subgroups of patients (96 treated with rituximab and 93 with cyclophosphamide) were individually analyzed, the authors did not find any potential predictor of treatment response among the genetic variants selected. However, when AAV patients were considered as a global cohort, the *FCGR2A* 519AA genotype was found to predict complete response independently of the induction treatment used.

In addition, a study published in 2017 evaluated the role of several candidate genes in the rituximab response in two independent cohorts of patients with AAV, including MPA and GPA (43). Only one (rs3759467) of the 18 analyzed SNPs showed a consistent association with treatment efficacy. Interestingly, this association was specific for the subgroup of patients PR3-ANCA positive. The associated SNP is located in the 5′ regulatory region of the *TNFSF13B* gene, encoding the B-cell activating factor BAFF, which has been reported to increase the production of PR3-ANCA in GPA patients (99), as previously mentioned.

Finally, pharmacogenetic studies performed in BD were focused on analyzing genetic factors implicated in the response to colchicine. This drug is the most frequently and widely used for oral and genital ulcers, papulopustular lesions, and arthralgias; however, some patients do not respond to this therapy.

Until now, two genes have been associated with colchicine response in BD. A study published in 2012 identified an association between two SNPs, C3435T and G2677T/A, of the *ABCB1* (ATP binding cassette subfamily B member 1) gene and the efficacy of this treatment in a candidate-gene study including a cohort of 68 responder and 37 non-responder patients (44). *ABCB1*, also known as *MDR1* (multidrug resistance), is implicated in drug metabolism by encoding an ATP-dependent drug efflux pump for different xenobiotic compounds, including colchicine (143).

A second pharmacogenetic study, in which 165 responder and 215 non-responder patients were analyzed, reported a role of the *MTHFR* (methylenetetrahydrofolate reductase) locus in the response to colchicine treatment (45). This gene

encodes an enzyme that catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a co-substrate for homocysteine remethylation to methionine. In this case, the polymorphism associated with the response, C677T, causes an amino acid substitution from alanine to valine leading to reduced activity and increased thermolability of the enzyme, which in turn results in increased levels of homocysteine (144). It has been described the existence of hyperhomocysteinemia in BD patients, which correlates with thrombosis and ocular involvement (145).

SHARED GENETIC COMPONENT IN VASCULITIS

Nowadays, it is widely accepted that autoimmune disorders in general and vasculitides in particular share susceptibility genes and molecular pathways influencing their development (146, 147). Indeed, a large number of susceptibility loci described here are common to different vasculitides. The combination of different diseases as a single phenotype in large-scale studies, such as GWAS and Immunochip, has proven to be very useful in the evaluation of this shared genetic component and in the identification of potential drug targets that could be repurposed in related conditions (148–151).

To date, two studies have been conducted combining different forms of vasculitides. In the first one, Carmona et al. (152) combined data from large-vessel vasculitis, namely GCA and TAK, and found a significant genetic correlation within the *IL12B* locus. Considering this, ustekinumab, which has been successfully used to treat refractory TAK, could be of potential clinical use in GCA. Similarly, Ortiz-Fernandez et al. (153) combined data of different vasculitides (GCA, TAK, AAV, IgA vasculitis, and BD) and identified a common signal within the lysine demethylase 4C (*KDM4C*) gene, which encodes a histone demethylase involved in epigenetic mechanisms and that could be of potential use in the treatment of these conditions.

PRECISION MEDICINE IN VASCULITIS: FROM GENETIC FINDINGS TO CLINICAL APPLICATION

The goal of precision medicine is to maximize treatment efficacy by developing more targeted drugs directed against biological pathways with a pathogenic role in the disease, as well as by optimizing the use of existing drugs, through the *a priori* selection of those patients who will benefit from a certain treatment.

As described in this review, it is now clear that genetic studies offer great potential for understanding the molecular mechanisms involved in vasculitis. Thus, insight into disease pathogenesis is progressively leading to new ways for targeted biologic treatment. Moreover, based on the moderate effects provided by the thousands of genome-wide SNPs identified by GWAS, nowadays it is possible to predict each individual susceptibility by means of the polygenic risk score (PRS) analysis, which have been recently performed in other diseases with remarkable results (154). Currently, PRSs are being calculated for different phenotypes separately and, as a potential next step, parallel calculation and disease comparisons of PRS could reflect shared and opposite mechanisms in different vasculitides. However, although these diseases have benefited from the genome-wide era, genetic studies conducted to date still lack enough statistical power to detect variants with moderate effects and, consequently, only a few consistent genetic risk loci have been identified so far. Therefore, further genetic studies in larger cohorts are crucial to obtain information on the missing heritability of these disorders.

Moreover, in recent years, the translation of GWAS/Immunochip findings into biological insights has been challenging, mainly due to the difficulty of identifying causal variants, as well as by the fact that many of the disease-associated SNPs are located in non-coding regions of the genome. Therefore, substantial effort is needed to move from association signals to understanding the functional implication of the genes. In this sense, integration of genomic data with other–omic information, such as epigenomic and transcriptomic data, has become a useful approach to unravel the mechanisms underlying complex diseases. Thus, a better understanding of the interaction between these factors will allow us to obtain a clearer picture of the molecular network involved in the pathogenesis of vasculitis, so that we may turn basic biological knowledge into targets for new therapeutic approaches.

On the other hand, it is likely that a better use of existing drugs will improve the clinical management of vasculitis. In this regard, prediction of those patients that will respond to a specific drug based on their molecular profiles results essential. Although several genetic variants have been described as potential predictor of drug efficacy, mainly in KD but also in AAV and BD, at present, no validated biological biomarker exists to predict treatment response in vasculitis. Again, large-scale genetic studies including well-powered cohorts will be essential to identify genetic profiles that help to classify vasculitis patients and to guide the selection of the most appropriate therapeutic intervention.

It is, therefore, expected that genetic findings in vasculitis continue to open new ways for targeted biologic therapies and improve the use of existing drugs, which will lead to a more personalized application of treatment in the future. However, multiple issues must be overcome before precision medicine can be effectively implemented, which will necessarily require great collaborative efforts among vasculitis expertise research groups.

AUTHOR CONTRIBUTIONS

MA-H and AM wrote this review. MG-G and JM critically read and edited the manuscript.

FUNDING

This work was supported by the following grants: PI18/00040 from Instituto de Salud Carlos III (ISCIII, Spanish Ministry of Economy, Industry, and Competitiveness) and the

Cooperative Research Thematic Network (RETICS) programme (RD16/0012/0013 and RD16/0012/0009) (RIER), from ISCIII (Spanish Ministry of Economy, Industry and Competitiveness). AM is recipient of a Miguel Servet fellowship (CP17/00008) from ISCIII (Spanish Ministry of Economy, Industry and Competitiveness).

REFERENCES

1. Katsuyama T, Sada KE, Makino H. Current concept and epidemiology of systemic vasculitides. *Allergol Int*. (2014) 63:505–13. doi: 10.2332/allergolint.14-RAI-0778
2. Carmona FD, Martin J, Gonzalez-Gay MA. Genetics of vasculitis. *Curr Opin Rheumatol*. (2015) 27:10–7. doi: 10.1097/BOR.0000000000000124
3. Saruhan-Direskeneli G, Hughes T, Aksu K, Keser G, Coit P, Aydin SZ, et al. Identification of multiple genetic susceptibility loci in Takayasu arteritis. *Am J Hum Genet*. (2013) 93:298–305. doi: 10.1016/j.ajhg.2013.05.026
4. Terao C, Yoshifuji H, Kimura A, Matsumura T, Ohmura K, Takahashi M, et al. Two susceptibility loci to Takayasu arteritis reveal a synergistic role of the IL12B and HLA-B regions in a Japanese population. *Am J Hum Genet*. (2013) 93:289–97. doi: 10.1016/j.ajhg.2013.05.024
5. Renauer PA, Saruhan-Direskeneli G, Coit P, Adler A, Aksu K, Keser G, et al. Identification of susceptibility loci in IL6, RPS9/LILRB3, and an intergenic locus on chromosome 21q22 in Takayasu arteritis in a genome-wide association study. *Arthritis Rheumatol*. (2015) 67:1361–8. doi: 10.1002/art.39035
6. Carmona FD, Mackie SL, Martin JE, Taylor JC, Vaglio A, Eyre S, et al. A large-scale genetic analysis reveals a strong contribution of the HLA class II region to giant cell arteritis susceptibility. *Am J Hum Genet*. (2015) 96:565–80. doi: 10.1016/j.ajhg.2015.02.009
7. Serrano A, Marquez A, Mackie SL, Carmona FD, Solans R, Miranda-Filloy JA, et al. Identification of the PTPN22 functional variant R620W as susceptibility genetic factor for giant cell arteritis. *Ann Rheum Dis*. (2013) 72:1882–6. doi: 10.1136/annrheumdis-2013-203641
8. Carmona FD, Vaglio A, Mackie SL, Hernandez-Rodriguez J, Monach PA, Castaneda S, et al. A genome-wide association study identifies risk alleles in plasminogen and P4HA2 associated with giant cell arteritis. *Am J Hum Genet*. (2017) 100:64–74. doi: 10.1016/j.ajhg.2016.11.013
9. Lyons PA, Rayner TF, Trivedi S, Holle JU, Watts RA, Jayne DR, et al. Genetically distinct subsets within ANCA-associated vasculitis. *N Engl J Med*. (2012) 367:214–23. doi: 10.1056/NEJMoa1108735
10. Merkel PA, Xie G, Monach PA, Ji X, Ciavatta DJ, Byun J, et al. Identification of functional and expression polymorphisms associated with risk for antineutrophil cytoplasmic autoantibody-associated vasculitis. *Arthritis Rheumatol*. (2017) 69:1054–66. doi: 10.1002/art.40034
11. Mahr AD, Edberg JC, Stone JH, Hoffman GS, St. Clair EW, Specks U, et al. Alpha$_1$-antitrypsin deficiency-related alleles Z and S and the risk of Wegener's granulomatosis. *Arthritis Rheum*. (2010) 62:3760–7. doi: 10.1002/art.27742
12. Gencik M, Meller S, Borgmann S, Fricke H. Proteinase 3 gene polymorphisms and Wegener's granulomatosis. *Kidney Int*. (2000) 58:2473–7. doi: 10.1046/j.1523-1755.2000.00430.x
13. Xie G, Roshandel D, Sherva R, Monach PA, Lu EY, Kung T, et al. Association of granulomatosis with polyangiitis (Wegener's) with HLA-DPB1*04 and SEMA6A gene variants: evidence from genome-wide analysis. *Arthritis Rheum*. (2013) 65:2457–68. doi: 10.1002/art.38036
14. Carr EJ, Niederer HA, Williams J, Harper L, Watts RA, Lyons PA, et al. Confirmation of the genetic association of CTLA4 and PTPN22 with ANCA-associated vasculitis. *BMC Med Genet*. (2009) 10:121. doi: 10.1186/1471-2350-10-121
15. Jagiello P, Aries P, Arning L, Wagenleiter SE, Csernok E, Hellmich B, et al. The PTPN22 620W allele is a risk factor for Wegener's granulomatosis. *Arthritis Rheum*. (2005) 52:4039–43. doi: 10.1002/art.21487
16. Mizuki N, Meguro A, Ota M, Ohno S, Shiota T, Kawagoe T, et al. Genome-wide association studies identify IL23R-IL12RB2 and IL10 as Behcet's disease susceptibility loci. *Nat Genet*. (2010) 42:703–6. doi: 10.1038/ng.624
17. Remmers EF, Cosan F, Kirino Y, Ombrello MJ, Abaci N, Satorius C, et al. Genome-wide association study identifies variants in the MHC class I, IL10, and IL23R-IL12RB2 regions associated with Behcet's disease. *Nat Genet*. (2010) 42:698–702. doi: 10.1038/ng.625
18. Ortiz-Fernandez L, Carmona FD, Montes-Cano MA, Garcia-Lozano JR, Conde-Jaldon M, Ortego-Centeno N, et al. Genetic analysis with the immunochip platform in behcet disease. Identification of residues associated in the HLA class I region and new susceptibility loci. *PLoS ONE*. (2016) 11:e0161305. doi: 10.1371/journal.pone.0161305
19. Kirino Y, Bertsias G, Ishigatsubo Y, Mizuki N, Tugal-Tutkun I, Seyahi E, et al. Genome-wide association analysis identifies new susceptibility loci for Behcet's disease and epistasis between HLA-B*51 and ERAP1. *Nat Genet*. (2013) 45:202–7. doi: 10.1038/ng.2520
20. Lee YJ, Horie Y, Wallace GR, Choi YS, Park JA, Choi JY, et al. Genome-wide association study identifies GIMAP as a novel susceptibility locus for Behcet's disease. *Ann Rheum Dis*. (2013) 72:1510–6. doi: 10.1136/annrheumdis-2011-200288
21. Kappen JH, Medina-Gomez C, van Hagen PM, Stolk L, Estrada K, Rivadeneira F, et al. Genome-wide association study in an admixed case series reveals IL12A as a new candidate in Behcet disease. *PLoS ONE*. (2015) 10:e0119085. doi: 10.1371/journal.pone.0119085
22. Khor CC, Davila S, Breunis WB, Lee YC, Shimizu C, Wright VJ, et al. Genome-wide association study identifies FCGR2A as a susceptibility locus for Kawasaki disease. *Nat Genet*. (2011) 43:1241–6. doi: 10.1038/ng.981
23. Onouchi Y, Ozaki K, Buns JC, Shimizu C, Hamada H, Honda T, et al. Common variants in CASP3 confer susceptibility to Kawasaki disease. *Hum Mol Genet*. (2010) 19:2898–906. doi: 10.1093/hmg/ddq176
24. Kim JJ, Yun SW, Yu JJ, Yoon KL, Lee KY, Kil HR, et al. A genome-wide association analysis identifies NMNAT2 and HCP5 as susceptibility loci for Kawasaki disease. *J Hum Genet*. (2017) 62:1023–9. doi: 10.1038/jhg.2017.87
25. Lee YC, Kuo HC, Chang JS, Chang LY, Huang LM, Chen MR, et al. Two new susceptibility loci for Kawasaki disease identified through genome-wide association analysis. *Nat Genet*. (2012) 44:522–5. doi: 10.1038/ng.2227
26. Onouchi Y, Ozaki K, Burns JC, Shimizu C, Terai M, Hamada H, et al. A genome-wide association study identifies three new risk loci for Kawasaki disease. *Nat Genet*. (2012) 44:517–21. doi: 10.1038/ng.2220
27. Nelson MR, Tipney H, Painter JL, Shen J, Nicoletti P, Shen Y, et al. The support of human genetic evidence for approved drug indications. *Nat Genet*. (2015) 47:856–60. doi: 10.1038/ng.3314
28. Onouchi Y, Suzuki Y, Suzuki H, Terai M, Yasukawa K, Hamada H, et al. ITPKC and CASP3 polymorphisms and risks for IVIG unresponsiveness and coronary artery lesion formation in Kawasaki disease. *Pharmacogenomics J*. (2013) 13:52–9. doi: 10.1038/tpj.2011.45
29. Kuo HC, Hsu YW, Wu CM, Chen SH, Hung KS, Chang WP, et al. A replication study for association of ITPKC and CASP3 two-locus analysis in IVIG unresponsiveness and coronary artery lesion in Kawasaki disease. *PLoS ONE*. (2013) 8:e69685. doi: 10.1371/journal.pone.0069685
30. Makowsky R, Wiener HW, Ptacek TS, Silva M, Shendre A, Edberg JC, et al. FcgammaR gene copy number in Kawasaki disease and intravenous immunoglobulin treatment response. *Pharmacogenet Genomics*. (2013) 23:455–62. doi: 10.1097/FPC.0b013e328363686e
31. Shrestha S, Wiener H, Shendre A, Kaslow RA, Wu J, Olson A, et al. Role of activating FcgammaR gene polymorphisms in Kawasaki disease susceptibility and intravenous immunoglobulin response. *Circ Cardiovasc Genet*. (2012) 5:309–16. doi: 10.1161/CIRCGENETICS.111.962464
32. Shrestha S, Wiener HW, Olson AK, Edberg JC, Bowles NE, Patel H, et al. Functional FCGR2B gene variants influence intravenous immunoglobulin response in patients with Kawasaki disease. *J Allergy Clin Immunol*. (2011) 128:677–80. doi: 10.1016/j.jaci.2011.04.027
33. Lee CP, Huang YH, Hsu YW, Yang KD, Chien HC, Yu HR, et al. TARC/CCL17 gene polymorphisms and expression associated with susceptibility and coronary artery aneurysm formation in Kawasaki disease. *Pediatr Res*. (2013) 74:545–51. doi: 10.1038/pr.2013.134

34. Mamtani M, Matsubara T, Shimizu C, Furukawa S, Akagi T, Onouchi Y, et al. Association of CCR2-CCR5 haplotypes and CCL3L1 copy number with Kawasaki Disease, coronary artery lesions, and IVIG responses in Japanese children. *PLoS ONE*. (2010) 5:e11458. doi: 10.1371/journal.pone.0011458
35. Weng KP, Hsieh KS, Ho TY, Huang SH, Lai CR, Chiu YT, et al. IL-1B polymorphism in association with initial intravenous immunoglobulin treatment failure in Taiwanese children with Kawasaki disease. *Circ J*. (2010) 74:544–51. doi: 10.1253/circj.CJ-09-0664
36. Huang YH, Hsu YW, Lu HF, Wong HS, Yu HR, Kuo HC, et al. Interferon-gamma Genetic Polymorphism and Expression in Kawasaki Disease. *Medicine (Baltimore)*. (2016) 95:e3501. doi: 10.1097/MD.0000000000003501
37. Ahn JG, Bae Y, Shin D, Nam J, Kim KY, Kim DS. HMGB1 gene polymorphism is associated with coronary artery lesions and intravenous immunoglobulin resistance in Kawasaki disease. *Rheumatology (Oxford)*. (2018) 2018:356. doi: 10.1093/rheumatology/key356
38. Kwon YC, Kim JJ, Yun SW, Yu JJ, Yoon KL, Lee KY, et al. BCL2L11 is associated with kawasaki disease in intravenous immunoglobulin responder patients. *Circ Genom Precis Med*. (2018) 11:e002020. doi: 10.1161/CIRCGEN.117.002020
39. Shendre A, Wiener HW, Zhi D, Vazquez AI, Portman MA, Shrestha S. High-density genotyping of immune loci in Kawasaki disease and IVIG treatment response in European-American case-parent trio study. *Genes Immun*. (2014) 15:534–42. doi: 10.1038/gene.2014.47
40. Kim JJ, Yun SW, Yu JJ, Yoon KL, Lee KY, Kil HR, et al. Identification of SAMD9L as a susceptibility locus for intravenous immunoglobulin resistance in Kawasaki disease by genome-wide association analysis. *Pharmacogenomics J*. (2019) 2019:85. doi: 10.1038/s41397-019-0085-1
41. Chang DY, Luo H, Zhou XJ, Chen M, Zhao MH. Association of HLA genes with clinical outcomes of ANCA-associated vasculitis. *Clin J Am Soc Nephrol*. (2012) 7:1293–9. doi: 10.2215/CJN.13071211
42. Cartin-Ceba R, Indrakanti D, Specks U, Stone JH, Hoffman GS, Kallenberg CG, et al. The pharmacogenomic association of Fcgamma receptors and cytochrome P450 enzymes with response to rituximab or cyclophosphamide treatment in antineutrophil cytoplasmic antibody-associated vasculitis. *Arthritis Rheumatol*. (2017) 69:169–75. doi: 10.1002/art.39822
43. Alberici F, Smith RM, Fonseca M, Willcocks LC, Jones RB, Holle JU, et al. Association of a TNFSF13B (BAFF) regulatory region single nucleotide polymorphism with response to rituximab in antineutrophil cytoplasmic antibody-associated vasculitis. *J Allergy Clin Immunol*. (2017) 139:1684–7 e10. doi: 10.1016/j.jaci.2016.08.051
44. Rustemoglu A, Gul U, Gumus-Akay G, Gonul M, Yigit S, Bozkurt N, et al. MDR1 gene polymorphisms may be associated with Behcet's disease and its colchicum treatment response. *Gene*. (2012) 505:333–9. doi: 10.1016/j.gene.2012.05.040
45. Karakus N, Yigit S, Kalkan G, Rustemoglu A, Inanir A, Gul U, et al. Association between the methylene tetrahydrofolate reductase gene C677T mutation and colchicine unresponsiveness in Behcet's disease. *Mol Vis*. (2012) 18:1696–700.
46. Keser G, Direskeneli H, Aksu K. Management of Takayasu arteritis: a systematic review. *Rheumatology (Oxford)*. (2014) 53:793–801. doi: 10.1093/rheumatology/ket320
47. Terao C, Yoshifuji H, Ohmura K, Murakami K, Kawabata D, Yurugi K, et al. Association of Takayasu arteritis with HLA-B 67:01 and two amino acids in HLA-B protein. *Rheumatology (Oxford)*. (2013) 52:1769–74. doi: 10.1093/rheumatology/ket241
48. Karageorgaki ZT, Bertsias GK, Mavragani CP, Kritikos HD, Spyropoulou-Vlachou M, Drosos AA, et al. Takayasu arteritis: epidemiological, clinical, and immunogenetic features in Greece. *Clin Exp Rheumatol*. (2009) 27:S33–9.
49. Lee SW, Kwon OJ, Park MC, Oh HB, Park YB, Lee SK. HLA alleles in Korean patients with Takayasu arteritis. *Clin Exp Rheumatol*. (2007) 25: S18–22.
50. Vargas-Alarcon G, Flores-Dominguez C, Hernandez-Pacheco G, Zuniga J, Gamboa R, Soto ME, et al. Immunogenetics and clinical aspects of Takayasu's arteritis patients in a Mexican Mestizo population. *Clin Exp Rheumatol*. (2001) 19:439–43.
51. Charoenwongse P, Kangwanshiratada O, Boonnam R, Hoomsindhu U. The association between the HLA antigens and Takayasu's arteritis in Thai patients. *Int J Cardiol*. (1998) 66(Suppl 1):S117–20.
52. Mehra NK, Jaini R, Balamurugan A, Kanga U, Prabhakaran D, Jain S, et al. Immunogenetic analysis of Takayasu arteritis in Indian patients. *Int J Cardiol*. (1998) 66(Suppl 1):S127–32; discussion S33.
53. Lv N, Wang Z, Dang A, Zhu X, Liu Y, Zheng D, et al. HLA-DQA1, DQB1 and DRB1 alleles associated with Takayasu arteritis in the Chinese Han population. *Hum Immunol*. (2015) 76:241–4. doi: 10.1016/j.humimm.2015.01.008
54. Dang A, Wang B, Zhang Y, Zhang P, Huang J, Liu G, et al. Association of the HLA-DRB1 gene with susceptibility to aortoarteritis in a Chinese Han population. *Hypertens Res*. (2002) 25:631–4. doi: 10.1291/hypres.25.631
55. Kimura A, Kishimoto T. IL-6: regulator of Treg/Th17 balance. *Eur J Immunol*. (2010) 40:1830–5. doi: 10.1002/eji.201040391
56. Sloane DE, Tedla N, Awoniyi M, Macglashan DW Jr, Borges L, Austen KF, et al. Leukocyte immunoglobulin-like receptors: novel innate receptors for human basophil activation and inhibition. *Blood*. (2004) 104:2832–9. doi: 10.1182/blood-2004-01-0268
57. Teng MW, Bowman EP, McElwee JJ, Smyth MJ, Casanova JL, Cooper AM, et al. IL-12 and IL-23 cytokines: from discovery to targeted therapies for immune-mediated inflammatory diseases. *Nat Med*. (2015) 21:719–29. doi: 10.1038/nm.3895
58. Tan Sardjono C, Mottram PL, Hogarth PM. The role of FcgammaRIIa as an inflammatory mediator in rheumatoid arthritis and systemic lupus erythematosus. *Immunol Cell Biol*. (2003) 81:374–81. doi: 10.1046/j.1440-1711.2003.01182.x
59. Samson M, Espigol-Frigole G, Terrades-Garcia N, Prieto-Gonzalez S, Corbera-Bellalta M, Alba-Rovira R, et al. Biological treatments in giant cell arteritis & Takayasu arteritis. *Eur J Intern Med*. (2018) 50:12–9. doi: 10.1016/j.ejim.2017.11.003
60. Nakaoka Y, Isobe M, Takei S, Tanaka Y, Ishii T, Yokota S, et al. Efficacy and safety of tocilizumab in patients with refractory Takayasu arteritis: results from a randomised, double-blind, placebo-controlled, phase 3 trial in Japan (the TAKT study). *Ann Rheum Dis*. (2018) 77:348–54. doi: 10.1136/annrheumdis-2017-211878
61. Terao C, Yoshifuji H, Nakajima T, Yukawa N, Matsuda F, Mimori T. Ustekinumab as a therapeutic option for Takayasu arteritis: from genetic findings to clinical application. *Scand J Rheumatol*. (2016) 45:80–2. doi: 10.3109/03009742.2015.1060521
62. Yachoui R, Kreidy M, Siorek M, Sehgal R. Successful treatment with ustekinumab for corticosteroid- and immunosuppressant-resistant Takayasu's arteritis. *Scand J Rheumatol*. (2018) 47:246–7. doi: 10.1080/03009742.2017.1278788
63. Pazzola G, Muratore F, Pipitone N, Crescentini F, Cacoub P, Boiardi L, et al. Rituximab therapy for Takayasu arteritis: a seven patients experience and a review of the literature. *Rheumatology (Oxford)*. (2017) 2017:249. doi: 10.1093/rheumatology/kex249
64. Salvarani C, Cantini F, Hunder GG. Polymyalgia rheumatica and giant-cell arteritis. *Lancet*. (2008) 372:234–45. doi: 10.1016/S0140-6736(08)61077-6
65. Carmona FD, Gonzalez-Gay MA, Martin J. Genetic component of giant cell arteritis. *Rheumatology (Oxford)*. (2014) 53:6–18. doi: 10.1093/rheumatology/ket231
66. Boiardi L, Casali B, Nicoli D, Farnetti E, Chen Q, Macchioni P, et al. Vascular endothelial growth factor gene polymorphisms in giant cell arteritis. *J Rheumatol*. (2003) 30:2160–4.
67. Enjuanes A, Benavente Y, Hernandez-Rodriguez J, Queralt C, Yague J, Jares P, et al. Association of NOS2 and potential effect of VEGF, IL6, CCL2 and IL1RN polymorphisms and haplotypes on susceptibility to GCA–a simultaneous study of 130 potentially functional SNPs in 14 candidate genes. *Rheumatology (Oxford)*. (2012) 51:841–51. doi: 10.1093/rheumatology/ker429
68. Marquez A, Hernandez-Rodriguez J, Cid MC, Solans R, Castaneda S, Fernandez-Contreras ME, et al. Influence of the IL17A locus in giant cell arteritis susceptibility. *Ann Rheum Dis*. (2014) 73:1742–5. doi: 10.1136/annrheumdis-2014-205261
69. Marquez A, Solans R, Hernandez-Rodriguez J, Cid MC, Castaneda S, Ramentol M, et al. A candidate gene approach identifies an IL33 genetic

variant as a novel genetic risk factor for GCA. *PLoS ONE*. (2014) 9:e113476. doi: 10.1371/journal.pone.0113476
70. Rueda B, Lopez-Nevot MA, Lopez-Diaz MJ, Garcia-Porrua C, Martin J, Gonzalez-Gay MA. A functional variant of vascular endothelial growth factor is associated with severe ischemic complications in giant cell arteritis. *J Rheumatol*. (2005) 32:1737–41.
71. Serrano A, Carmona FD, Castaneda S, Solans R, Hernandez-Rodriguez J, Cid MC, et al. Evidence of association of the NLRP1 gene with giant cell arteritis. *Ann Rheum Dis*. (2013) 72:628–30. doi: 10.1136/annrheumdis-2012-202609
72. Choi YS, Choi HJ, Min JK, Pyun BJ, Maeng YS, Park H, et al. Interleukin-33 induces angiogenesis and vascular permeability through ST2/TRAF6-mediated endothelial nitric oxide production. *Blood*. (2009) 114:3117–26. doi: 10.1182/blood-2009-02-203372
73. Schmitz J, Owyang A, Oldham E, Song Y, Murphy E, McClanahan TK, et al. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity*. (2005) 23:479–90. doi: 10.1016/j.immuni.2005.09.015
74. Weyand CM, Goronzy JJ. Immune mechanisms in medium and large-vessel vasculitis. *Nat Rev Rheumatol*. (2013) 9:731–40. doi: 10.1038/nrrheum.2013.161
75. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. *Nat Med*. (2003) 9:669–76. doi: 10.1038/nm0603-669
76. Tschopp J, Martinon F, Burns K. NALPs: a novel protein family involved in inflammation. *Nat Rev Mol Cell Biol*. (2003) 4:95–104. doi: 10.1038/nrm1019
77. Bottini N, Peterson EJ. Tyrosine phosphatase PTPN22: multifunctional regulator of immune signaling, development, and disease. *Annu Rev Immunol*. (2014) 32:83–119. doi: 10.1146/annurev-immunol-032713-120249
78. Miles LA, Parmer RJ. Plasminogen receptors: the first quarter century. *Semin Thromb Hemost*. (2013) 39:329–37. doi: 10.1055/s-0033-1334483
79. Gilkes DM, Bajpai S, Chaturvedi P, Wirtz D, Semenza GL. Hypoxia-inducible factor 1 (HIF-1) promotes extracellular matrix remodeling under hypoxic conditions by inducing P4HA1, P4HA2, and PLOD2 expression in fibroblasts. *J Biol Chem*. (2013) 288:10819–29. doi: 10.1074/jbc.M112.442939
80. Stone JH, Klearman M, Collinson N. Trial of tocilizumab in giant-cell arteritis. *N Engl J Med*. (2017) 377:1494–5. doi: 10.1056/NEJMc1711031
81. Villiger PM, Adler S, Kuchen S, Wermelinger F, Dan D, Fiege V, et al. Tocilizumab for induction and maintenance of remission in giant cell arteritis: a phase 2, randomised, double-blind, placebo-controlled trial. *Lancet*. (2016) 387:1921–7. doi: 10.1016/S0140-6736(16)00560-2
82. Conway R, O'Neill L, O'Flynn E, Gallagher P, McCarthy GM, Murphy CC, et al. Ustekinumab for the treatment of refractory giant cell arteritis. *Ann Rheum Dis*. (2016) 75:1578–9. doi: 10.1136/annrheumdis-2016-209351
83. Langford CA, Cuthbertson D, Ytterberg SR, Khalidi N, Monach PA, Carette S, et al. A randomized, double-blind trial of abatacept (CTLA-4Ig) for the treatment of giant cell arteritis. *Arthritis Rheumatol*. (2017) 69:837–45. doi: 10.1002/art.40044
84. Jarrot PA, Kaplanski G. Pathogenesis of ANCA-associated vasculitis: an update. *Autoimmun Rev*. (2016) 15:704–13. doi: 10.1016/j.autrev.2016.03.007
85. Segarra M, Ohnuki H, Maric D, Salvucci O, Hou X, Kumar A, et al. Semaphorin 6A regulates angiogenesis by modulating VEGF signaling. *Blood*. (2012) 120:4104–15. doi: 10.1182/blood-2012-02-410076
86. Wieczorek S, Holle JU, Cohen Tervaert JW, Harper L, Moosig F, Gross WL, et al. The SEM6A6 locus is not associated with granulomatosis with polyangiitis or other forms of antineutrophil cytoplasmic antibody-associated vasculitides in Europeans: comment on the article by Xie et al. *Arthritis Rheumatol*. (2014) 66:1400–1. doi: 10.1002/art.38367
87. Giscombe R, Wang X, Huang D, Lefvert AK. Coding sequence 1 and promoter single nucleotide polymorphisms in the CTLA-4 gene in Wegener's granulomatosis. *J Rheumatol*. (2002) 29:950–3.
88. Huang D, Giscombe R, Zhou Y, Lefvert AK. Polymorphisms in CTLA-4 but not tumor necrosis factor-alpha or interleukin 1beta genes are associated with Wegener's granulomatosis. *J Rheumatol*. (2000) 27:397–401.
89. Kamesh L, Heward JM, Williams JM, Gough SC, Chavele KM, Salama A, et al. CT60 and +49 polymorphisms of CTLA 4 are associated with ANCA-positive small vessel vasculitis. *Rheumatology (Oxford)*. (2009) 48:1502–5. doi: 10.1093/rheumatology/kep280
90. Lee YH, Choi SJ, Ji JD, Song GG. CTLA-4 and TNF-alpha promoter-308 A/G polymorphisms and ANCA-associated vasculitis susceptibility: a meta-analysis. *Mol Biol Rep*. (2012) 39:319–26. doi: 10.1007/s11033-011-0741-2
91. Slot MC, Sokolowska MG, Savelkouls KG, Janssen RG, Damoiseaux JG, Tervaert JW. Immunoregulatory gene polymorphisms are associated with ANCA-related vasculitis. *Clin Immunol*. (2008) 128:39–45. doi: 10.1016/j.clim.2008.03.506
92. Zhou Y, Huang D, Paris PL, Sauter CS, Prock KA, Hoffman GS. An analysis of CTLA-4 and proinflammatory cytokine genes in Wegener's granulomatosis. *Arthritis Rheum*. (2004) 50:2645–50. doi: 10.1002/art.20385
93. Vaglio A, Martorana D, Maggiore U, Grasselli C, Zanetti A, Pesci A, et al. HLA-DRB4 as a genetic risk factor for Churg-Strauss syndrome. *Arthritis Rheum*. (2007) 56:3159–66. doi: 10.1002/art.22834
94. Martorana D, Bonatti F, Alberici F, Gioffredi A, Reina M, Urban ML, et al. Fcgamma-receptor 3B (FCGR3B) copy number variations in patients with eosinophilic granulomatosis with polyangiitis. *J Allergy Clin Immunol*. (2016) 137:1597–9 e8. doi: 10.1016/j.jaci.2015.09.053
95. Wieczorek S, Hellmich B, Arning L, Moosig F, Lamprecht P, Gross WL, et al. Functionally relevant variations of the interleukin-10 gene associated with antineutrophil cytoplasmic antibody-negative Churg-Strauss syndrome, but not with Wegener's granulomatosis. *Arthritis Rheum*. (2008) 58:1839–48. doi: 10.1002/art.23496
96. Polzer K, Karonitsch T, Neumann T, Eger G, Haberler C, Soleiman A, et al. Eotaxin-3 is involved in Churg-Strauss syndrome–a serum marker closely correlating with disease activity. *Rheumatology (Oxford)*. (2008) 47:804–8. doi: 10.1093/rheumatology/ken033
97. Zwerina J, Bach C, Martorana D, Jatzwauk M, Hegasy G, Moosig F, et al. Eotaxin-3 in Churg-Strauss syndrome: a clinical and immunogenetic study. *Rheumatology (Oxford)*. (2011) 50:1823–7. doi: 10.1093/rheumatology/keq445
98. McClure M, Gopaluni S, Jayne D, Jones R. B cell therapy in ANCA-associated vasculitis: current and emerging treatment options. *Nat Rev Rheumatol*. (2018) 14:580–91. doi: 10.1038/s41584-018-0065-x
99. Lepse N, Land J, Rutgers A, Kallenberg CG, Stegeman CA, Abdulahad WH, et al. Toll-like receptor 9 activation enhances B cell activating factor and interleukin-21 induced anti-proteinase 3 autoantibody production *in vitro*. *Rheumatology (Oxford)*. (2016) 55:162–72. doi: 10.1093/rheumatology/kev293
100. Langford CA, Monach PA, Specks U, Seo P, Cuthbertson D, McAlear CA, et al. An open-label trial of abatacept (CTLA4-IG) in non-severe relapsing granulomatosis with polyangiitis (Wegener's). *Ann Rheum Dis*. (2014) 73:1376–9. doi: 10.1136/annrheumdis-2013-204164
101. Zeidan MJ, Saadoun D, Garrido M, Klatzmann D, Six A, Cacoub P. Behcet's disease physiopathology: a contemporary review. *Auto Immun Highlights*. (2016) 7:4. doi: 10.1007/s13317-016-0074-1
102. de Menthon M, Lavalley MP, Maldini C, Guillevin L, Mahr A. HLA-B51/B5 and the risk of Behcet's disease: a systematic review and meta-analysis of case-control genetic association studies. *Arthritis Rheum*. (2009) 61:1287–96. doi: 10.1002/art.24642
103. Hughes T, Coit P, Adler A, Yilmaz V, Aksu K, Duzgun N, et al. Identification of multiple independent susceptibility loci in the HLA region in Behcet's disease. *Nat Genet*. (2013) 45:319–24. doi: 10.1038/ng.2551
104. Iyer SS, Cheng G. Role of interleukin 10 transcriptional regulation in inflammation and autoimmune disease. *Crit Rev Immunol*. (2012) 32:23–63. doi: 10.1615/CritRevImmunol.v32.i1.30
105. Rossi D, Zlotnik A. The biology of chemokines and their receptors. *Annu Rev Immunol*. (2000) 18:217–42. doi: 10.1146/annurev.immunol.18.1.217
106. Watford WT, Hissong BD, Bream JH, Kanno Y, Muul L, O'Shea JJ. Signaling by IL-12 and IL-23 and the immunoregulatory roles of STAT4. *Immunol Rev*. (2004) 202:139–56. doi: 10.1111/j.0105-2896.2004.00211.x
107. Raulet DH. Roles of the NKG2D immunoreceptor and its ligands. *Nat Rev Immunol*. (2003) 3:781–90. doi: 10.1038/nri1199
108. Schnell S, Demolliere C, van den Berk P, Jacobs H. Gimap4 accelerates T-cell death. *Blood*. (2006) 108:591–9. doi: 10.1182/blood-2005-11-4616
109. Shimoda Y, Watanabe K. Contactins: emerging key roles in the development and function of the nervous system. *Cell Adh Migr*. (2009) 3:64–70. doi: 10.4161/cam.3.1.7764

110. Vitale A, Rigante D, Lopalco G, Emmi G, Bianco MT, Galeazzi M, et al. New therapeutic solutions for Behcet's syndrome. *Expert Opin Investig Drugs*. (2016) 25:827–40. doi: 10.1080/13543784.2016.1181751
111. Di Scala G, Bettiol A, Cojan RD, Finocchi M, Silvestri E, Emmi G. Efficacy of the anti-IL 17 secukinumab in refractory Behcet's syndrome: a preliminary study. *J Autoimmun*. (2019) 97:108–13. doi: 10.1016/j.jaut.2018.09.002
112. Mirouse A, Barete S, Monfort JB, Resche-Rigon M, Bouyer AS, Comarmond C, et al. Ustekinumab for Behcet's disease. *J Autoimmun*. (2017) 82:41–6. doi: 10.1016/j.jaut.2017.05.002
113. Shulman ST, Rowley AH. Kawasaki disease: insights into pathogenesis and approaches to treatment. *Nat Rev Rheumatol*. (2015) 11:475–82. doi: 10.1038/nrrheum.2015.54
114. Burgner D, Davila S, Breunis WB, Ng SB, Li Y, Bonnard C, et al. A genome-wide association study identifies novel and functionally related susceptibility Loci for Kawasaki disease. *PLoS Genet*. (2009) 5:e1000319. doi: 10.1371/journal.pgen.1000319
115. Kim JJ, Hong YM, Sohn S, Jang GY, Ha KS, Yun SW, et al. A genome-wide association analysis reveals 1p31 and 2p13.3 as susceptibility loci for Kawasaki disease. *Hum Genet*. (2011) 129:487–95. doi: 10.1007/s00439-010-0937-x
116. Tsai FJ, Lee YC, Chang JS, Huang LM, Huang FY, Chiu NC, et al. Identification of novel susceptibility Loci for kawasaki disease in a Han chinese population by a genome-wide association study. *PLoS ONE*. (2011) 6:e16853. doi: 10.1371/journal.pone.0016853
117. Kato S, Kimura M, Tsuji K, Kusakawa S, Asai T, Juji T, et al. HLA antigens in Kawasaki disease. *Pediatrics*. (1978) 61:252–5.
118. Matsuda I, Hattori S, Nagata N, Fruse A, Nambu H. HLA antigens in mucocutaneous lymph node syndrome. *Am J Dis Child*. (1977) 131:1417–8.
119. Kaslow RA, Bailowitz A, Lin FY, Koslowe P, Simonis T, Israel E. Association of epidemic Kawasaki syndrome with the HLA-A2, B44, Cw5 antigen combination. *Arthritis Rheum*. (1985) 28:938–40.
120. Krensky AM, Berenberg W, Shanley K, Yunis EJ. HLA antigens in mucocutaneous lymph node syndrome in New England. *Pediatrics*. (1981) 67:741–3.
121. Krensky AM, Grady S, Shanley KM, Berenberg W, Yunis EJ. Epidemic and endemic HLA-B and DR associations in mucocutaneous lymph node syndrome. *Hum Immunol*. (1983) 6:75–7.
122. Warmerdam PA, van de Winkel JG, Vlug A, Westerdaal NA, Capel PJ. A single amino acid in the second Ig-like domain of the human Fc gamma receptor II is critical for human IgG2 binding. *J Immunol*. (1991) 147:1338–43.
123. Onouchi Y, Gunji T, Burns JC, Shimizu C, Newburger JW, Yashiro M, et al. ITPKC functional polymorphism associated with Kawasaki disease susceptibility and formation of coronary artery aneurysms. *Nat Genet*. (2008) 40:35–42. doi: 10.1038/ng.2007.59
124. Alphonse MP, Duong TT, Shumitzu C, Hoang TL, McCrindle BW, Franco A, et al. Inositol-triphosphate 3-kinase C mediates inflammasome activation and treatment response in kawasaki disease. *J Immunol*. (2016) 197:3481–9. doi: 10.4049/jimmunol.1600388
125. Nemazee D, Weigert M. Revising B cell receptors. *J Exp Med*. (2000) 191:1813–7. doi: 10.1084/jem.191.11.1813
126. Peters AL, Stunz LL, Bishop GA. CD40 and autoimmunity: the dark side of a great activator. *Semin Immunol*. (2009) 21:293–300. doi: 10.1016/j.smim.2009.05.012
127. Wallace CA, French JW, Kahn SJ, Sherry DD. Initial intravenous gammaglobulin treatment failure in Kawasaki disease. *Pediatrics*. (2000) 105:E78. doi: 10.1542/peds.105.6.e78
128. Galeotti C, Kaveri SV, Cimaz R, Kone-Paut I, Bayry J. Predisposing factors, pathogenesis and therapeutic intervention of Kawasaki disease. *Drug Discov Today*. (2016) 21:1850–7. doi: 10.1016/j.drudis.2016.08.004
129. Lee Y, Schulte DJ, Shimada K, Chen S, Crother TR, Chiba N, et al. Interleukin-1beta is crucial for the induction of coronary artery inflammation in a mouse model of Kawasaki disease. *Circulation*. (2012) 125:1542–50. doi: 10.1161/CIRCULATIONAHA.111.072769
130. Wakita D, Kurashima Y, Crother TR, Noval Rivas M, Lee Y, Chen S, et al. Role of interleukin-1 signaling in a mouse model of kawasaki disease-associated abdominal aortic aneurysm. *Arterioscler Thromb Vasc Biol*. (2016) 36:886–97. doi: 10.1161/ATVBAHA.115.307072
131. Medvedev AE, Murphy M, Zhou H, Li X. E3 ubiquitin ligases Pellinos as regulators of pattern recognition receptor signaling and immune responses. *Immunol Rev*. (2015) 266:109–22. doi: 10.1111/imr.12298
132. Navon Elkan P, Pierce SB, Segel R, Walsh T, Barash J, Padeh S, et al. Mutant adenosine deaminase 2 in a polyarteritis nodosa vasculopathy. *N Engl J Med*. (2014) 370:921–31. doi: 10.1056/NEJMoa1307362
133. Zhou Q, Yang D, Ombrello AK, Zavialov AV, Toro C, Stone DL, et al. Early-onset stroke and vasculopathy associated with mutations in ADA2. *N Engl J Med*. (2014) 370:911–20. doi: 10.1056/NEJMoa1307361
134. Zavialov AV, Gracia E, Glaichenhaus N, Franco R, Lauvau G. Human adenosine deaminase 2 induces differentiation of monocytes into macrophages and stimulates proliferation of T helper cells and macrophages. *J Leukoc Biol*. (2010) 88:279–90. doi: 10.1189/jlb.1109764
135. Hashem H, Kumar AR, Muller I, Babor F, Bredius R, Dalal J, et al. Hematopoietic stem cell transplantation rescues the hematological, immunological, and vascular phenotype in DADA2. *Blood*. (2017) 130:2682–8. doi: 10.1182/blood-2017-07-798660
136. Hsu AP, West RR, Calvo KR, Cuellar-Rodriguez J, Parta M, Kelly SJ, et al. Adenosine deaminase type 2 deficiency masquerading as GATA2 deficiency: successful hematopoietic stem cell transplantation. *J Allergy Clin Immunol*. (2016) 138:628–30 e2. doi: 10.1016/j.jaci.2016.03.016
137. Van Eyck L Jr, Hershfield MS, Pombal D, Kelly SJ, Ganson NJ, Moens L, et al. Hematopoietic stem cell transplantation rescues the immunologic phenotype and prevents vasculopathy in patients with adenosine deaminase 2 deficiency. *J Allergy Clin Immunol*. (2015) 135:283-7 e5. doi: 10.1016/j.jaci.2014.10.010
138. Ombrello AK, Qin J, Hoffmann PM, Kumar P, Stone D, Jones A, et al. Treatment Strategies for Deficiency of Adenosine Deaminase 2. *N Engl J Med*. (2019) 380:1582–4. doi: 10.1056/NEJMc1801927
139. Samuelsson A, Towers tl, Ravetch JV. Anti-inflammatory activity of IVIG mediated through the inhibitory Fc receptor. *Science*. (2001) 291:484–6. doi: 10.1126/science.291.5503.484
140. Hughes P, Bouillet P, Strasser A. Role of Bim and other Bcl-2 family members in autoimmune and degenerative diseases. *Curr Dir Autoimmun*. (2006) 9:74–94. doi: 10.1159/000090773
141. Lemos de Matos A, Liu J, McFadden G, Esteves PJ. Evolution and divergence of the mammalian SAMD9/SAMD9L gene family. *BMC Evol Biol*. (2013) 13:121. doi: 10.1186/1471-2148-13-121
142. Kuo HC, Wong HS, Chang WP, Chen BK, Wu MS, Yang KD, et al. Prediction for intravenous immunoglobulin resistance by using weighted genetic risk score identified from genome-wide association study in kawasaki disease. *Circ Cardiovasc Genet*. (2017) 10:1625. doi: 10.1161/CIRCGENETICS.116.001625
143. Richaud-Patin Y, Soto-Vega E, Jakez-Ocampo J, Llorente L. P-glycoprotein in autoimmune diseases. *Autoimmun Rev*. (2004) 3:188–92. doi: 10.1016/j.autrev.2003.08.002
144. Liew SC, Gupta ED. Methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism: epidemiology, metabolism and the associated diseases. *Eur J Med Genet*. (2015) 58:1–10. doi: 10.1016/j.ejmg.2014.10.004
145. Aksu K, Turgan N, Oksel F, Keser G, Ozmen D, Kitapcioglu G, et al. Hyperhomocysteinaemia in Behçet's disease. *Rheumatology (Oxford)*. (2001) 40:687–90. doi: 10.1093/rheumatology/40.6.687
146. Parkes M, Cortes A, van Heel DA, Brown MA. Genetic insights into common pathways and complex relationships among immune-mediated diseases. *Nat Rev Genet*. (2013) 14:661–73. doi: 10.1038/nrg3502
147. Zhernakova A, Withoff S, Wijmenga C. Clinical implications of shared genetics and pathogenesis in autoimmune diseases. *Nat Rev Endocrinol*. (2013) 9:646–59. doi: 10.1038/nrendo.2013.161
148. Acosta-Herrera M, Kerick M, Gonzalez-Serna D, Wijmenga C, Franke A, Gregersen PK, et al. Genome-wide meta-analysis reveals shared new loci in systemic seropositive rheumatic diseases. *Ann Rheum Dis*. (2019) 78:311–9. doi: 10.1136/annrheumdis-2018-214127

149. Ellinghaus D, Jostins L, Spain SL, Cortes A, Bethune J, Han B, et al. Analysis of five chronic inflammatory diseases identifies 27 new associations and highlights disease-specific patterns at shared loci. *Nat Genet*. (2016) 48:510–8. doi: 10.1038/ng.3528
150. Li YR, Li J, Zhao SD, Bradfield JP, Mentch FD, Maggadottir SM, et al. Meta-analysis of shared genetic architecture across ten pediatric autoimmune diseases. *Nat Med*. (2015) 21:1018–27. doi: 10.1038/nm.3933
151. Marquez A, Kerick M, Zhernakova A, Gutierrez-Achury J, Chen WM, Onengut-Gumuscu S, et al. Meta-analysis of Immunochip data of four autoimmune diseases reveals novel single-disease and cross-phenotype associations. *Genome Med*. (2018) 10:97. doi: 10.1186/s13073-018-0604-8
152. Carmona FD, Coit P, Saruhan-Direskeneli G, Hernandez-Rodriguez J, Cid MC, Solans R, et al. Analysis of the common genetic component of large-vessel vasculitides through a meta-Immunochip strategy. *Sci Rep*. (2017) 7:43953. doi: 10.1038/srep43953
153. Ortiz-Fernandez L, Carmona FD, Lopez-Mejias R, Gonzalez-Escribano MF, Lyons PA, Morgan AW, et al. Cross-phenotype analysis of Immunochip data identifies KDM4C as a relevant locus for the development of systemic vasculitis. *Ann Rheum Dis*. (2018) 77:589–95. doi: 10.1136/annrheumdis-2017-212372
154. Khera AV, Chaffin M, Aragam KG, Haas ME, Roselli C, Choi SH, et al. Genome-wide polygenic scores for common diseases identify individuals with risk equivalent to monogenic mutations. *Nat Genet*. (2018) 50:1219–24. doi: 10.1038/s41588-018-0183-z

Conflict of Interest Statement: 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 handling editor declared a past co-authorship with one of the authors JM.

The reviewer DM declared a past co-authorship with one of the authors with the authors JM and AM to the handling editor.

frontiers in Immunology

MINI REVIEW
published: 20 September 2019
doi: 10.3389/fimmu.2019.02165

Streptococcal Enzymes as Precision Tools Against Pathogenic IgG Autoantibodies in Small Vessel Vasculitis

Mårten Segelmark[1]* *and Lars Björck*[2]

[1] Nephrology, Department of Clinical Sciences, Lund University, Lund, Sweden, [2] Infection Medicine, Department of Clinical Sciences, Lund University, Lund, Sweden

In primary systemic small vessel vasculitis autoantibodies are common and seem to play an important role in the pathogenesis. Autoantibodies in vasculitis are preferentially directed against components of the immune system or directly against components of the vessel wall. Plasmapheresis is often applied in emergency situationists when the function of vital organs is jeopardized, the level of clinical evidence to apply such therapy, however, varies between low and non-existing. Plasmapheresis is a blunt and unspecific instrument that requires several sessions to achieve a substantial reduction of autoantibody levels. IdeS and EndoS are two relatively recently discovered enzymes produced by *S. pyogenes*, that have a remarkable capacity to degrade and disarm IgG. They have shown positive results in several *in vivo* models of autoimmunity, and treatment with IdeS has successfully been used to inactivate HLA alloantibodies in patients undergoing renal transplantation. Both IdeS and EndoS have the potential to become precision tools to replace plasmapheresis in the treatment of vasculitic emergencies and a clinical trial of IdeS in anti-GBM vasculitis is now ongoing.

Keywords: vasculitis, ANCA, *Streptococcus pygenes*, anti-GBM antibody disease, autoantibodies

OPEN ACCESS

Edited by:
Giacomo Emmi,
University of Florence, Italy

Reviewed by:
Luca Quartuccio,
University of Udine, Italy
Haner Direskeneli,
Marmara University, Turkey

***Correspondence:**
Mårten Segelmark
marten.segelmark@med.lu.se

Specialty section:
This article was submitted to Autoimmune and Autoinflammatory Disorders, a section of the journal Frontiers in Immunology

Received: *13 June 2019*
Accepted: *28 August 2019*
Published: *20 September 2019*

Citation:
Segelmark M and Björck L (2019) Streptococcal Enzymes as Precision Tools Against Pathogenic IgG Autoantibodies in Small Vessel Vasculitis. Front. Immunol. 10:2165. doi: 10.3389/fimmu.2019.02165

AUTOANTIBODIES ARE COMMON

The association of autoantibodies and inflammatory diseases was established more than 60 years ago (1); and now there are several hundred described specificities associated with different diseases. They are utilized for diagnostic purposes to differentiate between diseases and sometimes also as markers of disease activity; and they may participate in the pathogenesis (2). Elevated levels of disease associated autoantibodies can sometimes be found long before onset of symptoms and any diagnosis can be established (3). Autoantibodies can also be found in healthy humans (2, 4) and in mice raised under germfree conditions (5). Such natural autoantibodies are probably instrumental in removing cellular debris and seems to be protective of both arteriosclerosis and autoimmune disease. Most of these natural autoantibodies are thought to be of IgM class with limited avidity and specificity. However, it has been shown that healthy humans also have low levels of IgG antibodies (4) with highly restricted epitope specificity (6).

Autoantibodies in Vasculitis

Vasculitides are broadly divided into primary and secondary forms, where primary vasculitides are diseases where inflamed blood vessel is the defining and most prominent feature. Primary

TABLE 1 | Autoantibodies in small vessel vasculitis.

Disease	Main autoantibody	Autoantigen
Microscopic polyangiitis (MPA)	MPO-ANCA	Myeloperoxidase
Granulomatous polyangiitis (GPA, formerly Wegener's granulomatosis)	PR3-ANCA	Proteinase 3
Eosinophilic granulomatous polyangiitis (EGPA, formerly Churg-Strauss Syndrome)	MPO-ANCA	Myeloperoxidase
Anti-GBM disease (formerly Goodpasture's disease)	Anti-GBM	α3 chain of Type IV collagen
IgA-vasculitis (formerly Henoch-Schönlein purpura)	IgG/IgA anti-IgA	Degalactoslylated IgA
Cryoglobulinemic vasculitis	IgM anti-IgG	Polyclonal immunoglobulin G
Hypocomplementemic urticarial vasculitis	Anti-C1q	Complement factor C1q

vasculitides are further divided based on vessel size into large vessel, medium-sized vessel and small vessel vasculitis (7). Autoantibodies are common in all diseases in the small vessel group but are rare or at least not yet discovered in large and medium-sized vessel vasculitis (8, 9) (**Table 1**). Autoantibodies are also common in secondary forms of small vessel vasculitis, such as in systemic lupus erythematosus (1) and drug induced vasculits (10, 11), but in secondary vasculitis treatment should be aimed at underlying condition.

Autoantibodies Take Part in the Pathogenesis

The role of the autoantibodies in the pathogenesis, and the implicated pathogenic mechanisms, varies between the different diseases. In anti-GBM disease the binding of autoantibodies along the capillary wall of the glomeruli and alveoli start the complement cascade through the classical pathway and attract neutrophils through the C5a fragment (12, 13). Transfer experiments of eluted human antibodies injected into primates show that anti-GBM alone can mediate the disease (14), but there are also reports of anti-GBM models driven by T cells in agammaglobulinemic animals (15). Several *in vitro* studies indicate a role for anti-neutrophil cytoplasm antibodies (ANCA) in the pathogenesis of small vessel vasculitides such as microscopic polyangiitis (MPA) and granulomatosis with polyangiitis (GPA) (16, 17). There are many ANCA specificities in different autoimmune diseases but only myeloperoxidase (MPO) and proteinase 3 (PR3) that are expressed on the surface of primed neutrophils are major ANCA-antigens in vasculitis (8). The most compelling evidence for a role of ANCA in the pathogenesis comes from animal models of MPO-ANCA, where antibodies alone or antibody producing cells can transfer the disease (18). However, there are also data that do not support a direct role for ANCA in the pathogenesis; all purified IgG preparations from patients do not active neutrophils in a consistent manner (19, 20). IgA vasculitis (21) and cryoglobulinemic vasculitis (22) are immune complex mediated diseases, where polyclonal or monoclonal autoantibodies react with other immunoglobulins to form complexes. In urticarial vasculitis there are often autoantibodies directed to the complement factor C1q, which also lead to immune complex formation (23). Immune complexes activate complement primarily through the classical pathway which results in neutrophil influx and vessel wall damage (23). Physiochemical properties such as size and temperature determine where and when they will deposit, in urticarial vasculitis the direct targeting of the complement system also affect symptoms and signs.

IdeS AND EndoS

Streptococcus pyogenes, one of the most significant bacterial pathogens in humans, has evolved multiple mechanisms to avoid antibody attack and complement activation. IdeS, **I**mmunoglobulin G **d**egrading **e**nzyme of ***S****treptococcus pyogenes*, is a secreted cysteine proteinase which cleaves all four human IgG subclasses with a unique degree of specificity; apart from IgG no other substrate has been identified (24). Before cleavage can occur in the hinge region of the heavy chain to generate two Fc and one F $(ab^1)_2$ fragment the enzyme has to bind to the Fc region, and the remarkable specificity lies in this initial protein-protein interaction (25). *S. pyogenes* infects only humans, and from an evolutionary point of view it is noteworthy that the cleavage of IgG in other species is more restricted; in mice for instance subclasses 2a/c and 3 are sensitive, but not 1 and 2b (26).

Human IgG contains one N-linked glycan attached to Asn237 on the heavy chain (27). It is of great importance for effector functions such as complement activation and neutrophil recruitment. There are several bacterial enzymes that modifies N-linked glycans, but the first IgG specific glycan hydrolase to be described was EndoS which is also produced *Streptococcus pyogenes* (28). EndoS cleaves most of the carbohydrate moiety from IgG but leaves an N-acetylglucosamine with an alpha-linked fucose on protein backbone. EndoS treatment *in vitro* leads to reduced complement activation and phagocytosis of bacteria.

IdeS and EndoS in Experimental Models

The species specificity hampers to use of IdeS in many rodent models. Not surprising is that pretreatment *in vitro* of pathogenic autoantibodies with IdeS can abolish disease in passive transfer models, such as immune thrombocytopenic purpura, neuromyelitis optica, and collagen induced arthritis (26, 29). What is more encouraging is that is that mice *in vivo* can be rescued from a lethal dose of rabbit anti-mouse thrombocytes and that arthritis induced by mouse IgG2a antibodies can be reduced in severity by IdeS *in vivo* (26). EndoS is easier to employ in experimental rodent models and have been shown to be effective to prevent or to treat disease in multiple settings, also in strains that spontaneously develop systemic inflammation (30).

The effect of IdeS and EndoS has also been investigated in experimental models of vasculitis. A mouse/rabbit model had been developed to mimic essential steps in the pathogenesis of anti-GBM disease. Here we took advantage of IgG species differences. Mice are first given a bolus dose of rabbit anti-mouse

IgG; since rabbit IgG cannot activate mouse complement (31) this has no consequences. A week later, when there is no longer any circulating rabbit IgG, the animals are challenged with mouse-anti rabbit IgG. This leads to a dose-dependent renal injury mediated by complement induced neutrophil recruitment. When IdeS was given between the two IgG injections, it completely inhibited the development of proteinuria. Histological examinations confirmed that Fc fragments but not F (ab')2 fragment had been removed from the GBM. This was accompanied by a reduction in the deposition of complement and influx of neutrophils in the glomeruli. EndoS was also employed in this model, even though the setup is not ideal for testing EndoS a positive effect was seen.

EndoS has also been used in a model of ANCA associated vasculitis (32). Pre-treatment of human MPO-ANCA containing IgG with EndoS prevented neutrophil respiratory burst. When mouse anti-MPO was exposed to EndoS before injection into mice, the antibodies did not induce disease. In addition, when EndoS was given to the mice after challenge with anti-MPO IgG antibodies this attenuated the disease (32).

IdeS in Humans

In a phase I trial, IdeS was given in different doses to healthy human volunteers (33). Doses as low as 0.12 mg/kg body weight led to a complete cleavage of not only plasma IgG but the entire extracellular IgG pool in all subjects without any obvious side effects. Intact IgG started to reappear after a few days and reached pre-treatment levels within a month. Varying titer of anti-IdeS antibodies were detected in the healthy volunteers; these levels rose after IdeS infusion, peaked after 2 weeks and was back to pre-treatment levels after 6 months.

IdeS is being developed as a pharmaceutical agent by Hansa-Biopharma; the non-proprietary name for the compound is Imlifidase. It has been tested in clinical trials to enable transplantation in patients with multiple HLA alloantibodies (34). A single dose given prior to transplantation enabled transplantations in 24 out 25 such sensitized patients who if they ultimately receive a kidney at all, may have to wait for years for a matching kidney (35). In many of the patients HLA antibodies rebounded, but 6 months after transplantation all 24 patients had functioning grafts.

IdeS like all other therapies can have side effects, total depletion of IgG take away an important protection against microorganisms. This is done also by plasma exchange but not as effective, on the other hand IdeS treated patients still have intact levels of IgA and IgM. Most individuals have measurable levels of anti-IdeS this introduces the risk of immune-complex formation and the development serum sickness. Furthermore, there is a theoretical risk of formation of neo-eptiopes by IdeS cleavage which can trigger autoantibody formation to IgG bound to different surfaces. Vigilance is always needed when introducing new pharma.

The Potential Role IdeS and EndoS in Treatment of Human Vasculitis

The rapid depletion of autoantibodies that can be achieved by IdeS is potentially beneficial in acute settings where vasculits threatens the function of vital organs. Today plasma exchange or immunadsorption therapy is used to lower levels of pathogenic IgG antibodies in such settings (36). With plasma exchange only about one third of the total body IgG is removed in each session. That means that it takes several days to reduce the levels with one order of magnitude, and many times a greater reduction is needed. Immunadsorption is more effective, but so far there are no randomized trials showing that this therapy leads to an improved clinical outcome in any condition, as compared to standard plasma exchange. The question is whether depleting autoantibodies with IdeS or disarming them with EndoS would make a clinical meaningful difference.

Most patients with anti-GBM disease have rapidly progressive glomerulonephritis (13). The standard therapy today is the combination of cyclophosphamide to stop autoantibody production, plasma exchange to remove autoantibodies and steroids to dampen inflammation (37, 38). This therapy is effective if started early, but most patients are diagnosed late. More than 2/3 are diagnosed when glomerular filtration rate is below 15 ml/min and in such cases <10% achieve renal survival. Anti-GBM disease is in most cases a monophasic disease, where autoantibodies are only produced during a few months. This period is substantially shortened by immunosuppression (39). We treated three patients on compassionate basis with refractory anti-GBM disease after an individual permit from the Swedish Medicinal Agency (40). All there were dialysis dependent and had high levels of circulating anti-GBM despite intense plasma-exchange. In all three cases anti-GBM levels dropped to levels within the normal range. Using Fc-gamma specific antisera we could show that IdeS had cleaved IgG bound to the kidney. None of three, however, regained kidney function enough to stop dialysis. We are now conducting an investigator driven clinical trial (EdraCT2016-004082-39) where 0.25 mg/kg of imlifidase (IdeS) is given as a single injection early in the course. Anti-GBM disease is rare, and considering inclusion and exclusion criteria, we need a very large catchment area to include the goal of 15 patients. So far 15 tertiary referral hospitals in major European cities participate in the study and 11 out 15 patients have been included.

In ANCA associated vasculitis plasma exchange is often used when complicated by rapidly progressive glomerulonephritis with severe renal failure or alveolar hemorrhage with respiratory distress (41). However, the use of plasma exchange in ANCA vasculitis is controversial. The MEPEX study published in 2003, show positive effect on 1-year renal survival (42). The more recently conducted PEXIVAS trial, that is so far only available as congress abstracts, did not show any benefit of plasma exchange, neither in cases with rapidly progressive glomerulonephritis nor in cases with alveolar hemorrhage. The reason for the negative results could be that plasma exchange is not effective enough to lower autoantibody levels in acute settings. In such cases treatment with IdeS or EndoS would be attractive treatment options. On the other hand, it also possible that the autoantibodies only play a minor role in the pathogenesis and that their removal does not lead to any meaningful clinical effect.

IgA vasculitis, urticarial vasculitis and cryoglobulinemic vasculitis are all considered to be immune complex mediated diseases. Plasma exchange is being used in all three diseases, but the evidence level is low (36, 43, 44). The potential effect of IdeS

and EndoS is only speculative and might not even be beneficial. IgA vasculitis is the most common form of primary systemic vasculitis in children. The yearly incidence is reported to be as high as 176 per million children (45). Most cases are, however, mild and heal without any treatment. The disease is much less common in adults, but on the other hand also more severe. The high rate of spontaneous recovery makes clinical trials difficult. IgA is not effective when it comes to complement activation; the presence of IgG in the complexes may therefore be instrumental for the development of vasculitis.

In cryoglobulinemic vasculitis IgG is the target of the autoantibodies. Removing them could also alleviate the inflammation. However, the short duration of the effect would only provide transient benefit, and severe vasculitic crises are rare in cryoglobulinemic vasculitis. The same is true for urticarial vasculitis. The role of the autoantibodies against complement component C1q is uncertain, and it must be kept in mind that F (ab')2 fragments can continue to form immune complexes also after losing their Fc tail.

CONCLUSION

IgG class autoantibodies are common in primary small vessel vasculitis and they seem to participate in the pathogenesis. Today plasma exchange is often employed to reduce levels, but this treatment is unselective and rather ineffective. IdeS and EndoS are novel precision tools the rapidly either cleaves or disarms the IgG molecules. Whether this would lead to meaningful clinical responses remains to be determined in each individual disease. Anti-GBM disease, where the pathogenesis seems to be most straight forward, is first in line and a clinical trial is already ongoing.

AUTHOR CONTRIBUTIONS

MS wrote the first draft of the manuscript and then both authors contributed on an equal basis.

FUNDING

LB's work was supported by the Foundations of Knut and Alice Wallenberg and Alfred Österlund, the Swedish Government Funds for Clinical Research (ALF), the Medical Faculty of Lund University, and Hansa Biopharma AB. MS work was supported the Swedish Rheumatic Disease Foundation, Swedish Government Funds for Clinical Research (ALF), The Ingrid Asp Foundation, Hansa Biopharma AB, Skåne University Hospital Funds.

REFERENCES

1. Holman HR, Deicher HR, Kunkel HG. The L. E. cell and the L. E. serum factors. *Bull NY Acad Med.* (1959) 35:409–18.
2. Elkon K, Casali P. Nature and functions of autoantibodies. *Nat Clin Pract Rheumatol.* (2008) 4:491–8. doi: 10.1038/ncprheum0895
3. Leslie D, Lipsky P, Notkins AL. Autoantibodies as predictors of disease. *J Clin Invest.* (2001) 108:1417–22. doi: 10.1172/JCI14452
4. Nagele EP, Han M, Acharya NK, DeMarshall C, Kosciuk MC, Nagele RG. Natural IgG autoantibodies are abundant and ubiquitous in human sera, and their number is influenced by age, gender, and disease. *PLoS ONE.* (2013) 8:e60726. doi: 10.1371/journal.pone.0060726
5. Haury M, Sundblad A, Grandien A, Barreau C, Coutinho A, Nobrega A. The repertoire of serum IgM in normal mice is largely independent of external antigenic contact. *Eur J Immunol.* (1997) 27:1557–63. doi: 10.1002/eji.1830270635
6. Yang R, Cui Z, Hellmark T, Segelmark M, Zhao MH, Wang HY. Natural anti-GBM antibodies from normal human sera recognize alpha3 (IV)NC1 restrictively and recognize the same epitopes as anti-GBM antibodies from patients with anti-GBM disease. *Clin Immunol.* (2007) 124:207–12. doi: 10.1016/j.clim.2007.05.001
7. Jennette JC, Falk RJ, Bacon PA, Basu N, Cid MC, Ferrario F, et al. 2012 revised International Chapel Hill Consensus Conference Nomenclature of Vasculitides. *Arthritis Rheum.* (2013) 65:1–11. doi: 10.1002/art.37715.
8. Weiner M, Segelmark M. The clinical presentation and therapy of diseases related to anti-neutrophil cytoplasmic antibodies (ANCA). *Autoimmun Rev.* (2016) 15:978–82. doi: 10.1016/j.autrev.2016.07.016
9. Kallenberg CG. Usefulness of antineutrophil cytoplasmic autoantibodies in diagnosing and managing systemic vasculitis. *Curr Opin Rheumatol.* (2016) 28:8–14. doi: 10.1097/BOR.0000000000000233
10. Jin Q, Kant S, Alhariri J, Geetha D. Levamisole adulterated cocaine associated ANCA vasculitis: review of literature and update on pathogenesis. *J Community Hosp Intern Med Perspect.* (2018) 8:339–44. doi: 10.1080/20009666.2018.1536242
11. Chen TK, Murakami C, Manno RL, Geetha D. Hematuria duration does not predict kidney function at 1 year in ANCA-associated glomerulonephritis. *Semin Arthritis Rheum.* (2014) 44:198–201. doi: 10.1016/j.semarthrit.2014.03.008
12. Hellmark T, Segelmark M. Diagnosis and classification of Goodpasture's disease (anti-GBM). *J Autoimmun.* (2014) 48–49:108–12. doi: 10.1016/j.jaut.2014.01.024
13. Hudson BG, Tryggvason K, Sundaramoorthy M, Neilson EG. Alport's syndrome, Goodpasture's syndrome, and type IV collagen. *N Engl J Med.* (2003) 348:2543–56. doi: 10.1056/NEJMra022296
14. Lerner RA, Glassock RJ, Dixon FJ. The role of anti-glomerular basement membrane antibody in the pathogenesis of human glomerulonephritis. *J Exp Med.* (1967) 126:989–1004. doi: 10.1084/jem.126.6.989
15. Bolton WK, Tucker FL, Sturgill BC. New avian model of experimental glomerulonephritis consistent with mediation by cellular immunity. Nonhumorally mediated glomerulonephritis in chickens. *J Clin Invest.* (1984) 73:1263–76. doi: 10.1172/JCI111328
16. van Rossum AP, Limburg PC, Kallenberg CG. Membrane proteinase 3 expression on resting neutrophils as a pathogenic factor in PR3-ANCA-associated vasculitis. *Clin Exp Rheumatol.* (2003) 21 (6 Suppl. 32):S64–8.
17. Falk RJ, Terrell RS, Charles LA, Jennette JC. Anti-neutrophil cytoplasmic autoantibodies induce neutrophils to degranulate and produce oxygen radicals *in vitro*. *Proc Natl Acad Sci USA.* (1990) 87:4115–9. doi: 10.1073/pnas.87.11.4115
18. 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/JCI0215918
19. Popat RJ, Robson MG. Neutrophils are not consistently activated by antineutrophil cytoplasmic antibodies *in vitro*. *Ann Rheum Dis.* (2019) 78:709–11. doi: 10.1136/annrheumdis-2018-214405
20. Ohlsson SM, Ohlsson S, Soderberg D, Gunnarsson L, Pettersson A, Segelmark M, et al. Neutrophils from vasculitis patients exhibit an increased propensity for activation by anti-neutrophil cytoplasmic antibodies. *Clin Exp Immunol.* (2014) 176:363–72. doi: 10.1111/cei.12301
21. Heineke MH, Ballering AV, Jamin A, Ben Mkaddem S, Monteiro RC, Van Egmond M. New insights in the pathogenesis of immunoglobulin A

vasculitis (Henoch-Schonlein purpura). *Autoimmun Rev.* (2017) 16:1246–53. doi: 10.1016/j.autrev.2017.10.009
22. Damoiseaux J. The diagnosis and classification of the cryoglobulinemic syndrome. *Autoimmun Rev.* (2014) 13:359–62. doi: 10.1016/j.autrev.2014.01.027
23. Mahler M, van Schaarenburg RA, Trouw LA. Anti-C1q autoantibodies, novel tests, and clinical consequences. *Front Immunol.* (2013) 4:117. doi: 10.3389/fimmu.2013.00117
24. von Pawel-Rammingen U, Johansson BP, Bjorck L. IdeS, a novel streptococcal cysteine proteinase with unique specificity for immunoglobulin G. *EMBO J.* (2002). 21:1607–15. doi: 10.1093/emboj/21.7.1607
25. Vincents B, von Pawel-Rammingen U, Bjorck L, Abrahamson M. Enzymatic characterization of the streptococcal endopeptidase, IdeS, reveals that it is a cysteine protease with strict specificity for IgG cleavage due to exosite binding. *Biochemistry*. (2004) 43:15540–9. doi: 10.1021/bi048284d
26. Nandakumar KS, Johansson BP, Bjorck L, Holmdahl R. Blocking of experimental arthritis by cleavage of IgG antibodies *in vivo*. *Arthritis Rheum.* (2007) 56:3253–60. doi: 10.1002/art.22930
27. Rudd PM, Elliott T, Cresswell P, Wilson IA, Dwek RA. Glycosylation and the immune system. *Science.* (2001) 291:2370–6. doi: 10.1126/science.291.5512.2370
28. Collin M, Olsen A. EndoS, a novel secreted protein from *Streptococcus pyogenes* with endoglycosidase activity on human IgG. *EMBO J.* (2001) 20:3046–55. doi: 10.1093/emboj/20.12.3046
29. Johansson BP, Shannon O, Bjorck L. IdeS: a bacterial proteolytic enzyme with therapeutic potential. *PLoS ONE.* (2008) 3:e1692. doi: 10.1371/journal.pone.0001692
30. Collin M, Shannon O, Bjorck L. IgG glycan hydrolysis by a bacterial enzyme as a therapy against autoimmune conditions. *Proc Natl Acad Sci USA.* (2008) 105:4265–70. doi: 10.1073/pnas.0711271105
31. Yang R, Otten MA, Hellmark T, Collin M, Bjorck L, Zhao MH, et al. Successful treatment of experimental glomerulonephritis with IdeS and EndoS, IgG-degrading streptococcal enzymes. *Nephrol Dial Transplant.* (2010) 25:2479–86. doi: 10.1093/ndt/gfq115
32. van Timmeren MM, van der Veen BS, Stegeman CA, Petersen AH, Hellmark T, Collin M, et al. IgG glycan hydrolysis attenuates ANCA-mediated glomerulonephritis. *J Am Soc Nephrol.* (2010) 21:1103–14. doi: 10.1681/ASN.2009090984
33. Winstedt L, Jarnum S, Nordahl EA, Olsson A, Runstrom A, Bockermann R, et al. Complete removal of extracellular IgG antibodies in a randomized dose-escalation phase I study with the bacterial enzyme IdeS–a novel therapeutic opportunity. *PLoS ONE.* (2015) 10:e0132011. doi: 10.1371/journal.pone.0132011
34. Lorant T, Bengtsson M, Eich T, Eriksson BM, Winstedt L, Jarnum S, et al. Safety, immunogenicity, pharmacokinetics, and efficacy of degradation of anti-HLA antibodies by IdeS (imlifidase) in chronic kidney disease patients. *Am J Transplant.* (2018) 18:2752–62. doi: 10.1111/ajt.14733
35. Jordan SC, Lorant T, Choi J, Kjellman C, Winstedt L, Bengtsson M, et al. IgG endopeptidase in highly sensitized patients undergoing transplantation. *N Engl J Med.* (2017) 377:442–53. doi: 10.1056/NEJMoa1612567
36. Black AK. Urticarial vasculitis. *Clin Dermatol.* (1999) 17:565–9. doi: 10.1016/S0738-081X (99)00062-0
37. Segelmark M, Hellmark T. Anti-glomerular basement membrane disease: an update on subgroups, pathogenesis and therapies. *Nephrol Dial Transplant.* (2018). doi: 10.1093/ndt/gfy327. [Epub ahead of print].
38. Pusey CD, Lockwood CM, Peters DK. Plasma exchange and immunosuppressive drugs in the treatment of glomerulonephritis due to antibodies to the glomerular basement membrane. *Int J Artif Organs.* (1983) 6 (Suppl. 1):15–8.
39. Pusey CD, Bowman C, Peters DK, Lockwood CM. Effects of cyclophosphamide on autoantibody synthesis in the Brown Norway rat. *Clin Exp Immunol.* (1983) 54:697–704.
40. Soveri I, Mölne J, Uhlin F, Nilsson T, Kjellman C, Sonesson E, et al. Rapid clearance of anti-GBM antibodies by IgG degrading enzyme of *streptococcus pyogenes* (IdeS) in three patients with refractory anti-GBM disease. *Kidney Int.* (2019). doi: 10.1016/j.kint.2019.06.019
41. Szpirt WM. Plasma exchange in antineutrophil cytoplasmic antibody-associated vasculitis–a 25-year perspective. *Nephrol Dial Transplant.* (2015) 30 (Suppl. 1):i146–9. doi: 10.1093/ndt/gfv051
42. Jayne DR, Gaskin G, Rasmussen N, Abramowicz D, Ferrario F, Guillevin L, et al. Randomized trial of plasma exchange or high-dosage methylprednisolone as adjunctive therapy for severe renal vasculitis. *J Am Soc Nephrol.* (2007) 18:2180–8. doi: 10.1681/ASN.2007010090
43. Augusto JF, Sayegh J, Delapierre L, Croue A, Tollis F, Cousin M, et al. Addition of plasma exchange to glucocorticosteroids for the treatment of severe Henoch-Schonlein purpura in adults: a case series. *Am J Kidney Dis.* (2012) 59:663–9. doi: 10.1053/j.ajkd.2011.12.015
44. Marson P, Monti G, Montani F, Riva A, Mascia MT, Castelnovo L, et al. Apheresis treatment of cryoglobulinemic vasculitis: a multicentre cohort study of 159 patients. *Transfus Apher Sci.* (2018) 57:639–45. doi: 10.1016/j.transci.2018.06.005
45. Mossberg M, Segelmark M, Kahn R, Englund M, Mohammad AJ. Epidemiology of primary systemic vasculitis in children: a population-based study from southern Sweden. *Scand J Rheumatol.* (2018) 47:295–302. doi: 10.1080/03009742.2017.1412497

Conflict of Interest: Hansa Biopharma AB owns the commercial rights for IdeS and EndoS as pharmaceuticals, and LB is listed as an inventor on the patents. MS and LB received research grants from Hansa Biopharma AB, and LB has an extensive research contract with the company.

frontiers in Immunology

ORIGINAL RESEARCH
published: 25 October 2019
doi: 10.3389/fimmu.2019.02467

Remote Activation of a Latent Epitope in an Autoantigen Decoded With Simulated B-Factors

Yuan-Ping Pang[1], Marta Casal Moura[2], Gwen E. Thompson[2], Darlene R. Nelson[2], Amber M. Hummel[2], Dieter E. Jenne[3], Daniel Emerling[4], Wayne Volkmuth[4], William H. Robinson[5] and Ulrich Specks[2]**

[1] Computer-Aided Molecular Design Laboratory, Mayo Clinic, Rochester, MN, United States, [2] Thoracic Disease Research Unit, Mayo Clinic, Rochester, MN, United States, [3] Comprehensive Pneumology Center, Helmholtz Zentrum München & Max-Planck Institute for Neuroimmunology, Martinsried, Germany, [4] Atreca, Inc., Redwood City, CA, United States, [5] Department of Medicine, Stanford University, Palo Alto, CA, United States

Mutants of a catalytically inactive variant of Proteinase 3 (PR3)—iPR3-Val^{103} possessing a Ser195Ala mutation relative to wild-type PR3-Val^{103}—offer insights into how autoantigen PR3 interacts with antineutrophil cytoplasmic antibodies (ANCAs) in granulomatosis with polyangiitis (GPA) and whether such interactions can be interrupted. Here we report that iHm5-Val^{103}, a triple mutant of iPR3-Val^{103}, bound a monoclonal antibody (moANCA518) from a GPA patient on an epitope remote from the mutation sites, whereas the corresponding epitope of iPR3-Val^{103} was latent to moANCA518. Simulated B-factor analysis revealed that the binding of moANCA518 to iHm5-Val^{103} was due to increased main-chain flexibility of the latent epitope caused by remote mutations, suggesting rigidification of epitopes with therapeutics to alter pathogenic PR3·ANCA interactions as new GPA treatments.

Keywords: autoimmunity, autoantigen, antigenicity, antineutrophil cytoplasmic antibody, Proteinase 3, B-factor

OPEN ACCESS

Edited by:
Andreas Kronbichler, Innsbruck Medical University, Austria

Reviewed by:
Anja Kerstein-Staehle, Universität zu Lübeck, Germany
Dana P. Ascherman, University of Pittsburgh, United States

****Correspondence:***
Yuan-Ping Pang
pang@mayo.edu
Ulrich Specks
specks.ulrich@mayo.edu

Specialty section:
This article was submitted to Autoimmune and Autoinflammatory Disorders, a section of the journal Frontiers in Immunology

Received: *07 August 2019*
Accepted: *03 October 2019*
Published: *25 October 2019*

Citation:
Pang Y-P, Casal Moura M, Thompson GE, Nelson DR, Hummel AM, Jenne DE, Emerling D, Volkmuth W, Robinson WH and Specks U (2019) Remote Activation of a Latent Epitope in an Autoantigen Decoded With Simulated B-Factors. Front. Immunol. 10:2467. doi: 10.3389/fimmu.2019.02467

INTRODUCTION

Proteinase 3 (PR3) is a neutrophil serine protease targeted by antineutrophil cytoplasmic antibodies (ANCAs) in the autoimmune disease granulomatosis with polyangiitis (GPA) (1–5). To investigate how PR3 interacts with the ANCAs during inflammation and whether these interactions can be intervened by therapeutics, we developed a human PR3 mutant (iPR3-Val^{103}) with a Val^{103}—the major polymorphic variant at the Val/Ile polymorphic site of wild-type human PR3 [Val/Ile in GPA patients: 64.7/35.3 (6)]—and a Ser195Ala mutation that alters the charge relay network of Asp102, His57, and Ser195 and thereby disables catalytic functioning in PR3 (7–10). This mutant recognized as many ANCA serum samples from patients with GPA as the wild-type human PR3-Val^{103} in both immunofluorescence assay and enzyme-linked immunosorbent assay (ELISA), while the Ser195Ala mutation is close to Epitope 5 of PR3 and remote from Epitopes 1, 3, and 4 as shown in **Figure 1** (8, 11). We also developed a number of variants of iPR3-Val^{103} in the course of our investigation (11).

One such variant, iHm5-Val^{103} (formerly referred to as Hm5), has Ala146, Trp218, and Leu223 from human PR3 replaced by Thr146, Arg218, and Gln223 from mouse PR3. Our initial intent of this chimeric triple mutant was to demonstrate reduced binding of ANCAs to Epitope 5 (and possibly Epitope 1 but not Epitopes 3 and 4) of the mutant because Trp218 and Leu223 reside in

Epitope 5 and Ala146 is in Epitope 1 as shown in **Figure 1** (11). However, as described below, we serendipitously found that a monoclonal ANCA (moANCA518) from a patient with GPA bound to Epitope 3 of iHm5-Val^{103} but not iPR3-Val^{103}, although Epitope 3 is distal to the three mutations that reside in Epitopes 1 and 5 (**Figure 1**). This finding indicates that Epitope 3, a mutation-free epitope of iHm5-Val^{103}, is latent in iPR3-Val^{103} but active in iHm5-Val^{103} for ANCA binding. It also indicates that the latent epitope of PR3 can be activated by remote mutations.

In this context, we raised a mechanistic question: How can a latent antibody binding site in iPR3-Val^{103} be activated by topologically distal mutations in iHm5-Val^{103}? The experimental and computational results described below offer insights into this mechanistic question and open a new perspective on a possible cause and novel therapy of GPA.

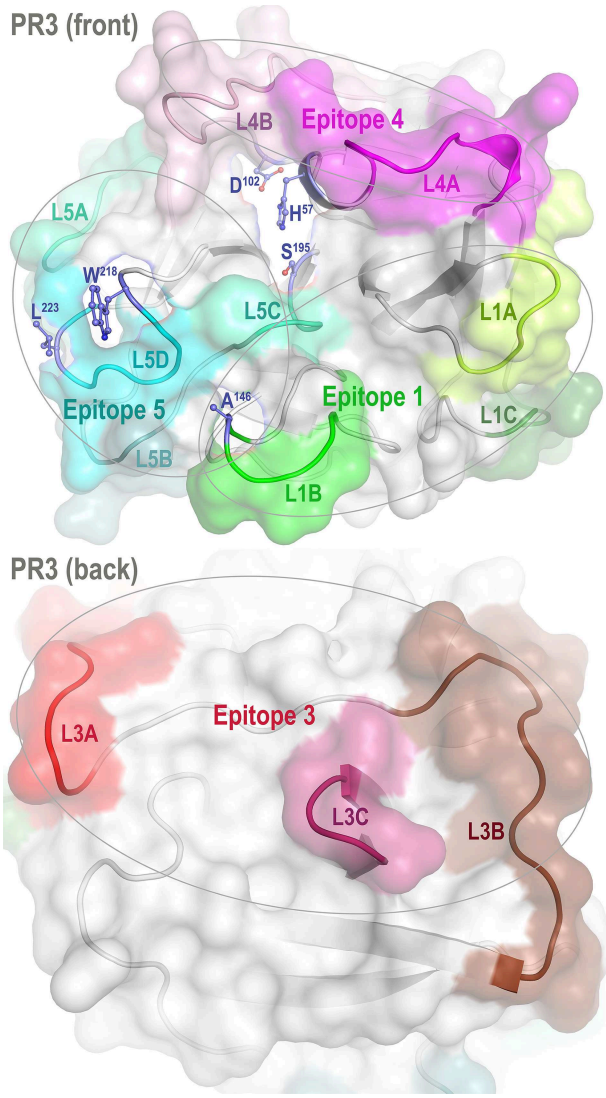


FIGURE 1 | Front and back views of PR3 depicting its four known epitopes, each comprising multiple surface loops with high Cα B-factors derived from simulations. L1A, Loop 1A of residues 36–38C; L1B, Loop 1B of residues 145–151; L1C, Loop 1C of residues 75–79; L3A, Loop 3A of residues 110–117; L3B, Loop 3B of residues 124–133; L3C, Loop 3C of residues 202–204; L4A, Loop 4A of residues 59–63C; L4B, Loop 4B of residues 92–99; L5A, Loop 5A of residues 165–178; L5B, Loop 5B of residues 186–187; L5C, Loop 5C of residues 192–194; and L5D, Loop 5D of residues 219–224; wherein the residue numbering here is identical to that of the PR3 crystal structure (PDB ID: 1FUJ).

MATERIALS AND METHODS

Materials

Reagents were obtained from Sigma (St. Louis, MO) unless specified otherwise. The human embryonic kidney cell line 293 (HEK293) used for the expression of recombinant PR3 mutants was obtained from ATCC (Rockville, MD).

iPR3-Val^{103} and iHm5-Val^{103}: The cDNA constructs coding for iPR3-Val^{103} and iHm5-Val^{103} and their expression in HEK293 cells were described in detail elsewhere (11, 12). Both mutants carry a carboxy-terminal cmyc-peptide extension and a poly-His peptide extension for purification using nickel columns from GE Healthcare (Chicago, IL) and for anchoring in ELISAs as previously described and specified below (11–15).

moANCA518: DNA barcode-enabled sequencing of the antibody repertoire was performed on plasmablasts derived from a PR3-targeting ANCA (PR3-ANCA) positive patient as described elsewhere for rheumatoid arthritis and Sjögren syndrome (16–18). Phylograms of the antibody repertoires revealed clonal families of affinity matured antibodies with shared heavy and light chain VJ usage. Twenty-five antibodies were selected for recombinant expression (18) and tested for reactivity with recombinant ANCA antigens [including myeloperoxidase (15), human neutrophil elastase (19–21), iPR3-Val^{103}, and iHm5-Val^{103}] using the ELISA. As described in Results, one antibody bound iHm5-Val^{103} but not iPR3-Val^{103} and is termed moANCA518, whereas none of the other 24 antibodies bound either of the two PR3 antigens or other ANCA antigens.

Epitope-specific anti-PR3 moAbs: PR3G-2 (22) was a gift from C.G.M. Kallenberg of the University of Groningen. WGM2 (11, 23) was purchased from Hycult Biotech Inc (Wayne, PA). MCPR3-3 was made as previously described (8, 11).

Enzyme-Linked Immunosorbent Assays

ELISAs used for detection of PR3-ANCA were described in detail elsewhere (12, 13, 15). In brief, either purified PR3 mutants or culture media supernatants from PR3 mutant-expressing HEK 293 cell clones diluted in the IRMA buffer (0.05 mM Tris-HCl, 0.1 M NaCl, pH 7.4, and 0.1% bovine serum albumin) were incubated in Pierce® nickel-coated plates from Thermo Fisher Scientific (Waltham, CA) for 1 h at room temperature; control wells were incubated with the IRMA buffer only. The plates were washed three times with Tris-buffered saline (TBS; 20 mM Tris-HCl, 500 mM NaCl, pH 7.5, and 0.05% Tween 20) in between steps. The ANCA-containing serum samples were diluted 1:20 in TBS with 0.5% bovine serum albumin and incubated in the plates with or without the PR3 mutants for 1 h at room temperature. The PR3·ANCA complexation was

detected after incubation for 1 h at room temperature with alkaline phosphatase-conjugated goat anti-human IgG (1:10,000 dilution). *P*-Nitrophenyl phosphate was used as substrate at a concentration of 1 mg/mL. The net UV absorbance was obtained by spectrophotometry at 405 nm after 30 min of exposure. Similarly, when epitope-specific anti-PR3 moAbs were used to immobilize iHm5-Val^{103} on Maxisorp® plates from Invitrogen (Carlsbad, CA), complexation of moANCA518 with the antigen was detected after incubation of HRP-conjugated anti-human IgG antibody (1:250 dilution) for 1 h at room temperature; 3,3′,5,5′-tetramethylbenzidine (Thermo Fisher Scientific®) was used as substrate, and the net UV absorbance was obtained by spectrophotometry at 450 nm after 15 min of exposure.

Western Blots

Non-reductive, purified PR3 mutant proteins were loaded (1 μg/lane) onto 12% Tris-HCl gels from BioRad (Hercules, CA). The SDS gel electrophoresis was performed at 180 V for 35 min. The proteins were transferred from gels to nitrocellulose membranes, which were subsequently washed with TBS, blocked for 45 min at room temperature with TBS with 0.2% non-fat dry milk. The membranes were then washed twice with TBS with 0.1% Tween 20. Monoclonal antibodies (0.5–1.0 μg/mL) were incubated on the membranes overnight at 4°C. The membranes were then washed twice with TBS with 0.1% Tween 20 and incubated with goat anti-human or anti-mouse IgG HRP conjugates, diluted to 1:20,000, for 20 min at room temperature. The membranes were washed again and developed with the Pierce ECL Western Blotting Substrate kit from Thermo Fisher Scientific (Waltham, MA).

Statistical Analysis

SPSS® Statistics for MacOS, version 25 from IBM (Armonk, NY, USA) was used to calculate the means and standard errors of 3–5 repeat experiments and to compare the means between groups with the two-tailed paired *t*-test.

Initial Conformations of PR3 Variants

The initial conformation of PR3-Ile^{103} (residues 16–239; truncated for atomic charge neutrality) was taken from the crystal structure of PR3 (24). The initial conformations of the corresponding PR3-Val^{103} and iPR3-Val^{103} (residues 16–239) were taken from the initial PR3-Ile^{103} conformation with mutations of Ile103Val alone and Ile103Val together with Ser195Ala, respectively. The initial conformation of iHm5-Val^{103} (residues 16–238; truncated for atomic charge neutrality) was taken from the initial PR3-Ile^{103} conformation with mutations of Ala146Thr, Trp218Arg, Leu223Gln, Ile103Val, and Ser195Ala. The crystallographically determined water molecules with residue identifiers of 246–249, 257–259, 261–263, 268, 270, 274–276, 279, 280, 291, 292, 296, 298, 307, 309, and 317 were included in all four initial conformations. The AMBER residue names of ASP, GLU, ARG, LYS, HID, and CYX were used for all Asp, Glu, Arg, Lys, His, and Cys residues, respectively. All initial conformations were refined via energy minimization using the SANDER module of AMBER 11 (University of California, San Francisco) and forcefield FF12MClm (25) with a dielectric constant of 1.0, a cutoff of 30.0 Å for non-bonded interactions, and 200 cycles of steepest descent minimization followed by 100 cycles of conjugate gradient minimization.

Molecular Dynamics Simulations

Each of the four energy-minimized conformations described above was solvated with 5578 (for iHm5-Val^{103}) or 5536 (for all other variants) TIP3P (26) water molecules (using "solvatebox PR3 TIP3BOX 8.2") and then energy-minimized for 100 cycles of steepest descent minimization followed by 900 cycles of conjugate gradient minimization using SANDER of AMBER 11 to remove close van der Waals contacts. The initial solvation box size was $58.268\times68.409\times65.657$ Å^3 (for iHm5-Val^{103}) or $67.337\times66.050\times58.335$ Å^3 (for all other variants). The resulting system was heated from 5 to 340 K at a rate of 10 K/ps under constant temperature and constant volume, then equilibrated for 10^6 timesteps under a constant temperature of 340 K and a constant pressure of 1 atm using the isotropic molecule-based scaling. Finally, 20 distinct, independent, unrestricted, unbiased, isobaric–isothermal, 316-ns molecular dynamics (MD) simulations of the equilibrated system with forcefield FF12MClm (25) were performed using PMEMD of AMBER 11 with a periodic boundary condition at 340 K and 1 atm. The 20 unique seed numbers for initial velocities of the 20 simulations were taken from Pang (27). All simulations used (i) a dielectric constant of 1.0, (ii) the Berendsen coupling algorithm (28), (iii) the particle mesh Ewald method to calculate electrostatic interactions of two atoms at a separation of >8 Å (29), (iv) $\Delta t = 1.00$ fs of the standard-mass time (25), (v) the SHAKE-bond-length constraint applied to all bonds involving hydrogen, (vi) a protocol to save the image closest to the middle of the "primary box" to the restart and trajectory files, (vii) a formatted restart file, (viii) the revised alkali and halide ion parameters (30), (ix) a cutoff of 8.0 Å for non-bonded interactions, (x) a uniform 10-fold reduction in the atomic masses of the entire simulation system (both solute and solvent), and (xi) default values of all other inputs of the PMEMD module. The forcefield parameters of FF12MClm are available in the Supporting Information of Pang (31). All simulations were performed on a cluster of 100 12-core Apple Mac Pros with Intel Westmere (2.40/2.93 GHz).

Alpha Carbon B-Factor Calculation

In a two-step procedure using PTRAJ of AmberTools 1.5, the B-factors of alpha carbon (Cα) atoms in PR3 were calculated from all conformations saved at every 10^3 timesteps during 20 simulations of the protein using the simulation conditions described above except that (i) the atomic masses of the entire simulation system (both solute and solvent) were uniformly increased by 100-fold relative to the standard atomic masses, (ii) the simulation temperature was lowered to 300 K, and (iii) the simulation time was reduced to 500 ps. The first step was to align all saved conformations onto the first saved conformation to obtain an average conformation using the root mean square fit of all Cα atoms. The second step was to perform root mean square fitting of all Cα atoms in all saved conformations onto the corresponding atoms of the average conformation. The Cα B-factors were then calculated using

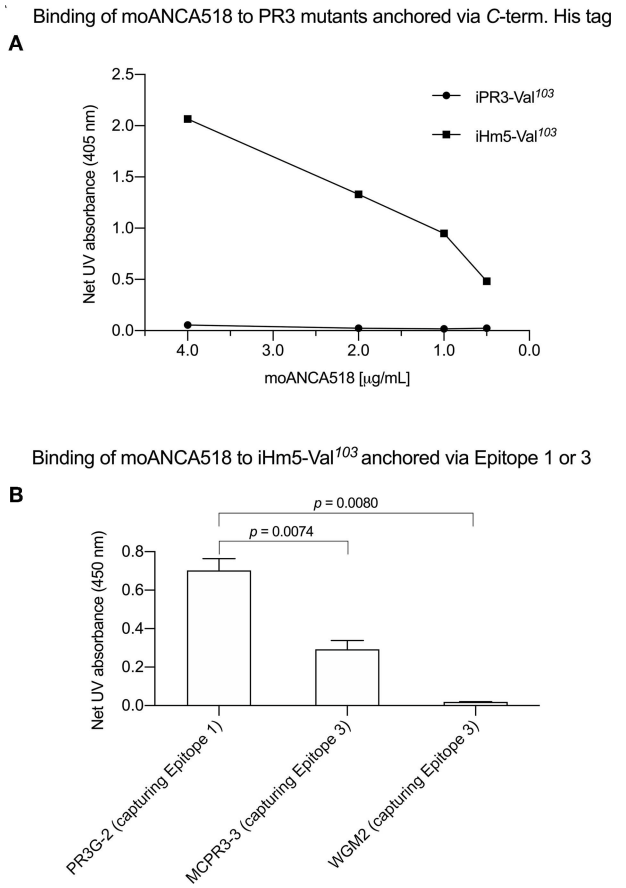


FIGURE 2 | Selective binding of moANCA518 to Epitope 3 of iHm5-Val^{103}. **(A)** Dilution curves show dose-dependent binding of moANCA518 to iHm5-Val^{103} but not iPR3-Val^{103} in the ELISA using an antigen whose C-terminal poly-His tag was anchored at the plate. The culture media supernatants from PR3 mutant expressing 293 cells were used in the ELISA. **(B)** Epitope-specific anti-PR3 moAbs PR3G-2, MCPR3-3, and WGM2 (2, 4, and 4 μg/mL, respectively), which were coated to the plate and used to capture iHm5-Val^{103} in the ELISA, show Epitope 3 of iHm5-Val^{103} as a major target site by the primary antibody moANCA518 (1.0 μg/mL). The purified PR3 mutants were used in the ELISA.

the "atomicfluct" command in PTRAJ. For each protein, the calculated B-factor of any atom in **Table S2** was the mean of all B-factors of the atom derived from 20 simulations of the protein. The standard error (SE) of a B-factor was calculated according to Equation 2 of Pang (32). The SE of the average Cα B-factor of each PR3 variant was calculated according to the standard method for propagation of errors of precision (33). The 95% confidence interval (95% CI) of the average Cα B-factor was obtained according to the formula mean ± 1.96 × SE because the sample size of each PR3 variant exceeded 100.

Conformational Cluster Analysis and Root Mean Square Deviation Calculation

The conformational cluster analyses were performed using CPPTRAJ of AmberTools 16 with the average-linkage algorithm (34), epsilon of 3.0 Å, and root mean square coordinate deviation on all Cα atoms of the proteins. Cα root mean square deviations (CαRMSDs) were manually calculated using ProFit V2.6 (http://www.bioinf.org.uk/software/profit/). The first unit of the crystal structure of the PR3 tetramer and the time-averaged conformation (without energy minimization) of the most populated cluster were used for the CαRMSD calculations.

RESULTS

In characterizing moAbs identified and cloned from B cells in patients with GPA, we found that one of these, moANCA518, bound to iHm5-Val^{103} but not iPR3-Val^{103} (**Figure 2A**) according to the ELISA using iHm5-Val^{103} and iPR3-Val^{103} both of which contain a *C*-terminal poly-His tag for anchoring the antigens without perturbing the folded conformations of the antigens and without blocking the epitopes of the antigens (12). Further, the binding of moANCA518 to iHm5-Val^{103} was dose dependent (**Figure 2A**) and confirmed by the Western blot under non-reducing conditions (**Figure S1**) as well as by ELISAs using untagged PR3 variants (data not shown). This serendipitous finding prompted us to investigate how the triple chimeric mutations in iHm5-Val^{103} changed the conformation of iPR3-Val^{103} and consequently the antigenicity to moANCA518.

Accordingly, we developed computer models of PR3-Val^{103}, iPR3-Val^{103}, and iHm5-Val^{103} to understand how mutations of these variants affect the ANCA-binding capabilities of the four reported epitopes of PR3 (11). These models were derived from MD simulations using our published forcefield and simulation protocol (25), which reportedly folded fast-folding proteins in isobaric–isothermal MD simulations to achieve agreements between simulated and experimental folding times within factors of 0.69–1.75 (35) and are hence suitable for predicting *in vivo* conformations of PR3 and its variants. The initial conformations of the three variants used in these simulations were derived from the PR3-Ile^{103} crystal structure (24) because experimentally determined structures of these variants have been unavailable to date. Although small differences in the time-averaged main-chain conformations of two surface loops (Loops 3 and 5) between iHm5-Val^{103} and PR3-Val^{103} (or between iHm5-Val^{103} and iPR3-Val^{103}) were observed (**Figure 3**), the overall conformations of the three variants resembled one another according to the Cα root mean square deviations of ≤1.63 Å (**Table S1**). Given these conformational properties, we could not determine how mutations of these variants affect the ANCA-binding capabilities of the PR3 epitopes, primarily because these surface loops are highly flexible and lack the time dimension (due to time-averaging) that is required for immunological function analysis (36).

To take the time dimension into account, we turned our attention to the dynamic properties of the PR3 variants. It is well-known that a folded protein is fluid-like with fluctuations in atomic position on the picosecond timescale and that the dynamics of these atomic displacements are dominated by collisions with neighboring atoms involving reorientation of side chains or localized portions of the backbone (37). Two

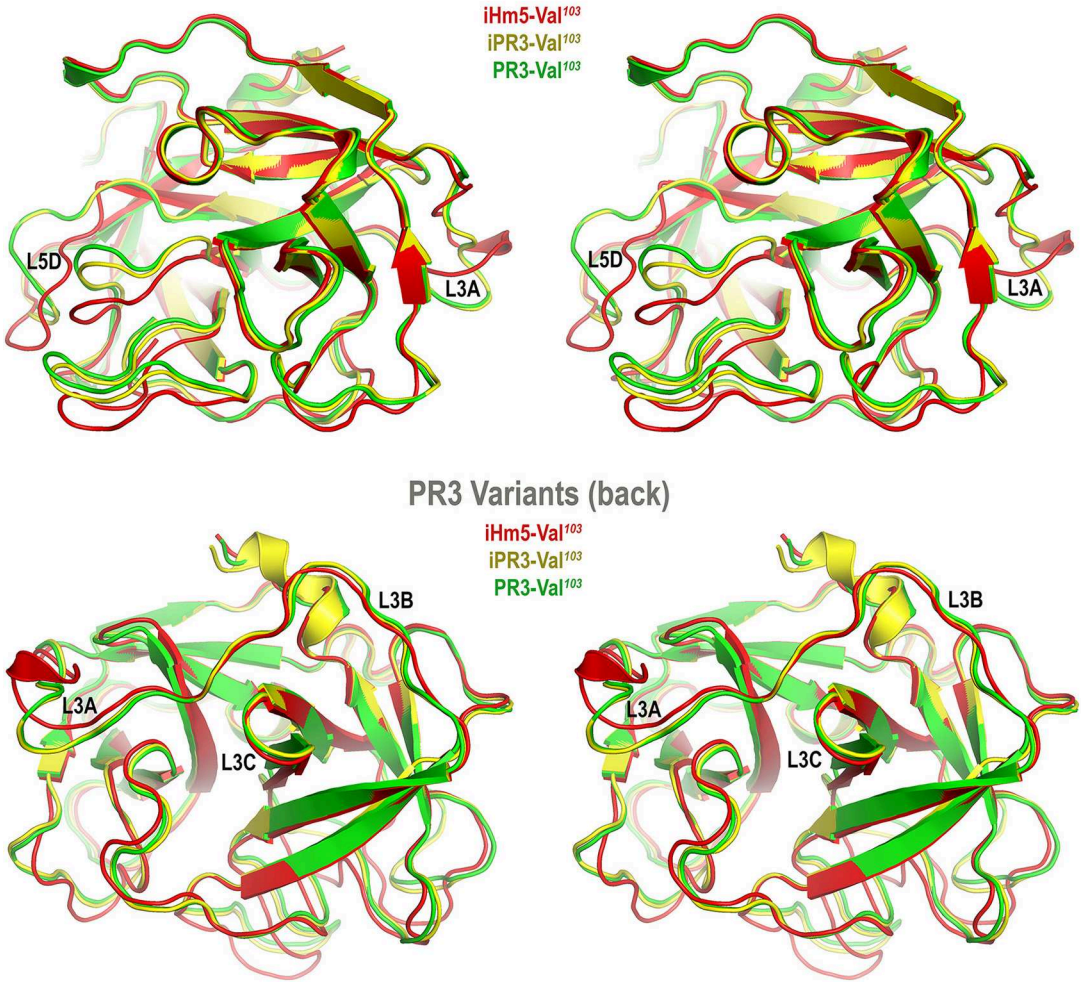


FIGURE 3 | Superimposed time-averaged conformations of three PR3 variants in cross-eye stereo view. The time-averaged conformations were obtained via cluster analyses (without energy minimization) from the most populated cluster in the three sets of molecular dynamics simulations. The L3A in iHm5-Val^{103} is slightly structured relative to that in two other variants, indicating that L3A in iHm5-Val^{103} is less mobile than that in the others. The L3B in iHm5-Val^{103} is slightly contracted (due to time-averaging) relative to that in two other variants, indicating that L3B is more mobile than that in the others. See **Figure 4** legend for definition of L3A, L3B, L3C, and L5D.

seminal studies have also shown that the crystallographically determined high B-factors of a protein fragment are linked to the antigenicity of the fragment (38, 39). This link indicates that the crystallographically determined B-factor—defined as $8\pi^2<u^2>$ to reflect the displacement u of the atom from its mean position, thermal motions, local mobility, or the uncertainty of the atomic mean position (40–48)—can be used to aid the identification and characterization of epitopes.

However, the crystallographically determined B-factor of an atom reflects not only the thermal motion or local mobility of the atom but also conformational and static lattice disorders of the atom, and even the refinement error in determining the mean position of the atom (43, 45, 47, 49). Therefore, using crystallographically determined B-factors to investigate epitopes requires the comparison of B-factors of different crystal structures of the same protein, which are in different space groups and obtained with different refinement procedures at different resolutions, in order to identify the B-factors that reflect the local mobility of the protein (49).

This requirement can be avoided by using simulated B-factors derived from MD simulations on a picosecond timescale because simulated B-factors are devoid of refinement errors and conformational and static lattice disorders. In addition, local motions, such as those of backbone N–H bonds, occur on the order of tens or hundreds of picoseconds (50).

In this context, we calculated the Cα B-factors of PR3-Val^{103}, iPR3-Val^{103}, and iHm5-Val^{103} from MD simulations on a 50-ps timescale using our published forcefield (25) and method (51). The mean Cα B-factors of PR3-Val^{103}, iPR3-Val^{103}, and iHm5-Val^{103} were 6.84 Å^2 (95% CI: 6.75–6.94 Å^2), 6.91 Å^2

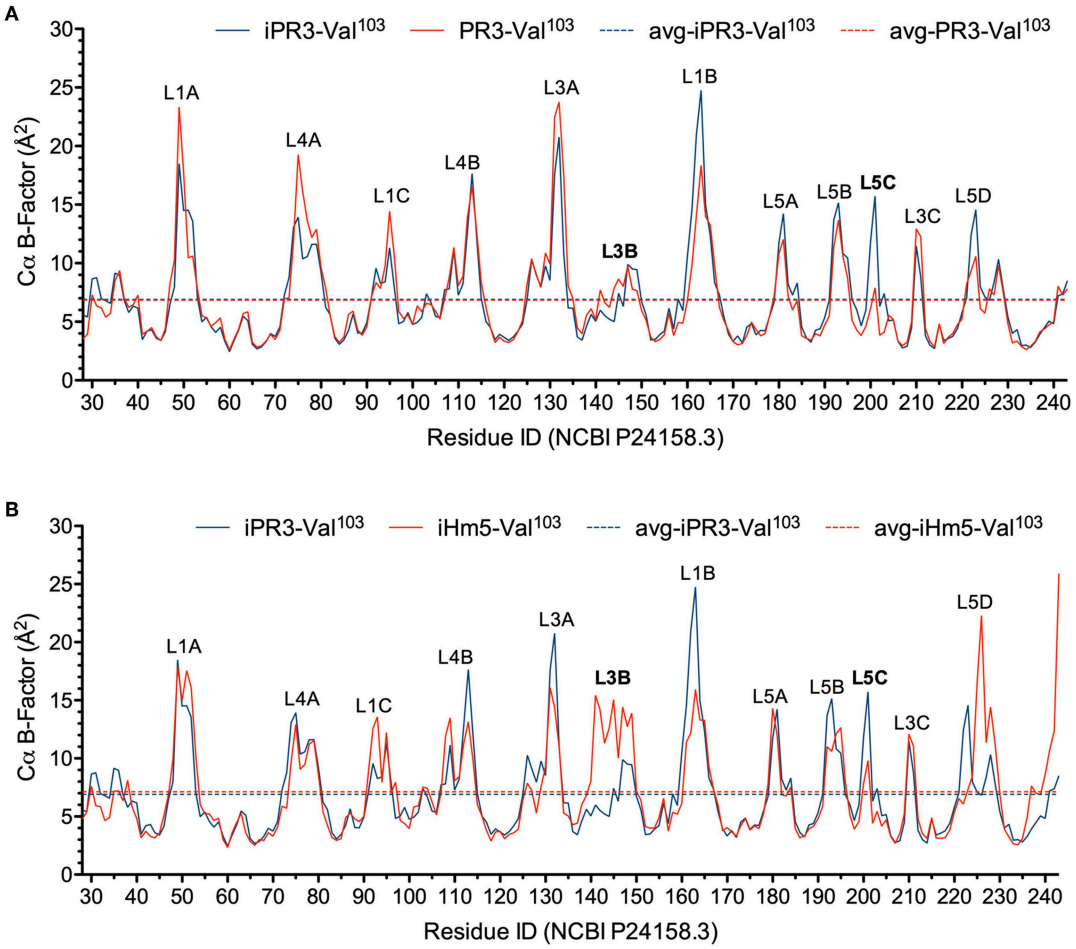


FIGURE 4 | Simulated Cα B-factors of PR3-Val^{103}, iPR3-Val^{103}, and iHm5-Val^{103}. **(A)** B-factor comparison of PR3-Val^{103} with iPR3-Val^{103}. **(B)** B-factor comparison of iHm5-Val^{103} with iPR3-Val^{103}. The simulated mean Cα B-factors of PR3-Val^{103}, iPR3-Val^{103}, and iHm5-Val^{103} are 6.84 Å^2 (95%CI: 6.75–6.94 Å^2; labeled as avg-PR3-Val^{103}), 6.91 Å^2 (95%CI: 6.82–7.00 Å^2; labeled as avg-iPR3-Va^{103}), and 7.13 Å^2 (95%CI: 7.03–7.24 Å^2; labeled as avg-iHm5-Val^{103}), respectively, wherein 95%CI is the abbreviation of 95% confidence interval. The simulated Cα B-factors were plotted using the human PR3 sequence (NCBI P24158.3) numbering because the PR3 crystal structure numbering is discontinuous. Therefore, the following loop residues are defined using the PR3 crystal structure numbering followed by the NCBI P24158.3 numbering in parenthesis. L1A, Loop 1A of residues 36–38C(48–52); L1B, Loop 1B of residues 145–151(161–166); L1C, Loop 1C of residues 75–79(92–96); L3A, Loop 3A of residues 110–117(126–133); L3B, Loop 3B of residues 124–133(140–149); L3C, Loop 3C of residues 202–204(210–212); L4A, Loop 4A of residues 59–63C(73–80); L4B, Loop 4B of residues 92–99(108–115); L5A, Loop 5A of residues 165–178(180–184); L5B, Loop 5B of residues 186–187(192–195); L5C, Loop 5C of residues 192–194(200–202); L5D, Loop 5D of residues 219–224(223–229).

(95% CI: 6.82–7.00 Å^2), and 7.13 Å^2 (95% CI: 7.03–7.24 Å^2), respectively. Given these findings, we concluded that any surface loop is highly mobile and hence potentially antigenic if the mean Cα B-factor of the loop was >9.00 Å^2. This conservative cutoff of 9.00 Å^2 was based on the mean Cα B-factors of all PR3 variants used in this study (6.84, 6.91, and 7.13 Å^2). According to this criterion, PR3-Val^{103} has 10 potentially antigenic surface loops, and iPR3-Val^{103} and iHm5-Val^{103} have 11 each (**Figure 4**). Consistent with the two seminal reports (38, 39), all of these potentially antigenic loops identified *a priori* by using simulated B-factors fall within all four known epitopes of PR3 (11), demonstrating a clear association between a loop with a high mean simulated Cα B-factor and the experimentally determined antigenicity of the loop.

Further, we found that the Ser195Ala mutation caused no significant reduction in the mean Cα B-factor of any of the 10 potentially antigenic surface loops in PR3-Val^{103} (**Figure 4A**). This finding implies that the Ser195Ala mutation does not impair the ANCA-binding capability of any of the four epitopes of iPR3-Val^{103}, and it explains our reported observation that iPR3-Val^{103} recognizes as many ANCA serum samples as PR3-Val^{103} does (8).

We also found the mean Cα B-factors of Loop 3B in iPR3-Val^{103} (possessing Ala146, Trp218, and Leu223) and iHm5-Val^{103} (possessing Thr146, Arg218, and Gln223) to be 6.9 Å^2 (95% CI: 6.8–7.0 Å^2) and 12.8 Å^2 (95% CI: 12.3–13.2 Å^2), respectively (**Figure 4B**). According to the afore-described antigenicity criterion of 9.00 Å^2, these means suggest that the three chimeric mutations make Loop 3B (a mutation-free loop)

more mobile in iHm5-Val^{103}, despite large separations between Epitope 3 of PR3 and the chimeric mutation sites (∼32, ∼32, and ∼31 Å from the Cα atom of Gln122 in Epitope 3 to the Cα atoms of Ala146, Trp218, and Leu223, respectively, at the chimeric mutation sites). The higher mobility of Loop 3B in iHm5-Val^{103} relative to that in iPR3-Val^{103} is also evident from the slight contraction (due to time-averaging) of Loop 3B in iHm5-Val^{103} shown in **Figure 3**. Therefore, Epitope 3 of iHm5-Val^{103} could bind ANCAs, whereas the ANCA-binding capability of Epitope 3 of iPR3-Val^{103} would be rather limited.

We subsequently repeated the afore-described ELISAs in the presence of epitope-specific moAbs that target either Epitope 1 or 3 of PR3. Consistently, we found that PR3G-2 that targets Epitope 1 of PR3 (22) did not affect the binding of moANCA518 to iHm5-Val^{103}, whereas MCPR3-3 and WGM2, both of which recognize Epitope 3 of PR3 (11), reduced and abolished the moANCA518 binding ($p < 0.01$; **Figure 2B**), respectively. We also confirmed the binding of moANCA518 primarily to Epitope 3 of iHm5-Val^{103} using Fabs from epitope-specific moAbs that target Epitope 2 or 5 of PR3 (8, 11, 52) (data not shown).

DISCUSSION

In view of the data above, we suggest a new mechanism for latent epitope activation of PR3: Remote mutations can increase the local mobility (i.e., main-chain flexibility) of a latent epitope of PR3, which facilitates the conformational adaptation required for antibody binding and thereby activate the latent epitope. This type of exquisite epitope activation—achieved either *in vitro* by remote mutations as we demonstrated or *in vivo* conceivably by remote polymorphisms or by remote protein·ligand binding including allosteric binding with an autoantibody—may be a fundamental feature of GPA. There is evidence that increased mobility of Epitope 3 occurs *in vivo* as more than 50% of serum samples from patients with GPA preferentially bind iHm5-Val^{103} (53). It is worth noting that the remote mutations do not significantly change the main-chain conformation of iHm5-Val^{103} as shown in **Figure 3**, although these mutations were introduced with the intent for inducing conformational changes to reduce binding of ANCAs to the mutant. Therefore, the latent epitope activation described here conceptually differs from the exposure of cryptic epitopes caused by citrullination (*viz.,* post-translational conversion of arginine to citrulline) (54). The latent epitope activation is due to the significant increase of main-chain flexibility of Loop 3B shown in **Figure 4B** caused by the mutations, whereas the cryptic epitope exposure is reportedly due to conformational changes triggered by multiple citrullinations (54). It is also worth noting that identifying PR3 mutations in patients with GPA that can increase the Epitope 3 mobility is difficult because other factors such as remote protein·ligand interactions may also increase the latent epitope mobility *in vivo*, namely, it is challenging to identify the cause of the latent epitope activation *in vivo*.

Nevertheless, knowing the increased mobility of Epitope 3 of iHm5-Val^{103} responsible for its binding to moANCA518 alone may have implications for the development of novel, effective treatments of GPA that aim to disrupt the pathogenic autoantibody·autoantigen interactions in GPA by reducing the mobility of epitopes targeted by PR3-ANCAs. For example, the present finding may explain in principle why a monoclonal antibody strategy (that targets native PR3 and prevents binding of pathogenic PR3-ANCAs to PR3 that is not in itself pathogenic) is of advantage for disrupting the autoantibody·autoantigen interactions over the molecular decoy strategy (that targets pathogenic autoantibodies). For the latter, large numbers of decoys are required to block a stock of distinct, pathogenic PR3-ANCAs. The DNA recombination and affinity maturation mechanisms, which create diversity and potency in specificity of antibodies, can potentially lead to resistance against the decoys. For the former, only one or a few small-molecule or protein (*e.g.*, monoclonal antibody) binders are required to rigidify B-cell epitopes of PR3 and consequently make the autoantigen inaccessible to a repertoire of distinct, pathogenic PR3-ANCAs, thus obviating mechanisms that could potentially lead to resistance against such binders.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

DN and US initiated the collaboration project. US and DJ designed the PR3 variants and ANCA-binding experiments. MC, GT, AH, and DN performed ANCA-binding experiments. Y-PP designed and performed B-factor calculations. DE, WV, and WR discovered moANCA518. Y-PP, US, and DJ wrote the manuscript. All authors reviewed or contributed to revisions of the manuscript.

FUNDING

This work was supported by the US Army Research Office (W911NF-16-1-0264; to Y-PP), the Connor Group Foundation (to US), the Mayo Foundation for Medical Education and Research (to Y-PP and US), and the European Union's Horizon 2020 research and innovation program under grant agreement No. 668036 (RELENT; to DJ). Responsibility for the information and views in this study lies entirely with the authors.

ACKNOWLEDGMENTS

The authors wish to thank Dr. C.G.M. Kallenberg of the University of Groningen for providing epitope-specific anti-PR3 moAb PR3G-2 as a gift. This manuscript has been released as a pre-print at bioRxiv (doi: 10.1101/559963).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02467/full#supplementary-material

REFERENCES

1. Wegener F. Über generalisierte septische Gefäßerkrankungen. *Verh Dtsch Pathol Ges*. (1936) 29:202–9.
2. Fahey JL, Leonard E, Churg J, Godman G. Wegener's granulomatosis. *Am J Med*. (1954) 17:168–79. doi: 10.1016/0002-9343(54)90255-7
3. Fauci AS, Wolff SM. Wegener's granulomatosis: studies in eighteen patients and a review of the literature. *Medicine*. (1973) 52:535–61. doi: 10.1097/00005792-197311000-00002
4. Jenne DE, Tschopp J, Ludemann J, Utecht B, Gross WL. Wegener's autoantigen decoded. *Nature*. (1990) 346:520. doi: 10.1038/346520a0
5. Gross WL, Csernok E. Immunodiagnostic and pathophysiologic aspects of antineutrophil cytoplasmic antibodies in vasculitis. *Curr Opin Rheumatol*. (1995) 7:11–9.
6. Gencik M, Meller S, Borgmann S, Fricke H. Proteinase 3 gene polymorphisms and Wegener's granulomatosis. *Kidney Int*. (2000) 58:2473–7. doi: 10.1046/j.1523-1755.2000.00430.x
7. Specks U, Fass DN, Fautsch MP, Hummel AM, Viss MA. Recombinant human proteinase 3, the Wegener's autoantigen, expressed in HMC-1 cells is enzymatically active and recognized by c-ANCA. *FEBS Lett*. (1996) 390:265–70. doi: 10.1016/0014-5793(96)00669-2
8. Sun J, Fass DN, Hudson JA, Viss MA, Wieslander J, Homburger HA, et al. Capture-ELISA based on recombinant PR3 is sensitive for PR3-ANCA testing and allows detection of PR3 and PR3-ANCA/PR3 immunecomplexes. *J Immunol Methods*. (1998) 211:111–23. doi: 10.1016/S0022-1759(97)00203-2
9. Sun J, Fass DN, Viss MA, Hummel AM, Tang H, Homburger HA, et al. A proportion of proteinase 3 (PR3)-specific anti-neutrophil cytoplasmic antibodies (ANCA) only react with PR3 after cleavage of its N-terminal activation dipeptide. *Clin Exp Immunol*. (1998) 114:320–6. doi: 10.1046/j.1365-2249.1998.00730.x
10. Specks U. What you should know about PR3-ANCA. Conformational requirements of proteinase 3 (PR3) for enzymatic activity and recognition by PR3-ANCA. *Arthritis Res*. (2000) 2:263–7. doi: 10.1186/ar99
11. Silva F, Hummel AM, Jenne DE, Specks U. Discrimination and variable impact of ANCA binding to different surface epitopes on proteinase 3, the Wegener's autoantigen. *J Autoimmun*. (2010) 35:299–308. doi: 10.1016/j.jaut.2010.06.021
12. Capizzi SA, Viss MA, Hummel AM, Fass DN, Specks U. Effects of carboxy-terminal modifications of proteinase 3 (PR3) on the recognition by PR3-ANCA. *Kidney Int*. (2003) 63:756–60. doi: 10.1046/j.1523-1755.2003.00765.x
13. Lee AS, Finkielman JD, Peikert T, Hummel AM, Viss MA, Specks U. A novel capture-ELISA for detection of anti-neutrophil cytoplasmic antibodies (ANCA) based on c-myc peptide recognition in carboxy-terminally tagged recombinant neutrophil serine proteases. *J Immunol Methods*. (2005) 307:62–72. doi: 10.1016/j.jim.2005.09.004
14. Finkielman JD, Lee AS, Hummel AM, Viss MA, Jacob GL, Homburger HA, et al. ANCA are detectable in nearly all patients with active severe Wegener's granulomatosis. *Am J Med*. (2007) 120:643.e9–14. doi: 10.1016/j.amjmed.2006.08.016
15. Oommen E, Hummel A, Allmannsberger L, Cuthbertson D, Carette S, Pagnoux C, et al. IgA antibodies to myeloperoxidase in patients with eosinophilic granulomatosis with polyangiitis (Churg-Strauss). *Clin Exp Rheumatol*. (2017) 35(Suppl 103):98–101.
16. Tan YC, Kongpachith S, Blum LK, Ju CH, Lahey LJ, Lu DR, et al. Barcode-enabled sequencing of plasmablast antibody repertoires in rheumatoid arthritis. *Arthritis Rheumatol*. (2014) 66:2706–15. doi: 10.1002/art.38754
17. Tan YC, Blum LK, Kongpachith S, Ju CH, Cai X, Lindstrom TM, et al. High-throughput sequencing of natively paired antibody chains provides evidence for original antigenic sin shaping the antibody response to influenza vaccination. *Clin Immunol*. (2014) 151:55–65. doi: 10.1016/j.clim.2013.12.008
18. DeFalco J, Harbell M, Manning-Bog A, Baia G, Scholz A, Millare B, et al. Non-progressing cancer patients have persistent B cell responses expressing shared antibody paratopes that target public tumor antigens. *Clin Immunol*. (2018) 187:37–45. doi: 10.1016/j.clim.2017.10.002
19. Peikert T, Finkielman JD, Hummel AM, McKenney ME, Gregorini G, Trimarchi M, et al. Functional characterization of antineutrophil cytoplasmic antibodies in patients with cocaine-induced midline destructive lesions. *Arthritis Rheum*. (2008) 58:1546–51. doi: 10.1002/art.23469
20. Wiesner O, Russell KA, Lee AS, Jenne DE, Trimarchi M, Gregorini G, et al. Antineutrophil cytoplasmic antibodies reacting with human neutrophil elastase as a diagnostic marker for cocaine-induced midline destructive lesions but not autoimmune vasculitis. *Arthritis Rheum*. (2004) 50:2954–65. doi: 10.1002/art.20479
21. Wiesner O, Litwiller RD, Hummel AM, Viss MA, McDonald CJ, Jenne DE, et al. Differences between human proteinase 3 and neutrophil elastase and their murine homologues are relevant for murine model experiments. *FEBS Lett*. (2005) 579:5305–12. doi: 10.1016/j.febslet.2005.08.056
22. Van Der Geld YM, Limburg PC, Kallenberg CG. Characterization of monoclonal antibodies to proteinase 3. (PR3) as candidate tools for epitope mapping of human anti-PR3 autoantibodies. *Clin Exp Immunol*. (1999) 118:487–96. doi: 10.1046/j.1365-2249.1999.01079.x
23. Csernok E, Ludemann J, Gross WL, Bainton DF. Ultrastructural localization of proteinase 3, the target antigen of anti-cytoplasmic antibodies circulating in Wegener's granulomatosis. *Am J Pathol*. (1990) 137:1113–20.
24. Fujinaga M, Chernaia MM, Halenbeck R, Koths K, James MN. The crystal structure of PR3, a neutrophil serine proteinase antigen of Wegener's granulomatosis antibodies. *J Mol Biol*. (1996) 261:267–78. doi: 10.1006/jmbi.1996.0458
25. Pang Y-P. FF12MC: a revised AMBER forcefield and new protein simulation protocol. *Proteins*. (2016) 84:1490–516. doi: 10.1002/prot.25094
26. Jorgensen WL, Chandreskhar J, Madura JD, Impey RW, Klein ML. Comparison of simple potential functions for simulating liquid water. *J Chem Phys*. (1983) 79:926–35. doi: 10.1063/1.445869
27. Pang Y-P. Use of 1–4 interaction scaling factors to control the conformational equilibrium between α-helix and β-strand. *Biochem Biophys Res Commun*. (2015) 457:183–6. doi: 10.1016/j.bbrc.2014.12.084
28. Berendsen HJC, Postma JPM, van Gunsteren WF, Di Nola A, Haak JR. Molecular dynamics with coupling to an external bath. *J Chem Phys*. (1984) 81:3684–90. doi: 10.1063/1.448118
29. Darden TA, York DM, Pedersen LG. Particle mesh Ewald: an N log(N) method for Ewald sums in large systems. *J Chem Phys*. (1993) 98:10089–92. doi: 10.1063/1.464397
30. Joung IS, Cheatham TE. Determination of alkali and halide monovalent ion parameters for use in explicitly solvated biomolecular simulations. *J Phys Chem B*. (2008) 112:9020–41. doi: 10.1021/jp8001614
31. Pang Y-P. Low-mass molecular dynamics simulation for configurational sampling enhancement: more evidence and theoretical explanation. *Biochem Biophys Rep*. (2015) 4:126–33. doi: 10.1016/j.bbrep.2015.08.023
32. Pang Y-P. At least 10% shorter C–H bonds in cryogenic protein crystal structures than in current AMBER forcefields. *Biochem Biophys Res Commun*. (2015) 458:352–5. doi: 10.1016/j.bbrc.2015.01.115
33. Andraos J. On the propagation of statistical errors for a function of several variables. *J Chem Educ*. (1996) 73:150–4. doi: 10.1021/ed073p150
34. Shao J, Tanner SW, Thompson N, Cheatham III TE. Clustering molecular dynamics trajectories: 1. Characterizing the performance of different clustering algorithms. *J Chem Theory Comput*. (2007) 3:2312–34. doi: 10.1021/ct700119m
35. Pang Y-P. How fast fast-folding proteins fold *in silico*. *Biochem Biophys Res Commun*. (2017) 492:135–9. doi: 10.1016/j.bbrc.2017.08.010
36. Van Regenmortel MH. Reductionism and the search for structure-function relationships in antibody molecules. *J Mol Recognit*. (2002) 15:240–7. doi: 10.1002/jmr.584
37. McCammon JA, Gelin BR, Karplus M. Dynamics of folded proteins. *Nature*. (1977) 267:585–90. doi: 10.1038/267585a0
38. Westhof E, Altschuh D, Moras D, Bloomer AC, Mondragon A, Klug A, et al. Correlation between segmental mobility and the location of antigenic determinants in proteins. *Nature*. (1984) 311:123–6. doi: 10.1038/311123a0
39. Tainer JA, Getzoff ED, Alexander H, Houghten RA, Olson AJ, Lerner RA, et al. The reactivity of anti-peptide antibodies is a function of the atomic mobility of sites in a protein. *Nature*. (1984) 312:127–34. doi: 10.1038/312127a0
40. Debye P. Interference of x rays and heat movement. *Ann Phys*. (1913) 43:49–95. doi: 10.1002/andp.19133480105
41. Waller I. On the effect of thermal motion on the interference of X-rays. *Z Phys*. (1923) 17:398–408. doi: 10.1007/BF01328696
42. Willis BTM, Pryor AW. *Thermal Vibrations in Crystallography*. London: Cambridge University Press (1975).

43. Yu HA, Karplus M, Hendrickson WA. Restraints in temperature-factor refinement for macromolecules: an evaluation by molecular dynamics. *Acta Crystallogr Sect B Struct Sci*. (1985) 41:191–201. doi: 10.1107/S0108768185001926
44. Kidera A, Go N. Normal mode refinement: crystallographic refinement of protein dynamic structure. 1. Theory and test by simulated diffraction data. *J Mol Biol*. (1992) 225:457–75. doi: 10.1016/0022-2836(92)90932-A
45. McRee DE. *Practical protein crystallography*. San Diego, CA: Academy Press (1993).
46. Trueblood KN, Burgi HB, Burzlaff H, Dunitz JD, Gramaccioli CM, Schulz HH, et al. Atomic displacement parameter nomenclature: report of a subcommittee on atomic displacement parameter nomenclature. *Acta Crystallogr, Sect A*. (1996) 52:770–81. doi: 10.1107/S0108767396005697
47. Tronrud DE. Knowledge-based B-factor restraints for the refinement of proteins. *J Appl Crystallogr*. (1996) 29:100–4. doi: 10.1107/S002188989501421X
48. Garcia AE, Krumhansl JA, Frauenfelder H. Variations on a theme by Debye and Waller: from simple crystals to proteins. *Proteins*. (1997) 29:153–60. doi: 10.1002/(SICI)1097-0134(199710)29:2<153::AID-PROT3>3.0.CO;2-E
49. Artymiuk PJ, Blake CC, Grace DE, Oatley SJ, Phillips DC, Sternberg MJ. Crystallographic studies of the dynamic properties of lysozyme. *Nature*. (1979) 280:563–8. doi: 10.1038/280563a0
50. Morin S. A practical guide to protein dynamics from ^{15}N spin relaxation in solution. *Prog Nucl Magn Reson Spectrosc*. (2011) 59:245–62. doi: 10.1016/j.pnmrs.2010.12.003
51. Pang Y-P. Use of multiple picosecond high-mass molecular dynamics simulations to predict crystallographic B-factors of folded globular proteins. *Heliyon*. (2016) 2:e00161. doi: 10.1016/j.heliyon.2016.e00161
52. Hinkofer LC, Seidel SA, Korkmaz B, Silva F, Hummel AM, Braun D, et al. A monoclonal antibody (MCPR3-7) interfering with the activity of proteinase 3 by an allosteric mechanism. *J Biol Chem*. (2013) 288:26635–48. doi: 10.1074/jbc.M113.495770
53. Thompson GE, Casal Moura M, Nelson DA, Hummel A, Jenne DE, Fervenza FC, et al. Characterization of preferential recognition of a chimeric recombinant proteinase 3 variant by anti-neutrophil cytoplasmic antibodies. *Arthritis Rheumatol*. (2018). doi: 10.1093/rheumatology/kez061.037
54. Travers TS, Harlow L, Rosas IO, Gochuico BR, Mikuls TR, Bhattacharya SK, et al. Extensive citrullination promotes immunogenicity of HSP90 through protein unfolding and exposure of cryptic epitopes. *J Immunol*. (2016) 197:1926–36. doi: 10.4049/jimmunol.1600162

Conflict of Interest: DE and WV were employed by Atreca, Inc.

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.

frontiers in Immunology

ORIGINAL RESEARCH
published: 07 November 2019
doi: 10.3389/fimmu.2019.02603

Low Density Granulocytes in ANCA Vasculitis Are Heterogenous and Hypo-Responsive to Anti-Myeloperoxidase Antibodies

*Aisling Ui Mhaonaigh[1], Alice M. Coughlan[1], Amrita Dwivedi[1], Jack Hartnett[2], Joana Cabral[3], Barry Moran[4], Kiva Brennan[2,5], Sarah L. Doyle[2,5], Katherine Hughes[1], Rosemary Lucey[1], Achilleas Floudas[6], Ursula Fearon[6], Susan McGrath[3], Sarah Cormican[3], Aine De Bhailis[1], Eleanor J. Molloy[7], Gareth Brady[1] and Mark A. Little[1,8]**

[1] Trinity Health Kidney Centre, Trinity Translational Medicine Institute, Trinity College Dublin, Dublin, Ireland, [2] Department of Clinical Medicine, School of Medicine, Trinity College Dublin, Dublin, Ireland, [3] The Regenerative Medicine Institute (REMEDI), National University of Ireland, Galway, Ireland, [4] School of Biochemistry and Immunology, Trinity Biomedical Sciences Institute, Trinity College Dublin, Dublin, Ireland, [5] National Children's Research Centre, Our Lady's Children's Hospital Crumlin, Dublin, Ireland, [6] Molecular Rheumatology, Trinity Biomedical Sciences Institute, Trinity College Dublin, Dublin, Ireland, [7] Department of Paediatrics, Trinity Translational Medicine Institute, Trinity College Dublin, Dublin, Ireland, [8] Irish Centre for Vascular Biology, Trinity College Dublin, Dublin, Ireland

OPEN ACCESS

Edited by:
Andreas kronbichler, Innsbruck Medical University, Austria

Reviewed by:
Joshua Daniel Ooi, Monash University, Australia
Dimitrios Vassilopoulos, National and Kapodistrian University of Athens, Greece

***Correspondence:**
Mark A. Little
mlittle@tcd.ie

Specialty section:
This article was submitted to Autoimmune and Autoinflammatory Disorders, a section of the journal Frontiers in Immunology

Received: *02 August 2019*
Accepted: *21 October 2019*
Published: *07 November 2019*

Citation:
Ui Mhaonaigh A, Coughlan AM, Dwivedi A, Hartnett J, Cabral J, Moran B, Brennan K, Doyle SL, Hughes K, Lucey R, Floudas A, Fearon U, McGrath S, Cormican S, De Bhailis A, Molloy EJ, Brady G and Little MA (2019) Low Density Granulocytes in ANCA Vasculitis Are Heterogenous and Hypo-Responsive to Anti-Myeloperoxidase Antibodies. Front. Immunol. 10:2603. doi: 10.3389/fimmu.2019.02603

Low Density Granulocytes (LDGs), which appear in the peripheral blood mononuclear cell layer of density-separated blood, are seen in cancer, sepsis, autoimmunity, and pregnancy. Their significance in ANCA vasculitis (AAV) is little understood. As these cells bear the autoantigens associated with this condition and have been found to undergo spontaneous NETosis in other diseases, we hypothesized that they were key drivers of vascular inflammation. We found that LDGs comprise a 3-fold higher fraction of total granulocytes in active vs. remission AAV and disease controls. They are heterogeneous, split between cells displaying mature (75%), and immature (25%) phenotypes. Surprisingly, LDGs (unlike normal density granulocytes) are hyporesponsive to anti-myeloperoxidase antibody stimulation, despite expressing myeloperoxidase on their surface. They are characterized by reduced CD16, CD88, and CD10 expression, higher LOX-1 expression and immature nuclear morphology. Reduced CD16 expression is like that observed in the LDG population in umbilical cord blood and in granulocytes of humanized mice treated with G-CSF. LDGs in AAV are thus a mixed population of mature and immature neutrophils. Their poor response to anti-MPO stimulation suggests that, rather than being a primary driver of AAV pathogenesis, LDGs display characteristics consistent with generic emergency granulopoiesis responders in the context of acute inflammation.

Keywords: ANCA associated vasculitis, low density granulocytes, anti-MPO, reactive oxygen species, neutrophil heterogeneity

INTRODUCTION

Neutrophils have conventionally been considered a uniform, short-lived, and functionally-restricted population of immune cells (1). Recent evidence suggests that they feature a plasticity that allows them to respond and adapt to different disease situations (2, 3). Anti-neutrophil cytoplasm autoantibody (ANCA) vasculitis (AAV) is a systemic autoimmune disease in which

neutrophils play a pivotal role (4). It is characterized by autoantibodies directed against neutrophil proteins myeloperoxidase (MPO) and proteinase-3 (PR3) and is associated clinically with rapidly progressive glomerulonephritis and inflammatory necrosis of small blood vessels in lungs, skin, and other organs (5–7). Neutrophils obtained from patients with active AAV aberrantly transcribe the autoantigens MPO and PR3, a feature that correlates with subsequent clinical outcome (8).

Low density granulocytes (LDGs) are distinct from normal density granulocytes (NDGs) with a density below 1.07 g/ml and sediment in the PBMC layer after density gradient fractionation of whole blood (3, 9, 10). LDGs are expanded in animal models of viral-infection (11) and arthritis (12) and in humans with cancer (13), sepsis (14), HIV (9, 15) and various autoimmune conditions, including systemic lupus erythematosus (SLE) (3), rheumatoid arthritis (RA) (16), and psoriasis (17). There is lack of clarity in the phenotypical and functional characteristics of LDGs, and in the relationship of LDGs to myeloid-derived suppressor cells (MDSCs) (**Table 1**). Most studies in autoimmune diseases suggest that LDGs are pro-inflammatory, relatively long-lived and undergo NETosis more readily than NDGs (3, 23). Therefore, it has been postulated that these cells are a key pathogenic force of autoimmunity (25).

Traditional flow cytometric markers to identify neutrophil populations within highly granulated populations include CD66b, CD15, CD11b, and CD16. However, the expression of these surface receptors can be altered upon neutrophil activation and following density centrifugation (26, 27). Despite recent work to consolidate phenotypic description of these cells, many different ways of identifying LDGs are present in the literature (28). Potentially useful distinguishing markers include CD10, which distinguishes mature from immature neutrophils, and lectin-type oxidized LDL receptor 1 (LOX-1) (20, 24).

To investigate the role of LDGs in AAV, we combined traditional and imaging flow cytometric analysis with functional assays. We found that, in active AAV, the LDG population is expanded and comprised of a heterogeneous population of neutrophils, with differential expression of CD16 and CD10. A substantial fraction of LDGs are immature neutrophils, likely released in response to emergency granulopoiesis. We found that, unlike NDGs, LDGs are hyporesponsive to stimulation with monoclonal antibodies directed against MPO, suggesting that they may not have an important pathogenic role in AAV.

MATERIALS AND METHODS

Patients

We recruited AAV patients with acute disease ($n = 13$), those in remission ($n = 6$), age matched healthy controls (HC, $n = 5$) and disease controls (DC, a mix of renal impairment and non-AAV systemic inflammation, $n = 11$, Chronic kidney disease $n = 3$, Coronary artery disease $n = 1$, Stroke $n = 1$, Colorectal carcinoma $n = 1$, IgA vasculitis $n = 1$, rheumatoid arthritis $n = 4$) (**Table 2**). All patients with AAV fulfilled the revised Chapel Hill Consensus Conference classification (29). Active AAV was defined by a Birmingham vasculitis activity score (BVAS) ≥ 2 and remission by BVAS $= 0$. Disease/healthy controls and patients with AAV were recruited from the Rare Kidney Disease Registry and Biobank (www.tcd.ie/medicine/thkc/research/rare.php). Umbilical cord blood (UCB) was obtained from mothers undergoing vaginal deliveries with healthy term pregnancy; the babies had normal Apgar scores. The study was approved by institutional ethics committees of Tallaght, St Vincent's, St James and Beaumont Hospitals, and all recruits provided written informed consent.

Density Centrifugation

Venous blood samples were collected in lithium-heparin vacutainers (Becton Dickinson, New Jersey, USA). PBMC/LDGs and NDGs were isolated by a modified Percoll (GE healthcare, Uppsala, Sweden) gradient centrifugation procedure (3, 9, 24) and stained immediately for surface markers as listed in **Supplementary Table 1**. Arm to stain time was <4 h in all cases. Briefly, an equal volume of 2% Dextran (Sigma-Aldrich, Missouri, USA) was added to 6–12 ml blood and inverted 20 times. Erythrocytes were left to sediment by gravity for 30 min; the supernatant was then spun at 200 g with no brake. The pellet was re-suspended in 3 ml 55% Percoll, slowly layered over 4.5 ml 65% Percoll and spun with no brake for 30 min at 1,500 g (**Supplementary Figure 1**). The PBMC/LDG and NDG layers were carefully removed to fresh tubes, cells were washed with PBS and the resulting cell pellets incubated with 10 ml of RBC lysis buffer (155 mM NH_4Cl, 0.1 mM EDTA, 12 mM $NaHCO_3$ pH 7.4) for 5 min. After washing, the cells were re-suspended in 1 ml of FACS buffer (2% fetal calf serum in PBS). Viability was determined using Trypan blue (Gibco) and in all cases was >90%.

Phenotypic Analysis by Traditional Flow Cytometry

The appropriate antibodies (**Supplementary Table 1**) were added and incubated in the dark at room temperature for 20 min. Cells were washed with PBS before being resuspended in 500 μl FACS buffer (if run immediately) or 2% paraformaldehyde (PFA) (Santa Cruz, Texas, USA) if being stored overnight at 4°C. Fluorescence minus one (FMO) controls were prepared for each fluorophore and used to define positive staining. Compensation was performed with OneComp beads (eBioscience, California, USA) stained with appropriate antibodies. A minimum of 10,000 events were collected for each sample. Cells were acquired on a FACS Canto II flow cytometer (BD, San Jose, USA) and the data were analyzed using Kaluza software (Beckman Coulter, USA). To assess the fraction and absolute cell count of LDGs in the different patient and control groups, LDGs were defined as SSC^{hi} and $CD15^{+}$ after gating on singlets (**Figure 1B**). We defined the LDG fraction in three ways: (1) as a fraction of PBMCs, (2) the absolute LDG count per mL of blood, and (3) as a fraction of total granulocyte count (LDG + NDG). The latter allowed us to distinguish whether LDGs were expanded preferentially in AAV, or simply increased in proportion to total neutrophil expansion, as acute AAV is known to be associated with peripheral neutrophil leucocytosis. To further delineate the phenotypic characteristics of LDGs in comparison to paired

TABLE 1 | LDG population characteristics in various disease conditions.

Disease area	Specific condition	Population	Surface marker expression	Arginase	Nuclear Morphology	ROS	Reference
Infection	HIV	LDGs	$CD15^+$,$CD11b^+$,$CD13^+$,$CD33^+$, $CD16^{int/lo}$,$CD66b^+CD63^+$	Decreased	Mature		(9)
	TB	LDGs	$CD15^+$,$CD14^{low}$ $CD16^+$, $CD33^+$, $CD66b^+$ and $CD62L^{low}$,		Mature	Increased in LDGs	(18)
	Sepsis	Interphase neutrophils	$CD16^{int}$, $CD11b^+$, $CD15^+$, $CD33^{-/low}$, $CD54^{-/low}$, $CD62L^{-/low}$, $CD66b^+$ and $CD14^{-/low}$ $HLA\text{-}DR^{-/low}$	Increased	Heterogeneous mixed banded and segmented	ND	(14)
Malignancy	Cancer	LDGs	$CD66b^+$, $CD33^+$, $CD16^{var}$, $CD11b^{Var}$, $CD125^-$ $HLA\text{-}DR^-$		Immature		(19)
	Cancer	G-MDSC	$CD11b^+$, $CD14^-$,$CD15^+$, $CD66b^+$, $LOX\text{-}1^{var}$	Increased expression in $Lox\text{-}1^+$	$Lox\text{-}1^+$ mature, $Lox\text{-}1^-$ immature	$LOX\text{-}1^+$ increased	(20)
	Hepatocellular carcinoma	G-MDSC	$CD11b^+$,$CD14^-$,$HLA\text{-}DR^{-/low}$,$CD15^+$, $LOX\text{-}1^+$	Increased on CD15+ Lox-1+	ND	Increased	(21)
	Renal cell carcinoma	MDSC	$CD66b^+$,$CD11b^+$,$VEGFR1^+$, $CD62l^{low}$,$CD16^{low}$	Decreased	Heterogeneous, 90% segmented	ND	(22)
Autoimmunity	Rheumatoid arthritis	LDGs	$CD10^+$,$CD14^+$,$CD15^+$ $CD16^{int/low}$	ND	ND	Lower than NDGs	(16)
	Psoriasis	LDGs	$CD10^+CD14^{low}$	ND	ND		(17)
	SLE	LDGs	$CD10^+$,$CD11c^{lo}$,$CD14^{lo}$, $CD15^{hi}$,$CD16^{hi}$, $CD31^+$, $CD114^+$, $CD116^-$	ND*	Heterogeneous, Mature, less segmented	ND	(3, 23)
Other	G-CSF treated donors	LDNs	$CD66b^+$,$CD11b^{var}$,$CD10^{var}$, $CD16^{var}$	Increased mRNA, decreased activity	Heterogeneous mixed banded and segmented	Not involved in T Cell suppression	(24)
	Pregnancy	LDGs	$CD15^+$, $CD66b^+$,$CD63^+$,$CD33^+$, $CD16^{int/low}$	Increased on cord vs maternal	ND	ND	(10)

HIV, Human Immunodeficiency Virus; TB, Tuberculosis; LDG, Low Density Granulocytes; G-MDSC, Granulocytic Myeloid Derived Suppressor Cells; LDN, Low Density Neutrophils; SLE, Systemic Lupus Erythematosus; NDG, Normal Density Granulocytes; ND, Not determined; LOX-1, Lectin-type Oxidized LDL receptor-1; var, variable; G-CSF, Granulocyte Colony Stimulating factor.

NDGs using markers listed in **Supplementary Table 1**, we defined the cells as $SSC^{hi}CD15^+CD14^-$ (30). Having observed differential CD16 expression we defined a $CD16^-$ population (based on FMO) and $CD16^+$ and $CD16^{int}$ populations.

Phenotypic Analysis by Imaging Flow Cytometry

After isolation from whole blood, LDGs and NDGs were immediately stained with combinations of monoclonal antibodies as detailed in **Supplementary Table 1**. DAPI 0.2 μg/ml (Sigma-Aldrich, Missouri, USA) was used for nuclear staining. One million cells were stained and re-suspended in 50 μl FACS buffer (2% fetal calf serum in PBS) before analysis. Images were acquired on an ImageStream X MkII imaging flow cytometer (Amnis Corporation, Seattle, WA) using INSPIRE data acquisition software (Amnis). Compensation and data analysis were performed using IDEAS 5.0 software (Amnis).

ROS Production

ROS production was measured using the dihydrorhodamine123 (DHR123) assay as described previously (31). Briefly, 2 × 106 cells/ml from PBMCs and NDGs were suspended in separate 15 ml falcon tubes in HBH buffer (0.01% HEPES in Hank's buffered salt solution (HBSS). The cell suspension was incubated with 20 μg/ml DHR123 (Molecular Probes, D-632) and 5 μg/ml Cytochalasin B (Sigma) for 15 min at 37°C in the dark. Cells were then stimulated with 5 μg/ml anti-MPO mAb (Clone B3147M, Meridian Life sciences, Tennessee, USA) or isotype control IgG (IgG1, Origene technologies, Hanford, Germany) for 1 h at 37°C in the dark. 0.5 μg/ml Phorbol 12-myristate 13-acetate (PMA, Sigma) treated cells served as positive control. The reaction was stopped by adding 2 ml of cold HBSS (Gibco) containing 1% BSA and, after washing, the cells were stained for flow cytometric analysis as described above. Intracellular ROS production was determined by quantifying the fraction of Rhodamine123 positive cells.

G-CSF Treatment of Humanized Mice

To assess the impact of granulocyte colony stimulating factor (G-CSF) on human CD16 granulocyte expression, we generated humanized mice as described previously (32). Briefly, NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjlt}Tg (PGK1-KITLG*220)441Daw/SzJ

TABLE 2 | Baseline characteristics of the study subjects, by disease classification.

Characteristics			HC	DC	AAV-Active	AAV-Remission
n			5	11	13	6
Age, median (range), years			70 (66–72)	53 (43–87)	73 (40–85)	57 (41–70)
Male/Female			3/2	5/6	6/7	4/2
ANCA status, *n* (%)	Anti-MPO		0	0	9 (69)	3 (50)
	Anti-PR3		0	0	4 (31)	3 (50)
Diagnosis, *n* (median disease duration at sampling, month)	GPA		0	0	4 (0)	3 (143)
	MPA		0	0	9 (0)	3 (35.2)
BVAS, median (range)			N/A	N/A	16 (3–25)	0
CRP (mg/dL), median (IQR)			N/A	9 (3–26)	24 (4–60)	6 (1.8–14)
Creatinine (μmol/L), mean (SEM)			N/A	187 (63)	253 (69)	153 (52)
eGFR (mL/min), mean (SEM)			N/A	57.1 (8.3)	17.0 (7.9)	36.0 (6.9)
Immunosuppression treatment, *n* (%)	Treatment naïve		5 (100)	5 (45)	5 (38)	0
	CYC	0-6 months	0	1 (9)	1 (8)	4 (67)
		6-12 months	0	0	0	0
		>12 months	0	1 (9)	0	2 (33)
	Aza	Current	0	0	1 (8)	2 (33)
	MMF	Current	0	0	0	2 (33)
	MTX	Current	0	0	0	1 (17)
	Anti-TNF	Current	0	4 (36)	0	0
	Corticosteroids	Current	0	2 (18)	8 (62)	6 (100)
	Corticosteroids	Median duration (days, range)			1.5 (1–25)	
	Corticosteroids	Median cum dose (mg, range)			500 (60–1,780)	

AAV, Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis; BVAS, Birmingham Vasculitis Activity Score; CRP, C-reactive protein; eGFR, Estimated glomerular filtration rate; GPA, Granulomatosis with polyangiitis; MPA, Microscopic polyangiitis; CYC, duration since cyclophosphamide exposure; Aza, Azathioprine; MMF, mycophenolate mofetil; MTX, Methotrexate.

(hu-mSCF) mice were obtained from Jax (Bar Harbor, Maine, USA) and engrafted by injecting 1×10^5 purified human cord blood derived CD34+ stem cells (Lonza, Slough, Berkshire, UK) into the lateral tail veins of 10–14 week-old mice ∼24 h post irradiation (2.4 Gy). Following confirmation of engraftment, mice were injected subcutaneously with 50 μg pegylated filgrastim (Neulasta®, Amgen, Cambridge, UK), with repeat peripheral cell granulocyte phenotype assessed by flow cytometry 4 days later. Cells were stained with appropriate antibodies (**Supplementary Table 2**) after blocking with 2.5 μg/ml human BD Fc Block (clone: Fc1.3070) and 1 μg/ml mouse BD Fc Block (clone: 2.4G2). Flow cytometric analysis was performed on a CyAn ADP Analyzer (Beckman Coulter, California, USA) using Summit software (Beckman Coulter). Data were analyzed using Kaluza software. Human granulocytes were identified as $hCD45^+CD66b^+$.

Statistical Analysis

All statistical analysis was performed using GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA, USA). The LDG fraction and absolute LDG cell count were compared between groups using the Kruskal Wallis test, with comparison between individual groups using Dunn's multiple comparison test. The fraction of $CD16^+$ cells between LDGs and NDGs was assessed with a Wilcoxon ranked sum test, with sub-group analysis performed using ANOVA with correction for multiple comparisons using Tukey's test. The change in CD16 expression on granulocytes in humanized mice and the variation in DHR response to stimulation were assessed with 2-way ANOVA and Sidak's multiple comparison tests. Differences between LDG CD16 subsets, and ROS production in $CD10^+$ and $CD10^-$ neutrophils were tested using Friedman's paired test, with *post hoc* comparison of groups using Dunn's test. The number of neutrophil lobes in $CD16^+$ and $CD16^{int/-}$ cells was compared using the Chi square test and the correlation between CD16 and CD10 expression using Spearman correlation.

RESULTS

Low Density Granulocytes Are Expanded in Patients With Acute AAV

To determine whether LDGs were elevated in acute AAV, PBMC were isolated from peripheral blood of AAV patients and healthy controls (HC) by density gradient centrifugation. LDGs were initially identified as a population of high side scatter cells in acute AAV that was not present in HC (**Figure 1A**). These cells were further defined by their expression of CD15 (**Figure 1B**). We found that the LDG fraction (17.4%, IQR 11.2–40.7) and the absolute number of LDG /ml of blood (1.9×10^5/ mL, IQR 1.0–3.5 $\times 10^5$) were significantly increased in acute AAV (**Figures 1C,D**). For comparison, blood containing a high fraction of immature neutrophils, umbilical cord blood

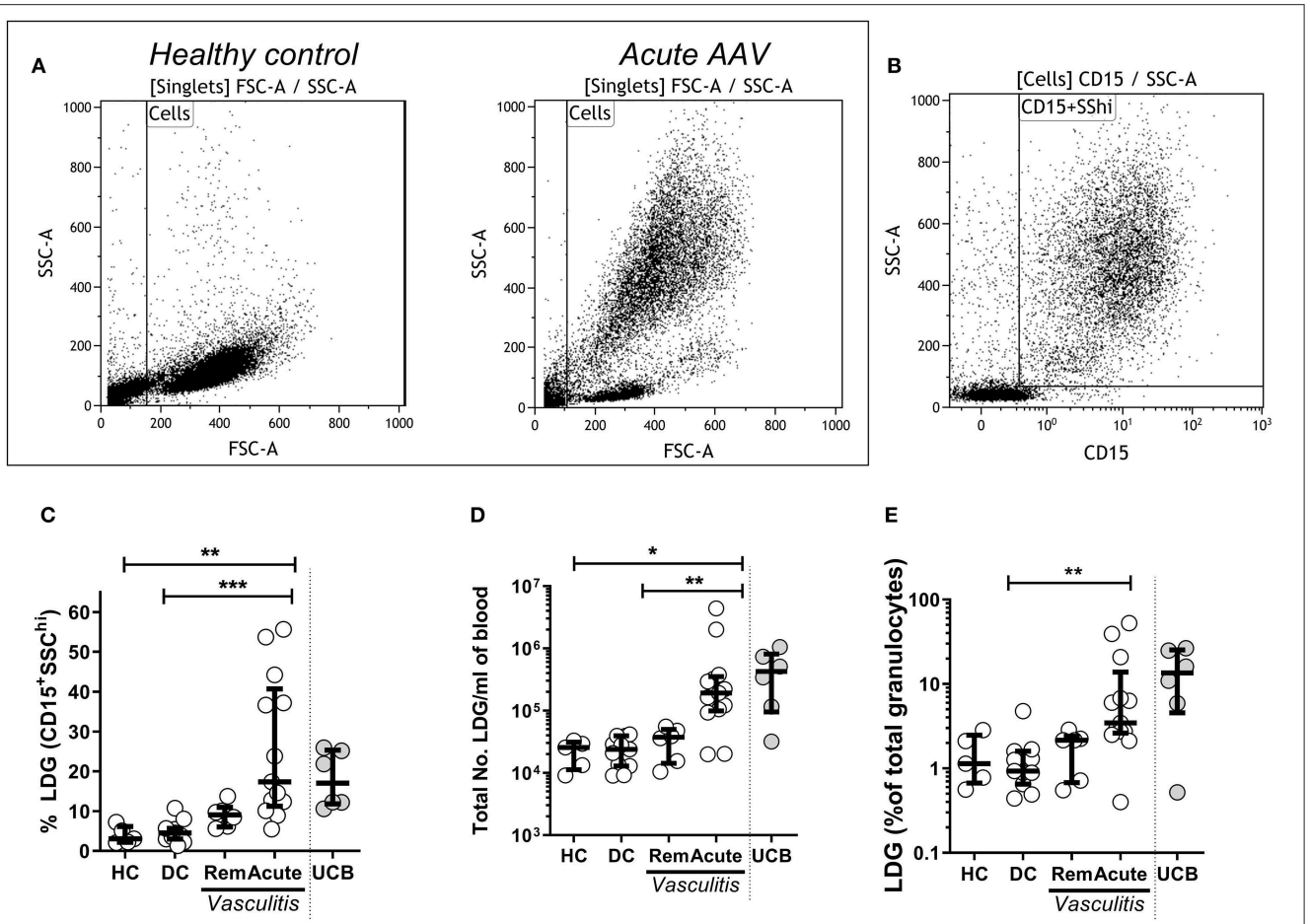


FIGURE 1 | Low density granulocytes (LDGs) are elevated in patients with Acute AAV. PBMC were isolated from peripheral blood of patients with AAV by density gradient centrifugation. Representative flow cytometry dot plots are shown from healthy control PBMC and acute AAV patient PBMC. **(A)** LDGs were classified as live $SSC^{hi}CD15^{+}$ singlets (data shown from a representative patient with acute AAV). **(B)** LDGs were quantified as the percentage of PBMC in the peripheral blood of 13 acute AAV patients, 6 remission (rem), 11 disease controls (DC), and 5 age matched healthy controls (HC). Each symbol represents an individual donor. The values from 6 samples of umbilical cord blood (UCB) are shown for comparison **(C)**. The absolute numbers of LDGs/mL of blood **(D)** and percentage of total granulocytes **(E)** were also quantified. *Median with interquartile range. Kruskal-Wallis test, with post hoc analysis with Dunn's multiple comparison.* $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

(UCB), contained a median LDG fraction of 17.0% (IQR 11.8–25.4, **Figure 1D**). The LDG fraction represents the low-density tail of the neutrophil density distribution. As acute AAV is characterized by neutrophil leucocytosis, we assessed whether the observed LDG expansion was merely in proportion to the overall granulocyte expansion. Median LDG fraction of total granulocytes in acute AAV (3.5%, IQR 2.6–13.8) was significantly higher than in DC (**Figure 1E**), indicating that although total granulocyte (LDG and NDG) numbers are increased in acute AAV, there is preferential expansion of the LDG fraction.

CD16 Expression Defines Subpopulations of LDGs in AAV

CD16 (Fcγ receptor III) is a low affinity receptor for IgG, expressed on neutrophils in a glycosylphosphatidyl inositol (GPI) linked form. It appears late during neutrophilic maturation. It is faintly expressed on metamyelocytes while banded and segmented stages of neutrophilic development show the highest expression (33–35). Therefore, to investigate the sub-populations within the LDG fraction CD16 expression was quantified by flow cytometric analysis. LDGs and NDGs (classified as $SSC^{hi}CD15^{+}CD14^{-}$ singlets in the low density (PBMC) and high-density layer, respectively) were categorized as $CD16^{+}$, $CD16^{int}$ (clearly separated from $CD16^{+}$ cells) and $CD16^{-}$ (defined by FMO). Representative CD16 plots from LDGs (**Figure 2A**) and NDGs (**Figure 2B**) illustrate the difference in CD16 expression. In the NDG fraction in adult patients and healthy controls, 95.2% (IQR 91.0–97.2) of neutrophils were $CD16^{+}$ which fell to 65.2% (IQR 52.1–75.7) in the LDG fraction (**Figure 2C**). In comparison, only 5.4% (IQR 3.2–15.9) of LDGs from UCB were $CD16^{+}$ (**Figure 2D**). Consequently, there was a significant increase in $CD16^{int/-}$ cells in the LDG fraction (34.2%, IQR 23.8–48.4) from adult patients and

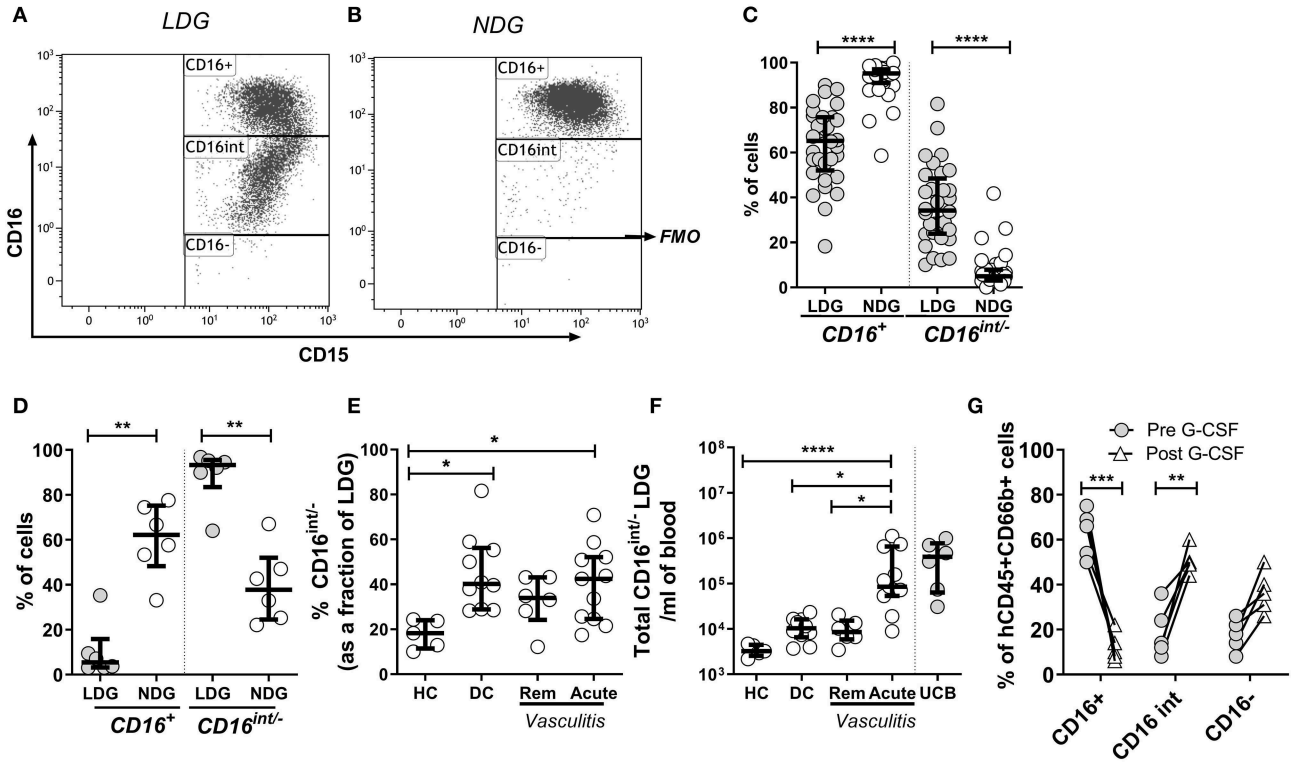


FIGURE 2 | CD16 expression defines subpopulations of LDGs. Mixed leukocyte populations were separated by density centrifugation; LDGs and NDGs were classified as $SSC^{hi}CD15^{+}CD14^{-}$ singlets in the low density (PBMC) and high-density layers, respectively. LDGs and NDGs were categorized as $CD16^{+}$, $CD16^{int}$ and $CD16^{-}$ (FMO, fluorescence minus one), with a representative LDG sample illustrated in **(A)**. A representative NDG sample is shown in **(B)**. Most NDGs were $CD16^{+}$, whereas approximately one third of LDGs were $CD16^{int/-}$ **(C)** *Wilcoxon ranked sum test*. For comparison, virtually all LDGs in UCB were $CD16^{int/-}$ **(D).** The percentage **(E)** and absolute number **(F)** of $CD16^{int/-}$ cells in the LDG fraction was compared across disease phenotypes. *ANOVA with post hoc Tukey's multiple comparison test,* $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. Administration of G-CSF to mice ($n = 5$) with a humanized immune system caused dramatic reduction in peripheral blood $CD16^{+}$ neutrophils, with an associated increase in $CD16^{int/-}$ neutrophils. *2-way ANOVA and Sidak's multiple comparison test* $^{**}p < 0.01$, $^{***}p < 0.001$ **(G)**.

healthy controls compared to NDG fraction (4.9%, IQR 3.0–7.8). We hypothesized that this LDG expansion of the $CD16^{int/-}$ population was a non-specific feature of acute illness. We found that these cells made up a greater fraction of LDGs in DC (40.2%, IQR 28.9–56.2) and patients with acute AAV (42.5%, IQR 24.6–52.1) than HC (18.3%, IQR 11.5–24.0, **Figure 2E**) and absolute $CD16^{int/-}$ cell count was markedly expanded in acute AAV (**Figure 2F**). This non-specific expansion of the $CD16^{int/-}$ LDG fraction in AAV suggested that these cells may be arising as a result of acute granulopoiesis leading to an increase in the number of circulating immature neutrophils (36). To test this hypothesis, we administered G-CSF to mice with a humanized immune system; this caused a dramatic reduction of peripheral blood $CD16^{+}$ neutrophils from 62.8 ± 4.7 to 12.0 ± 2.8%, with an associated increase in $CD16^{int/-}$ neutrophils (**Figure 2G**).

LDG Surface Immune Markers Vary According to CD16 Expression

LDGs are characterized by a population of cells with high side scatter and variably low surface expression of Fc receptor CD16. To examine the phenotype of these cells in the context of AAV, we went on to define surface expression of the relevant immune markers on LDGs in detail (**Table 3**) and then stratified populations according to CD16 expression, with marker expression on NDGs shown alongside for comparison (**Figure 3**). We observed a small but statistically significant reduction in expression of the classical granulocyte marker CD66b in both $CD16^{int}$ and $CD16^{-}$ cells, although a high proportion of $CD16^{int}$

TABLE 3 | Percentage expression of phenotypic markers on LDG and NDG.

Marker	LDG (Median, IQR)	NDG (Median, IQR)	*p-value*
CD66b	98.7% (97.199.5)	99.8% (99.0–99.9)	0.001
CD88	71.2% (64.8–82.2)	95% (88.6–97.6)	0.003
HLA-DR	2.2% (1.1–3.4)	0.3% (0.17–1.6)	<0.0001
MPO	6.6% (3.2–11.1)	5.5% (3.9–10.8)	0.0674
PR3	14.4% (10.3–19.6)	7% (3.6–11.3)	0.0002
CD177	55.4% (45.5–70.7)	70.8% (52.4–83.1)	<0.0001
PR3+CD177+	10.4% (3.7–14.5)	3.7% (1.6–8.05)	0.0091
PR3+CD177−	5.4% (1.7–7.6)	1.7% (0.7–5.2)	0.008
CD10	75.8% (54.8–89.2)	86.7% (81.5–96.6)	0.01

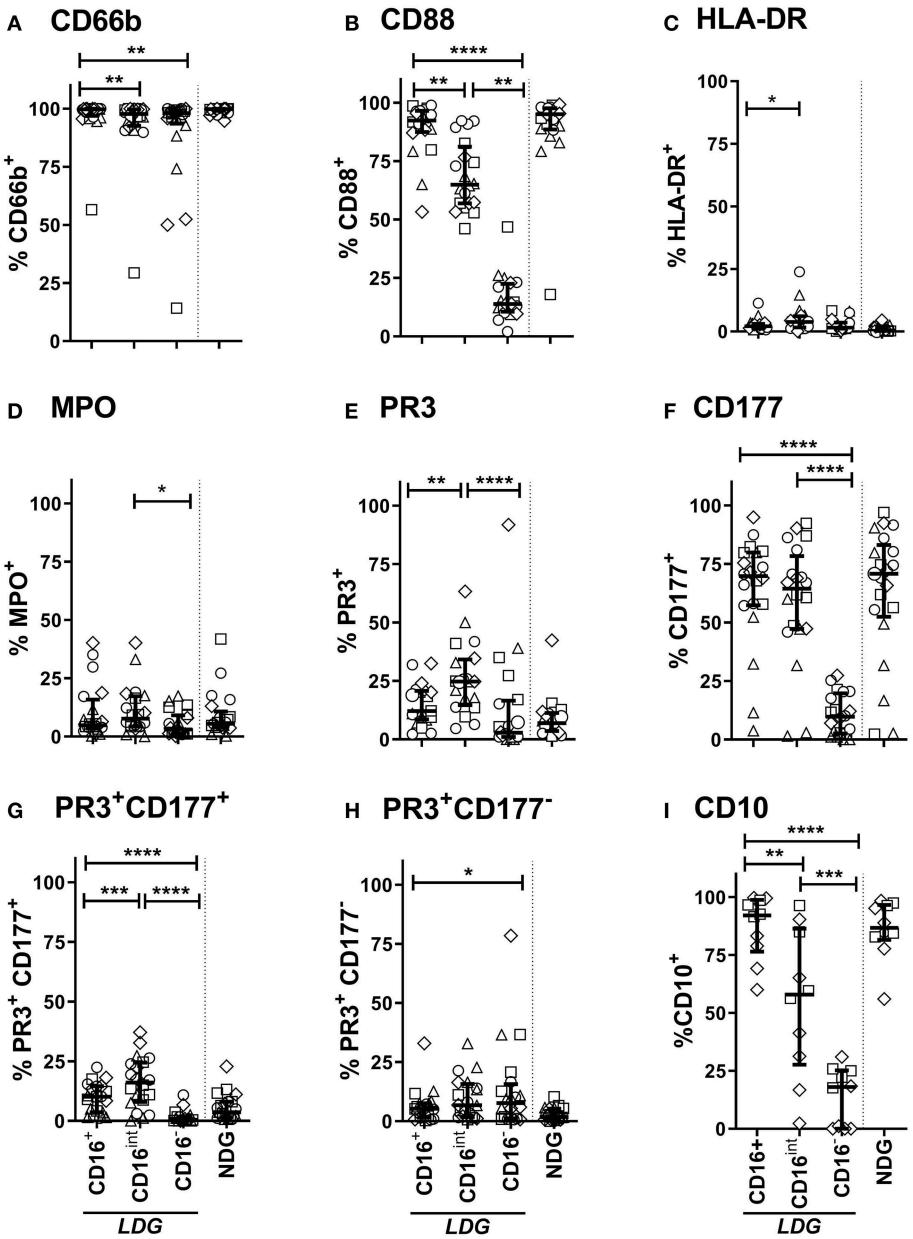


FIGURE 3 | LDG Surface immune markers vary according to CD16 expression. Surface expression of CD66b **(A),** CD88 (C5Ar) **(B),** HLA-DR **(C),** MPO **(D),** PR3 **(E),** CD177 **(F),** PR3 CD177 co-expression **(G),** PR3 independent of CD177 **(H),** and CD10 **(I)** on $CD16^{+}$, $CD16^{int}$and $CD16^{-}$ LDG subsets are represented. Equivalent surface expression on NDG is represented beyond the dotted line for visual comparison. Data are from Age-matched healthy control ($\triangle n = 5$), Disease control ($\square$ $n = 5$), Remission AAV ($\circ$ $n = 6$), and Acute AAV ($\diamond$ $n = 4$) and presented as median with IQR. *Differences between LDG subsets were analyzed using Friedman's paired test, with post hoc comparison of groups using Dunn's test.* $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

and $CD16^{-}$ LDGs (98%) were CD66b positive (**Figure 3A**). The alternative pathway of complement activation has recently been identified as a key pathogenic force in AAV (37). Expression of the C5a receptor (CD88), was markedly reduced on $CD16^{-}$ cells compared to $CD16^{+}$ (13.8%, IQR 10.6–22.5, vs. 92.3%, 87.4–96.5, respectively), with an intermediate phenotype in $CD16^{int}$ cells (**Figure 3B**). Although thought to be restricted to professional antigen-presenting cells HLA-DR is present on LDGs (**Figure 3C**) with $CD16^{int}$ LDGs (3.85% IQR 1.6–6.0) showing highest expression, compared to 0.3% (IQR 0.1–1.6) in NDGs. Attention was then focused on the autoantigens MPO and PR3 as they are presumed to be directly involved in cellular activation by ANCA. MPO expression was similar between LDGs and NDGs, with the highest observed in the $CD16^{int}$ population (7.7% IQR 4.3–17.3) (**Figure 3D**). However, when the source of cells was stratified by disease, we observed that MPO surface

expression on the CD16$^-$ LDGs fraction was virtually absent in patients with AAV (1.4% IQR 0.9–4.2, **Supplementary Figure 2**). Expression of the autoantigen PR3 on LDGs was largely similar to NDGs, although again, a relative reduction of PR3 expression was observed in CD16- cells (**Figure 3E**). To explore this further, we examined expression of CD177, which is required for surface presentation of PR3 on neutrophils (38). Interestingly, CD177 was markedly reduced on CD16$^-$ LDGs (9.8% IQR 2.5–19.8), compared to CD16+ LDGs (69.8%, IQR 57.3–79.9), which appeared similar to NDGs (**Figure 3F**). Accordingly, PR3/CD177 surface co-expression was virtually absent on CD16$^-$ LDGs (0.5% IQR 0.0–1.7, **Figure 3G**), whereas any PR3 that was expressed on the surface of CD16$^-$ cells appeared to be independent of CD177 (**Figure 3H**). Taken together, these findings indicate that the CD16$^-$ LDG population is phenotypically distinct from other LDGs and NDGs. To address the hypothesis that these cells were immature neutrophils, we examined expression of CD10, a marker of granulocyte maturity only expressed at the segmented stage of neutrophil development. We found that CD10 expression (**Figure 3I**) mirrored that of CD16 and CD88. CD16$^+$ LDGs (98.1% IQR 76.4–99.7) had the highest expression (similar to NDGs), while CD16$^-$ LDGs (18.0% IQR 0–25.2) had the lowest.

Nuclear Morphology Defines the Maturity of LDG Subsets

About one third of the LDG population was CD16 and CD10 negative, suggesting that these cells are immature

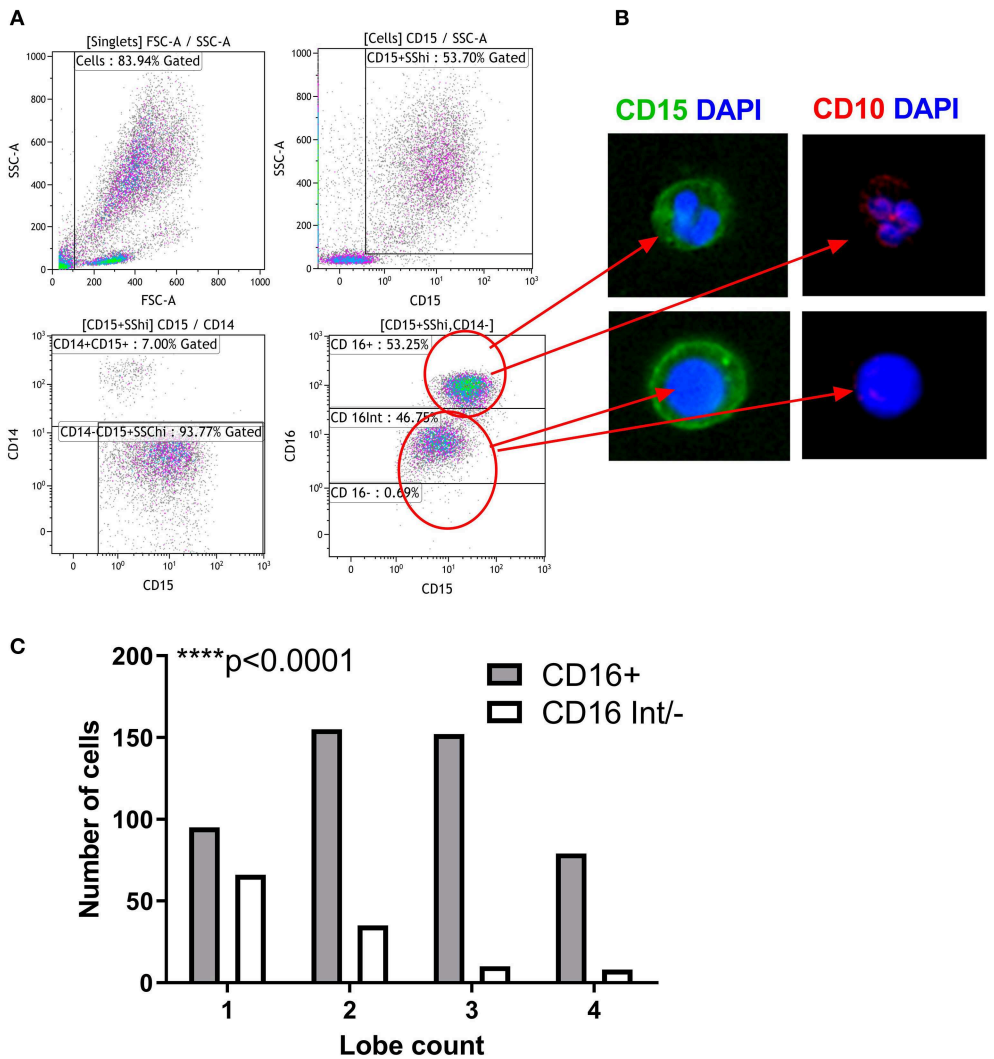


FIGURE 4 | Nuclear Morphology defines the maturity of LDG subsets. Gating strategy for identification of LDG subsets by imaging flow cytometry: after gating on SSC^{hi}CD15$^+$CD14$^-$ singlets, LDG subsets were defined as CD16$^+$ and CD16$^{int/-}$. **(A)** Representative images of LDG subsets shown. Images obtained at 60x magnification on ImageStream X MkII using DAPI nuclear stain (blue), CD15 (green), and CD10 (red). Merged images show the multilobed CD16$^+$ population and the circular/kidney-shaped nuclei of the CD16$^{int/-}$ population and the CD10 staining of the CD16$^+$ subpopulations. **(B)** LDG subsets can be defined by their nuclear lobe count, a marker of granulocyte maturity. CD16$^+$ LDGs have predominantly multilobed nuclei while CD16$^{int/-}$ have mostly single lobed nuclei. *Chi square test* **(C)**. $^{****}p < 0.0001$.

neutrophils possibly representing myeloblasts, promyelocytes, or metamyelocytes. Therefore, we used imaging flow cytometry to further characterize the LDG subsets simultaneously by both surface marker expression and nuclear morphology (**Figure 4**). Merged images clearly show that the $CD15^{+}CD16^{+}$ cells are multilobed while the $CD15^{+}CD16^{int/-}$ cells have circular or kidney-shaped nuclei (**Figures 4A,B**), with the latter combining an immature nuclear shape with absence of CD10 staining, compared to the $CD15^{+}CD16^{+}$ population, which is strongly positive for CD10. To further validate these findings, we quantified the number of nuclear lobes in all cells in the $CD15^{+}CD16^{+}$ and $CD15^{+}CD16^{int/-}$ populations using automated analysis of an imaging cytometry dataset. $CD16^{+}$ LDGs had a median of 2 lobes whereas the $CD16^{int/-}$ LDGs had a median of 1 lobe (**Figure 4C**).

Imaging Flow Cytometry Allows Definitive Phenotyping of PBMC Cell Populations

Having established that LDGs are heterogeneous based on nuclear morphology and CD16/CD10 expression, we then sought to link nuclear morphology to additional surface markers to more accurately define LDGs in the context of the PBMC population and to confirm that the $CD16^{-}$ population are not eosinophils, which are also $CD15+CD16^{-}$. We found that $CD15^{+}CD16^{+}CD10^{+}$ cells with multi-lobed nuclei had low expression of the putative myeloid-derived suppressor cell marker LOX-1 (**Figure 5A**). Conversely, the corresponding $CD16^{int/-}$ LDG population with round or bean-shaped nuclei had high LOX-1 expression. Eosinophils, with classical hinged nuclei, were clearly identified as siglec-8 positive and, like monocytes, were distinct from $CD15^{+}CD16^{-}$ LDGs (**Figure 5A**). CD10 and CD16 expression was highly correlated on both LDGs and NDGs (**Figure 5B**), suggesting that the observed low CD16 expression was due to neutrophil immaturity rather than down-regulation or shedding of this Fc receptor due to neutrophil activation or apoptosis (39, 40). The bimodal surface expression of CD16 was mirrored by CD10, whereas LOX-1 expression was unimodal (**Supplementary Figure 3**). LOX-1 was highly expressed on LDGs when compared to NDGs (**Figure 5C**); this marker may thus be useful for whole blood identification of the LDG population.

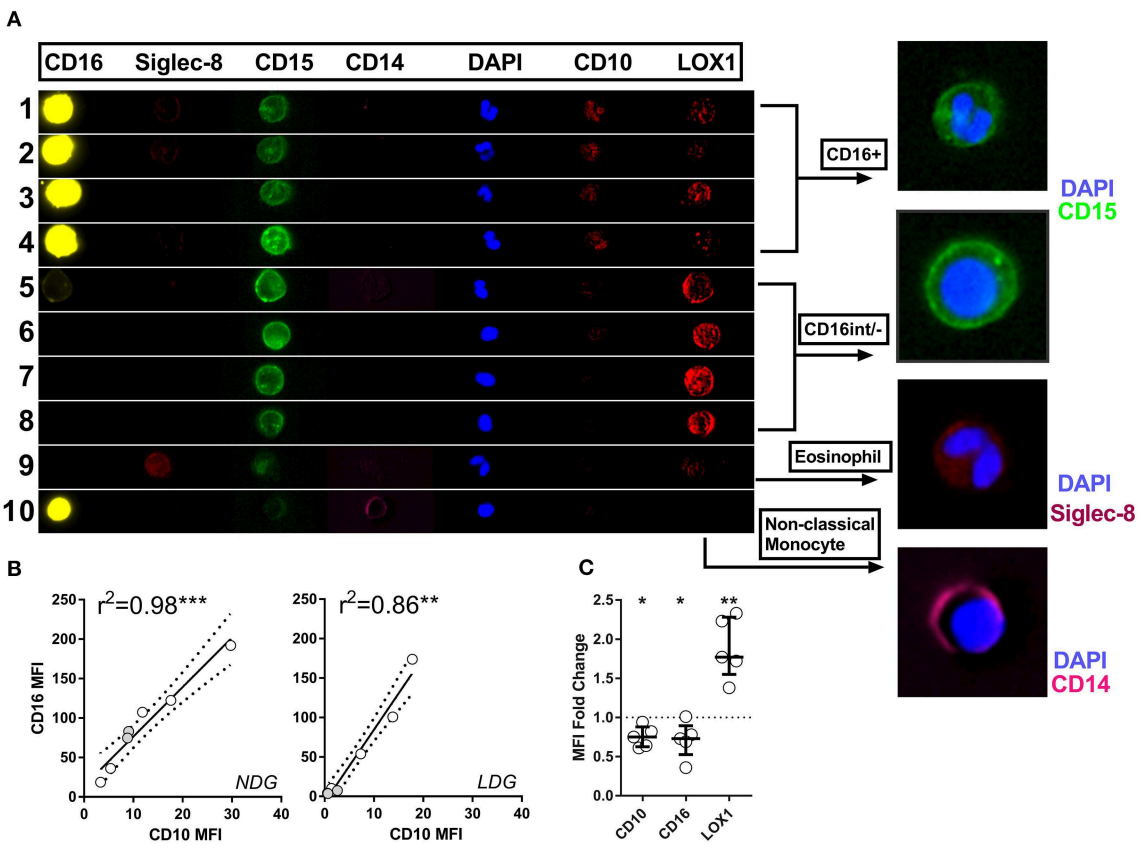


FIGURE 5 | Imaging flow cytometry allows definitive phenotyping of PBMC cell populations. Representative images are shown of various cell populations from a patient with active AAV, with each row illustrating the separate channels, alongside merged images **(A)**. Rows 1–4 show $CD15^{+}CD16^{+}$ granulocytes, rows 5–8 $CD16^{int/-}$ granulocytes, row 9 eosinophil and row 10 monocyte. CD10 expression correlates closely with CD16 expression in both LDGs and NDGs. *Spearman correlation* **(B)**. Differential CD10, CD16 and LOX-1 expression between LDG and NDG was then assessed by analyzing the fold change in MFI between the two populations. *T test* $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ **(C)**.

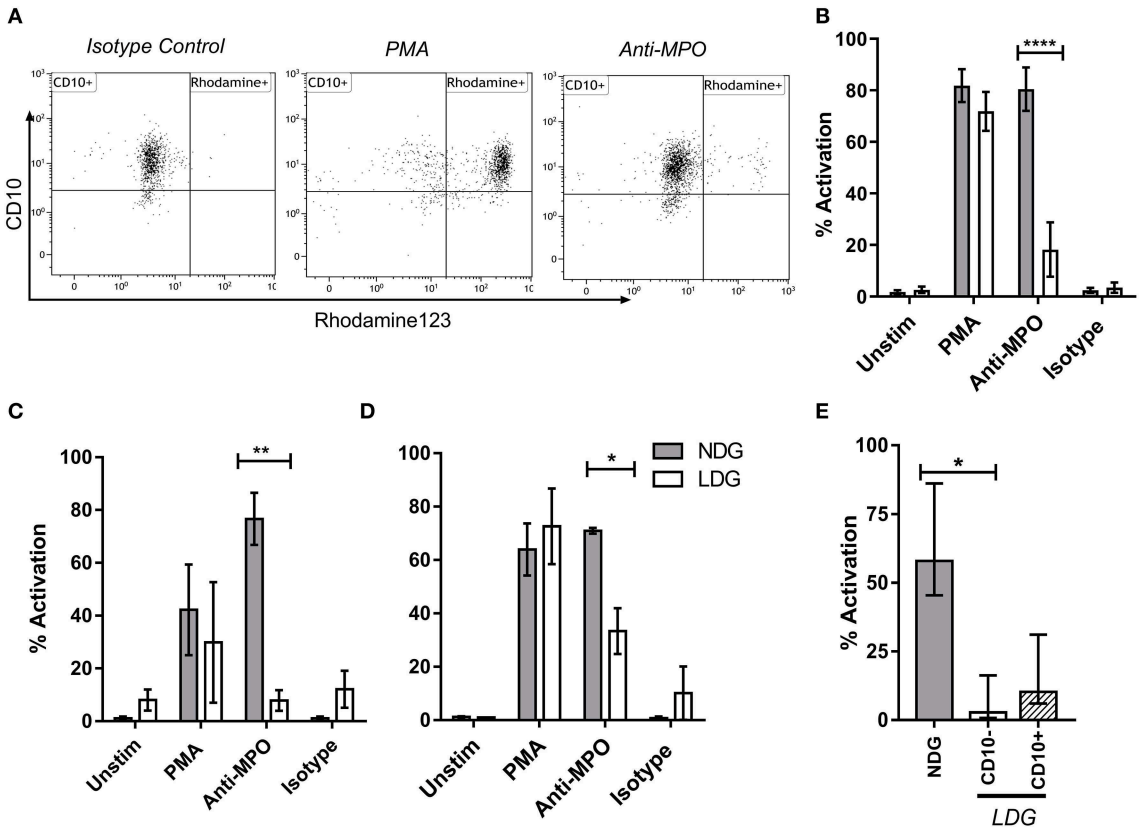


FIGURE 6 | LDG stimulated with anti-MPO display decreased ROS production compared to NDG. LDG and NDG samples were stimulated with isotype control, anti-MPO antibodies, and with PMA, after loading with DHR123. ROS production was quantified as the % of Rhodamine123$^+$ cells. Representative dot plots are shown demonstrating the % rhodamine123$^+$ LDGs from a patient with active AAV following exposure to isotype control, PMA and anti-MPO **(A)**. ROS production by LDGs and NDGs from Acute AAV patients **(B)**, healthy controls **(C),** and umbilical cord blood **(D)**, treated with DHR123 alone (unstim), or with DHR123 plus PMA, anti-MPO, or isotype control is shown. As ROS production was reduced in LDGs following anti-MPO stimulation, we tested whether this effect was restricted to the CD10$^-$ subset **(E)**, *Friedman test with Dunn's multiple comparison test, n = 5. Differences between LDG and NDG response to stimulus were analyzed using 2-way ANOVA with Sidak's multiple comparison test, n = 3 healthy control*; *n = 5 Acute AAV, n = 2 Cord blood* $^*p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$.

LDGs Stimulated With Anti-MPO Antibodies Are Hypo-Responsive Compared to NDGs

The production of reactive oxygen species (ROS) by neutrophils in response to anti-MPO and anti-PR3 antibodies is a well-defined functional readout of relevance to AAV (41). Dihydro-rhodamine123 is a non-fluorescent molecule which gets converted to a fluorescent molecule, rhodamine123 in the presence of ROS. As expected, NDGs produced high levels of ROS, as determined by conversion of di-hydro rhodamine to rhodamine, when stimulated with anti-MPO antibodies. However, unexpectedly, LDGs responded relatively poorly to this stimulus, despite having a good response to PMA (**Figures 6A,B**). We confirmed that LDGs from different clinical settings were relatively hypo-responsive to anti-MPO antibodies using healthy control and umbilical cord blood (**Figures 6C,D**). To test whether this response correlated with neutrophil maturity, we stratified ROS production in the LDG fraction by CD10 expression. CD10$^-$ cells were completely unresponsive to anti-MPO antibodies, while CD10$^+$ cells displayed an intermediate response (**Figure 6E**). When autoantigen surface availability was separated by CD16 expression and disease status, CD16$^-$ cells from patients with AAV lacked surface MPO (**Supplementary Figure 2**). However, this was not observed in control cells, so cannot fully explain the lack of response in CD10$^-$ cells.

DISCUSSION

Low density granulocytes that appear in the PBMC layer of peripheral leukocytes are recognized in diseases ranging from cancer to sepsis and autoimmunity. However, no definitive surface or functional markers for LDGs have been defined so the literature pertaining to these cells is inconsistent. We have studied LDGs in the severe autoimmune condition AAV, identifying a clear expansion in active AAV. These LDGs were phenotypically characterized by two broad cell types: CD16$^+$/CD10$^+$ LDGs that shared many characteristics of NDGs, and CD16$^{int/-}$/CD10$^-$ that displayed features consistent with immature neutrophils. Although other LDG work in autoimmune disease settings has classified LDGs as pro-inflammatory, mainly attributing to their ability to undergo

NETosis rapidly, their response to autoantibodies hasn't been examined. Using a disease-specific ROS production assay we have shown that LDGs are unresponsive to anti-MPO stimulation, thus suggesting that LDGs unlike NDGs do not contribute to vascular damage via ROS production. Our findings suggest that the LDGs in AAV are heterogeneous, comprise a significant fraction of immature granulocytes and are unresponsive to autoantibody stimulation despite expressing MPO.

Recently, due to a surge in studies suggesting neutrophil plasticity, the concept of neutrophils as terminally differentiated innate immune cells has been brought into question and key immunomodulatory roles have been ascribed to them. Several gene expression profiling studies in AAV identified granulocyte signatures in PBMC fractions isolated by density gradient (42, 43). The neutrophil related gene expression in AAV overlapped with LDG gene expression identified in lupus and was associated with disease activity and response to treatment (44). Additionally, granulocyte transcripts detected in the blood of patients with AAV were preferentially observed in the PBMC layer, with changes in this expression correlating with subsequent relapse risk (8). It is possible that expansion of the LDG population during emergency granulopoiesis in the setting of acute disease accounts for this granulocyte signal, with transcriptionally active myelocytes and metamyelocytes exiting the bone marrow in response to G-CSF, which is known to be elevated in active AAV (45). Interestingly, in the autoimmune disease systemic lupus erythematosus, the principal upregulated genes in LDGs include serine proteases, bactericidal proteins, and other peptides present in azurophilic granules and involved in neutrophil regulation of inflammatory responses (23). These findings also suggest an immature LDG phenotype in this condition, as transcription of neutrophil serine proteases is greatest at the promyelocytic stage of neutrophil differentiation and is down regulated as neutrophils mature (46). Interestingly, a recent study utilized large scale bioinformatics approach that combined gene expression data and clinical measurements in SLE, found a core signature of 10 granulopoeisis-related genes in LDGs (47).

The accumulation of relatively immature and pathologically activated granulocytic MDSCs with potent immunosuppressive activity is well-recognized in cancer and linked to poor clinical outcome (20, 48). These have also been identified in the blood of patients with sepsis (14), cancer (26, 49), HIV (15), graft vs. host disease (50), and in pregnant women (51). Interestingly, the expression of CD10 correlates with T-cell suppression, with $CD10^-$ LDGs causing T cell activation (24). An obvious question that arises is how the LDG population observed in studies in autoimmune disease relates to these granulocytic MDSCs. Attempts to answer this question have been hampered by a lack of consensus on immunophenotypic definition of these low-density cell populations (**Table 1**). Reliance upon density centrifugation to identify LDGs introduces a difficult to control variable and speaks to an urgent requirement for whole blood staining mechanisms that would allow for a concerted comparison of these cells across various diseases.

Table 1 compares in detail the characteristics of LDGs in various pathological conditions. LDGs from SLE patients have a pro-inflammatory phenotype. They secrete increased levels of type 1 interferon, TNF α and IFN-γ but show impaired phagocytic potential (3). A recent study found that SLE LDGs display an activated phenotype, exert proinflammatory effects on T cells and do not exhibit MDSC function (52). On the other hand, tumor associated neutrophils (TANs) are divided into two subgroups with anti-tumor (N1) or pro-tumor (N2) activity (53). A recent study shows that cancer-cell-derived G-CSF is necessary, but not sufficient, to mobilize immature low-density neutrophils (LDNs) that promote liver metastasis. In contrast, mature high-density neutrophils (HDNs) inhibit the formation of liver metastases (54). Interestingly, in multiple sclerosis (MS) the use of G-CSF to promote the recruitment and activation of neutrophils can exacerbate symptoms (55). Further work is necessary to determine the role of neutrophil subsets in different pathological settings, which is inhibited by the lack of standardization of nomenclature and classification of LDGs in these different fields.

Our observation of a lack of response of LDGs to anti-MPO stimulation casts doubt on their role as an active driver of vascular inflammation. It is conceivable that these cells are released through the action of G-CSF in the context of acute inflammation as part of a counter-regulatory homeostatic mechanism that helps to bring the immune system back to a resting state. However, given the presence of the autoantigens MPO and PR3 in this cell fraction and in immature neutrophils (56), it may also be reasonable to attribute to them a role in driving autoimmunity to these proteins. Indeed, G-CSF has been found to prime neutrophils to respond to ANCA stimulation and pre-treatment of a mouse model of MPO-AAV with G-CSF greatly exacerbates disease (45). Thus, the question as to whether LDGs arise after the onset of acute vasculitis, in response to systemic inflammation signals, or whether they act as drivers or initiators of endothelial injury, remains unanswered. Our data support the concept that they follow rather than initiate acute vasculitis, but this could only be addressed using *in-vivo* models or detailed study of relapsing patients.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by St. Vincent's Hospital Research ethics committee, Tallaght Hospital Research ethics committee, St. James' Hospital Research ethics committee, and Beaumont Hospital Research ethics committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ML, AC, GB, and EM designed and managed the project, and collated and analyzed results. AU, AC, and AD performed *in vitro* and *in vivo* experiments and analyzed data. JC and BM analyzed imaging cytometry data. KB and SD provided UCB samples. KH and JH derived clinical phenotype data.

AF and UF provided rheumatoid arthritis data and samples. SM, SC, and ADB recruited patients and collected samples. All authors contributed to manuscript preparation. RL facilitated recruitment and sample collection.

FUNDING

AC was supported by Irish Research Council grant GOIPD/2014/252. The project was funded by Science Foundation Ireland grant 11/Y/B2093 and Meath Foundation grant 203170.13161. SC was supported by an Irish Clinical Academic Training (ICAT) fellowship, funded by the Wellcome Trust and the Health Research Board (Grant Number 203930/B/16/Z).

ACKNOWLEDGMENTS

Imaging Cytometry was performed at the Flow Cytometry Facility, School of Biochemistry and Immunology, Trinity College Dublin.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02603/full#supplementary-material

Supplementary Figure 1 | Schematic Diagram depicting the difference between Standard, modified Percoll, and lymphoprep isolations. LDGs co-localize with PBMC layer at a density of <1.077 g/ml during density gradient preparations. Schematic representation of PBMC/LDG, neutrophil and RBC isolation from whole blood using three density (55, 65, and 85%) standard Percoll method **(A)**. PBMC/LDG and neutrophil isolation using two density (55 and 65%) modified Percoll density gradient separation **(B)** and PBMC/LDG and neutrophil isolation using 2 density Lymphoprep density gradient separation.

Supplementary Figure 2 | CD16- LDG from patients with active AAV lack surface expression of the autoantigen MPO. Surface expression of MPO on CD16 subsets of LDG were analyzed for healthy control (n = 5) **(A)**, disease control (n = 5) **(B)** and AAV patients (n = 10) **(C)**. MPO surface expression was increased on $CD16^+$ LDG in AAV patients, compared to healthy and disease control. Although, no differences were observed in MPO expression within CD16 subsets in healthy and disease controls, $CD16^-$ LDG from AAV patients had very low MPO expression compared to $CD16^+$ and $CD16^{int}$, groups were compared using repeated-measures one-way ANOVA, $^*p < 0.05$.

Supplementary Figure 3 | CD16 and CD10, but not LOX-1, have a bimodal distribution in LDG. Histogram overlays of $CD15^+SSC^{hi}CD14^-$ singlets in LDG (dark gray) and NDG (light gray) preparations are displayed from a representative acute AAV patient sample showing surface expression of CD16 **(A)**, CD10 **(B)**, and LOX-1 **(C)**.

Supplementary Table 1 | Panel of antibodies used in flow cytometry experiments.

Supplementary Table 2 | Panel of antibodies used in G-CSF treated mice flow cytometric experiments.

REFERENCES

1. Mócsai A. Diverse novel functions of neutrophils in immunity, inflammation, and beyond. *J Exp Med*. (2013) 210:1283. doi: 10.1084/jem.20122220
2. Fridlender ZG, Sun J, Kim S, Kapoor V, Cheng G, Ling L, et al. Polarization of tumor-associated neutrophil phenotype by TGF-beta: N1 versus N2 TAN. *Cancer Cell*. (2009) 16:183–94. doi: 10.1016/j.ccr.2009.06.017
3. Denny MF, Yalavarthi S, Zhao W, Thacker S G, Anderson M, Kaplan MJ. A distinct subset of proinflammatory neutrophils isolated from patients with systemic lupus erythematosus induces vascular damage and synthesizes type I IFNs. *J Immunol*. (2010) 184:3284–97. doi: 10.4049/jimmunol.0902199
4. Schonermarck U, Csernok E, Gross WL. Pathogenesis of anti-neutrophil cytoplasmic antibody-associated vasculitis: challenges and solutions 2014. *Nephrol Dial Transplant*. (2015) 30 (Suppl 1): i46–52. doi: 10.1093/ndt/gfu398
5. Falk RJ, Jennette J C. Anti-neutrophil cytoplasmic autoantibodies with specificity for myeloperoxidase in patients with systemic vasculitis and idiopathic necrotizing and crescentic glomerulonephritis. *N Engl J Med*. (1988) 318:1651–7. doi: 10.1056/NEJM198806233182504
6. Williams RC, Staud R, Malone CC, Payabyab J, Byres L, Underwood D. Epitopes on proteinase-3 recognized by antibodies from patients with Wegeners granulomatosis. *J Immunol*. (1994) 152:4722–37.
7. Booth AD, Almond M K, Burns A, Ellis P, Gaskin G, Jayne D RW. Outcome of ANCA-associated renal vasculitis: a 5-year retrospective study. *Am J Kidney Dis*. (2003) 41:776–84. doi: 10.1016/S0272-6386(03)00025-8
8. Jones BE, Yang J, Muthigi A, Hogan S L, Hu Y, Ciavatta D J. Gene-specific DNA methylation changes predict remission in patients with ANCA-associated vasculitis. *J Am Soc Nephrol*. (2017) 28:1175–87. doi: 10.1681/ASN.2016050548
9. Cloke T, Munder M, Taylor G, Müller I, Kropf P. Characterization of a novel population of low-density granulocytes associated with disease severity in HIV-1 infection. *PLoS ONE*. (2012) 7:e48939. doi: 10.1371/journal.pone.0048939
10. Ssemaganda A, Kindinger L, Bergin P, Nielsen L, Mpendo J, Ssetaala A, Müller I. Characterization of neutrophil subsets in healthy human pregnancies. *PLoS ONE*. (2014) 9:e85696. doi: 10.1371/journal.pone.0085696
11. Porntrakulpipat S, Depner K R, Moennig V. Are low-density granulocytes the major target cells of classical swine fever virus in the peripheral blood? *J Vet Med Ser*. (2001) 48:593–602. doi: 10.1046/j.1439-0450.2001.00477.x
12. Hoffmann MH, Bruns H, Bäckdahl L, Neregård P, Niederreiter B, Herrmann M, Holmdahl R. The cathelicidins LL-37 and rCRAMP are associated with pathogenic events of arthritis in humans and rats. *Ann Rheumat Dis*. (2013) 72:1239–48. doi: 10.1136/annrheumdis-2012-202218
13. Sagiv JY, Voels S, Granot Z. Isolation and characterization of low- vs. high-density neutrophils in cancer. *Methods Mol Biol*. (2016) 1458:179–93. doi: 10.1007/978-1-4939-3801-8_13
14. Darcy CJ, Minigo G, Piera KA, Davis JS, McNeil YR, Woodberry T. Neutrophils with myeloid derived suppressor function deplete arginine and constrain T cell function in septic shock patients. *Crit Care*. (2014) 18:R163. doi: 10.1186/cc14003
15. Cloke T, Munder M, Bergin P, Herath S, Modolell M, Taylor G, et al. Phenotypic alteration of neutrophils in the blood of HIV seropositive patients. *PLoS ONE*. (2013) 8:e72034. doi: 10.1371/journal.pone.0072034
16. Wright HL, Makki FA, Moots RJ, Edwards SW. Low-density granulocytes: functionally distinct, immature neutrophils in rheumatoid arthritis with altered properties and defective TNF signalling. *J Leukoc Biol*. (2016) 101:599–611. doi: 10.1189/jlb.5A0116-022R
17. Lin AM, Rubin C J, Khandpur R, Wang J Y, Riblett M, Bruce AT. Mast cells and neutrophils release IL-17 through extracellular trap formation in psoriasis. *J. Immunol*. (2011) 187:490–500. doi: 10.4049/jimmunol.1100123
18. Deng Y, Ye J, Luo Q, Huang Z, Peng Y, Xiong G, et al. Low-density granulocytes are elevated in mycobacterial infection and associated

with the severity of tuberculosis. *PLoS ONE*. (2016) 11:e0153567. doi: 10.1371/journal.pone.0153567
19. Brandau S, Trellakis S, Bruderek K, Schmaltz D, Steller G, Elian M, et al. Myeloid-derived suppressor cells in the peripheral blood of cancer patients contain a subset of immature neutrophils with impaired migratory properties. *J Leukoc Biol*. (2011) 89:311–7. doi: 10.1189/jlb.0310162
20. Condamine T, Dominguez GA,Youn JI, Kossenkov AV, Mony S, Gabrilovich DI. Lectin-type oxidized LDL receptor-1 distinguishes population of human polymorphonuclear myeloid-derived suppressor cells in cancer patients. *Sci Immunol*. (2016) 1:aaf8943. doi: 10.1126/sciimmunol.aaf8943
21. Nan JY, Xing F, Hu BJ, Tang XH, Dong MY, Li X. Endoplasmic reticulum stress induced LOX-1+ CD15+ polymorphonuclear myeloid-derived suppressor cells in hepatocellular carcinoma. *Immunology*. (2018) 154:144–55. doi: 10.1111/imm.12876
22. Rodriguez PC, Ernstoff M S, Hernandez C, Atkins M, Zabaleta J, Ochoa AC. Arginase I-producing myeloid-derived suppressor cells in renal cell carcinoma are a subpopulation of activated granulocytes. *Cancer Res*. (2009) 69:1553–60. doi: 10.1158/0008-5472.CAN-08-1921
23. Villanueva E, Yalavarthi S, Berthier CC, Hodgin JB, Khandpur R, Kaplan MJ. Netting neutrophils induce endothelial damage, infiltrate tissues, and expose immunostimulatory molecules in systemic lupus erythematosus. *J Immunol*. (2011) 187:538–52. doi: 10.4049/jimmunol.1100450
24. Marini O, Costa S, Bevilacqua D, Calzetti F, Tamassia N, Spina C, Scapini P. Mature $CD10^+$ and immature CD10– neutrophils present in G-CSF–treated donors display opposite effects on T cells. *Blood*. (2017) 129:1343–56. doi: 10.1182/blood-2016-04-713206
25. Carmona-Rivera C, Kaplan MJ. Low-density granulocytes: a distinct class of neutrophils in systemic autoimmunity. *Semin Immunopathol*. (2013) 35:455–63. doi: 10.1007/s00281-013-0375-7
26. Dumitru CA, Moses K, Trellakis S, Lang S, Brandau S. Neutrophils and granulocytic myeloid-derived suppressor cells: immunophenotyping, cell biology and clinical relevance in human oncology. *Cancer Immunol Immunother*. (2012) 61:1155–67. doi: 10.1007/s00262-012-1294-5
27. Zhou L, Somasundaram R, Nederhof R F, Dijkstra G, Faber KN, Fuhler GM. Impact of human granulocyte and monocyte isolation procedures on functional studies. *Clin Vacc Immunol*. (2012) 19:1065–74. doi: 10.1128/CVI.05715-11
28. Scapini P, Marini O, Tecchio C, Cassatella MA. Human neutrophils in the saga of cellular heterogeneity: insights and open questions. *Immunol Rev*. (2016) 273:48–60. doi: 10.1111/imr.12448
29. Jennette JC, Falk RJ, Bacon PA, Basu N, Cid MC, Watts RA. 2012 revised International Chapel Hill Consensus Conference Nomenclature of Vasculitides. *Arthritis Rheum*. (2013) 65:1–11. doi: 10.1002/art.37715
30. Antal-Szalmas P, van Strijp JAG, Weersink AJL, Verhoef J, van Kessel KPM. Quantitation of surface CD14 on human monocytes and neutrophils. *J Leukocyte Biol*. (1997) 61:721–8. doi: 10.1002/jlb.61.6.721
31. Hu N, Westra J, Huitema MG, Bijl M, Brouwer E, Kallenberg CG. Coexpression of CD177 and membrane proteinase 3 on neutrophils in antineutrophil cytoplasmic autoantibody-associated systemic vasculitis: anti-proteinase 3-mediated neutrophil activation is independent of the role of CD177-expressing neutrophils. *Arthritis Rheum*. (2009) 60:1548–57. doi: 10.1002/art.24442
32. Coughlan AM, Harmon C, Whelan S, O'Brien E C, O'Reilly VP, Little MA. Myeloid engraftment in humanized mice: impact of granulocyte-colony stimulating factor treatment and transgenic mouse strain. *Stem Cells Dev*. (2016) 25:530–41. doi: 10.1089/scd.2015.0289
33. Fleit HB, Wright SD, Durie CJ, Valinsky JE, Unkeless JC. Ontogeny of Fc receptors and complement receptor (CR3) during human myeloid differentiation. *J Clin Invest*. (1984) 73:516–25. doi: 10.1172/JCI111238
34. Lund-Johansen F, Terstappen LW? Differential surface expression of cell adhesion molecules during granulocyte maturation. *J Leukoc Biol*. (1993) 54:47–55. doi: 10.1002/jlb.54.1.47
35. Elghetany MT. Surface antigen changes during normal neutrophilic development: a critical review. *Blood Cells Mol Dis*. (2002) 28:260–74. doi: 10.1006/bcmd.2002.0513
36. Pedersen CC, Borup R, Fischer-Nielsen A, Mora-Jensen H, Fossum A, Cowland J, et al. (2016). Changes in gene expression during G-CSF–induced emergency granulopoiesis in humans. *J Immunol*. 1:1502690. doi: 10.4049/jimmunol.1502690
37. Charles Jennette J, Xiao H, Hu P. Complement in ANCA-associated vasculitis. *Semin Nephrol*. (2013) 33:557–64. doi: 10.1016/j.semnephrol.2013.08.006
38. Eulenberg-Gustavus C, Bähring S, Maass PG, Luft FC, Kettritz R. Gene silencing and a novel monoallelic expression pattern in distinct CD177 neutrophil subsets. *J Exp Med*. (2017) 214:2089–101. doi: 10.1084/jem.20161093
39. Werfel T, Sonntag G, Weber M H, Götze O. Rapid increases in the membrane expression of neutral endopeptidase (CD10), aminopeptidase N (CD13), tyrosine phosphatase (CD45), and Fc gamma-RIII (CD16) upon stimulation of human peripheral leukocytes with human C5a. *J Immunol*. (1991) 147:3909.
40. Hart SP, Ross J A, Ross K, Haslett C, Dransfield I. Molecular characterization of the surface of apoptotic neutrophils: implications for functional downregulation and recognition by phagocytes. *Cell Death Different*. (2000) 7:493–503. doi: 10.1038/sj.cdd.4400680
41. Falk RJ, Terrell R S, Charles L A, Jennette JC. Anti-neutrophil cytoplasmic autoantibodies induce neutrophils to degranulate and produce oxygen radicals *in vitro*. *Proc Natl Acad Sci USA*. (1990) 87:4115–9. doi: 10.1073/pnas.87.11.4115
42. Cheadle C, Berger A E, Andrade F, James R, Johnson K, Levine SM. Transcription of proteinase 3 and related myelopoiesis genes in peripheral blood mononuclear cells of patients with active Wegener's granulomatosis. *Arthritis Rheumat*. (2010) 62:1744–54. doi: 10.1002/art.27398
43. Lyons PA, McKinney EF, Rayner TF, Hatton A, Woffendin HB, Smith KG. Novel expression signatures identified by transcriptional analysis of separated leucocyte subsets in systemic lupus erythematosus and vasculitis. *Ann Rheum Dis*. (2010) 69:1208–13. doi: 10.1136/ard.2009.108043
44. Grayson PC, Carmona-Rivera C, Xu L, Lim N, Gao Z, Asare A. Neutrophil-related gene expression and low-density granulocytes associated with disease activity and response to treatment in antineutrophil cytoplasmic antibody-associated vasculitis. *Arthr Rheumatol*. (2015) 67:1922–32. doi: 10.1002/art.39153
45. Freeley SJ, Coughlan AM, Popat RJ, Dunn-Walters DK, Robson MG. Granulocyte colony stimulating factor exacerbates antineutrophil cytoplasmic antibody vasculitis. *Ann Rheumat Dis*. (2013) 72:1053–8. doi: 10.1136/annrheumdis-2012-202160
46. Cowland JB, Borregaard N. The individual regulation of granule protein mRNA levels during neutrophil maturation explains the heterogeneity of neutrophil granules. *J Leukoc Biol*. (1999) 66:989–95. doi: 10.1002/jlb.66.6.989
47. Kegerreis BJ, Catalina MD, Geraci NS, Bachali P, Lipsky PE, Grammer AC. Genomic identification of low-density granulocytes and analysis of their role in the pathogenesis of systemic lupus erythematosus. *J Immunol*. (2019) 24:ji1801512. doi: 10.4049/jimmunol.1801512
48. Messmer MN, Netherby CS, Banik D, Abrams SI. Tumor-induced myeloid dysfunction and its implications for cancer immunotherapy. *Cancer Immunol Immunother*. (2015) 64:1–13. doi: 10.1007/s00262-014-1639-3
49. Solito S, Marigo I, Pinton L, Damuzzo V, Mandruzzato S, Bronte V. Myeloid-derived suppressor cell heterogeneity in human cancers. *Ann N Y Acad Sci*. (2014) 1319:47–65. doi: 10.1111/nyas.12469
50. Rieber N, Wecker I, Neri D, Fuchs K, Schafer I, Brand A, et al. Extracorporeal photopheresis increases neutrophilic myeloid-derived suppressor cells in patients with GvHD. *Bone Marrow Transplant*. (2014) 49:545–52. doi: 10.1038/bmt.2013.236
51. Kostlin N, Kugel H, Spring B, Leiber A, Marme A, Henes M, et al. Granulocytic myeloid derived suppressor cells expand in human pregnancy and modulate T-cell responses. *Eur J Immunol*. (2014) 44:2582–91. doi: 10.1002/eji.201344200
52. Rahman S, Sagar D, Hanna R N, Lightfoot YL, Mistry P, Casey KA. Low-density granulocytes activate T cells and demonstrate a non-suppressive role in systemic lupus erythematosus. *Ann Rheum Dis*. (2019) 78:957–66. doi: 10.1136/annrheumdis-2018-214620

53. Sagiv JY, Michaeli J, Assi S, Mishalian I, Kisos H, Levy L, Granot Z. Phenotypic diversity and plasticity in circulating neutrophil subpopulations in cancer. *Cell Rep*. (2015) 10:562–73. doi: 10.1016/j.celrep.2014.12.039
54. Hsu BE, Tabariès S, Johnson RM, Andrzejewski S, Senecal J, Siegel PM. Immature low-density neutrophils exhibit metabolic flexibility that facilitates breast cancer liver metastasis. *Cell Rep*. (2019) 27:3902–15.e3906. doi: 10.1016/j.celrep.2019.05.091
55. Casserly CS, Nantes J C, Whittaker Hawkins RF, Vallières L. Neutrophil perversion in demyelinating autoimmune diseases: Mechanisms to medicine. *Autoimmun Rev*. (2017) 16:294–307. doi: 10.1016/j.autrev.2017.01.013
56. Strobl H, Takimoto M, Majdic O, Fritsch G, Scheinecker C, Hocker P, et al. Myeloperoxidase expression in CD34+ normal human hematopoietic cells. *Blood*. (1993) 82:2069. doi: 10.1182/blood.V82.7.2069.2069

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.

frontiers in Immunology

REVIEW
published: 06 December 2019
doi: 10.3389/fimmu.2019.02855

Personalized Medicine in ANCA-Associated Vasculitis ANCA Specificity as the Guide?

Zachary S. Wallace[1,2,3,4]* *and John H. Stone*[2,3,4]

[1] Clinical Epidemiology Program, Mongan Institute, Boston, MA, United States, [2] Rheumatology Unit, Division of Rheumatology, Allergy, and Immunology, Boston, MA, United States, [3] Massachusetts General Hospital, Boston, MA, United States, [4] Harvard Medical School, Boston, MA, United States

Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is a small- to medium-vessel necrotizing vasculitis responsible for excess morbidity and mortality (1). The AAVs, which include granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA), and eosinophilic granulomatosis with polyangiitis (EGPA), are among the most difficult types of vasculitis to treat. Although clinicopathologic disease definitions have been used traditionally to categorize patients into one of these three diagnoses, more recently ANCA specificity for either proteinase 3 (PR3) or myeloperoxidase (MPO) has been advocated for the purpose of disease classification (2). This is because differences in genetics, pathogenesis, risk factors, treatment responses, and outcomes align more closely with PR3- or MPO-ANCA type than with the clinocopathologic diagnosis. Moreover, classifying patients as GPA or MPA can be challenging because biopsies are not obtained routinely in most cases and existing classification systems can provide discrepant classification for the same patient (3). In this review, we address the recent literature supporting the use of ANCA specificity to study and personalize the care of AAV patients (**Table 1**). We focus particularly on patients with GPA or MPA.

Keywords: ANCA–associated vasculitis, vasculilis, personalized medicine, genetics, pathogenesis

OPEN ACCESS

Edited by:
Federico Alberici,
University of Milan, Italy

Reviewed by:
Annette Bruchfeld,
Karolinska Institutet (KI), Sweden
Maria I. Bokarewa,
University of Gothenburg, Sweden

***Correspondence:**
Zachary S. Wallace
zswallace@mgh.harvard.edu;
@zach_wallace_md

Specialty section:
This article was submitted to Autoimmune and Autoinflammatory Disorders, a section of the journal Frontiers in Immunology

Received: *01 October 2019*
Accepted: *20 November 2019*
Published: *06 December 2019*

Citation:
Wallace ZS and Stone JH (2019) Personalized Medicine in ANCA-Associated Vasculitis ANCA Specificity as the Guide? Front. Immunol. 10:2855. doi: 10.3389/fimmu.2019.02855

GENETIC DIFFERENCES BETWEEN PR3- AND MPO-ANCA+ PATIENTS

An estimated 20% of AAV risk is due to genetic factors (4). Several genome-wide association studies (GWAS) have identified functional genetic variants leading to altered gene expression or protein function that are thought to be relevant to AAV pathogenesis, presumably explaining the association between these variants and AAV risk (4–6). Across these studies, AAV risk has been most strongly associated with gene variants in the MHC class II region, but non-MHC associations have also been identified. The preponderance of evidence suggests that ANCA type distinguishes two groups characterized by unique genetics better than clinical phenotype (4–6).

A recent study confirmed previous reports that PR3-ANCA+ but not MPO-ANCA+ disease is associated with gene variants in *HLA-DPA1* and *DPB1*. A tri-allelic *HLA-DPB1* haplotype explained much of the genetic risk in patients with AAV. In contrast, MPO-ANCA+ disease is associated with *HLA-DQA2* and *DQB1* variants (4, 5).

TABLE 1 | Distinguishing features between PR3-ANCA+ and MPO-ANCA+ AAV.

Feature	PR3-ANCA (associated with C-ANCA pattern)	MPO-ANCA (associated with P-ANCA pattern)
Age at Diagnosis	• 45–55 years	• 60–65 years
Racial/Geographic Differences	• ↑ Northern Europe and Americas	• ↑ Asia and Southern Europe
Genetic Differences	• *HLA-DPA1* and *DPB1* • *SERPINA1* • *PRTN3*	• *HLA-DQA2* and *DQB1*
Pathogenesis	• Normally expressed on PMN cell surface • Granulomatous inflammation common • ↑ IL-6, IL-15, IL-18, IL-18 binding protein, sIL-2 receptor a, gCSF, and mCSF	• Not normally expressed on PMN cell surface • Fibrotic manifestations observed (e.g., ILD) • ↑ sIL-6 receptor, sTNF-receptor type II, neutrophil gelatinase–associated lipocalin, and soluble intercellular adhesion molecule
Risk Factors	• ± *S. aureus*	• Drugs (e.g., hydralazine, levamisole, propylthiouracil)
Clinical Phenotype	• >> GPA (~90%)	• >>> MPA (~100%)
Organ Involvement	• Ear, nose, and sinus disease • Upper airway involvement • Pulmonary nodules/cavitary lesions	• Pulmonary fibrosis • Bronchiectasis • Renal disease (↑ severity and chronicity)
Response to RTX or CYC	• RTX > CYC for remission induction	• RTX = CYC for remission induction
Outcomes	• ↑ Relapse and treatment failure • Rising titer may predict flare if RTX-treated	• ↑ Non-fatal CVD events • ↑ Death to CVD

Non-MHC variants such as those in the *SERPINA1* and *PRTN3* genes have been associated with PR3-ANCA+ but not MPO-ANCA+ disease, but variants in *PTPN22* are observed in both MPO- and PR3-ANCA+ disease (4, 5). Functional studies have expanded upon previous GWAS studies and confirmed the potential pathogenic link between genetic variants and AAV (6).

Given the associations between genetic variants and ANCA specificity, genetic testing may play a future role in identifying patients at risk for AAV. In fact, the presence of several of these variants (e.g., MHC and non-MHC) in the same individual increases the odds that the individual will develop AAV (4). However, additional studies are necessary to understand how genetic testing might be used in the clinical setting. Moreover, our knowledge of genetic associations in AAV stems from studies of patients of European descent and may be difficult to extrapolate to patients with other ancestry. One previous case-control study found that genetic variants at *DRB1* might predispose African American patients to PR3-ANCA+ AAV (7), but additional studies in patients of non-European descent are needed.

PATHOGENESIS OF PR3- AND MPO-ANCA+ AAV

The pathogenesis of AAV is complex and the precise cause or causes remain unknown, but MPO- and PR3-ANCA are generally considered to have substantial roles in the pathophysiology of most patients' disease (8). Direct proof of a relationship between the presence of these antibodies and the initiation of disease in humans, however, remains lacking, despite the fact that compelling animal models for AAV exist. This is particularly true for MPO-ANCA, as discussed below (9).

MPO- and PR3-ANCA+ AAV appear to share many features of pathogenesis, yet certain differences have also been observed. Myeloperoxidase and proteinase 3, the targets of MPO- and PR3-ANCA, respectively, are both found in neutrophil granules and monocyte lysosomes. PR3 is normally expressed on the neutrophil cell surface, more so in PR3-ANCA+ patients than healthy controls. In contrast, MPO is not spontaneously expressed on neutrophil cell surfaces but surface MPO expression is detectable after neutrophil activation (10).

In AAV, the binding of MPO- or PR3-ANCA to neutrophils induces activation and degranulation as well as adhesion and transmigration of neutrophils across the vascular endothelium, culminating in endothelial cell damage. The role of monocytes in AAV is less well understood. The pathogenic importance of MPO-ANCA is supported by the ability of these antibodies to induce a vasculitis syndrome resembling AAV when MPO-ANCA are transferred into experimental mouse models (9). The development of a similar animal model for PR3-ANCA+ AAV has been elusive to date, in part due to differences in PR3 expression in mice and humans.

Several additional observations support the importance of PR3- and MPO-ANCA in the pathogenesis of AAV. These include: (1) the great majority of patients with AAV are MPO- or PR3-ANCA+ (2, 11) there are consistent differences in clinical features of AAV according to ANCA type (see below); (3) B-cell targeted therapies and/or plasma exchange are efficacious in both PR3- and MPO-ANCA+ AAV (4, 12, 13) there is some correlation between ANCA titer and disease activity (see below); (5) transplacental transfer of MPO-ANCA is reported to have caused AAV in a newborn (6, 14); PR3-ANCA+ antibodies are known to appear in patients' blood years before clinical presentation (15); and (7) genetic variants in proteinase 3, the antigenic target of PR3-ANCA, are associated with PR3-ANCA+ AAV (see above). However, the presence of MPO- or PR3-ANCA positivity does not always correlate with disease activity, suggesting that multiple factors are necessary to induce vasculitic and granulomatous features of AAV. Such factors include genes, infections, medications, environmental exposures, the epitope specificity of ANCA, and almost certainly others (8).

Neutrophil extracellular traps (NETs) are increasingly recognized as important for the pathogenesis of autoimmune conditions, including both MPO- and PR3-ANCA+ AAV (16, 17). In normal individuals, NETs are immunogenic and have a role in trapping and killing invading extracellular microbes. Notably, NETs can activate certain immune cells, including

autoreactive B cells (16, 17), and cause end-organ damage. Spontaneous NET formation is observed more often in AAV patients than in healthy controls, likely because of stimulation of neutrophils by ANCA (16), and correlates with disease activity (17). Upon stimulation, NETs containing PR3 and MPO (16) are released in both the circulation as well as in damaged tissues.

Complement has traditionally not been thought to play a role in the pathogenesis of these "pauci-immune" vasculitides. Neither immunoglobulins nor complement components are observed prominently in the biopsy specimens from patients with AAV. The lack of immunoglobulin and complement in the renal lesions of AAV, for example, contrasts strikingly with the glomerular lesions observed in systemic lupus erythematosus, for example. However, mounting evidence suggests that activation of the alternative pathway is important to the pathogenesis of MPO-ANCA+ and, more recently, PR3-ANCA+ AAV (18, 19). A recent study by Wu et al. suggested that the classical or lectin complement pathways are activated in PR3-ANCA+ but not MPO-ANCA+ AAV (18). Moreover, avacopan, a C5a receptor inhibitor, was found in early phase trials to have efficacy in AAV and have a potential role as a glucocorticoid-sparing drug in remission induction (20). The results of an ongoing phase 3 randomized controlled trial evaluating its efficacy for remission induction will be an important proof-of-concept advance in our understanding of the role of complement activation in AAV (21).

Cytokine profiles may highlight potential differences in pathogenesis between MPO- and PR3-ANCA+ patients. Berti et al. recently compared differences in serum cytokine profiles associated with inflammation, proliferation, vascular injury, and tissue damage and repair among AAV patients grouped according to ANCA type or clinical diagnosis (22). Differences according to phenotype (e.g., PR3- vs. MPO-ANCA+ and GPA vs. MPA) were observed regardless of whether ANCA type or clinicopathologic condition was used to group patients, but the differences were more striking when PR3- and MPO-ANCA patients were compared to one another.

In the study by Berti et al., nine biomarkers were higher among the PR3-ANCA+ subset (22). These included interleukin (IL)-6, granulocyte–macrophage colony-stimulating factor, IL-15, IL-18, CXCL8/IL-8, CCL17/thymus and activation–regulated chemokine, IL-18 binding protein, soluble IL-2 receptor a, and nerve growth factor b. Four cytokines were higher in the MPO-ANCA+ subset, including soluble IL-6 receptor, soluble tumor necrosis factor receptor type II, neutrophil gelatinase–associated lipocalin, and soluble intercellular adhesion molecule. In multivariate-adjusted analyses, no cytokine levels remained significantly associated with either GPA or MPA, but several associations between cytokines and ANCA-type persisted. Additional studies are necessary to further validate these observations, particularly in larger MPO-ANCA+ cohorts.

In conclusion, the current pathogenic model of AAV suggest that MPO- and PR3-ANCA+ vasculitis share many similar pathogenic features. However, recent studies suggest that there may also be differences in complement activation and cytokine profiles according to ANCA type. Additional studies are necessary to clarify how pathogenesis may differ according to ANCA type. Differences in pathogenesis between PR3- and MPO-ANCA+ patients may identify novel treatments guided by ANCA specificity.

AAV RISK FACTORS

Several potential risk factors have been associated with the development of AAV, including environmental, drug, and infectious exposures.

Silica

Silica exposure, typically related to occupational history, has been associated with AAV in several studies. Indeed, a recent meta-analysis found that silica exposure was associated with a 2.6-fold higher odds (OR 2.6, 95% CI: 1.5–4.4) of AAV (23). This observation was true for MPA and GPA patients, suggesting that similar risk exists for both MPO- and PR3-ANCA+ subjects. In another study, MPO-ANCA+ disease was more common than PR3-ANCA+ disease (24) among cases with high silica exposure, but additional studies of this question would be useful.

Staphylococcus aureus

There is a long-standing interest in understanding potential associations between microbes, particularly chronic nasal carriage of *Staphylococcus aureus*, and the risk of AAV and flare. These suspected associations date back to early observations of infectious symptoms and secondary sinonasal infections in GPA patients with sinonasal disease (25). Subsequently, a small clinical trial in GPA, the majority of whom were presumably PR3-ANCA+, found that trimethoprim/sulfamethoxazole was associated with a 70% (HR 0.3, 95% CI: 0.1–0.8) reduction in risk of flare compared to placebo. These findings have been interpreted as support of the hypothesized role of *S. aureus* or other microbes as risk factors for AAV relapse (26). However, it has been noted that the effects of trimethoprim/sulfamethoxazole on disease activity might be mediated through mechanisms other than reducing *S. aureus* carriage, given that changes in *S. aureus* carriage on antibiotics did not necessarily relate to subsequent flare.

More recently, in a sub-study of two randomized clinical trials, GPA patients with chronic nasal *S. aureus* carriage were observed to have a higher risk of relapse than GPA patients without chronic *S. aureus* carriage (27). Again, these findings suggest an association between chronic *S. aureus* carriage and relapse risk, but the authors propose that that an underlying genetic confounder might be responsible for this observation. In GPA, and therefore likely PR3-ANCA+ AAV, we can therefore only surmise that chronic nasal carriage of *S. aureus* may be associated with the risk of flare, but further studies are needed to account for potential confounders of this observed association. There is no strong evidence base to suggest that *S. aureus* or other infections, however, are risk factors for GPA or AAV generally.

Medication-Induced AAV

A number of drug exposures, including prescribed medications and illicit substances, have been associated with AAV, though well-designed studies assessing the association between these exposures and risk of AAV are lacking. Case series and anecdotal

experience strongly suggest potential associations between drug exposures, particularly hydralazine (28), propylthiouracil (28, 29), and levamisole (typically when in adulterated cocaine) (30). The link between these medications and AAV appears to be far stronger for MPO-ANCA+ AAV than for PR3-ANCA+ AAV. Extremely high titers of MPO are often reported in these cases. In one single-center study, 13 of 30 (43%) patients with the highest MPO-ANCA titers in a large hospital's ANCA lab had been exposed to hydralazine or propylthiouracil (28).

Levamisole-contaminated cocaine has also been associated with AAV. This drug-induced syndrome is manifested often by large-joint arthralgias and cutaneous lesions, purpuric earlobe lesions, and frequently MPO-ANCA positivity but often dual positivity (50% were PR3- and MPO-ANCA+ in one study) (30). The presence of both MPO- and PR3-ANCA positivity is not seen in all cases of drug-induced AAV, but dual-positivity should raise suspicion for a drug culprit. It is important to note that the presence of ANCA positivity in the setting of drug exposure can occur without clinical features of vasculitis and is not diagnostic of AAV. The MPO-ANCA in propylthiouracil therapy, for instance, may have features that distinguish it from the pathogenic MPO-ANCA seen in classic AAV (29).

In summary, several risk factors for AAV have been proposed and these may differ according to ANCA type (e.g., *S. aureus* in PR3-ANCA+, drugs in MPO-ANCA+). However, environmental exposures, particularly to silica, appear to be a common risk factor in both PR3- and MPO-ANCA+ AAV. Additional well-designed studies are needed to better characterize environmental, infectious, and other exposure-related risk factors in AAV, particularly according to ANCA type.

ANCA TESTING FOR THE DIAGNOSIS AND MONITORING OF AAV

The initial discovery of ANCA among patients with clinical syndromes that would be characterized as GPA or MPA was a major milestone in the diagnosis and management of these conditions (31). Following the discovery of ANCA and spreading availability of testing, the diagnosis of GPA or MPA was increasingly made with confidence in the proper clinical setting, often without a biopsy.

The classic approach to ANCA testing is a two-step process (32). First, indirect immunofluorescence (IIF) is performed to detect a cytoplasmic or peri-nuclear ANCA pattern. Second, immunoassays of samples positive for ANCA by IIF are performed to confirm the IIF results and to detect ANCA specificity (e.g., PR3-ANCA or MPO-ANCA). However, accumulating evidence suggests that the test performance (e.g., receiver operating characteristic curves) of contemporary immunoassays is quite strong and less susceptible to inter-reader variability and other potential sources of imprecision than IIF (33). For instance, in a study by Damoiseaux et al., the area under the curve (AUC) of immunoassays for PR3- or MPO-ANCA was between 94 and 96%, whereas the AUC for IIF was between 84 and 92% (33). A two-step process for ANCA testing has not been found to improve test performance (33, 34). Therefore, a one-step process using only immunoassay testing for PR3- or MPO-ANCA without IIF is sufficient for diagnosing AAV. In addition to test performance, it is also important to consider the test results appropriately. Though PR3- and MPO-ANCA test results are often interpreted as positive or negative, the test performance may vary according to titer such that increasing titers may more accurately classify patients according to the correct diagnosis (34).

The role of serial ANCA testing in the management, as opposed to diagnosis, of AAV patients remains poorly defined and controversial. In a *post-hoc* analysis of the Wegener Granulomatosis Etanercept Trial (WGET) trial in which patients with GPA were randomized to conventional therapy (cyclophosphamide or methotrexate) or conventional therapy plus etanercept (35), PR3-ANCA titers correlated with disease activity and both PR3- and MPO-ANCA titers decreased during remission induction (36). Notably, the vast majority (∼73%) of patients in WGET were PR3-ANCA+ (35). A meta-analysis that includes *post-hoc* analyses of WGET as well as other studies found that a rise in ANCA levels in patients in remission was associated with a positive likelihood ratio of 2.8 (95% CI: 1.7–4.9) of a future relapse; the absence of a rise in ANCA was associated a negative likelihood ratio of 0.5 (95% CI: 0.3–0.9) of having a future relapse (37). Becoming ANCA negative, and even staying ANCA negative during follow-up, has not been observed to be a reliable indicator that a patient will achieve or maintain remission (36, 37).

The utility of repeat testing may differ according to ANCA type, especially with contemporary treatment strategies. Findings from the Rituximab in ANCA-Associated Vasculitis (RAVE) trial provided additional insights into the potential value of serial ANCA testing. In the RAVE trial, MPO- and PR3-ANCA+ patients were randomized to remission induction with either rituximab (RTX) or cyclophosphamide followed by azathioprine (CYC/AZA) (12). Approximately 67% of patients were PR3-ANCA+ in RAVE. Similar to observations from WGET, RAVE patients who became ANCA negative were not more likely to achieve clinical remission at 6 months (12). However, differences in the likelihood of becoming ANCA negative were observed according to ANCA type and treatment. In particular, PR3-ANCA+ patients treated with RTX were more likely than those treated with CYC/AZA to become ANCA negative. There was no difference in the rate of becoming ANCA negative among MPO-ANCA+ patients treated with RTX or CYC/AZA (12).

Among PR3-ANCA+ patients treated with RTX in RAVE, a *post-hoc* analysis found that a rise (defined as a doubling) in the PR3-ANCA titer was associated with a higher risk of severe relapse within 1 year, especially in those with a history of renal involvement or alveolar hemorrhage (38). This was not observed among PR3-ANCA+ patients treated with CYC/AZA in RAVE and was not observed in a *post-hoc* analysis of WGET where most patients were PR3-ANCA+ and received CYC for severe disease (36). Thus, the potential utility of serial PR3-ANCA testing may be specific to patients treated with rituximab, as opposed to other therapies.

In summary, the significance of an isolated increase in an ANCA titer without an associated change in symptoms or

findings otherwise suggestive of a disease flare is of unclear significance. Certainly not all patients who experience an increase in their ANCA titers will go on to have a disease flare and, if they do, the timing of a flare could be many months to even more than a year following the ANCA titer rise. Therefore, one must weigh the risks and benefits of treatment decisions guided by only ANCA titers (36). The ANCA type and treatment exposure may influence the predictive ability of changes in titers so the utility of serial ANCA measurements may evolve over time as our treatment regimens change. It is important to note that most studies to date evaluating the predictive value of changes in ANCA titers have been limited because of frequency of titer measurements, variations in outcome definition, and the inclusion of mostly PR3-ANCA+ patients.

CLINICAL FEATURES

Demographics

MPO-ANCA+ patients are more likely to be female and, on average, 10 years older than PR3-ANCA+ patients at presentation (39). There are also differences in the distribution of ANCA type according to race and geography such that Japanese, Chinese, and Southern European AAV patients are more likely to be MPO- rather than PR3-ANCA+ when compared with non-Japanese, non-Chinese, and Northern European AAV patients (40). In a population-based study comparing AAV incidence and features in defined geographic regions of the UK and Japan, more than 80% of cases in Japan were MPO-ANCA+. In contrast, more than 66% of cases in the UK were PR3-ANCA+ (40).

Clinical Phenotype

With regard to clinical phenotype, those who are PR3-ANCA+ more often have a presentation consistent with GPA whereas those who are MPO-ANCA+ tend to have features of MPA. However, ∼10% of patients with GPA are MPO-ANCA+; PR3-ANCA+ MPA seems to be a rarer phenomenon (41, 42).

In contrast to MPO-ANCA+ patients, those who are PR3-ANCA+ are more likely to have involvement of ears, nose, sinuses, and throat (3, 39, 43). Whereas both MPO- and PR3-ANCA+ patients can have lung involvement, those who are MPO-ANCA+ more often present with features of interstitial lung disease (e.g., fibrosing lung disease) rather than cavitary lesions and/or nodules characteristic of PR3-ANCA+ disease (44, 45). Evolving literature suggests that MPO-ANCA+ patients are at higher risk for bronchiectasis, which is often present prior to AAV presentation. In two recent cohort studies, MPO-ANCA+ subjects were found to have bronchiectasis more often than PR3-ANCA+ subjects (44, 46). In one, only MPO-ANCA+ subjects had bronchiectasis (46). In the other, MPO-ANCA+ subjects were twice as likely to have bronchiectasis (31% vs. 15%) and the bronchiectasis was more severe among the MPO-ANCA+ subjects (44). The high proportion of MPO-ANCA+ patients with bronchiectasis raises the question of whether it might predispose to MPO-ANCA+ AAV, be more likely to complicate MPO-ANCA+ AAV, or go undetected for some time before AAV comes to medical attention.

In addition to differences in respiratory tract involvement, MPO-ANCA+ patients more often have renal involvement than PR3-ANCA+ patients. Moreover, among MPO- and PR3-ANCA+ patients with renal involvement, those who are MPO-ANCA+ often present with more severe renal disease, characterized by a lower glomerular filtration rate, greater need for renal replacement therapy (31% vs. 20%), and more chronic appearing lesions on renal biopsy (47). However, ANCA type does not consistently predict the risk of end-stage renal disease (3).

Features Among Patients With Discordant ANCA Types and Clinical Phenotypes

Though ANCA type is increasingly recognized as a clinically-meaningful and standardized approach to characterizing AAV patients, the combination of ANCA type with clinical phenotype (e.g., GPA or MPA) may identify additional subtypes with unique features (**Table 2**). Several studies have suggested that there may be differences between MPO-ANCA+ GPA patients compared with those who are PR3-ANCA+ or those who are MPO-ANCA+ and have presentations consistent with MPA (45).

In one single-center cohort study by Schirmer et al., MPO-ANCA+ GPA patients were found to have limited disease more often, to have higher rates of subglottic stenosis, and to have lower rates of renal involvement compared with PR3-ANCA+ GPA patients (45). In a nephrology clinic-based cohort study by Chang et al., Chinese patients with MPO-ANCA+ GPA were found to have less severe renal disease than PR3-ANCA+ GPA patients and a lower risk of progressive renal failure (42). In contrast, disease manifestations did not differ between MPO-ANCA+ and PR3-ANCA+ GPA patients who had been enrolled in two large clinical trials (41) and studied in a *post-hoc* analysis by Miloslavsky et al. These conflicting results with regard to disease manifestations may be related to differences in study design (clinical trial vs. single center cohort study) (48), classification of GPA and MPA, and enrollment criteria. They may also reflect the limitations of attempting to address these questions in studies of small sample sizes.

Discordant associations between ANCA type and clinical phenotype may also have implications for relapse rates. In the study by Miloslavsky et al., MPO-ANCA+ GPA patients flared more often than MPO-ANCA+ MPA patients (41). Due to statistical limitations, this question could not be addressed in the

TABLE 2 | Potential differences between PR3-ANCA+ GPA and MPO-ANCA+ GPA.

Feature	PR3-ANCA+ GPA	MPO-ANCA+ GPA
Manifestations	• Renal disease more common	• Limited disease more common • Subglottic stenosis more common
Flares	• Higher flare rate	• Lower flare rate

study by Schirmer et al. (45). In the study by Chang et al., MPO-ANCA+ GPA patients had a lower flare rate than PR3-ANCA+ GPA (42).

In summary, reliable interpretations of the results of these small studies that often provide disparate results is difficult. Nevertheless, it is important to note that MPO-ANCA+ GPA patients may have a unique natural history, especially when compared with PR3-ANCA+ GPA patients.

RESPONSE TO TREATMENT ACCORDING TO ANCA TYPE

The Rituximab in ANCA-Associated Vasculitis (RAVE) trial randomized patients with severe PR3- or MPO-ANCA+ AAV to either rituximab (RTX) or cyclophosphamide/azathioprine (CYC/AZA) for induction therapy. RTX was found to be non-inferior to CYC/AZA for remission induction. In a *post-hoc* analysis of the RAVE trial, however, PR3-ANCA+ patients treated with RTX had a 2-fold higher odds (OR 2.1, 95% CI: 1.0–4.3) of achieving remission at 6 months than those treated with CYC/AZA (39). This was also true among those PR3-ANCA+ patients who were randomized in the setting of relapsing disease. There was no difference between the efficacy of RTX or CYC/AZA among MPO-ANCA+ patients with regard to achieving remission.

There may also be a difference in the efficacy of mycophenolate mofetil for remission induction in MPO-ANCA+ AAV compared with PR3-ANCA+ AAV patients without life-threatening disease (49). In the recent open-label, non-inferiority MYCYC trial, patients were randomized to mycophenolate mofetil or cyclophosphamide for remission induction. Both arms received azathioprine for maintenance therapy after remission induction. Remission rates at 6 months were similar in the mycophenolate mofetil and cyclophosphamide groups (67% vs. 61%) such that the two were found to be non-inferior to one another. Following remission, more patients in the mycophenolate mofetil group relapsed when compared with those in the cyclophosphamide group (33% vs. 19%). This difference, however, was strongly driven by relapses in PR3-ANCA+ patients, 48% of whom relapsed following mycophenolate mofetil compared with 24% following cyclophosphamide. Therefore, it may be that mycophenolate mofetil is a reasonable option for remission induction in patients who are MPO-ANCA+ but may not be ideal for patients who are PR3-ANCA+.

PR3-ANCA+ patients have been found in multiple studies to relapse more often than MPO-ANCA+ patients following remission induction (3, 45, 50). For instance, in one large United States community-based cohort, PR3-ANCA+ patients have been consistently found to have a nearly 2-fold higher risk of relapse than MPO-ANCA+ patients (3, 51). Though this cohort is largely composed of patients with renal involvement, similar observations regarding differences in the risk of relapse between PR3-ANCA+ and MPO-ANCA+ patients have been made in the RAVE trial (12); a cohort composed of patients from several large European clinical trials (52); as well as a recently described large multi-center Spanish cohort (53). All of those studies included patients with both renal and non-renal manifestations.

Patients with PR3-ANCA+ disease may also be more likely to have treatment-refractory disease. The term "treatment-refractory" is often challenging to define and differing definitions have been used across studies. In the RAVE trial, however, the term "early treatment failure" was used to describe patients whose disease was not responding to therapy at the 1 month time point. Eleven of the 12 early treatment failures in the RAVE trial were PR3-ANCA+ (54). Patients with PR3-ANCA+ disease in the RAVE trial also had a 29% chance of failing the primary outcome at 6 months because of the recurrence of active disease (54).

These observations suggest that different treatment approaches may be indicated for patients depending on ANCA type. PR3-ANCA+ patients, in contrast to MPO-ANCA+ patients, may benefit from rituximab rather than cyclophosphamide for remission induction and may also benefit from continued immunosuppression following remission given their increased risk of relapse. It may be reasonable, for example, to consider an extra one-gram infusion of rituximab at 4 months of treatment in the interest of inducing a solid disease remission. Flare rates, however, vary significantly depending on the regimen used to maintain remission.

In the recent MAINRITSAN trials comparing different contemporary maintenance strategies, those using rituximab at fixed doses had relatively low flare rates (3% at 22 months and 10% at 28 months) (55, 56) compared with the approximate 32% rate of relapse at 18 months without maintenance therapy (50) and 29% relapse rate at 28 months with azathioprine as maintenance (55). The vast majority of patients enrolled in these trials were PR3-ANCA+ so it is difficult to assess how flare rates may vary between PR3- and MPO-ANCA+ patients using contemporary maintenance strategies. One single-center experience using continuous B cell depletion with rituximab in MPO- and PR3-ANCA+ AAV patients reported a relapse rate of 20% but the duration of follow-up in this study is not reported (43), nor is relapse rate according to ANCA type. Additional studies are necessary to determine flare rates according to ANCA type using contemporary maintenance strategies and to understand the optimal long-term management of AAV according to ANCA type.

LONG-TERM OUTCOMES ACCORDING TO ANCA TYPE

As short-term AAV outcomes are optimized, increasing attention has shifted toward improving long-term outcomes. Particular focus has been paid to reducing the incidence of end-stage renal disease (ESRD) and death in AAV.

Over the last two decades, renal survival in AAV has improved, such that fewer patients are developing ESRD (57). As mentioned, MPO-ANCA+ patients with biopsy-proven disease typically have more chronic, as opposed to active, renal lesions at the time of diagnosis (47, 58–60) when compared to PR3-ANCA+ patients. However, in a large cohort study by Rhee et al., there was no difference in renal survival when MPO-

TABLE 3 | Future direction of research regarding ANCA type in AAV.

Topic	Research Question
Genetics and Pathogenesis	• Can unique genetic risk factors be used to identify patients at risk of MPO- or PR3-ANCA+ AAV before the onset of clinical symptoms or findings?
Risk Factors	• Are there modifiable risk factors for AAV that differ according to ANCA type? • How do certain drugs induce an MPO-ANCA+ AAV?
Organ Involvement	• Why might fibrotic lung disease and bronchiectasis be more common in MPO-ANCA+ AAV? • Why is renal disease more severe at presentation in MPO-ANCA+ AAV? • Why does PR3-ANCA+ AAV tend to affect the upper airway more often?
Remission Induction	• Should remission induction treatment decisions be influenced by ANCA type? • Should clinical trials be powered to detect significant differences in treatment arms stratified by ANCA type?
Remission Maintenance	• Should maintenance strategies (continuous vs. tailored immunosuppression) be driven by ANCA type? • Does monitoring PR3- or MPO-ANCA+ titer predict flares depending on treatment strategy used?
Long-Term Outcomes	• Are MPO-ANCA+ patients at higher risk for chronic lung disease like pulmonary fibrosis or chronic respiratory symptoms? • Should cardiovascular disease risk be managed differently in patients who are MPO-ANCA+?

and PR3-ANCA+ patients were compared both in unadjusted and adjusted analyses (aHR 0.92, 95% CI: 0.6–1.5) (57). In that study, the most important predictor of long-term renal survival was renal function at presentation. Similar observations have been made in other studies of ESRD outcomes associated with AAV (61).

Overall, mortality among patients with AAV is approximately 3-fold higher than that of the general population (62) but the gap in survival has improved over the last two decades (63, 64). Both PR3- and MPO-ANCA+ AAV patients are at similarly increased risk of death compared to the general population (47, 65). In other words, PR3- and MPO-ANCA+ AAV patients have a similar risk of death after accounting for differences in age- and sex- distributions between the subgroups (3). However, a recent study suggested that there may be differences in cause-specific death according to ANCA type. While more studies are needed, MPO-ANCA+ patients may be at higher risk for death due to cardiovascular disease even after accounting for differences in renal involvement, age, and sex (65). This observation is also consistent with the results of a prior study which found that MPO-ANCA+ patients may be at higher risk of non-fatal CVD events (66).

Collectively, these findings suggest that to further improve long-term survival in AAV, PR3-, and MPO-ANCA+ patients may benefit from different targeted interventions. Additional studies are necessary to determine whether the management of CVD risk should differ according to ANCA type.

ANCA-NEGATIVE AAV

While ANCA type is increasingly used to classify patients with AAV, it is important to note that a portion of patients with AAV are ANCA negative because the diagnosis of AAV remains based on clinicopathologic features rather than a positive ANCA test. This is especially true in patients with limited AAV and/or non-renal AAV (67). Rates of ANCA negativity in AAV are difficult to estimate because ANCA positivity is often used in AAV diagnostic algorithms. However, ∼20% of patients with AAV are thought to be ANCA negative; rates may be as high as 40% in those with limited AAV in historic studies (33, 67, 68). It is important to note that there are an increasing number of methods that can be used to detect PR3- or MPO-ANCA positivity and that the diagnostic test performance characteristics of these methods can vary (33). Therefore, in the setting of high diagnostic suspicion but negative ANCA testing, it may be useful to test for ANCA positivity using an alternative method for ANCA detection (68). There is limited data on the comparison of patients with ANCA negative AAV vs. PR3-ANCA+ AAV vs. MPO-ANCA+ AAV (41). Moreover, many contemporary AAV trials exclude patients who have no history ANCA positivity. Studies of ANCA negative AAV are an important avenue of future investigation.

CONCLUSIONS

ANCA testing is a useful test to establish a diagnosis of AAV in the appropriate clinical setting. ANCA testing also provides important insights into differences in genetic risk, pathogenesis, and response to treatment between PR3- and MPO-ANCA positive patients. A growing body of evidence supports the hypothesis that PR3- and MPO-ANCA+ AAV might represent distinct diseases rather than a single spectrum of disease. A number of research questions can be addressed to further advance our understanding of the potential use of ANCA type for guiding AAV care (**Table 3**). The available evidence suggests that AAV treatment might be optimized using a personalized approach guided by a patient's ANCA type.

AUTHOR CONTRIBUTIONS

ZW and JS contributed to conception of the review, literature search, manuscript drafting and revision, read the review for publication, and agree to be accountable for all aspects of the review.

FUNDING

This work was funded by NIH/NIAMS [K23AR073334 and L30 AR070520 to ZW].

REFERENCES

1. Jennette JC, Falk RJ, Bacon PA, Basu N, Cid MC, Ferrario F, et al. 2012 revised International Chapel Hill Consensus Conference Nomenclature of Vasculitidesa. *Arthritis Rheum*. (2013) 65:1–11. doi: 10.1002/art.37715
2. Cornec D, Cornec-Le Gall E, Fervenza FC, Specks U. ANCA-associated vasculitis - clinical utility of using ANCA specificity to classify patients. *Nat Rev Rheumatol*. (2016) 12:570–9. doi: 10.1038/nrrheum.2016.123
3. Lionaki S, Blyth ER, Hogan SL, Hu Y, Senior BA, Jennette CE, et al. Classification of antineutrophil cytoplasmic autoantibody vasculitides: the role of antineutrophil cytoplasmic autoantibody specificity for myeloperoxidase or proteinase 3 in disease recognition and prognosis. *Arthritis Rheum*. (2012) 64:3452–62. doi: 10.1002/art.34562
4. Merkel PA, Xie G, Monach PA, Ji X, Ciavatta DJ, Byun J, et al. Identification of functional and expression polymorphisms associated with risk for antineutrophil cytoplasmic autoantibody-associated vasculitis. *Arthritis Rheumatol*. (2017) 69:1054–66. doi: 10.1002/art.40034
5. Lyons PA, Rayner TF, Trivedi S, Holle JU, Watts RA, Jayne DR, et al. Genetically distinct subsets within ANCA-associated vasculitis. *N Engl J Med*. (2012) 367:214–23. doi: 10.1056/NEJMoa1108735
6. Xie G, Roshandel D, Sherva R, Monach PA, Lu EY, Kung T, et al. Association of granulomatosis with polyangiitis (Wegener's) with HLA-DPB1*04 and SEMA6A gene variants: evidence from genome-wide analysis. *Arthritis Rheum*. (2013) 65:2457–68. doi: 10.1002/art.38036
7. Cao Y, Schmitz JL, Yang J, Hogan SL, Bunch D, Hu Y, et al. DRB1*15 allele is a risk factor for PR3-ANCA disease in African Americans. *J Am Soc Nephrol*. (2011) 22:1161–7. doi: 10.1681/ASN.2010101058
8. Jennette JC, Falk RJ. Pathogenesis of antineutrophil cytoplasmic autoantibody-mediated disease. *Nat Rev Rheumatol*. (2014) 10:463–73. doi: 10.1038/nrrheum.2014.103
9. 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/JCI0215918
10. Hess C, Sadallah S, Schifferli JA. Induction of neutrophil responsiveness to myeloperoxidase antibodies by their exposure to supernatant of degranulated autologous neutrophils. *Blood*. (2000) 96:2822–7. doi: 10.1182/blood.V96.8.2822
11. Finkielman JD, Lee AS, Hummel AM, Viss MA, Jacob GL, Homburger HA, et al. ANCA are detectable in nearly all patients with active severe Wegener's granulomatosis. *Am J Med*. (2007) 120:643.e9–14. doi: 10.1016/j.amjmed.2006.08.016
12. Stone JH, Merkel PA, Spiera R, Seo P, Langford CA, Hoffman GS, et al. Rituximab versus cyclophosphamide for ANCA-associated vasculitis. *N Engl J Med*. (2010) 363:221–32. doi: 10.1056/NEJMoa0909905
13. Jayne DR, Gaskin G, Rasmussen N, Abramowicz D, Ferrario F, Guillevin L, et al. Randomized trial of plasma exchange or high-dosage methylprednisolone as adjunctive therapy for severe renal vasculitis. *J Am Soc Nephrol*. (2007) 18:2180–8. doi: 10.1681/ASN.2007010090
14. Bansal PJ, Tobin MC. Neonatal microscopic polyangiitis secondary to transfer of maternal myeloperoxidase-antineutrophil cytoplasmic antibody resulting in neonatal pulmonary hemorrhage and renal involvement. *Ann Allergy Asthma Immunol*. (2004) 93:398–401. doi: 10.1016/S1081-1206(10)61400-7
15. Olson SW, Owshalimpur D, Yuan CM, Arbogast C, Baker TP, Oliver D, et al. Relation between asymptomatic proteinase 3 antibodies and future granulomatosis with polyangiitis. *Clin J Am Soc Nephrol*. (2013) 8:1312–8. doi: 10.2215/CJN.10411012
16. 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
17. van Dam LS, Kraaij T, Kamerling SWA, Bakker JA, Scherer UH, Rabelink TJ, et al. Neutrophil extracellular trap formation is intrinsically distinct in ANCA-associated vasculitis and systemic lupus erythematosus. *Arthritis Rheumatol*. (2019) 71:2047–58. doi: 10.1002/art.41047
18. Wu EY, McInnis EA, Boyer-Suavet S, Mendoza CE, Aybar LT, Kennedy KB, et al. Measuring circulating complement activation products in myeloperoxidase and proteinase 3 antineutrophil cytoplasmic antibody vasculitis. *Arthritis Rheumatol*. (2019) 71:1894–903. doi: 10.1002/art.41011
19. Xiao H, Dairaghi DJ, Powers JP, Ertl LS, Baumgart T, Wang Y, et al. C5a receptor (CD88) blockade protects against MPO-ANCA GN. *J Am Soc Nephrol*. (2014) 25:225–31. doi: 10.1681/ASN.2013020143
20. Jayne DR, Bruchfeld AN, Harper L, Schaier M, Venning MC, Hamilton P, et al. Randomized trial of C5a receptor inhibitor avacopan in ANCA-associated vasculitis. *J Am Soc Nephrol*. (2017) 28:2756–67. doi: 10.1681/ASN.2016111179
21. *A Phase 3 Clinical Trial of CCX168 (Avacopan) in Patients With ANCA-Associated Vasculitis (ADVOCATE)*. Identifier NCT02994927. National Library of Medicine (2019). Available online at: https://clinicaltrials.gov/ct2/show/NCT02994927 (accessed October 1, 2019).
22. Berti A, Warner R, Johnson K, Cornec D, Schroeder D, Kabat B, et al. Brief report: circulating cytokine profiles and antineutrophil cytoplasmic antibody specificity in patients with antineutrophil cytoplasmic antibody-associated vasculitis. *Arthritis Rheumatol*. (2018) 70:1114–21. doi: 10.1002/art.40471
23. Gomez-Puerta JA, Gedmintas L, Costenbader KH. The association between silica exposure and development of ANCA-associated vasculitis: systematic review and meta-analysis. *Autoimmun Rev*. (2013) 12:1129–35. doi: 10.1016/j.autrev.2013.06.016
24. Hogan SL, Cooper GS, Savitz DA, Nylander-French LA, Parks CG, Chin H, et al. Association of silica exposure with anti-neutrophil cytoplasmic autoantibody small-vessel vasculitis: a population-based, case-control study. *Clin J Am Soc Nephrol*. (2007) 2:290–9. doi: 10.2215/CJN.03501006
25. Stegeman CA, Tervaert JW, Sluiter WJ, Manson WL, de Jong PE, Kallenberg CG. Association of chronic nasal carriage of Staphylococcus aureus and higher relapse rates in Wegener granulomatosis. *Ann Intern Med*. (1994) 120:12–7. doi: 10.7326/0003-4819-120-1-199401010-00003
26. Stegeman CA, Tervaert JW, de Jong PE, Kallenberg CG. Trimethoprim-sulfamethoxazole (co-trimoxazole) for the prevention of relapses of Wegener's granulomatosis. Dutch Co-Trimoxazole Wegener Study Group. *N Engl J Med*. (1996) 335:16–20. doi: 10.1056/NEJM199607043350103
27. Salmela A, Rasmussen N, Tervaert JWC, Jayne DRW, Ekstrand A, Group EVS. Chronic nasal Staphylococcus aureus carriage identifies a subset of newly diagnosed granulomatosis with polyangiitis patients with high relapse rate. *Rheumatology*. (2017) 56:965–72. doi: 10.1093/rheumatology/kex001
28. Choi HK, Merkel PA, Walker AM, Niles JL. Drug-associated antineutrophil cytoplasmic antibody-positive vasculitis: prevalence among patients with high titers of antimyeloperoxidase antibodies. *Arthritis Rheum*. (2000) 43:405–13. doi: 10.1002/1529-0131(200002)43:2<405::AID-ANR22>3.0.CO;2-5
29. Chen M, Gao Y, Guo XH, Zhao MH. Propylthiouracil-induced antineutrophil cytoplasmic antibody-associated vasculitis. *Nat Rev Nephrol*. (2012) 8:476–83. doi: 10.1038/nrneph.2012.108
30. McGrath MM, Isakova T, Rennke HG, Mottola AM, Laliberte KA, Niles JL. Contaminated cocaine and antineutrophil cytoplasmic antibody-associated disease. *Clin J Am Soc Nephrol*. (2011) 6:2799–805. doi: 10.2215/CJN.03440411
31. Falk RJ, Jennette CE. Anti-neutrophil cytoplasmic autoantibodies with specificity for myeloperoxidase in patients with systemic vasculitis and idiopathic necrotizing and crescentic glomerulonephritis. *N Engl J Med*. (1988) 318:1651–7. doi: 10.1056/NEJM198806233182504
32. Savige JA, Gillis D, Benson E, Davies D, Esnault V, Falk RJ, et al. International consensus statement on testing and reporting of Antineutrophil Cytoplasmic Antibodies (ANCA). *Am J Clin Pathol*. (1999) 111:507–13. doi: 10.1093/ajcp/111.4.507
33. Damoiseaux J, Csernok E, Rasmussen N, Moosig F, Van Paassen P, Baslund B, et al. Detection of antineutrophil cytoplasmic antibodies (ANCAs): a multicentre European Vasculitis Study Group (EUVAS) evaluation of the value of indirect immunofluorescence (IIF) versus antigen-specific immunoassays. *Ann Rheum Dis*. (2017) 76:647–53. doi: 10.1136/annrheumdis-2016-209507

34. Bossuyt X, Rasmussen N, Van Paassen P, Hellmich B, Baslund B, Vermeersch P, et al. A multicentre study to improve clinical interpretation of proteinase-3 and myeloperoxidase anti-neutrophil cytoplasmic antibodies. *Rheumatology*. (2017) 56:1533–41. doi: 10.1093/rheumatology/kex170
35. Wegener's Granulomatosis Etanercept Trial (WGET) Research Group. Etanercept plus standard therapy for Wegener's granulomatosis. *N Engl J Med*. (2005) 352:351–61. doi: 10.1056/NEJMoa041884
36. Finkielman JD, Merkel PA, Schroeder D, Hoffman GS, Spiera R, St. Clair EW, et al. Antiproteinase 3 antineutrophil cytoplasmic antibodies and disease activity in Wegener granulomatosis. *Ann Intern Med*. (2007) 147:611–9. doi: 10.7326/0003-4819-147-9-200711060-00005
37. Tomasson G, Grayson PC, Mahr AD, Lavalley M, Merkel PA. Value of ANCA measurements during remission to predict a relapse of ANCA-associated vasculitis–a meta-analysis. *Rheumatology*. (2012) 51:100–9. doi: 10.1093/rheumatology/ker280
38. Fussner LA, Hummel AM, Schroeder DR, Silva F, Cartin-Ceba R, Snyder MR, et al. Factors determining the clinical utility of serial measurements of antineutrophil cytoplasmic antibodies targeting proteinase 3. *Arthritis Rheumatol*. (2016) 68:1700–10. doi: 10.1002/art.39637
39. Unizony S, Villarreal M, Miloslavsky EM, Lu N, Merkel PA, Spiera R, et al. Clinical outcomes of treatment of anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis based on ANCA type. *Ann Rheum Dis*. (2016) 75:1166–9. doi: 10.1136/annrheumdis-2015-208073
40. Fujimoto S, Watts RA, Kobayashi S, Suzuki K, Jayne DR, Scott DG, et al. Comparison of the epidemiology of anti-neutrophil cytoplasmic antibody-associated vasculitis between Japan and the U.K. *Rheumatology*. (2011) 50:1916–20. doi: 10.1093/rheumatology/ker205
41. Miloslavsky EM, Lu N, Unizony S, Choi HK, Merkel PA, Seo P, et al. Myeloperoxidase-Antineutrophil Cytoplasmic Antibody (ANCA)-positive and ANCA-negative patients with granulomatosis with polyangiitis (Wegener's): distinct patient subsets. *Arthritis Rheumatol*. (2016) 68:2945–52. doi: 10.1002/art.39812
42. Chang DY, Li ZY, Chen M, Zhao MH. Myeloperoxidase-ANCA-positive granulomatosis with polyangiitis is a distinct subset of ANCA-associated vasculitis: a retrospective analysis of 455 patients from a single center in China. *Semin Arthritis Rheum*. (2019) 48:701–6. doi: 10.1016/j.semarthrit.2018.05.003
43. Pendergraft WF III, Cortazar FB, Wenger J, Murphy AP, Rhee EP, Laliberte KA, et al. Long-term maintenance therapy using rituximab-induced continuous B-cell depletion in patients with ANCA vasculitis. *Clin J Am Soc Nephrol*. (2014) 9:736–44. doi: 10.2215/CJN.07340713
44. Mohammad AJ, Mortensen KH, Babar J, Smith R, Jones RB, Nakagomi D, et al. Pulmonary involvement in Antineutrophil Cytoplasmic Antibodies (ANCA)-associated vasculitis: the influence of ANCA subtype. *J Rheumatol*. (2017) 44:1458–67. doi: 10.3899/jrheum.161224
45. Schirmer JH, Wright MN, Herrmann K, Laudien M, Noelle B, Reinhold-Keller E, et al. Myeloperoxidase-Antineutrophil Cytoplasmic Antibody (ANCA)-positive granulomatosis with polyangiitis (Wegener's) is a clinically distinct subset of ANCA-associated vasculitis: a retrospective analysis of 315 patients from a German vasculitis referral center. *Arthritis Rheumatol*. (2016) 68:2953–63. doi: 10.1002/art.39786
46. Neel A, Espitia-Thibault A, Arrigoni PP, Volteau C, Rimbert M, Masseau A, et al. Bronchiectasis is highly prevalent in anti-MPO ANCA-associated vasculitis and is associated with a distinct disease presentation. *Semin Arthritis Rheum*. (2018) 48:70–6. doi: 10.1016/j.semarthrit.2017.12.002
47. de Joode AA, Sanders JS, Stegeman CA. Renal survival in proteinase 3 and myeloperoxidase ANCA-associated systemic vasculitis. *Clin J Am Soc Nephrol*. (2013) 8:1709–17. doi: 10.2215/CJN.01020113
48. Pagnoux C, Carette S, Khalidi NA, Walsh M, Hiemstra TF, Cuthbertson D, et al. Comparability of patients with ANCA-associated vasculitis enrolled in clinical trials or in observational cohorts. *Clin Exp Rheumatol*. (2015) 33(Suppl 89):S77–83.
49. Jones RB, Hiemstra TF, Ballarin J, Blockmans D, Brogan P, Bruchfeld A, et al. Mycophenolate mofetil versus cyclophosphamide for remission induction in ANCA-associated vasculitis: a randomised, non-inferiority trial. *Ann Rheum Dis*. (2019) 78:399–405. doi: 10.1136/annrheumdis-2018-214245
50. Specks U, Merkel PA, Seo P, Spiera R, Langford CA, Hoffman GS, et al. Efficacy of remission-induction regimens for ANCA-associated vasculitis. *N Engl J Med*. (2013) 369:417–27. doi: 10.1056/NEJMoa1213277
51. Hogan SL, Falk RJ, Chin H, Cai J, Jennette CE, Jennette JC, et al. Predictors of relapse and treatment resistance in antineutrophil cytoplasmic antibody-associated small-vessel vasculitis. *Ann Intern Med*. (2005) 143:621–31. doi: 10.7326/0003-4819-143-9-200511010-00005
52. Walsh M, Flossmann O, Berden A, Westman K, Hoglund P, Stegeman C, et al. Risk factors for relapse of antineutrophil cytoplasmic antibody-associated vasculitis. *Arthritis Rheum*. (2012) 64:542–8. doi: 10.1002/art.33361
53. Solans-Laque R, Fraile G, Rodriguez-Carballeira M, Caminal L, Castillo MJ, Martinez-Valle F, et al. Clinical characteristics and outcome of Spanish patients with ANCA-associated vasculitides: impact of the vasculitis type, ANCA specificity, and treatment on mortality and morbidity. *Medicine*. (2017) 96:e6083. doi: 10.1097/MD.0000000000006083
54. Miloslavsky EM, Specks U, Merkel PA, Seo P, Spiera R, Langford CA, et al. Clinical outcomes of remission induction therapy for severe antineutrophil cytoplasmic antibody-associated vasculitis. *Arthritis Rheum*. (2013) 65:2441–9. doi: 10.1002/art.38044
55. Guillevin L, Pagnoux C, Karras A, Khouatra C, Aumaitre O, Cohen P, et al. Rituximab versus azathioprine for maintenance in ANCA-associated vasculitis. *N Engl J Med*. (2014) 371:1771–80. doi: 10.1056/NEJMoa1404231
56. Charles P, Terrier B, Perrodeau E, Cohen P, Faguer S, Huart A, et al. Comparison of individually tailored versus fixed-schedule rituximab regimen to maintain ANCA-associated vasculitis remission: results of a multicentre, randomised controlled, phase III trial (MAINRITSAN2). *Ann Rheum Dis*. (2018) 77:1143–9. doi: 10.1136/annrheumdis-2017-212878
57. Rhee RL, Hogan SL, Poulton CJ, McGregor JA, Landis JR, Falk RJ, et al. Trends in long-term outcomes among patients with antineutrophil cytoplasmic antibody-associated vasculitis with renal disease. *Arthritis Rheumatol*. (2016) 68:1711–20. doi: 10.1002/art.39614
58. Franssen CF, Stegeman CA, Kallenberg CG, Gans RO, De Jong PE, Hoorntje SJ, et al. Antiproteinase 3- and antimyeloperoxidase-associated vasculitis. *Kidney Int*. (2000) 57:2195–206. doi: 10.1046/j.1523-1755.2000.00080.x
59. Hauer HA, Bajema IM, Van Houwelingen HC, Ferrario F, Noel LH, Waldherr R, et al. Determinants of outcome in ANCA-associated glomerulonephritis: a prospective clinico-histopathological analysis of 96 patients. *Kidney Int*. (2002) 62:1732–42. doi: 10.1046/j.1523-1755.2002.00605.x
60. Rihova Z, Jancova E, Merta M, Rysava R, Reiterova J, Zabka J, et al. Long-term outcome of patients with antineutrophil cytoplasmic autoantibody-associated vasculitis with renal involvement. *Kidney Blood Press Res*. (2005) 28:144–52. doi: 10.1159/000085854
61. Mohammad AJ, Segelmark M. A population-based study showing better renal prognosis for proteinase 3 antineutrophil cytoplasmic antibody (ANCA)-associated nephritis versus myeloperoxidase ANCA-associated nephritis. *J Rheumatol*. (2014) 41:1366–73. doi: 10.3899/jrheum.131038
62. Tan JA, Dehghan N, Chen W, Xie H, Esdaile JM, Avina-Zubieta JA. Mortality in ANCA-associated vasculitis: a meta-analysis of observational studies. *Ann Rheum Dis*. (2017) 76:1566–74. doi: 10.1136/annrheumdis-2016-210942
63. Wallace ZS, Lu N, Unizony S, Stone JH, Choi HK. Improved survival in granulomatosis with polyangiitis: A general population-based study. *Semin Arthritis Rheum*. (2016) 45:483–9. doi: 10.1016/j.semarthrit.2015.07.009
64. Wallace ZS, Lu N, Miloslavsky E, Unizony S, Stone JH, Choi HK. Nationwide trends in hospitalizations and in-hospital mortality of granulomatosis with polyangiitis. *Arthritis Care Res*. (2017) 69:915–21. doi: 10.1002/acr.22976
65. Wallace Z, Fu X, Zhang Y, Stone JH, Choi H. Cardiovascular disease is the most common cause of death in ANCA-associated vasculitis. *Rheumatology*. (2019) 58(Suppl 2). doi: 10.1093/rheumatology/kez059.048
66. Suppiah R, Judge A, Batra R, Flossmann O, Harper L, Hoglund P, et al. A model to predict cardiovascular events in patients with newly diagnosed

Wegener's granulomatosis and microscopic polyangiitis. *Arthritis Care Res.* (2011) 63:588–96. doi: 10.1002/acr.20433

67. Nolle B, Specks U, Ludemann J, Rohrbach MS, DeRemee RA, Gross WL. Anticytoplasmic autoantibodies: their immunodiagnostic value in Wegener granulomatosis. *Ann Intern Med.* (1989) 111:28–40. doi: 10.7326/0003-4819-111-1-28
68. Bossuyt X, Cohen Tervaert JW, Arimura Y, Blockmans D, Flores-Suarez LF, Guillevin L, et al. Position paper: revised 2017 international consensus on testing of ANCAs in granulomatosis with polyangiitis and microscopic polyangiitis. *Nat Rev Rheumatol.* (2017) 13:683–92. doi: 10.1038/nrrheum.2017.140

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.

frontiers in Immunology

MINI REVIEW
published: 06 December 2019
doi: 10.3389/fimmu.2019.02830

Treating the Different Phenotypes of Behçet's Syndrome

*Alessandra Bettiol[1,2], Gulen Hatemi[3], Lorenzo Vannozzi[4], Alessandro Barilaro[5], Domenico Prisco[1] and Giacomo Emmi[1]**

[1] Department of Experimental and Clinical Medicine, University of Florence, Florence, Italy, [2] Department of Neurosciences, Psychology, Drug Research and Child Health (NEUROFARBA), University of Florence, Florence, Italy, [3] Division of Rheumatology, Department of Internal Medicine, Istanbul University–Cerrahpaşa, Istanbul, Turkey, [4] Eye Clinic, Careggi Teaching Hospital, University of Florence, Florence, Italy, [5] Department of Neurology 2 and Multiple Sclerosis Regional Referral Centre, Careggi University Hospital, Florence, Italy

Behçet's syndrome (BS) is a multisystemic vasculitis, characterized by different clinical involvements, including mucocutaneous, ocular, vascular, neurological, and gastrointestinal manifestations. Based on this heterogeneity, BS can be hardly considered as a single clinical entity. Growing evidence supports that, within BS, different phenotypes, characterized by clusters of co-existing involvements, can be distinguished. Namely, three major BS phenotypes have been reported: (a) the mucocutaneous and articular phenotype, (b) the extra-parenchymal neurological and peripheral vascular phenotype, and (c) the parenchymal neurological and ocular phenotype. To date, guidelines for the management of BS have been focused on the pharmacological treatment of each specific BS manifestation. However, tailoring the treatments on patient's specific phenotype, rather than on single disease manifestation, could represent a valid strategy for a personalized therapeutic approach to BS. In the present literature review, we summarize current evidence on the pharmacological treatments for the first-, second-, and third-line treatment of the major BS phenotypes.

Keywords: Behçet's syndrome, phenotypes, cluster analysis, anti-TNF-α, DMARDs

OPEN ACCESS

Edited by:
Myung-Shik Lee, Yonsei University Health System, South Korea

Reviewed by:
Åsa Andersson, Halmstad University, Sweden
Dimitrios Vassilopoulos, National and Kapodistrian University of Athens, Greece

***Correspondence:**
Giacomo Emmi
giacomo.emmi@unifi.it

Specialty section:
This article was submitted to Autoimmune and Autoinflammatory Disorders, a section of the journal Frontiers in Immunology

Received: *09 August 2019*
Accepted: *18 November 2019*
Published: *06 December 2019*

Citation:
Bettiol A, Hatemi G, Vannozzi L, Barilaro A, Prisco D and Emmi G (2019) Treating the Different Phenotypes of Behçet's Syndrome. Front. Immunol. 10:2830. doi: 10.3389/fimmu.2019.02830

INTRODUCTION

Behçet's syndrome (BS) is a multisystemic vasculitis (1, 2), characterized by a broad spectrum of clinical involvements, including mucocutaneous, ocular, vascular, neurological, and gastrointestinal manifestations (1, 3). The different clinical manifestations may present alone, or co-exist in the same patient (4, 5). Cluster analyses and multivariate techniques have been applied to identify common clusters of BS manifestations, and, to date, three main disease phenotypes have been described: (a) the mucocutaneous and articular phenotype, (b) the extra-parenchymal neurological and peripheral vascular phenotype, and (c) the parenchymal neurological and ocular phenotype (**Table 1**).

While extensive and updated literature reviews and recommendations exist for the treatment of the single BS involvements (6, 54), to date, poor attention has been given to the management of the different clusters of BS manifestations. The present review aims to provide clinicians evidence-based data to guide the choice of the most appropriate first-, second-, and third-line therapeutic approaches of the major BS phenotypes. Namely, first-line treatments should be considered as first options for naïve patients, based on current EULAR recommendations and on the extensive literature evidence on their efficacy (55). In patients intolerant or resistant to first-line drugs (or with severe BS forms), second or

TABLE 1 | Major clusters of Behçet's manifestations and therapeutic options for the different disease phenotypes.

Phenotypes	Evidence for the phenotype	Type of study; Cluster of manifestations	Treatment	Major evidence for the treatment
Mucocutaneous and articular phenotype	Diri et al. (6)	Analysis of variance; Papulopustular lesions and arthritis	Colchicine (+/– steroids)	Clinical trials: (7–9)
	Tunc et al. (10)	Factor analysis; Genital ulcers, and erythema nodosum	Azathioprine	Clinical trial: (11)
	Hatemi et al. (12)	Analysis of variance; Enthesopathy, acne and arthritis	IFN α	Clinical trial: (13) Observational study: (14)
	Karaca et al. (15)	Factor analysis; Genital ulcers, and erythema nodosum with or without oral ulcers; papulopustular skin lesions and joint involvement with or without oral ulcers	Anti TNF-α	Clinical trial (for etanercept): (16). Observational studies and case series (for adalumumab and infliximab): (17, 18)
	Yazici et al. (4).		Anti Interleukin-1	Clinical trial: (19) Case series: (20)
	Kurosawa et al. (21)	Correspondence analysis; Onset age: 30–39 years, skin lesions, arthritis	Secukinumab	Case series: (22)
Extra-parenchymal neurological and peripheral vascular involvement phenotype	Tunc et al. (23)	Chi-square test; Cerebral venous sinus thrombosis and peripheral major vessel disease	Anticoagulant + immunosuppressant +/– steroids	Retrospective studies and case series (for anticoagulation): (24–26)
	Saadoun et al. (27)	Chi-square test; central nervous system involvement and extraneurologic vascular lesions		Retrospective studies (for immunosuppressants in general): (28–30) (for anti TNF-α): (31, 32)
	Tascilar et al. (33)	Correspondence analysis; Cerebral venous sinus thrombosis and pulmonary artery involvement		
	Shi et al. (24)	Chi-square test; extra cranial vascular involvement and cerebral venous sinus thrombosis.		
Parenchymal central nervous system and ocular phenotype	Bitik et al. (34)	Chi-square test; posterior uveitis and parenchymal neurological involvement	Steroid pulses	Clinical trial (for ocular involvement): (35)
	Kurosawa et al. (21)	Correspondence analysis; male, eye disease, HLA-B51 (+), neurologic involvement	Azathioprine	Clinical trial: (11, 36) Observational evidence (for azathioprine alone or in combination): (37, 38)
			Anti TNF-α	Interventional study (for infliximab): (39, 40) Observational studies (for infliximab): (41, 42) Clinical trials (for adalimumab): (43, 44) Observational studies (for adalimumab): (41, 45, 46)
			Cyclophosphamide	Observational study: (47–49)
			Tocilizumab	Observational study: (50) Case report/series: (51–53)

further lines of treatment should be considered, based on the availability of literature evidence to guide their use.

Abbreviations: ADA, adalimumab; ANA, anakinra; AZA, azathioprine; BS, Behçet's syndrome; CANA, canakinumab; CNS, central nervous system; CSA, cyclosporine; CVST, cerebral venous sinus thrombosis; CYC, cyclophosphamide; DMARDs, disease modifying anti-rheumatic drugs; DVT, deep vein thrombosis; ETN, etanercept; HLA, human leukocyte antigen; IFN, interferon; IFX, infliximab; IL, interleukin; MHC, major histocompatibility complex; RCT, randomized controlled trial; SpA, spondyloarthritides; SVT, superficial venous thrombosis; TAL, thrombosis of atypical locations; TCZ, tocilizumab.

MUCOCUTANEOUS AND ARTICULAR PHENOTYPE

Evidence on the Phenotype

Skin-mucosa ulcerations are the most common, and usually the earliest, manifestations of BS, and recurrent oral and genital lesions are the hallmark of this syndrome (1). While one third of the BS population presents with only recurrent mucocutaneous symptoms (56, 57), a not negligible proportion of patients presents both mucocutaneous and articular involvements. The association between acne and arthritis has been demonstrated in

past decades (6), but it is suggested that also enthesitis was part of this clinical association (4, 21).

Indeed, BS shares with seronegative spondyloarthritides (SpA) common pathogenetic mechanisms and genetic susceptibility, including the interleukin (IL)-23 and IL-17 pathways (1). Moreover, the involvement of major histocompatibility complex (MHC) class I alleles both in BS and in SpA [human leukocyte antigen (HLA)-B*51 and HLA-B*27, respectively] led to the unifying concept of "MHC-I-opathies" (58).

First- and Second-Line Treatments

In patients newly diagnosed with BS and presenting this "mucocutaneous and articular phenotype," first-line treatment should be based on colchicine (**Figure 1A**). Colchicine has long been used in BS, with first evidence on its beneficial results for the treatment of erythema nodosum and arthralgia dating back to 1980 (7). Later on, two randomized controlled trials (RCTs) showed that colchicine led to a significant improvement of oral and genital ulcers, erythema nodosum, and articular symptoms (8, 9). The 2018 EULAR recommendations support the use of colchicine as first-line systemic treatment, especially when the dominant lesions are erythema nodosum or genital ulcers (55).

In patients intolerant or resistant to colchicine, azathioprine (AZA) can represent an effective second-line treatment. Efficacy of AZA for oral and genital ulcers and for arthritis was documented in a 2-year RCT of AZA (2.5 mg per kilogram of body weight per day) (11). In addition, AZA was superior to placebo in preventing new eye disease involvement (11). Based on this evidence, AZA can be considered as a first-line treatment in patient carrying also mild ocular involvement.

Third Line Treatments

In patients inadequately controlled with, or intolerant to, the aforementioned synthetic immunosuppressive regimen, the use of biologic strategies, namely, with anti-TNF-α, or interferon (IFN) α should be considered. Among anti-TNF-α agents, only etanercept (ETN) 25 mg twice a week for 4 weeks has been studied in a trial on 40 BS patients with mucocutaneous disease and/or arthritis, showing a significant decrease of oral ulcers, nodular, and papulopustular lesions (16). However, data on the efficacy of ETN on arthritis were not conclusive, and the effects of this drug on genital ulcers were comparable with those in the placebo group. Conversely, the use of adalimumab (ADA) and infliximab (IFX) is supported by different observational studies and case series (17). Among them, a multicenter study on 124 BS patients showed that the clinical response to the treatment with either ADA or IFX was 88% for mucocutaneous involvement and 77.8% for articular involvement (18).

The efficacy of IFN α in the "mucocutaneous and articular phenotype" was reported in a retrospective observational study on 18 BS patients, treated for 12 weeks (14). Later on, in an RCT, IFN α was shown to control oral and genital ulcers, papulopustular lesions, erythema nodosum-like manifestations, and articular symptoms, while improving the severity and the frequency of ocular attacks (13). Of note, the safety profile of this drug deserves some attention, since adverse events including flu-like syndrome, leukopenia, transient elevation of liver enzymes, as well as psychiatric disorders have been reported (13). Bone marrow suppression may be even more pronounced when used together with AZA (37).

Fourth-Line Treatments

In patients resistant, refractory, or intolerant to anti-TNF-α agents or IFN α, evidence supports the use of other biologic treatments for this phenotype. Specifically, some evidence (although not consistent) supports the use of IL-1 inhibitors anakinra (ANA) or canakinumab (CANA) (19, 20, 59). Specifically, in an adaptive, two-phase pilot open label study conducted on six BS patients with active mucocutaneous manifestations and with concomitant arthritis, ANA at an optimal dose of 200 mg daily provided partial control of resistant mucocutaneous and articular involvements (19).

In a recent case series of five BS patients with active and refractory mucocutaneous and articular manifestations, the anti-IL17 agent secukinumab (either 150 mg and 300 mg/month) was associated with a consistent improvement of both mucocutaneous and articular involvements (22).

Regarding other promising treatments, growing evidence supports the use of ustekinumab (60–62) and apremilast (63, 64) for the control of mucocutaneous involvements. Of note, following a phase 2, placebo-controlled trial and a phase 3, multicenter, placebo-controlled study on 207 patients with active BS (64, 65), apremilast is the only drug currently approved by the Food and Drug Administration (FDA) for the treatment of mucocutaneous manifestations in BS. However, as no clear evidence exists on the efficacy of apremilast for the control of articular BS involvement, the role of this drug for the management of the mucocutaneous and articular BS phenotype is yet unclear.

On the other hand, the use of the anti-IL6R tocilizumab (TCZ) should be avoided in patients presenting this phenotype, considering that TCZ-induced exacerbation of mucosal ulcers has been reported (66, 67).

EXTRA-PARENCHYMAL NEUROLOGICAL AND PERIPHERAL VASCULAR PHENOTYPE

Evidence on the Phenotype

Superficial venous thrombosis (SVT) and deep vein thrombosis (DVT) are the most frequent vascular manifestations of BS, affecting altogether up to 40% of patients (31, 68–70). DVT mainly involves the inferior, but also the superior limbs, while venous thrombosis of atypical locations (TAL) have been described (31, 69–71). At the cerebral level, non-parenchymal vascular central nervous system (CNS) involvements include cerebral venous sinus thrombosis (CVST), arterial occlusion, and/or aneurysms (72). CVST represents 10–30% of all neurological BS manifestations (73). The concomitant presence of both cerebral arterial manifestations and CVST is extremely rare (74). In an analysis of 88 patients with CNS disease, a

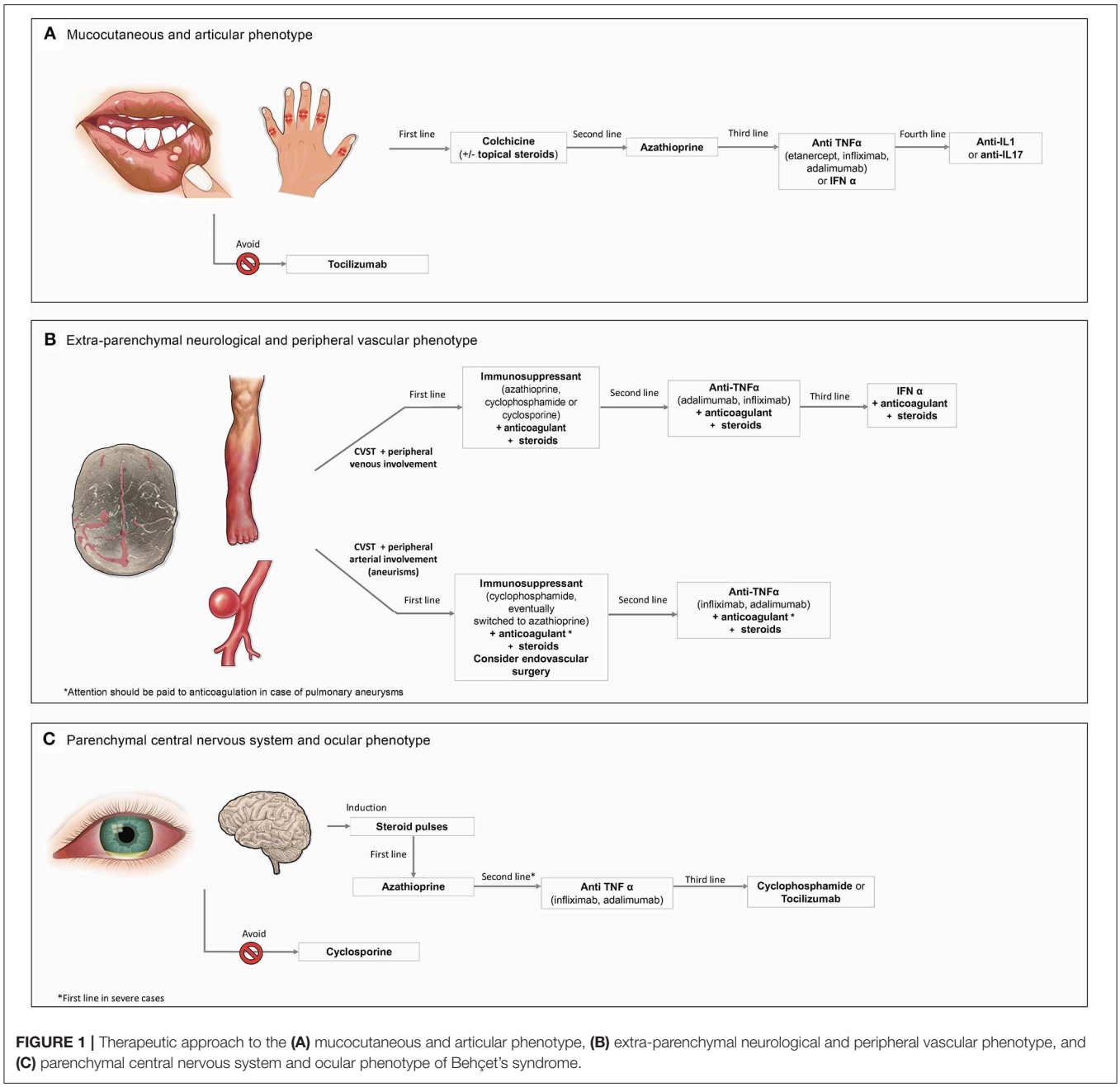


FIGURE 1 | Therapeutic approach to the **(A)** mucocutaneous and articular phenotype, **(B)** extra-parenchymal neurological and peripheral vascular phenotype, and **(C)** parenchymal central nervous system and ocular phenotype of Behçet's syndrome.

significant association was found between peripheral vascular disease and extra-parenchymal CNS involvement (i.e., dural sinus thrombi), while a poor association was found between parenchymal neurological and peripheral vascular involvements (23). In a retrospective study involving 21 BS patients with CVST, the presence of extra cranial thrombosis was documented in 52% of patients (24). In a cohort study on 820 patients, CVST was reported in 64 cases. Among them, the presence of concomitant extra-neurological vascular lesions was significantly more frequent than in patients without CVST (27).

The concomitant presence of central and peripheral vascular involvements is probably sustained by common thrombogenic mechanisms. Namely, inflammation-induced thrombosis has been described in BS, with neutrophils playing a critical role in promoting oxidative stress, inflammation, and consequent endothelial dysfunctions (31, 75, 76). In this context, immunosuppression represents a key strategy for the therapeutic management of central and peripheral vascular involvements (31, 71).

CVST and Peripheral Venous Involvements

First-Line Treatments

High-dose glucocorticoids are the mainstay treatment for rapid induction of remission in CVST (60). There is no consensus

on the use of additional anticoagulants or immunosuppressants, since recurrence is infrequent with this manifestation. In a recent literature review (31), we reported that anticoagulation has a predominant role in the management of BS-related CVST (24, 25, 31, 77), while it is yet unclear if the use of concomitant immunosuppressants influences the risk of sequalae or relapses (24). A recent case series of 7 patients with BS-associated CVST suggested that anticoagulant therapy might be safely discontinued during follow-up, in the presence of optimal BS therapeutic management with steroids alone or in combination with immunosuppressive drugs (26). On the other hand, the use of immunosuppressants is pivotal in the control of DVT and SVT (28–31), while concomitant use of anticoagulants in these peripheral associations has been associated with controversial benefits (31), except for preventing the occurrence of severe post-thrombotic syndrome (78).

Thus, the first-line treatment of patients carrying the "extra-parenchymal neurological and peripheral vascular phenotype" should be based on immunosuppressants with the addition of anticoagulants in selected patients (**Figure 1B**). Specifically, in CVST associated with SVT and/or DVT, evidence suggests as first-line treatment AZA, cyclophosphamide (CYC) or cyclosporine (CSA) (31, 66).

Second- and Third-Line Treatments

In patients with refractory peripheral venous thrombosis, anti-TNF-α, namely, ADA, or IFX, should be used, alone or in combination with traditional disease-modifying anti-rheumatic drugs (DMARDs) (1, 31).

Eventually, IFN α can be considered a therapeutic approach in selected cases (79). In a prospective study on patients with lower-extremity DVT, the treatment with IFN α accounted for a good recanalization and low relapse rates (80). According to the current EULAR recommendations, the treatment with IFN α can be considered in selected cases (55). However, the role of this treatment for the control of CNS vascular involvements is still unclear.

CVST and Arterial Involvements

First-Line Treatments

First-line treatment of patients carrying the CVST and peripheral arterial involvements should be based on immunosuppressants, mainly CYC, in association with high-dose steroid and (after excluding pulmonary aneurysms) with anticoagulants in selected patients (55). According to the last EULAR recommendations, CYC can be administered as monthly intravenous pulses, while glucocorticoids are given as three intravenous methylprednisolone pulses followed by oral prednisolone (or prednisone) at the dose of 1 mg/kg/day (55) (**Figure 1B**). For the maintenance treatment, CYC can be replaced by AZA (1).

Notably, sometimes peripheral aneurysms require emergency surgery or stenting (55). The use of prednisone alone or in combination with AZA is recommended also in patients with pseudoaneurysm, before endovascular treatment (81, 82), while in the days after surgery, successful use of hydrocortisone plus CSA has been reported (81).

Second-Line Treatments

In patients with arterial involvements refractory to conventional DMARDs, second-line treatment with anti-TNF-α (namely IFX or ADA) should be considered (32, 55). In an observational study on 13 BS patients with refractory pulmonary artery involvement, anti-TNF-α effectively controlled these involvements, although it did not prevent their development (32).

An effective use of ADA following unsuccessful treatment with prednisone, CYC, and conventional immunosuppression was reported also in a patient with right ventricular thrombus and large aneurysms of the pulmonary arteries leading to recurrent episodes of hemoptysis (83), as well as in a case of life threatening bilateral pulmonary artery aneurysms and thrombotic disease (84).

PARENCHYMAL CNS AND OCULAR PHENOTYPE

Evidence on the Phenotype

The involvement of the parenchymal CNS is a major cause of morbidity and mortality in BS (73, 85). In a study conducted on 200 neuro-BS out-patients, 162 had parenchymal CNS involvement (72). In a first post-mortem study on a BS patient with parenchymal involvement, a cell infiltration was found around the central retinal artery within the optic nerve (86). Eye involvement is present in around half of BS patients, with a higher prevalence in males, and a lower prevalence among elderly (87). Ocular involvement is one of the most disabling complication in BS (87). In a retrospective observational study on 295 BS patients, a significant association between posterior uveitis and parenchymal CNS involvement was reported (34). Furthermore, male sex, eye disease, HLA-B51 positivity, and neurologic involvement are features identifying a specific cluster of BS patients (21).

Of note, in a recent study on 30 BS patients with ocular involvement without overt neurological symptoms, silent neurologic manifestations, including neuropsychological deficits, subcortical magnetic resonance imaging (MRI) lesions, and non-structural headache, were found in a relevant proportion of patients (88).

Although the pathogenetic mechanisms sustaining the concomitant occurrence of ocular and neurological BS involvements have never been described, the embryogenic process and the involvement of the neural tube and neural crest in the organogenesis of the eye might account for this association (89).

First-Line Treatments

No RCT has determined the optimal therapeutic management of neurological BS, nor for its association with ocular involvement (90). The induction treatment of acute severe neuro-BS is mainly based on high-dose corticosteroids, followed by the gradual tapering of the oral doses over 3–6 months (90–92) (**Figure 1C**). As first-line treatment for the "parenchymal neurological and ocular phenotype," AZA should be used (90). Specifically, according to current EULAR recommendations,

AZA at the dosage of 2.5 mg/kg per day is recommended as first-line immunosuppressive agent for both ocular and parenchymal manifestations (1, 55). In case of severe ocular and parenchymal CNS involvements, the use of second-line options, namely, anti-TNF-α drugs, should be considered as first-line treatment.

Second-Line Treatments

In refractory cases, the use of anti-TNF-α can be considered (54). Indeed, consistent observational evidence supports the use of IFX (at the dose of 5 mg/kg) in both neurological and ocular BS involvements (1, 39, 55).

ADA at the dose of 40 mg every other week represents a valid second-line alternative (1). Effective use of ADA for non-infectious uveitis was first reported in two RCTs on few BS patients (43, 44, 93). Later observational evidence confirmed the benefits of this treatment in BS-related uveitis. In four Italian multicenter observational studies, treatment with either ADA or IFX proved effective for the treatment of refractory retinal vasculitis (45, 94–96). In another recent observational study on 106 patients with uveitis, ADA was associated with high rates of ocular control, effective steroid tapering, and good preservation of visual acuity, also in the absence of concomitant DMARDs treatment (46). Similarly, increasing observational evidence supports the use of ADA or IFX in neuro-BS (41).

Third-Line Treatments

Further therapeutic options for this phenotype are CYC or TZC. According to a 10-year longitudinal study, CYC (1 g/month for 6 months and then every 2–3 months), in association with AZA and prednisolone, was the best treatment for retinal vasculitis, before opting for biologic agents (47). Nevertheless, in a single masked trial (97), CYC was found to be inferior to CSA in controlling ocular involvements; however, CSA cannot be considered as a valid approach for this phenotype, as it is contraindicated in active neuro-BS.

CYC (1 g/month for 6–12 months or 0.8 g/m^2) has been associated also with some benefits in parenchymal neuro-BS (79, 98). In a French study on 115 patients with parenchymal neuro-BS, the use of CYC ($n = 53$) resulted as effective as AZA ($n = 40$) and steroids alone ($n = 19$) in preventing relapses (48). Furthermore, in patients with moderate to severe disability (i.e., with moderate to severe disability scoring 3 or more in the modified Rankin scale for the assessment of the disability), CYC was associated with slightly higher event-free survival rates at 1 to 10 years as compared to AZA, although without statistical significance. In a Korean study on 22 patients with parenchymal neuro-BS, a treatment with CYC associated with steroids was found to be as effective as treatment with steroids alone in preventing relapses (49).

The anti-IL6R TCZ is a promising treatment in the "parenchymal neurological and ocular phenotype." Results from case reports and case series suggest its effectiveness for refractory neuro-BS (51–53), while a recent retrospective study on 11 patients with refractory uveitis reported rapid and sustained ocular improvement in all the patients (50). However, the use in daily clinical practice of TZC for treating this phenotype still needs more studies for further confirmation. As for other non-biologic alternatives, IFN α is highly effective for ocular control (55), and might have a potential role also for refractory neuro-BS (99, 100). Notably, the use of CSA should be avoided in the "parenchymal neurological and ocular phenotype" (55). In fact, while effective in ocular manifestations, an increased risk of CNS manifestations in patients taking this drug has been reported (101–103).

CONCLUSIONS

Growing evidence supports that, within the definition of BS, different clinical phenotypes can be distinguished. Thus, therapeutic strategies could be tailored on patient's specific phenotype, rather than on single disease manifestations.

Based on available literature, patients carrying the "mucocutaneous and articular" BS phenotype should start a first-line treatment with colchicine, alone or in combination with corticosteroids, while AZA can be considered in patients resistant or intolerant to colchicine. The use of anti-TNF-α or IFN α should be reserved to truly refractory or severe forms.

In patients presenting the "extra-parenchymal and peripheral vascular phenotype," use of immunosuppressants and additional anticoagulants in selected patients should be recommended. Traditional immunosuppressants (mainly AZA) should be started as first-line treatment, while anti-TNF-α agents represent a valid second-line treatment. IFN α may be a promising alternative.

As for the "parenchymal neurological and ocular phenotype," first-line treatment with AZA is recommended after an induction therapy with high-dose steroids. In patients with a severe presentation, or those who are intolerant or refractory to AZA, anti-TNF-α drugs should be used.

However, comparative studies should be performed to evaluate whether this phenotype-based therapeutic approach is associated with a better effectiveness as compared to the classic organ-based approach.

AUTHOR CONTRIBUTIONS

GE and ABe conceived the work. ABe performed the literature review, assisted by GE and GH. ABe and GE drafted the paper. GH, LV, ABa, and DP critically revised and implemented the manuscript. All authors approved the final version of this manuscript.

REFERENCES

1. Yazici H, Seyahi E, Hatemi G, Yazici Y. Behçet syndrome: a contemporary view. *Nat Rev Rheumatol*. (2018) 14:107–19. doi: 10.1038/nrrheum.2018.3

2. Mumcu G, Direskeneli H. Triggering agents and microbiome as environmental factors on Behçet's syndrome. *Intern Emerg Med*. (2018) 14:653–60. doi: 10.1007/s11739-018-2000-1

3. Emmi G, Silvestri E, Squatrito D, D'Elios MM, Ciucciarelli L, Prisco D, et al. Behçet's syndrome pathophysiology and potential therapeutic targets. *Intern Emerg Med*. (2014) 9:257–65. doi: 10.1007/s11739-013-1036-5
4. Yazici H, Ugurlu S, Seyahi E. Behçet syndrome: is it one condition? *Clin Rev Allergy Immunol*. (2012) 43:275–80. doi: 10.1007/s12016-012-8319-x
5. Seyahi E. Phenotypes in Behçet's syndrome. *Intern Emerg Med*. (2019) 14:677–89. doi: 10.1007/978-3-030-24131-5_11
6. Diri E, Mat C, Hamuryudan V, Yurdakul S, Hizli N, Yazici H. Papulopustular skin lesions are seen more frequently in patients with Behçet's syndrome who have arthritis: a controlled and masked study. *Ann Rheum Dis*. (2001) 60:1074–6. doi: 10.1136/ard.60.11.1074
7. Aktulga E, Altaç M, Müftüoglu A, Ozyazgan Y, Pazarli H, Tüzün Y, et al. A double blind study of colchicine in Behçet's disease. *Haematologica*. (1980) 65:399–402.
8. Yurdakul S, Mat C, Tüzün Y, Ozyazgan Y, Hamuryudan V, Uysal O, et al. A double-blind trial of colchicine in Behçet's syndrome. *Arthritis Rheum*. (2001) 44:2686–92. doi: 10.1002/1529-0131(200111)44:11<2686::aid-art448>3.0.co;2-h
9. Davatchi F, Sadeghi Abdollahi B, Tehrani Banihashemi A, Shahram F, Nadji A, et al. Colchicine versus placebo in Behçet's disease: randomized, double-blind, controlled crossover trial. *Mod Rheumatol*. (2009) 19:542–9. doi: 10.1007/s10165-009-0200-2
10. Tunc R, Keyman E, Melikoglu M, Fresko I, Yazici H. Target organ associations in Turkish patients with Behçet's disease: a cross sectional study by exploratory factor analysis. *J Rheumatol*. (2002) 29:2393–6.
11. Yazici H, Pazarli H, Barnes CG, Tüzün Y, Özyazgan Y, Silman A, et al. A controlled trial of azathioprine in Behçet's syndrome. *N Engl J Med*. (1990) 322:281–5. doi: 10.1056/NEJM199002013220501
12. Hatemi G, Fresko I, Tascilar K, Yazici H. Increased enthesopathy among Behçet's syndrome patients with acne and arthritis: an ultrasonography study. *Arthritis Rheum*. (2008) 58:1539–4. doi: 10.1002/art.23450
13. Alpsoy E, Durusoy C, Yilmaz E, Ozgurel Y, Ermis O, Yazar S, et al. Interferon alfa-2a in the treatment of Behçet disease: a randomized placebo-controlled and double-blind study. *Arch Dermatol*. (2002) 138:467–71. doi: 10.1001/archderm.138.4.467
14. Azizlerli G, Sarica R, Köse A, Ovül C, Kavala M, Kayabali M, et al. Interferon alfa-2a in the treatment of Behçet's disease. *Dermatology*. (1996) 192:239–41. doi: 10.1159/000246374
15. Karaca M, Hatemi G, Sut N, Yazici H. The papulopustular lesion/arthritis cluster of Behcet's syndrome also clusters in families. *Rheumatology*. (2012) 51:1053–60. doi: 10.1093/rheumatology/ker423
16. Melikoglu M, Fresko I, Mat C, Ozyazgan Y, Gogus F, Yurdakul S, et al. Short-term trial of etanercept in Behçet's disease: a double blind, placebo controlled study. *J Rheumatol*. (2005) 32:98–105.
17. Arida A, Fragiadaki K, Giavri E, Sfikakis PP. Anti-TNF agents for Behçet's disease: analysis of published data on 369 patients. *Semin Arthritis Rheum*. (2011) 41:61–70. doi: 10.1016/j.semarthrit.2010.09.002
18. Vallet H, Riviere S, Sanna A, Deroux A, Moulis G, Addimanda O, et al. Efficacy of anti-TNF alpha in severe and/or refractory Behçet's disease: multicenter study of 124 patients. *J Autoimmun*. (2015) 62:67–74. doi: 10.1016/j.jaut.2015.06.005
19. Grayson PC, Yazici Y, Merideth M, Sen HN, Davis M, Novakovich E, et al. Treatment of mucocutaneous manifestations in Behçet's disease with anakinra: a pilot open-label study. *Arthritis Res Ther*. (2017) 19:69. doi: 10.1186/s13075-017-1222-3
20. Cantarini L, Vitale A, Scalini P, Dinarello CA, Rigante D, Franceschini R, et al. Anakinra treatment in drug-resistant Behcet's disease: a case series. *Clin Rheumatol*. (2015) 34:1293–301. doi: 10.1007/s10067-013-2443-8
21. Kurosawa M, Takeno Y, Kirino Y, Soejima N, Mizuki M. Subgroup classification of Behçet's disease using clinical information: analysis of a clinical database of patients receiving financial aid for treatment. In: *ISBD Conf 2018*. Rotterdam (2018).
22. Di Scala G, Bettiol A, Cojan RD, Finocchi M, Silvestri E, Emmi G. Efficacy of the anti-IL 17 secukinumab in refractory Behçet's syndrome: a preliminary study. *J Autoimmun*. (2018) 97:108–13. doi: 10.1016/j.jaut.2018.09.002
23. Tunc R, Saip S, Siva A, Yazici H. Cerebral venous thrombosis is associated with major vessel disease in Behçet's syndrome. *Ann Rheum Dis*. (2004) 63:1693–4. doi: 10.1136/ard.2003.018515
24. Shi J, Huang X, Li G, Wang L, Liu J, Xu Y, et al. Cerebral venous sinus thrombosis in Behçet's disease: a retrospective case-control study. *Clin Rheumatol*. (2018) 37:51–7. doi: 10.1007/s10067-017-3718-2
25. Uluduz D, Midi I, Duman T, Colakoglu S, Tüfekci A, Bakar M, et al. Behçet's disease as a causative factor of cerebral venous sinus thrombosis: subgroup analysis of data from the VENOST study. *Rheumatology*. (2018) 58:600–8. doi: 10.1093/rheumatology/key153
26. Roriz M, Crassard I, Lechtman S, Saadoun D, Champion K, Wechsler B, et al. Can anticoagulation therapy in cerebral venous thrombosis associated with Behçet's disease be stopped without relapse? *Rev Neurol*. (2018) 174:162–6. doi: 10.1016/j.neurol.2017.06.021
27. Saadoun D, Wechsler B, Resche-Rigon M, Trad S, Le Thi Huong D, Sbai A, et al. Cerebral venous thrombosis in Behçet's disease. *Arthritis Rheum*. (2009) 61:518–26. doi: 10.1002/art.24393
28. Ahn JK, Lee YS, Jeon CH, Koh E-M, Cha H-S. Treatment of venous thrombosis associated with Behcet's disease: immunosuppressive therapy alone versus immunosuppressive therapy plus anticoagulation. *Clin Rheumatol*. (2008) 27:201–5. doi: 10.1007/s10067-007-0685-z
29. Alibaz-Oner F, Karadeniz A, Ylmaz S, Balkarl A, Kimyon G, Yazc A, et al. Behçet disease with vascular involvement. *Medicine*. (2015) 94:e494. doi: 10.1097/MD.0000000000000494
30. Desbois AC, Wechsler B, Resche-Rigon M, Piette JC, Huong DLT, Amoura Z, et al. Immunosuppressants reduce venous thrombosis relapse in Behçet's disease. *Arthritis Rheum*. (2012) 64:2753–60. doi: 10.1002/art.34450
31. Emmi G, Bettiol A, Silvestri E, Di Scala G, Becatti M, Fiorillo C, et al. Vascular Behçet's syndrome: an update. *Intern Emerg Med*. (2018) 14:645–65. doi: 10.1007/s11739-018-1991-y
32. Hamuryudan V, Seyahi E, Ugurlu S, Melikoglu M, Hatemi G, Ozguler Y, et al. Pulmonary artery involvement in Behçet's syndrome: effects of anti-Tnf treatment. *Semin Arthritis Rheum*. (2015) 45:369–73. doi: 10.1016/j.semarthrit.2015.06.008
33. Tascilar K, Melikoglu M, Ugurlu S, Sut N, Caglar E, Yazici H. Vascular involvement in Behçet's syndrome: a retrospective analysis of associations and the time course. *Rheumatology*. (2014) 53:2018–22. doi: 10.1093/rheumatology/keu233
34. Bitik B, Tufan A, Sahin K, Sucullu Karadag Y, Can Sandikci S, Mercan R, et al. The association between the parenchymal neurological involvement and posterior uveitis in Behçet's syndrome. *Clin Exp Rheumatol*. (2016) 34:82–5.
35. Mohammadi M, Shahram F, Shams H, Akhlaghi M, Ashofteh F, Davatchi F. High-dose intravenous steroid pulse therapy in ocular involvement of Behcet's disease: a pilot double-blind controlled study. *Int J Rheum Dis*. (2017) 20:1269–76. doi: 10.1111/1756-185X.13095
36. Hamuryudan V, Ozyazgan Y, Hizli N, Mat C, Yurdakul S, Tüzün Y, et al. Azathioprine in Behcet's syndrome: effects on long-term prognosis. *Arthritis Rheum*. (1997) 40:769–74. doi: 10.1002/art.1780400425
37. Hamuryudan V, Ozyazgan Y, Fresko Y, Mat C, Yurdakul S, Yazici H. Interferon alfa combined with azathioprine for the uveitis of Behçet's disease: an open study. *Isr Med Assoc J*. (2002) 4:928–30.
38. Shahram F, Davatchi F, Chams H, Nadji A, Jamshidi A, Akbarian M, et al. Azathioprine and low dose pulse cyclophosphamide in severe ocular lesions of Behçet's disease. A preliminary report. *Adv Exp Med Biol*. (2003) 528:571–3. doi: 10.1007/0-306-48382-3_116
39. Giardina A, Ferrante A, Ciccia F, Vadalà M, Giardina E, Triolo G. One year study of efficacy and safety of infliximab in the treatment of patients with ocular and neurological Behçet's disease refractory to standard immunosuppressive drugs. *Rheumatol Int*. (2011) 31:33–7. doi: 10.1007/s00296-009-1213-z
40. Kikuchi H, Aramaki K, Hirohata S. Effect of infliximab in progressive Neuro-Behçet's syndrome. *J Neurol Sci*. (2008) 272:99–105. doi: 10.1016/j.jns.2008.05.002
41. Desbois AC, Addimanda O, Bertrand A, Deroux A, Pérard L, Depaz R, et al. Efficacy of anti-TNFα in severe and refractory neuro-Behcet disease. *Medicine*. (2016) 95:e3550. doi: 10.1097/MD.0000000000003550
42. Barešić M, Reihl M, Habek M, Vukojević N, Anić B. Improvement of neurological and ocular symptoms of Behçet's disease after the introduction of infliximab. *Rheumatol Int*. (2018) 38:1301–6. doi: 10.1007/s00296-018-4054-9

43. Jaffe GJ, Dick AD, Brézin AP, Nguyen QD, Thorne JE, Kestelyn P, et al. Adalimumab in Patients with Active Noninfectious Uveitis. *N Engl J Med*. (2016) 375:932–43. doi: 10.1056/NEJMoa1509852
44. Nguyen QD, Merrill PT, Jaffe GJ, Dick AD, Kurup SK, Sheppard J, et al. Adalimumab for prevention of uveitic flare in patients with inactive non-infectious uveitis controlled by corticosteroids (VISUAL II): a multicentre, double-masked, randomised, placebo-controlled phase 3 trial. *Lancet*. (2016) 388:1183–92. doi: 10.1016/S0140-6736(16)31339-3
45. Fabiani C, Vitale A, Emmi G, Vannozzi L, Lopalco G, Guerriero S, et al. Efficacy and safety of adalimumab in Behçet's disease-related uveitis: a multicenter retrospective observational study. *Clin Rheumatol*. (2017) 36:183–9. doi: 10.1007/s10067-016-3480-x
46. Bitossi A, Bettiol A, Silvestri E, Di Scala G, Bacherini D, Lopalco G, et al. Adalimumab accounts for long-term control of noninfectious uveitis also in the absence of concomitant DMARD treatment: a multicenter retrospective study. *Mediat Inflamm*. (2019) 2019:1623847. doi: 10.1155/2019/1623847
47. Davatchi F, Sadeghi Abdollahi B, Shams H, Shahram F, Nadji A, et al. Combination of pulse cyclophosphamide and azathioprine in ocular manifestations of Behcet's disease: longitudinal study of up to 10 years. *Int J Rheum Dis*. (2014) 17:444–52. doi: 10.1111/1756-185X.12248
48. Noel N, Bernard R, Wechsler B, Resche-Rigon M, Depaz R, Le Thi Huong Boutin D, et al. Long-term outcome of neuro-Behçet's disease. *Arthritis Rheumatol*. (2014) 66:1306–14. doi: 10.1002/art.38351
49. Yoon D-L, Kim YJ, Koo BS, Kim Y-G, Lee C-K, Yoo B. Neuro-behçet's disease in South Korea: clinical characteristics and treatment response. *Int J Rheum Dis*. (2014) 17:453–8. doi: 10.1111/1756-185X.12265
50. Atienza-Mateo B, Calvo-Río V, Beltrán E, Martínez-Costa L, Valls-Pascual E, Hernández-Garfella M, et al. Anti-interleukin 6 receptor tocilizumab in refractory uveitis associated with Behçet's disease: multicentre retrospective study. *Rheumatology*. (2018) 57:856–64. doi: 10.1093/rheumatology/kex480
51. Shapiro LS, Farrell J, Borhani Haghighi A. Tocilizumab treatment for neuro-Behcet's disease, the first report. *Clin Neurol Neurosurg*. (2012) 114:297–8. doi: 10.1016/j.clineuro.2011.10.024
52. Urbaniak P, Hasler P, Kretzschmar S. Refractory neuro-Behçet treated by tocilizumab: a case report. *Clin Exp Rheumatol*. (2012) 30:S73–5.
53. Addimanda O, Pipitone N, Pazzola G, Salvarani C. Tocilizumab for severe refractory neuro-Behçet: three cases IL-6 blockade in neuro-Behçet. *Semin Arthritis Rheum*. (2015) 44:472–5. doi: 10.1016/j.semarthrit.2014.08.004
54. Davatchi F, Chams-Davatchi C, Shams H, Shahram F, Nadji A, Akhlaghi M, et al. Behcet's disease: epidemiology, clinical manifestations, and diagnosis. *Expert Rev Clin Immunol*. (2017) 13:57–65. doi: 10.4172/2472-4971.1000141
55. Hatemi G, Christensen R, Bang D, Bodaghi B, Celik AF, Fortune F, et al. 2018 update of the EULAR recommendations for the management of Behçet's syndrome. *Ann Rheum Dis*. (2018) 77:808–18. doi: 10.1136/annrheumdis-2018-213225
56. Kural-Seyahi E, Fresko I, Seyahi N, Ozyazgan Y, Mat C, Hamuryudan V, et al. The long-term mortality and morbidity of Behçet syndrome: a 2-decade outcome survey of 387 patients followed at a dedicated center. *Medicine*. (2003) 82:60–76. doi: 10.1097/00005792-200301000-00006
57. Alibaz-Oner F, Mumcu G, Kubilay Z, Ozen G, Celik G, Karadeniz A, et al. Unmet need in Behcet's disease: most patients in routine follow-up continue to have oral ulcers. *Clin Rheumatol*. (2014) 33:1773–6. doi: 10.1007/s10067-014-2585-3
58. Giza M, Koftori D, Chen L, Bowness P. Is Behçet's disease a 'class 1-opathy'? The role of HLA-B*51 in the pathogenesis of Behçet's disease. *Clin Exp Immunol*. (2018) 191:11–18. doi: 10.1111/cei.13049
59. Emmi G, Talarico R, Lopalco G, Cimaz R, Cantini F, Viapiana O, et al. Efficacy and safety profile of anti-interleukin-1 treatment in Behçet's disease: a multicenter retrospective study. *Clin Rheumatol*. (2016) 35:1281–6. doi: 10.1007/s10067-015-3004-0
60. Lopalco G, Fabiani C, Venerito V, Lapadula G, Iannone F, Cantarini L. Ustekinumab efficacy and safety in mucocutaneous multi-refractory Behçet's disease. *Clin Exp Rheumatol*. (2017) 108:130–1. doi: 10.3389/fimmu.2017.00200
61. Mirouse A, Barete S, Monfort JB, Resche-Rigon M, Bouyer AS, Comarmond C, et al. Ustekinumab for Behçet's disease. *J Autoimmun*. (2017) 82:41–6. doi: 10.1016/j.jaut.2017.05.002
62. Mirouse A, Barete S, Desbois A, Comarmond C, Sène D, Domont F, et al. Long term outcome of ustekinumab therapy for Behçet's disease. *Arthritis Rheumatol*. (2019) 71:1727–32. doi: 10.1002/art.40912
63. Saini A, Ferguson C, Salkey K. Use of apremilast for aphthous ulcers in a patient with Behçet's syndrome. *J Drugs Dermatol*. (2018) 17:1328–9.
64. Hatemi G, Melikoglu M, Tuncay R, Korkmaz C, Ozturk BT, Mat C, et al. Apremilast for Behçet's syndrome - a phase 2, placebo-controlled study. *N Engl J Med*. (2015) 372:1510–18. doi: 10.1056/NEJMoa1408684
65. Hatemi G, Mahr A, Ishigatsubo Y, Song YW, Takeno M, Kim D, Melikoğlu M, et al. Trial of apremilast for oral ulcers in Behçet's syndrome. *N Engl J Med*. (2019) 381:1918–28. doi: 10.1056/NEJMoa1816594
66. Emmi G, Silvestri E, Squatrito D, Emmi L, Cantarini L, Prisco D. Tocilizumab-induced exacerbation of mucosal ulcers in a patient with multi-refractory Behçet's disease. *Semin Arthritis Rheum*. (2016) 46:e1-2. doi: 10.1016/j.semarthrit.2016.03.006
67. Cantarini L, Lopalco G, Vitale A, Coladonato L, Rigante D, Lucherini OM, et al. Paradoxical mucocutaneous flare in a case of Behçet's disease treated with tocilizumab. *Clin Rheumatol*. (2015) 34:1141–3. doi: 10.1007/s10067-014-2589-z
68. Cantarini L, Vitale A, Lucherini OM, De Clemente C, Caso F, Costa L, et al. The labyrinth of autoinflammatory disorders: a snapshot on the activity of a third-level center in Italy. *Clin Rheumatol*. (2015) 34:17–28. doi: 10.1007/s10067-014-2721-0
69. Becatti M, Emmi G, Bettiol A, Silvestri E, Di Scala G, Taddei N, et al. Behçet's syndrome as a tool to dissect the mechanisms of thrombo-inflammation: clinical and pathogenetic aspects. *Clin Exp Immunol*. (2018) 195:322–33. doi: 10.1111/cei.13243
70. Silvestri E, Emmi G, Prisco D. Vascular Behçet's disease: new insights in the management of thrombosis. *Expert Rev Cardiovasc Ther*. (2013) 11:1583–5. doi: 10.1586/14779072.2013.836449
71. Emmi G, Silvestri E, Squatrito D, Amedei A, Niccolai E, D'Elios MM, et al. Thrombosis in vasculitis: from pathogenesis to treatment. *Thromb J*. (2015) 13:15. doi: 10.1186/s12959-015-0047-z
72. Akman-Demir G, Serdaroglu P, Tasçi B. Clinical patterns of neurological involvement in Behçet's disease: evaluation of 200 patients. The Neuro-Behçet Study Group. *Brain*. (1999) 122(Pt 11):2171–82. doi: 10.1093/brain/122.11.2171
73. Noel N, Drier A, Wechsler B, Piette J-C, De Paz R, Dormont D, et al. [Neurological manifestations of Behçet's disease]. *La Rev Med Interne*. (2014) 35:112–20. doi: 10.1016/j.revmed.2013.10.332
74. Brenière C, Blanc C, Devilliers H, Samson M, Delpont B, Bielefeld P, et al. Associated arterial and venous cerebral manifestations in Behçet's disease. *Rev Neurol*. (2018) 174:337–41. doi: 10.1016/j.neurol.2017.06.031
75. Becatti M, Emmi G, Silvestri E, Bruschi G, Ciucciarelli L, Squatrito D, et al. Neutrophil activation promotes fibrinogen oxidation and thrombus formation in Behçet disease. *Circulation*. (2016) 133:302–11. doi: 10.1161/CIRCULATIONAHA.115.017738
76. Becatti M, Mannucci A, Taddei N, Fiorillo C. Oxidative stress and inflammation: new molecular targets for cardiovascular diseases. *Intern Emerg Med*. (2018) 13:647–9. doi: 10.1007/s11739-018-1865-3
77. Prisco D, Silvestri E, Di Scala G, Emmi G. Behçet's disease as a cause of cerebral sinus vein thrombosis: an emerging role. *Rheumatology*. (2018) 58:563–56. doi: 10.1093/rheumatology/key279
78. Seyahi E, Cakmak OS, Tutar B, Arslan C, Dikici AS, Sut N, et al. Clinical and ultrasonographic evaluation of lower-extremity vein thrombosis in Behcet syndrome: an observational study. *Medicine*. (2015) 94:e1899. doi: 10.1097/MD.0000000000001899
79. Esatoglu SN, Hatemi G. Update on the treatment of Behçet's syndrome. *Intern Emerg Med*. (2019) 14:661–75. doi: 10.1007/s11739-019-02035-1
80. Ozgurel Y, Hatemi G, Cetinkaya F, Tascilar K, Ugurlu S, SeyahI E, et al. Interferon-alpha for the management of lower extremity deep vein thrombosis in Behcet's syndrome: a case series. *Ann Rheum Dis*. (2018) 77:1496. doi: 10.1136/annrheumdis-2018-eular.7197
81. Balcioglu O, Ertugay S, Bozkaya H, Parildar M, Posacioglu H. Endovascular repair and adjunctive immunosuppressive therapy of aortic involvement in Behçet's disease. *Eur J Vasc Endovasc Surg*. (2015) 50:593–8. doi: 10.1016/j.ejvs.2015.07.011

82. Kwon Koo B, Shim W-H, Yoon Y-S, Kwon Lee B, Choi D, Jang Y, et al. Endovascular therapy combined with immunosuppressive treatment for pseudoaneurysms in patients with Behçet's disease. *J Endovasc Ther*. (2003) 10:75–80. doi: 10.1177/152660280301000116
83. Aamar S, Peleg H, Leibowitz D, Chajek-Shaul T, Hiller N, Heyman SN. Efficacy of adalimumab therapy for life-threatening pulmonary vasculitis in Behçet's disease. *Rheumatol Int*. (2014) 34:857–60. doi: 10.1007/s00296-013-2693-4
84. Lee S-W, Lee S-Y, Kim K-N, Jung J-K, Chung W-T. Adalimumab treatment for life threatening pulmonary artery aneurysm in Behçet disease: a case report. *Clin Rheumatol*. (2010) 29:91–3. doi: 10.1007/s10067-009-1272-2
85. Uygunoglu U, Siva A. Behçet's syndrome and nervous system involvement. *Curr Neurol Neurosci Rep*. (2018) 18:35. doi: 10.1007/s11910-018-0843-5
86. Berlin C. Behçet's syndrome with involvement of central nervous system. *Arch Derm Syph*. (1944) 49:227–33. doi: 10.1001/archderm.1944.01510100003001
87. Ozyazgan Y, Ucar D, Hatemi G, Yazici Y. Ocular involvement of Behçet's syndrome: a comprehensive review. *Clin Rev Allergy Immunol*. (2015) 49:298–306. doi: 10.1007/s12016-014-8425-z
88. Altunkaynak Y, Usta S, Ertem DH, Köksal A, Dirican AC, Baybaş S. Cognitive functioning and silent neurological manifestations in Behçet's disease with ocular involvement. *Noro Psikiyatr Ars*. (2019) 56:173–7. doi: 10.5152/npa.2017.19406
89. Graw J. Eye development. *Curr Top Dev Biol*. (2010) 90:343–86. doi: 10.1016/S0070-2153(10)90010-0
90. Caruso P, Moretti R. Focus on neuro-Behçet's disease: a review. *Neurol India*. (2018) 66:1619–28. doi: 10.4103/0028-3886.246252
91. Siva A, Kantarci OH, Saip S, Altintas A, Hamuryudan V, Islak C, et al. Behçet's disease: diagnostic and prognostic aspects of neurological involvement. *J Neurol*. (2001) 248:95–103. doi: 10.1007/s004150170242
92. Kalra S, Silman A, Akman-Demir G, Bohlega S, Borhani-Haghighi A, Constantinescu CS, et al. Diagnosis and management of Neuro-Behçet's disease: international consensus recommendations. *J Neurol*. (2014) 261:1662–76. doi: 10.1007/s00415-013-7209-3
93. Fortin E, van Calster J, van, Goto H, Namba K, Douglas K, Kron M, et al. Long-term efficacy and safety of adalimum-ab in Behçet's disease patients with non-infectious uveitis in the VISUAL III trial. *Clin Exp Rheumatol*. (2018) 36:S174.
94. Fabiani C, Vitale A, Rigante D, Emmi G, Lopalco G, Sota J, et al. Predictors of sustained clinical response in patients with Behçet's disease-related uveitis treated with infliximab and adalimumab. *Clin Rheumatol*. (2018) 37:1715–20. doi: 10.1007/s10067-018-4092-4
95. Fabiani C, Vitale A, Emmi G, Bitossi A, Lopalco G, Sota J, et al. Long-term retention rates of adalimumab and infliximab in non-infectious intermediate, posterior, and panuveitis. *Clin Rheumatol*. (2018) 38:63–7. doi: 10.1007/s10067-018-4069-3
96. Fabiani C, Sota J, Vitale A, Rigante D, Emmi G, Vannozzi L, et al. Cumulative retention rate of adalimumab in patients with Behçet's disease-related uveitis: a four-year follow-up study. *Br J Ophthalmol*. (2018) 102:637–41. doi: 10.1136/bjophthalmol-2017-310733
97. Ozyazgan Y, Yurdakul S, Yazici H, Tüzün B, Işçimen A, Tüzün Y, et al. Low dose cyclosporin A versus pulsed cyclophosphamide in Behçet's syndrome: a single masked trial. *Br J Ophthalmol*. (1992) 76:241–3. doi: 10.1136/bjo.76.4.241
98. Kürtüncü M, Tüzün E, Akman-Demir G. Behçet's disease and nervous system involvement. *Curr Treat Options Neurol*. (2016) 18:19. doi: 10.1007/s11940-016-0405-6
99. Bolek EC, Sari A, Kilic L, Karadag O. Interferon alpha might be an alternative therapeutic choice for refractory Neuro-Behçet's disease. *Mult Scler Relat Disord*. (2019) 29:153. doi: 10.1016/j.msard.2019.01.015
100. London F, Duprez T, van Pesch V. Response to correspondence: interferon alpha might be an alternative therapeutic choice for refractory neuro-Behçet's disease. *Mult Scler Relat Disord*. (2019) 29:154. doi: 10.1016/j.msard.2018.12.027
101. Kato Y, Numaga J, Kato S, Kaburaki T, Kawashima H, Fujino Y. Central nervous system symptoms in a population of Behçet's disease patients with refractory uveitis treated with cyclosporine A. *Clin Experiment Ophthalmol*. (2001) 29:335–6. doi: 10.1046/j.1442-9071.2001.00445.x
102. Akman-Demir G, Ayranci O, Kurtuncu M, Vanli EN, Mutlu M, Tugal-Tutkun I. Cyclosporine for Behçet's uveitis: is it associated with an increased risk of neurological involvement? *Clin Exp Rheumatol*. (2008) 26:S84–90.
103. Kötter I, Günaydin I, Batra M, Vonthein R, Stübiger N, Fierlbeck G, et al. CNS involvement occurs more frequently in patients with Behçet's disease under cyclosporin A (CSA) than under other medications–results of a retrospective analysis of 117 cases. *Clin Rheumatol*. (2006) 25:482–6. doi: 10.1007/s10067-005-0070-8

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.

frontiers in Immunology

ORIGINAL RESEARCH
published: 10 December 2019
doi: 10.3389/fimmu.2019.02843

The Co-inhibitor BTLA Is Functional in ANCA-Associated Vasculitis and Suppresses Th17 Cells

*Kai Werner[1], Sebastian Dolff[2], Yang Dai[1], Xin Ma[1], Alexandra Brinkhoff[1], Johannes Korth[1], Anja Gäckler[1], Hana Rohn[2], Ming Sun[1], Jan Willem Cohen Tervaert[3], Pieter van Paassen[4], Andreas Kribben[1], Oliver Witzke[2] and Benjamin Wilde[1]**

[1] Department of Nephrology, University Hospital Essen, University of Duisburg-Essen, Essen, Germany, [2] Department of Infectious Diseases, University Hospital Essen, University of Duisburg-Essen, Essen, Germany, [3] Division of Rheumatology, Department of Medicine, University of Alberta, Edmonton, AB, Canada, [4] Section of Nephrology and Immunology, Department of Internal Medicine, Maastricht University Medical Center, Maastricht, Netherlands

OPEN ACCESS

Edited by:
Federico Alberici,
University of Milan, Italy

Reviewed by:
Vladimir Tesar,
Charles University, Czechia
Augusto Vaglio,
University of Parma, Italy

***Correspondence:**
Benjamin Wilde
benjamin.wilde@uk-essen.de

Specialty section:
This article was submitted to Autoimmune and Autoinflammatory Disorders, a section of the journal Frontiers in Immunology

Received: 04 September 2019
Accepted: 19 November 2019
Published: 10 December 2019

Citation:
Werner K, Dolff S, Dai Y, Ma X, Brinkhoff A, Korth J, Gäckler A, Rohn H, Sun M, Cohen Tervaert JW, van Paassen P, Kribben A, Witzke O and Wilde B (2019) The Co-inhibitor BTLA Is Functional in ANCA-Associated Vasculitis and Suppresses Th17 Cells. Front. Immunol. 10:2843. doi: 10.3389/fimmu.2019.02843

Objectives: The activation and inhibition of T-cells has been well-studied under physiological conditions. Co-inhibition is an important mechanism to keep effector T-cells in check. Co-inhibitors mediate peripheral self-tolerance and limit the immune response. Dysfunctional co-inhibition is associated with loss of T-cell regulation and induction of autoimmunity. Therefore, we investigated the co-inhibitor B- and T-Lymphocyte attenuator (BTLA) in ANCA-associated vasculitis (AAV).

Methods: Fifty-six AAV patients and 32 healthy controls (HC) were recruited. Flow cytometry was performed to investigate the expression of BTLA on T-cells. Double negative T-cells were defined as $CD3^+CD4^-CD8^-$. To assess the functionality of BTLA, CFSE-labeled T-cells were stimulated in presence or absence of an agonistic anti-BTLA antibody. In addition, impact of BTLA-mediated co-inhibition on Th17 cells was studied.

Results: AAV patients in remission had a decreased expression of BTLA on double negative T-cells ($CD3^+CD4^-CD8^-$). On all other subtypes of T-cells, expression of BTLA was comparable to healthy controls. TCR-independent stimulation of T-cells resulted in down-regulation of BTLA on Th cells in AAV and HC, being significantly lower in HC. Co-inhibition via BTLA led to suppression of T-cell proliferation in AAV as well as in HC. As a result of BTLA mediated co-inhibition, Th17 cells were suppressed to the same extent in AAV and HC.

Conclusion: BTLA expression is altered on double negative T-cells but not on other T-cell subsets in quiescent AAV. BTLA-induced co-inhibition has the capacity to suppress Th17 cells and is functional in AAV. Thus, BTLA-mediated co-inhibition might be exploited for future targeted therapies in AAV.

Keywords: ANCA vasculitis, BTLA, co-inhibition, immune checkpoint, Th17 cells

INTRODUCTION

Anti-neutrophil cytoplasmatic antibody (ANCA)-associated vasculitis (AAV) is an autoimmune disease characterized by the presence of autoantibodies directed against myeloperoxidase (MPO) or Proteinase-3 (PR3) expressed by neutrophils (1). AAV mainly affects small- to medium- sized vessels. T-cells have an important role in the pathogenesis of AAV and persistent T-cell activation is frequently observed (2, 3). Th17 cells and effector memory T-cells are expanded which appears to be independent of disease activity (2, 4, 5). T-cell infiltrates are frequently found in affected organs (6–9). Moreover, defective function of regulatory T-cells has been demonstrated in AAV indicating disturbed immune regulation (10, 11). T-cells are not only restrained by regulatory cell subsets but are also controlled by a system of co-stimulation (12). Positive co-stimulation promotes T-cell activation whereas co-inhibition limits and suppresses activation of T-cells (12). Co-inhibitory signals may induce anergy or cell death of T-cells (12, 13). These mechanisms are important to maintain immune tolerance. Dysfunctional co-stimulation and co-inhibition promote the break of tolerance and are associated with autoimmunity. In human, a defective co-inhibitory PD1/PDL-1 axis is associated with a number of autoimmune diseases such as systemic lupus erythematosus and AAV (14, 15). In addition, targeted blockade of this system promotes the development of AAV (16). The B- and T-Lymphocyte Attenuator (BTLA) is an Ig superfamily member molecule and interacts with the herpes virus entry mediator (HVEM), a member of the TNFR family (13, 17, 18). BTLA is a co-inhibitor which is predominantly expressed on B-cells, T-cells and dendritic cells. BTLA ligation with HVEM results in a reduction of T-cell proliferation (13, 17, 18). Mice with BTLA deficiency show increased T-cell activation and higher levels of circulating TNF-α, IFN-γ, IL-2, and IL-4 (19, 20). However, BTLA as a co-inhibitor is scarcely studied in human autoimmune diseases and its role in disease pathogenesis is unclear. Therefore, it was our goal to examine the expression as well as function of BTLA on T-cells and B-cells in AAV.

MATERIALS AND METHODS

Patients

We enrolled 56 AAV patients who visited the outpatient clinic of the department of Nephrology at the University Hospital Essen. Eleven patients were measured twice. Forty-eight patients were in remission at the time of sampling, eight patients suffered from active vasculitis. The mean Birmingham vasculitis activity score of the active patients was 10 ± 3. None of the active patients were treatment naïve at the time of sampling; all patients had already received low dose steroids. One patient had received one dose of rituximab 2 days before sampling and one treatment cycle with plasma exchange. None of the active patients had received cyclophosphamide recently or before sampling. Two patients suffered from new-onset disease, the remaining six patients had a relapse. The clinical and laboratory characteristics of the quiescent patients at the time of sampling are given in **Table 1**. As a control cohort, we enrolled 32 persons [18 men and 14 women with median age of 51 (47–54) years] who had no history of immunological, infectious, rheumatic, or malignant disease. The Watts criteria were used to differentiate between GPA, EGPA and MPA (21). EULAR-criteria were used to classify the stage of diseases (22). All patients provided written informed consent. The study was approved by the local institutional review board.

TABLE 1 | Clinical characteristics of AAV patients in remission.

Total, *n*	**48**
Age, median (IQR), years	55 (19–84)
Gender, female/male, *n*	24/24
PR3/MPO/neg, *n*	25/22/1
Disease duration, median (IQR), months	36 (2–236)
Localized/systemic disease, *n*	12/36
CMV anti-IgG, +/−/na, *n*	20/15/13
Prednisone +/−, *n*	42/6
Azathioprine +/−, *n*	19/29
Mycophenolate Mofetil +/−, *n*	19/29
Methotrexate +/−, *n*	1/47
Rituximab +/−, *n*	2/46
Leflunomide +/−, *n*	1/47

na, not available; neg, negative.

Flow Cytometric Analysis of T-Cells and B-Cells

Separation of peripheral blood mononuclear cells (PBMC) out of whole blood was performed by Ficoll-density gradient centrifugation. PBMC were counted by BLAUBRAND® counting chamber. For surface staining, the following monoclonal antibodies were used: anti-human CD19 (PB, Beckman Coulter, Krefeld, Germany), anti-human CD3 (HORV450, BD Biosciences, Heidelberg, Germany), anti-human CD4 (PerCP, Biolegend, Koblenz, Germany), anti-human CD8 (APC-H7, BD Biosciences), anti-human CD45RA (APC, Beckman Coulter), CD272 (BTLA, PE, Biolegend). Appropriate isotype-controls and fluorescence-minus-one controls were used. After isolation, PBMC were incubated with monoclonal antibodies for 30 min at room temperature in the dark followed by washing steps with PBS. Double negative T-cells were defined as $CD3^+CD4^-CD8^-$ (DN), naïve double negative T-cells were defined as $CD3^+CD4^-CD8^-CD45RA^+$ and memory double negative T-cells were defined as $CD3^+CD4^-CD8^-CD45RA^-$. Memory T-helper-cells were defined as $CD3^+CD4^+CD45RA^-$. The analysis of the samples was carried out on a FACS Navios flow cytometer from Beckman Coulter. Where indicated, PBMC were stimulated by phorbol 12-myristat 13-acetat (PMA) (50 ng/ml, Sigma Aldrich, Taufkirchen, Germany), and Ionomycin (1 μg/ml) (Sigma Aldrich) in RPMI 1640 medium (Gibco Invitrogen, Darmstadt, Germany) supplemented with 10% heat-inactivated fetal calf serum (Greiner Bio-One, Frickenhausen, Germany), 100 U/mL penicillin and 100 μg/mL Streptomycin (both Gibco Invitrogen). PBMC were incubated for 4 h in a 5% CO_2 atmosphere at 37°C in culture medium. Unstimulated samples served as controls and were incubated

without stimulus. After incubation, surface staining was performed and cells were analyzed on a flow cytometer (Navios, Beckman Coulter). Kaluza Analysis Software (Version 1.5, Beckman Coulter) was used for analysis of flow cytometric data.

Functional Studies of BTLA

B-cells were isolated by using a negative selection method based on a magnetic bead technology (B cell isolation kit II, Miltenyi Biotec, Bergisch Gladbach, Germany), typical purity of isolated B-cells was above 95%. In order to quantify the proliferation of lymphocytes, isolated PBMCs or isolated B-cells were labeled with CFSE. CFSE was used at a concentration of 2 μM (Thermo Fisher Scientific, Dreieich, Germany). PBMC labeled with CFSE were then stimulated with anti-CD3 (0.5 ng/ml, clone HIT3a, Biolegend) and anti-CD28 (0.5 ng/ml, clone 28.2, Biolegend) in the presence or absence of an agonistic anti-BTLA antibody (50 ug/ml, clone MIH26, BioLegend) for 72 h. Isolated B-cells were stimulated with the TLR-agonist CpG ODN 2006 (Invivogen Toulouse, France) in the presence or absence of an agonistic anti-BTLA antibody (50 ug/ml, clone MIH26, BioLegend) for 72 h. In conditions without anti-BTLA antibody, an isotype control was used instead at the same concentration (50 ug/ml, mouse IgG2a, Invitrogen). Incubation was carried out at 37°C in 5% CO_2 atmosphere. After 72 h, PBMC were stained with anti-CD3 (Pacific Blue, Beckman Coulter), anti-CD4 (APC, Beckman Coulter), anti-CD8 (APC-H7, Becton Dickinson), and 7AAD (Biolegend). B-cells were restimulated with PMA (10 ng/ml, Sigma–Aldrich, Taufkirchen, Germany), Ionomycin (1 μg/ml, Sigma–Aldrich) in the presence of Brefeldin A (5 μg/ml, BD Biosciences) for 6 h followed by surface staining, fixation and permeabilization (CytoFix/CytoPerm kit, BD Biosciences, Erembodegen, Belgium). For intracellular flow cytometric analysis the Breg staining was performed with: anti-CD3(HorV450), anti-CD19(PB), anti-7AAD and IL-10 (APC). After fixation and permeabilization, PBMCs were stained intracellularly for IL-10 (APC) and CD69 (PE-CY7). Appropriate isotype controls were used to confirm specificity of staining and to discriminate background staining. The suppressive capacity of BTLA was determined as the relative inhibition of cell proliferation and was calculated as follows: (proliferated fraction of cells without anti-BTLA [isotype] MINUS proliferated fraction of cells with anti-BTLA) DIVIDED by proliferation of PBMCs without anti-BTLA [isotype] MULTPLIED by 100. In addition, cell culture supernatants were collected. All samples were stored at −20°C until bulk analysis. Human IL-17A and IFN-γ ELISA immunoassays were purchased by R&D systems Europe, Ltd. (Quantikine ELISA). The test was performed according to the manufacturer's instructions. IL-17A and IFN-γ levels are expressed as pg/mL.

Statistical Analysis

All values are given as mean ± standard error of the mean. The Mann-Whitney *U* test was used to detect statistically significant differences between two unpaired groups. The Wilcoxon test was performed to assess paired groups. $P < 0.05$ were considered as significant. GraphPad Prism 6.0c (GraphPad Software, Inc., California) was used for statistical analysis.

RESULTS

Reduced Expression of BTLA on Double Negative T-Cells in AAV

In quiescent AAV patients (AAV-r), the BTLA expression did not differ from HC on peripheral T-cells (AAV-r vs. HC, $CD3^+$ T-cells: $\%BTLA^{pos}$, 85.2 ± 1.7% vs. 86.6 ± 2.4%, $p = 0.19$, **Figure 1**). the same was found for T-helper cells (Th cells, AAV-r vs. HC, $\%BTLA^{pos}$ within $CD3^+CD4^+$ T-cells: 91.5 ± 1.2% vs. 92.2 ± 1.4%, $p = 0.21$), memory Th cells (AAV-r vs. HC, $\%BTLA^{pos}$ within $CD3^+CD4^+CD45RA^-$ T-cells: 90.1 ± 1.1 vs. 92.3 ± 1.6%, $p = 0.2$), and cytotoxic T-cells (AAV-r vs. HC, $\%BTLA^{pos}$ within $CD3^+CD8^+$ T-cells: 84.9 ± 2.5% vs. 81.6 ± 3.7%, $p = 0.54$). On double negative T-cells (DN, $CD3^+CD4^-CD8^-$) the expression of BTLA was significantly decreased in AAV (AAV-r vs. HC, $\%BTLA^{pos}$ within $CD3^+CD4^-CD8^-$ T-cells: 64.9 ± 3.6% vs. 84.0 ± 2.7%, $p < 0.001$, **Figure 1**). The lower BTLA expression in AAV-r could also be found on naïve DN T-cells (AAV-r vs. HC, $\%BTLA^{pos}$ within $CD3^+CD4^-CD8^-CD45RA^+$, $n = 34/27$; 91 ± 1.8% vs. 94 ± 2.1%, $p < 0.05$), and memory DN T-cells (AAV-r vs. HC, $\%BTLA^{pos}$ within $CD3^+CD4^-CD8^-CD45RA^-$, $n = 34/27$; 67.1 ± 3.4% vs. 85.5 ± 2.9%, $p < 0.05$). The frequency of DN T-cells was comparable between AAV und HC (AAV-r vs. HC, $\%CD4^-CD8^-$ within $CD3^+$ T-cells: 4.2 ± 0.4 vs. 5.1 ± 0.5%, $p > 0.05$). It was further studied whether the BTLA expression pattern was dependent on disease activity. For this purpose, patients with active ANCA-vasculitis (AAV-a) were recruited. Interestingly, BTLA was downregulated on T-helper-cells in patients with active disease as compared to HC and patients in remission ($\%BTLA^{pos}$ within $CD4^+$ T-helper-cells, AAV-a vs. HC: 85.9 ± 1.6% vs. 92.2 ± 1.4%, $p = 0.006$; AAV-a vs. AAV-r: 85.9 ± 1.6% vs. 91.5 ± 1.2%, $p = 0.001$). Cytotoxic T-cells showed reduced BTLA expression in active patients when compared to patients in remission ($\%BTLA^{pos}$ within $CD8^+$ T-cells: 78.6 ± 4.8% vs. 84.9 ± 2.5%, $p = 0.02$). In contrast, BTLA was upregulated on DN T-cells in active disease as compared to quiescent disease ($\%BTLA^{pos}$ within DN T-cells, 82.2 ± 7.5% vs. 64.9 ± 3.6%, $p = 0.03$). BTLA expression seemed to be dependent on disease activity and was differentially expressed on the specific T-cell subsets.

Longitudinal Assessment of BTLA Expression on T-Cells in AAV

To detect variability of BTLA expression, eleven AAV-r patients were measured twice over a period of 1 year (**Figure 2**). In AAV patients, the expression of BTLA did not change significantly between the first and the second visit on Th cells (AAV-r patients at the first visit vs. second visit, 93.1 ± 3.3% vs. 95.1 ± 6.9%, $p = 0.7$) and on cytotoxic T-cells (AAV-r patients at the first visit vs. second visit, $CD3^+CD8^+$ T-cells: $\%BTLA^{pos}$, 85.1 ± 18.6% vs. 83 ± 19.4%, $p = 0.41$). On double negative T-cells, the variability of BTLA expression was not significantly altered (AAV-r patients at the first visit vs. second visit, $CD3^+CD4^-CD8^-$: $\%BTLA^{pos}$, 66.7 ± 16.7% vs. 72.4 ± 25.2%, $p = 0.76$).

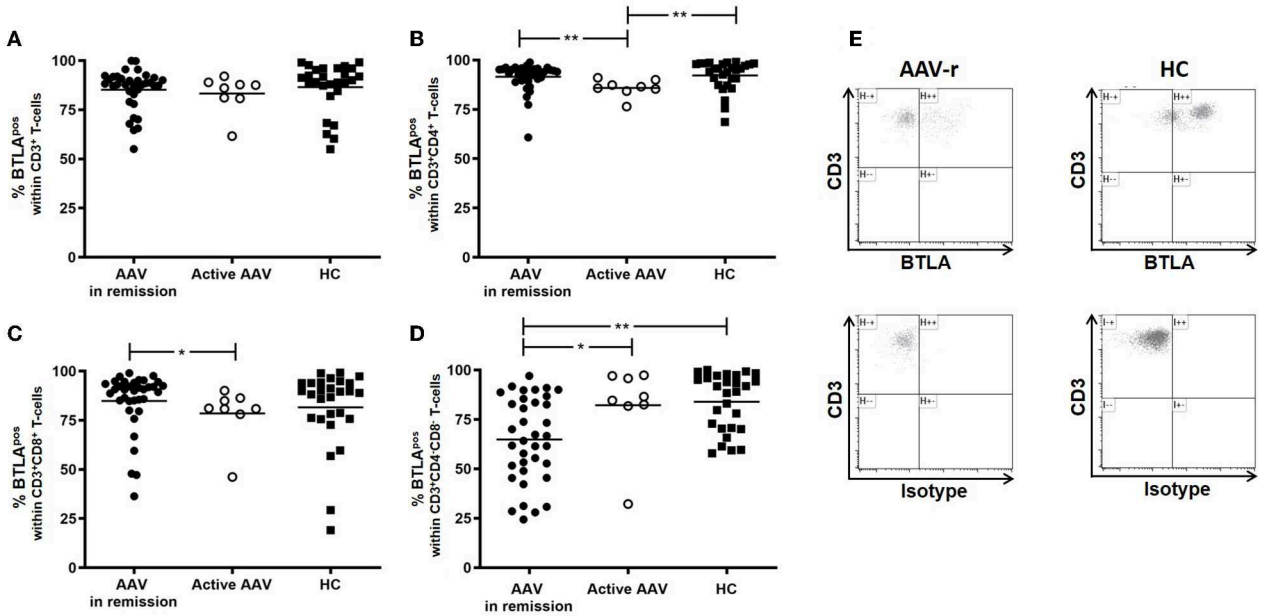


FIGURE 1 | BTLA expression on circulating T-cells in AAV and HC. **(A)** Expression of BTLA was comparable between AAV und HC on $CD3^+$ T-cells. **(B)** BTLA expression did not differ on Th cells and on **(C)** cytotoxic T-cells in quiescent AAV vs. HC. Patients with active disease showed diminished BTLA expression on Th cells and cytotoxic T-cells. **(D)** On $CD3^+CD4^-CD8^-$ T-cells, BTLA was diminished in quiescent AAV as compared to HC. In active patients, BTLA expression was enhanced as compared to patients in remission. **(E)** Representative flow cytometric data is depicted. The plots are gated on $CD3^+CD4^-CD8^-$ T-cells. Significant differences as calculated by the Mann-Whitney *U*-test are indicated: $^*p < 0.05$, $^{**}p < 0.01$.

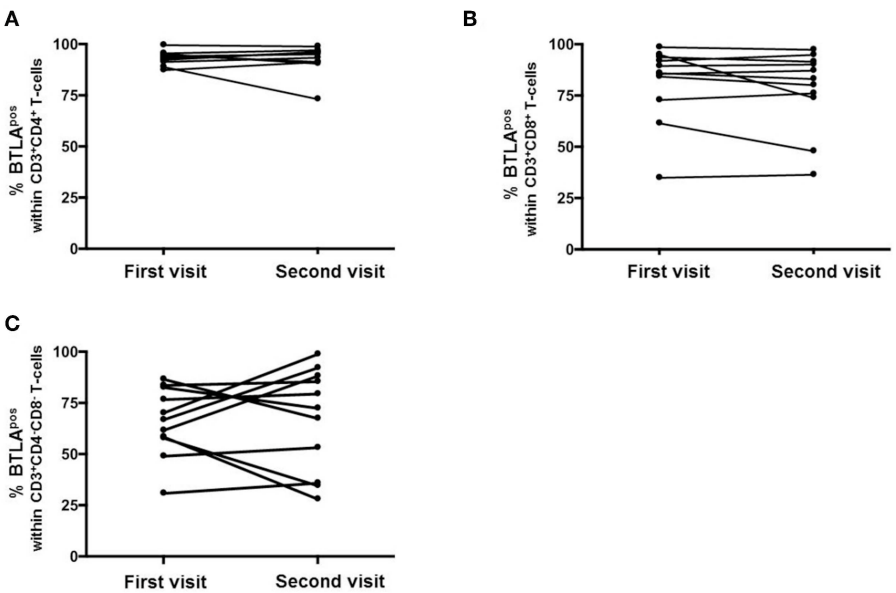


FIGURE 2 | Longitudinal assessment of BTLA expression on T-cells. The expression of BTLA on Th cells **(A)**, cytotoxic T-cells **(B)**, and DN T-cells **(C)** was comparable at the first vs. the second visit.

BTLA Expression Is Elevated on Stimulated T-Cells in AAV

BTLA expression decreased after stimulation on $CD3^+$ T-cells in AAV-r (AAV-r before stimulation vs. after stimulation, $CD3^+$ T-cells: $\%BTLA^{pos}$, 91.3 ± 1.3% vs. 82.3 ± 2.9%, $p < 0.05$, **Figure 3**) and in HC (HC before stimulation vs. after stimulation, $CD3^+$ T-cells: $\%BTLA^{pos}$, 88.9 ± 2.1% vs. 62.2 ± 6.1%, $p < 0.05$). $CD3^+CD8^-$ Th cells also showed decreased expression of BTLA after stimulation in AAV-r (AAV-r before stimulation vs. after stimulation, $CD3^+CD8^-$ T-cells: $\%BTLA^{pos}$, 91.3 ± 1.4%

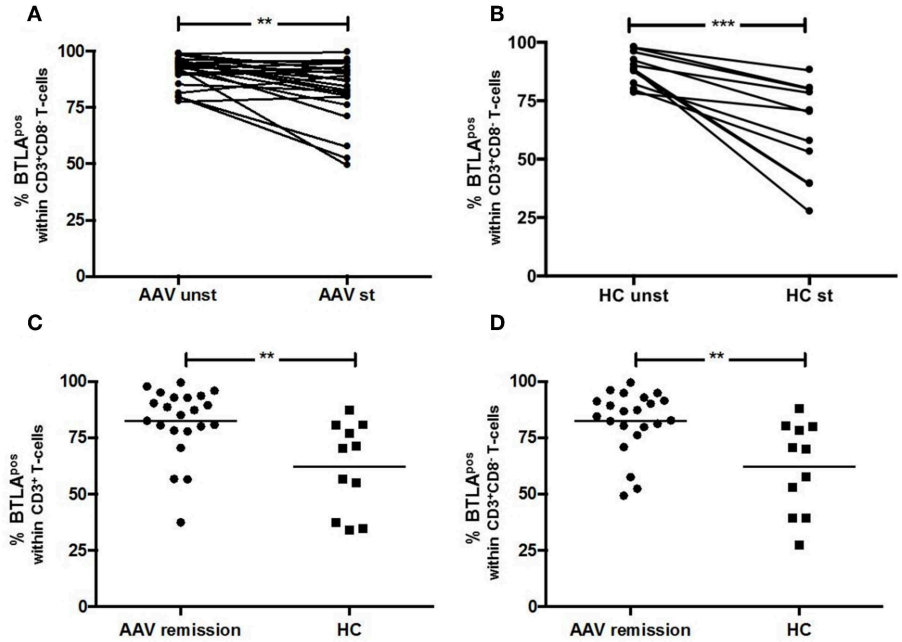


FIGURE 3 | BTLA expression on T-cells after TCR-independent stimulation. **(A,B)** BTLA was decreased on $CD3^+CD8^-$ T-cells after stimulation with PMA/Ionomycin. **(C,D)** After stimulation, the expression of BTLA was significantly more reduced on T-cells derived from HC as compared to AAV patients. Significant differences as calculated by the Wilcoxon-test for paired samples are indicated as $^{***}p < 0.001$. Significant differences as calculated by the Mann-Whitney *U* test for unpaired samples are indicated: $^{**}p < 0.0024$.

vs. 82.3 ± 3.1%, $p < 0.05$, **Figure 3**) and in HC (HC before stimulation vs. after stimulation, $CD3^+CD8^-$ T-cells: $\%BTLA^{pos}$, 88.9 ± 2.1% vs. 62.2 ± 6.1%, $p < 0.05$). In direct comparison, BTLA expression was more reduced on T-cells in HC after stimulation. This applied to the $CD3^+$ (AAV-r vs. HC, $CD3^+$ T-cells: $\%BTLA^{pos}$, 82.3 ± 3.3% vs. 62.3 ± 6.0%, $p < 0.05$) and the $CD3^+CD8^-$ (AAV-r vs. HC, $CD3^+CD8^-$ T-cells: $\%BTLA^{pos}$ 82.3 ± 2.9% vs. 62.2 ± 6.1%, $p < 0.05$) T-cell population. Likewise, the ratio of BTLA expression (BTLA expression on stimulated $CD3^+$ T-cells divided by BTLA expression on unstimulated $CD3^+$ T-cells) was increased in AAV compared to HC (AAV-r vs. HC, BTLA ratio on $CD3^+$ T-cells: 0.89 ± 0.003 vs. 0.71 ± 0.06, $p < 0.05$; AAV-r vs. HC, BTLA ratio on $CD3^+CD8^-$ T-cells: 0.9 ± 0.03 vs. 0.69 ± 0.06, $p < 0.05$), indicating a less pronounced downregulation of BTLA in AAV patients.

BTLA Suppresses T-Cell Proliferation and Th17 Cells

The function of BTLA was examined in 17 AAV-r patients and 10 HC. CFSE labeled PBMC were stimulated with anti-CD3/CD28 in the presence and absence of an agonistic anti-BTLA antibody. Stimulation of T-cells in presence of an agonistic anti-BTLA antibody resulted in suppression of T-cell proliferation in AAV and HC (relative inhibition of T-cell proliferation in %, AAV vs. HC, 32.1 ± 5.2% vs. 39.0 ± 6.3%, $p = 0.33$, **Figure 4**). Th cells (relative inhibition of Th cell proliferation in %, AAV vs. HC, 31.4 ± 5.9% vs. 38.63 ± 6.3%, $p = 0.36$, **Figure 4**) and cytotoxic T-cells (relative inhibition of the cytotoxic T-cell proliferation in %, AAV vs. HC, 32.1 ± 5.1% vs. 28.5 ± 7.9%, $p = 0.81$) were suppressed to the same extent in AAV and HC. Interestingly, the proliferation of DN T-cell was also suppressed by BTLA-induced co-inhibition (relative inhibition of the T-cell proliferation in %, AAV-r vs. HC, 36.4 ± 7.4% vs. 58.2 ± 9.1%, $p = 0.11$). In both groups, BTLA-mediated suppression inhibited IL-17A secretion (AAV-r vs. HC, suppression of IL-17A secretion, 29.9 ± 5.0% vs. 24.1 ± 14.3%, $p = 0.9$, **Figure 4**). The levels of INF-γ secretion were reduced by BTLA-mediated suppression in AAV and HC (AAV-r vs. HC, suppression of INF-γ secretion, 63.2 ± 3.4% vs. 74.2 ± 8.1%, $p = 0.17$).

Role of BTLA Expression on B-Cells in HC and Patients

The BTLA expression pattern was analyzed on B-cells in HC ($n = 16$), patients in remission (AAV-r, $n = 27$) and patients with active disease (AAV-a, $n = 8$, **Figure 5**). The fraction of BTLA expressing B-cells was comparable between patients in remission, active patients, and HC ($\%BTLA^{pos}$ within $CD19^+$ B-cells, AAV-r vs. AAV-a: 99.53 ± 0.3% vs. 96.45 ± 3.5%, $p = 0.63$; HC vs. AAV-r: 99.95 ± 0.04% vs. 99.53 ± 0.3%, $p = 0.2$; HC vs. AAV-a: 99.95 ± 0.04% vs. 96.45 ± 3.5%, $p = 0.13$, **Figure 5**). Furthermore, the functional role of BTLA on B-cells was assessed (**Figure 5**). For this purpose, isolated B-cells from HC were stimulated *ex vivo* in presence or absence of agonistic anti-BTLA. B-cell proliferation was assessed by CFSE dilution. Moreover, IL-10 production by B-cells was determined to assess whether agonistic anti-BTLA treatment hampers the function of anti-inflammatory regulatory

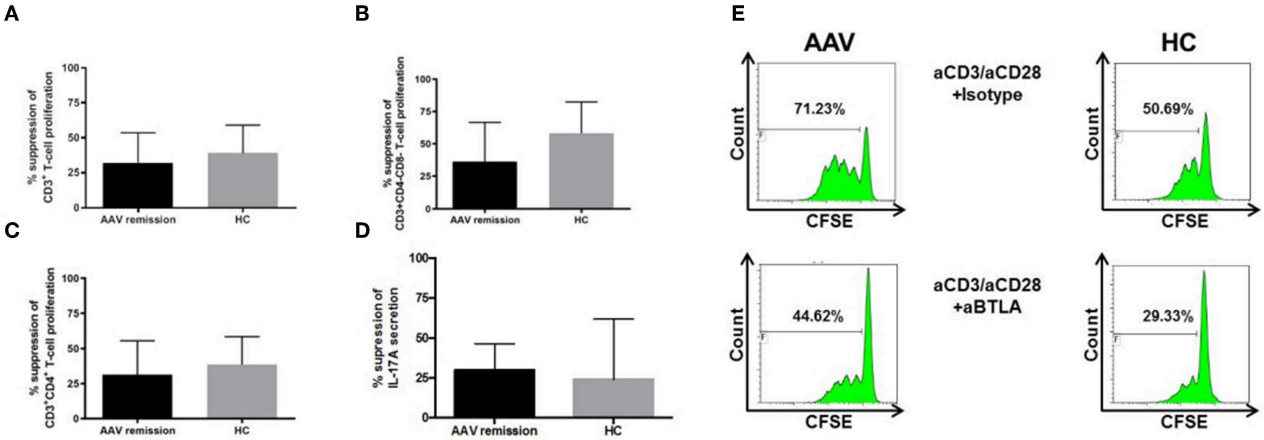


FIGURE 4 | Functional assessment of BTLA. CFSE-labeled PBMC were stimulated by anti-CD3/anti-CD28 (each 0.5 ng/ml) in presence of agonistic anti-BTLA (50 ug/ml) or isotype (mouse IgG2a, 50 ug/ml). Proliferated fraction was determined by CFSE dilution. IL-17A levels were determined from cell culture supernatants. **(A)** T-cell proliferation was inhibited by agonistic anti-BTLA treatment in AAV und HC. **(B)** Likewise, Th cell and **(C)** DN T-cell proliferation was suppressed. **(D)** Th17 cells were inhibited by agonistic anti-BTLA treatment as indicated by reduced IL-17A secretion. Suppression was similar between AAV patients and HC. IL-17A levels were determined from cell culture supernatants. **(E)** Representative flow cytometric data is shown. Plots are gated on viable $CD3^+$ T-cells. Suppression was calculated as follows: [proliferated fraction of T-cells/cytokine levels without anti-BTLA [isotype] MINUS proliferated fraction of T-cells/cytokine levels with anti-BTLA] DIVIDED by proliferation of PBMC without anti-BTLA [isotype] MULTIPLIED by 100. Data is depicted as mean ± standard deviation.

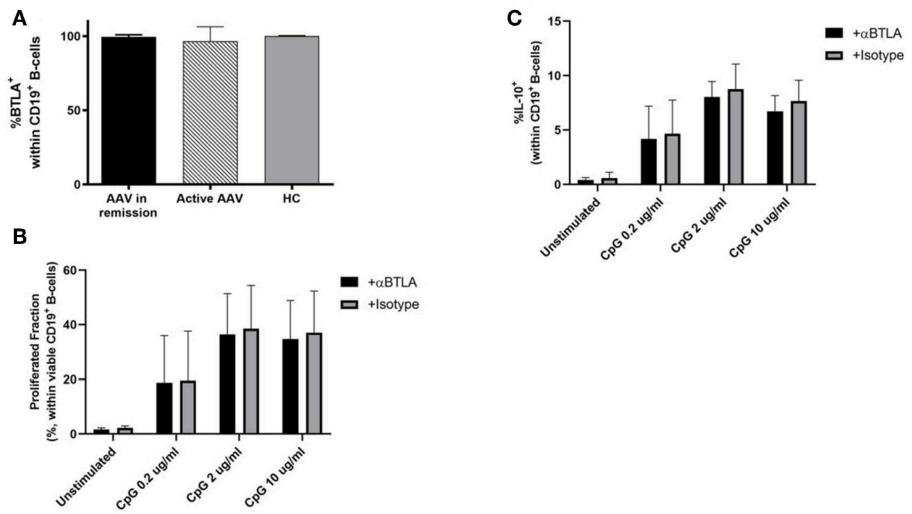


FIGURE 5 | BTLA expression and function on B-cells. **(A)** Expression of BTLA was comparable between AAV und HC on $CD19^+$ B-cells. **(B,C)** CFSE-labeled isolated $CD19^+$ B-cell from HC were stimulated by CpG ODN 2006 for 72 h in presence of agonistic anti-BTLA (50 ug/ml) or isotype (mouse IgG2a, 50 ug/ml). Proliferated fraction was determined by CFSE dilution, IL-10 production was determined by intracellular flow cytometry after restimulation. Agonistic anti-BTLA treatment did not suppress B-cell proliferation. IL-10 production by regulatory B-cells was not affected by agonistic anti-BTLA treatment. Data is depicted as mean ± standard deviation.

B-cells. Agonistic anti-BTLA treatment did not suppress B-cell proliferation. Even sub-optimal stimulation in presence of agonistic anti-BTLA did not result in a significant inhibition of CpG-induced B-cell proliferation. Moreover, differentiation of regulatory B-cells and IL-10 production were not suppressed by activation of BTLA (**Figure 5**). Thus, in contrast with the findings on T-cells, B-cell activation and regulatory B-cell differentiation were not susceptible to agonistic anti-BTLA treatment suggesting control by other co-inhibitory pathways.

Association of BTLA and Clinical Parameters: BTLA Expression on DN T-Cells Correlates With Relapse Rate

Further analyses were performed to unravel associations of BTLA expression with clinical parameters. Interestingly, relapse rate was associated negatively with BTLA expression on DN T-cells ($r = -0.3$, $p = 0.04$). There was no association of relapse rate with BTLA expression on $CD4^+$ T-helper-cells or $CD8^+$ cytotoxic T-helper-cells. Thus, increased BTLA expression on

DN T-cells correlated with better disease control and outcome. In addition, patients were stratified by history of biopsy proven renal involvement (RI) to study whether BTLA expression differs between these two groups. There was no difference comparing BTLA expression between both patient groups (with RI vs. without RI, %BTLA^{pos} within CD4$^+$ T-cells: 91.86 ± 1.4% vs. 90.59 ± 1.9%, $p = 0.2$; %BTLA^{pos} within CD8$^+$ T-cells: 86.53 ± 2.3% vs. 79.91 ± 7.3%, $p = 0.5$; %BTLA^{pos} within DN T-cells: 67.89 ± 3.9% vs. 55.86 ± 7.9%, $p = 0.2$). Likewise, renal function at the time of measurement -determined as glomerular filtration rate (GFR) estimated by CKD-EPI formula- did not correlate with BTLA expression on T-cell subsets (CD4$^+$ T-cells: $r = 0$–16, $p = 0.34$; CD8$^+$ T-cells: $r = -0.15$, $p = 0.38$; DN T-cells: $r = 0.14$, $p = 0.4$). Furthermore, it was assessed if differences can be detected between patients with MPO-ANCA as compared to patients with PR3-ANCA. Patients were stratified by antibody-status at time of diagnosis. There was no difference with regard to BTLA expression on T-cell subsets (PR3 vs. MPO patients, %BTLA^{pos} within CD4$^+$ T-cells: 90.53 ± 1.7% vs. 93.13 ± 1.2%, $p = 0.3$; %BTLA^{pos} within CD8$^+$ T-cells: 86.53 ± 2.4% vs. 83.11 ± 5.3%, $p = 0.6$; %BTLA^{pos} within DN T-cells: 68.25 ± 3.8% vs. 59.59 ± 7.0%, $p = 0.33$). It was further studied whether treatment impacted BTLA expression. Thus, cumulative cyclophosphamide (CYC) dosage and steroid dosage at time of sampling were correlated with BTLA expression on T-cell subsets. A significant positive correlation was found regarding BTLA expression on DN T-cells and cumulative CYC dosage ($r = 0.36$, $p = 0.04$); there was no association between cumulative CYC dosage and BTLA expression on T-helper-cells or cytotoxic T-cells (CYC/%BTLA on CD4$^+$ T-cells: $r = 0.09$, $p = 0.6$; CYC/%BTLA on CD8$^+$ T-cells: $r = -0.14$, $p = 0.42$). Steroid dosage at time of sampling was not significantly associated with BTLA expression on CD4$^+$, CD8$^+$, or DN T-cells (CD4$^+$ T-cells: $r = -0.12$, $p = 0.5$; CD8$^+$ T-cells: $r = 0.32$, $p = 0.06$; DN T-cells: $r = 0.09$, $p = 0.6$).

DISCUSSION

The expression of the negative co-stimulator BTLA was diminished on double negative T-cells in AAV-r and correlated with disease activity as well as relapse rate. BTLA expression was unaltered on Th-, cytotoxic -T-cells, and B-cells in quiescent AAV. After stimulation with PMA and Ionomycin, BTLA expression persisted in AAV and was downregulated in HC. The co-inhibition of T-cells via BTLA during TCR-mediated stimulation led to suppression of T-cell proliferation and inhibited secretion of IL-17 as well as INFγ. Thus, the BTLA axis seems intact and functional in AAV.

T-cell regulation is an essential feature of a healthy immune system (23). T-cell regulation is driven by negative co-stimulation mediated via a number of different systems such as the CTLA4-axis, BTLA-axis and PD1-axis (24). The CTLA4- and the PD1-axis have both been well-studied and the importance for maintaining immune tolerance has been shown (12, 13, 15, 25). In addition, both co-stimulatory systems are blocked for therapeutic purposes in malignant diseases to boost immune responses. BTLA has been investigated in experimental models. Otsuki et al. showed that BTLA ligation transmits an inhibitory signal to T-cells and thus might play an important role in T-cell tolerance (26). Krieg et al. noticed that the stimulation of murine T-cells in presence of an agonistic BTLA antibody results in decreased IL-2 production and diminished occurrence of CD25$^+$ T-cells (27). There is evidence from animal studies that BTLA knockout leads to autoimmune diseases (20, 28, 29).

In our study, we found that BTLA expression on Th cells, cytotoxic T-cells and B-cells was comparable between quiescent patients and HC when assessed under basal conditions. Interestingly, the expression of BTLA was significantly decreased on double negative T-cells in AAV-r. This was found on naïve DN- and on memory DN T-cells. BTLA expression on DN T-cells correlated with disease activity as well as relapse rate indicating a probable role in disease pathogenesis. Altered expression of other co-inhibitory molecules such as CTLA4 and PD-1 on T-cells has been reported in AAV. Wilde et al. found that the expression of PD-1 on T-helper was increased on Th cells from AAV patients as compared to HCs (15). Steiner et al. could show that expression levels of CTLA-4 were significantly increased on CD4$^+$ and on DN T-cells in AAV (25). After stimulation by PMA and Ionomycin, the CTLA-4 levels were increased on T-cells derived from HC but T-cells from AAV patients had an impaired response (25). Ye et al. also found a decreased expression of BTLA in Behcet's disease on Th cells and this was associated with an abnormal Th17 and Th1 immune response (30). In a recent publication by Sawaf et al., expression of BTLA on T-cell subsets was comparable in patients with SLE and HC (31). However, despite this finding, the authors showed that BTLA functionality was significantly impaired in SLE patients.

DN T-cells are poorly studied yet, but it is known that these cells are expanded in systemic lupus erythematosus and that their relative proportion correlates with disease activity (32). In patients with lupus nephritis, DN T-cell showed pro-inflammatory features producing IL-17A and were found in renal lesions (33). In Sjogren's disease, DN-T-cells have been identified as the cell population that is primarily involved in the production of IL-17A and plays an important role in the maintenance of inflammatory processes (34).

In contrast, in murine models of acute kidney injury, DN were found as tissue-resident anti-inflammatory T-cell population in acute kidney injury (35). In our study, the relative proportion of circulating DN T-cells was comparable between AAV and HC. The aberrant expression of BTLA on DN T-cells in AAV could nevertheless disrupt the co-inhibitory function and thereby contribute to systemic- and renal inflammation. Furthermore, we found that after stimulation of PBMC with PMA and Ionomycin, BTLA was downregulated in HC. Downregulation was less pronounced in AAV. Han et al. showed that BTLA was upregulated in mice after TCR-mediated stimulation (36). Sedy et al. have also shown in a mouse model that the expression of BTLA was variable after stimulation via TCR (37).In contrast to these studies, we stimulated T-cells in a TCR-independent manner, possibly explaining the different expression pattern. Data on human T-cells being stimulated short-term with PMA/Iono is lacking. Less downregulation of BTLA on

T-cells in AAV may cause an increased susceptibility to BTLA-mediated suppression which my counterbalance persistent T-cell activation.

Next, we further tested the function of the BTLA-axis. Suprisingly, B-cell proliferation and differentiation of regulatory B-cells were not susceptible to treatment with agonistic anti-BTLA. There is conflicting data on the functional meaning of BTLA expression on B-cells. HVEM, a cognate ligand of BTLA, has been demonstrated to suppress B-cell proliferation (38). In support of our own data, another study failed to show a suppressive effect on B-cell proliferation when using agonistic anti-BTLA treatment (39). In contrast, Co-inhibition of T-cells with an agonistic anti-BTLA antibody suppressed anti-CD3/-CD28 induced proliferation of HC and AAV patients to the same extent. Thus, the BTLA pathway appears to be intact in AAV. As mentioned above, Sawaf et al. found defective functionality of BTLA in SLE patients (31). T-cell stimulation via TCR in presence of agonistic anti-BTLA was less efficient suppressing proliferation and CD25 upregulation of T-cells in SLE patients as compared to HC (31). It is conceivable that other autoimmune diseases harbor a different pattern of functional co-inhibitors as multiple, different and redundant co-inhibitory systems exist to control T-cell immunity. Therefore, it is not surprising that another co-inhibitor, the PD1/PDL-1 pathway, is reported to be dysfunctional in AAV (15).

From our data, we also gain important novel information on the physiological role of BTLA. Agonistic treatment of TCR stimulated T-cells reduced not only proliferation –as has been reported previously- but also suppressed IL-17A and INFγ secretion in HC as well as in patients. Thus, BTLA-induced suppression seems to impact effector T-cells such as Th1 and Th17 cells efficiently. IL-17A is a key factor in pathogenesis of AAV and IL-17A knock out protects from disease in murine models (3, 40). As BTLA ligation led to suppression of *ex vivo* stimulated Th17 cells in HC and AAV, this co-inhibitor might be exploited in future for therapeutic purposes. Similar approaches have already been tested in experimental animal transplant models and agonistic anti-BTLA treatment conferred protection from allograft rejection (41, 42). However, it has not been unraveled whether agonistic BTLA treatment also counteracts established tissue inflammation by regulating lesional T-cells. Regulating circulating vs. lesional T-cells has important implications. If lesional T-cells are not functionally regulated by BTLA, the therapeutic potency of targeting BTLA might be limited to prophylactic purposes such as remission maintenance. It is a limitation of our study that we did not investigate lesional T-cells. However, we were focused on the role of BTLA in circulating T-cells to unravel whether this co-inhibitory pathway is in principle functional in ANCA-vasculitis. As the access to lesional T-cells is very limited in human disease and may not allow functional studies, the role of BTLA in regulating lesional T-cells could be addressed in future by employing one of the animal models available for ANCA-vasculitis.

In summary, the BTLA axis seems functional and intact in AAV. As BTLA ligation suppresses Th17 cells efficiently, this pathway should be investigated further as potential therapeutic target in AAV.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the local ethics committee of the University Hospital Essen. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

KW designed the study, performed the experiments, performed data analysis, and wrote the manuscript. SD, YD, AB, MS, and XM performed the experiments, performed data analysis, and wrote the manuscript. JK, AG, and HR performed data analysis and wrote the manuscript. JC, PP, and AK designed the study and wrote the manuscript. OW and BW designed the study, performed data analysis, and wrote the manuscript.

FUNDING

BW was funded by the Dr. Werner Jackstädt-Stfitung, OW received funding from the Rudolf-Ackermann-Stiftung. Open Access Fees were provided by the University of Duisburg-Essen.

ACKNOWLEDGMENTS

The authors thank Barbara Nilewski-Kühl and Tanja Hinkeldein for excellent technical assistance.

REFERENCES

1. Wilde B, Van Paassen P, Witzke O, Tervaert JWC. New pathophysiological insights and treatment of ANCA-associated vasculitis. *Kidney Int.* (2011) 79:599–612. doi: 10.1038/ki.2010.472
2. Wilde B, Thewissen M, Damoiseaux J, Van Paassen P, Witzke O, Tervaert JW. T cells in ANCA-associated vasculitis: what can we learn from lesional versus circulating T cells? *Arthritis Res Ther.* (2010) 12:204. doi: 10.1186/ar2923
3. Dolff S, Witzke O, Wilde B. Th17 cells in renal inflammation and autoimmunity. *Autoimmun Rev.* (2019) 18:129–36. doi: 10.1016/j.autrev.2018.08.006
4. Lamprecht P Mueller A, Gross WL. CD28- T cells display features of effector memory T cells in Wegener's granulomatosis. *Kidney Int.* (2004) 65:1113–4. doi: 10.1111/j.1523-1755.2004.501_5.x
5. Abdulahad WH, Van Der Geld YM, Stegeman CA, Kallenberg CG. Persistent expansion of CD4+ effector memory T cells in Wegener's granulomatosis. *Kidney Int.* (2006) 70:938–47. doi: 10.1038/sj.ki.5001670

6. Cunningham MA, Huang XR, Dowling JP, Tipping PG, Holdsworth SR. Prominence of cell-mediated immunity effectors in "pauci-immune" glomerulonephritis. *J Am Soc Nephrol.* (1999) 10:499–506.
7. Komocsi A, Lamprecht P, Csernok E, Mueller A, Holl-Ulrich K, Seitzer U, et al. Peripheral blood and granuloma CD4(+)CD28(-) T cells are a major source of interferon-gamma and tumor necrosis factor-alpha in Wegener's granulomatosis. *Am J Pathol.* (2002) 160:1717–24. doi: 10.1016/S0002-9440(10)61118-2
8. Lamprecht P, Bruhl H, Erdmann A, Holl-Ulrich K, Csernok E, Seitzer U, et al. Differences in CCR5 expression on peripheral blood CD4+CD28- T-cells and in granulomatous lesions between localized and generalized Wegener's granulomatosis. *Clin Immunol.* (2003) 108:1–7. doi: 10.1016/S1521-6616(03)00121-9
9. Wilde B, Van Paassen P, Damoiseaux J, Heerings-Rewinkel P, Van Rie H, Witzke O, et al. Dendritic cells in renal biopsies of patients with ANCA-associated vasculitis. *Nephrol Dial Transplant.* (2009) 24:2151–6. doi: 10.1093/ndt/gfp019
10. Abdulahad WH, Stegeman CA, Van Der Geld YM, Doornbos-Van Der Meer B, Limburg PC, Kallenberg CG. Functional defect of circulating regulatory CD4+ T cells in patients with Wegener's granulomatosis in remission. *Arthritis Rheum.* (2007) 56:2080–91. doi: 10.1002/art.22692
11. Morgan MD, Day CJ, Piper KP, Khan N, Harper L, Moss PA, et al. Patients with Wegener's granulomatosis demonstrate a relative deficiency and functional impairment of T-regulatory cells. *Immunology.* (2010) 130:64–73. doi: 10.1111/j.1365-2567.2009.03213.x
12. Bour-Jordan H, Esensten JH, Martinez-Llordella M, Penaranda C, Stumpf M, Bluestone JA. Intrinsic and extrinsic control of peripheral T-cell tolerance by costimulatory molecules of the CD28/ B7 family. *Immunol Rev.* (2011) 241:180–205. doi: 10.1111/j.1600-065X.2011.01011.x
13. De Sousa Linhares A, Leitner J, Grabmeier-Pfistershammer K, Steinberger P. Not all immune checkpoints are created equal. *Front Immunol.* (2018) 9:1909. doi: 10.3389/fimmu.2018.01909
14. Bertsias GK, Nakou M, Choulaki C, Raptopoulou A, Papadimitraki E, Goulielmos G, et al. Genetic, immunologic, and immunohistochemical analysis of the programmed death 1/programmed death ligand 1 pathway in human systemic lupus erythematosus. *Arthr Rheum.* (2009) 60:207–18. doi: 10.1002/art.24227
15. Wilde B, Hua F, Dolff S, Jun C, Cai X, Specker C, et al. Aberrant expression of the negative costimulator PD-1 on T cells in granulomatosis with polyangiitis. *Rheumatology.* (2012) 51:1188–97. doi: 10.1093/rheumatology/kes034
16. Van Den Brom RR, Abdulahad WH, Rutgers A, Kroesen BJ, Roozendaal C, De Groot DJ, et al. Rapid granulomatosis with polyangiitis induced by immune checkpoint inhibition. *Rheumatology.* (2016) 55:1143–5. doi: 10.1093/rheumatology/kew063
17. Murphy TL, Murphy KM. Slow down and survive: enigmatic immunoregulation by BTLA and HVEM. *Annu Rev Immunol.* (2010) 28:389–411. doi: 10.1146/annurev-immunol-030409-101202
18. Shui J-W, Steinberg MW, Kronenberg M. Regulation of inflammation, autoimmunity, and infection immunity by HVEM-BTLA signaling. *J Leukoc Biol.* (2011) 89:517–23. doi: 10.1189/jlb.0910528
19. Liu X, Alexiou M, Martin-Orozco N, Chung Y, Nurieva RI, Ma L, et al. Cutting edge: a critical role of B and T lymphocyte attenuator in peripheral T cell tolerance induction. *J Immunol.* (2009) 182:4516. doi: 10.4049/jimmunol.0803161
20. Oya Y, Watanabe N, Kobayashi Y, Owada T, Oki M, Ikeda K, et al. Lack of B and T lymphocyte attenuator exacerbates autoimmune disorders and induces Fas-independent liver injury in MRL-lpr/lpr mice. *Int Immunol.* (2011) 23:335–44. doi: 10.1093/intimm/dxr017
21. Watts R, Lane S, Hanslik T, Hauser T, Hellmich B, Koldingsnes W, et al. Development and validation of a consensus methodology for the classification of the ANCA-associated vasculitides and polyarteritis nodosa for epidemiological studies. *Ann Rheum Dis.* (2007) 66:222–7. doi: 10.1136/ard.2006.054593
22. Hellmich B, Flossmann O, Gross WL, Bacon P, Cohen-Tervaert JW, Guillevin L, et al. EULAR recommendations for conducting clinical studies and/or clinical trials in systemic vasculitis: focus on anti-neutrophil cytoplasm antibody-associated vasculitis. *Ann Rheum Dis.* (2007) 66:605–17. doi: 10.1136/ard.2006.062711
23. Alroqi FJ, Chatila TA. T regulatory cell biology in health and disease. *Curr Allergy Asthma Rep.* (2016) 16:27. doi: 10.1007/s11882-016-0606-9
24. Khoury SJ, Sayegh MH. The roles of the new negative T cell costimulatory pathways in regulating autoimmunity. *Immunity.* (2004) 20:529–38. doi: 10.1016/S1074-7613(04)00116-5
25. Steiner K, Moosig F, Csernok E, Selleng K, Gross WL, Fleischer B, et al. Increased expression of CTLA-4 (CD152) by T and B lymphocytes in Wegener's granulomatosis. *Clin Exp Immunol.* (2001) 126:143–50. doi: 10.1046/j.1365-2249.2001.01575.x
26. Otsuki N, Kamimura Y, Hashiguchi M, Azuma M. Expression and function of the B and T lymphocyte attenuator (BTLA/CD272) on human T cells. *Biochem Biophys Res Commun.* (2006) 344:1121–7. doi: 10.1016/j.bbrc.2006.03.242
27. Krieg C, Han P, Stone R, Goularte OD, Kaye J. Functional analysis of B and T lymphocyte attenuator engagement on CD4+ and CD8+ T cells. *J Immunol.* (2005) 175:6420–7. doi: 10.4049/jimmunol.175.10.6420
28. Steinberg MW, Turovskaya O, Shaikh RB, Kim G, Mccole DF, Pfeffer K, et al. A crucial role for HVEM and BTLA in preventing intestinal inflammation. *J Exp Med.* (2008) 205:1463–76. doi: 10.1084/jem.20071160
29. Sadasivam M, Noel S, Lee SA, Gong J, Allaf ME, Pierorazio P, et al. Activation and proliferation of PD-1+ kidney double-negative T cells is dependent on nonclassical MHC proteins and IL-2. *J Am Soc Nephrol.* (2019) 30:277. doi: 10.1681/ASN.2018080815
30. Ye Z, Deng B, Wang C, Zhang D, Kijlstra A, Yang P. Decreased B and T lymphocyte attenuator in Behcet's disease may trigger abnormal Th17 and Th1 immune responses. *Sci Rep.* (2016) 6:20401. doi: 10.1038/srep20401
31. Sawaf M, Fauny JD, Felten R, Sagez F, Gottenberg JE, Dumortier H, et al. Defective BTLA functionality is rescued by restoring lipid metabolism in lupus CD4+ T cells. *JCI Insight.* (2018) 3:99711. doi: 10.1172/jci.insight.99711
32. Lai ZW, Borsuk R, Shadakshari A, Yu J, Dawood M, Garcia R, et al. Mechanistic target of rapamycin activation triggers IL-4 production and necrotic death of double-negative T cells in patients with systemic lupus erythematosus. *J Immunol.* (2013) 191:2236–46. doi: 10.4049/jimmunol.1301005
33. Crispín JC, Oukka M, Bayliss G, Cohen RA, Van Beek CA, Stillman IE, et al. Expanded double negative T cells in patients with systemic lupus erythematosus produce IL-17 and infiltrate the kidneys. *J Immunol.* (2008) 181:8761. doi: 10.4049/jimmunol.181.12.8761
34. Alunno A, Bistoni O, Bartoloni E, Caterbi S, Bigerna B, Tabarrini A, et al. IL-17-producing CD4-CD8- T cells are expanded in the peripheral blood, infiltrate salivary glands and are resistant to corticosteroids in patients with primary Sjogren's syndrome. *Ann Rheum Dis.* (2013) 72:286–92. doi: 10.1136/annrheumdis-2012-201511
35. Martina MN, Noel S, Saxena A, Bandapalle S, Majithia R, Jie C, et al. Double-negative alphabeta T cells are early responders to AKI and are found in human kidney. *J Am Soc Nephrol.* (2016) 27:1113–23. doi: 10.1681/ASN.2014121214
36. Han P, Goularte OD, Rufner K, Wilkinson B, Kaye J. An inhibitory Ig superfamily protein expressed by lymphocytes and APCs is also an early marker of thymocyte positive selection. *J Immunol.* (2004) 172:5931–9. doi: 10.4049/jimmunol.172.10.5931
37. Sedy JR, Gavrieli M, Potter KG, Hurchla MA, Lindsley RC, Hildner K, et al. B and T lymphocyte attenuator regulates T cell activation through interaction with herpesvirus entry mediator. *Nat Immunol.* (2005) 6:90–8. doi: 10.1038/ni1144
38. Vendel AC, Calemine-Fenaux J, Izrael-Tomasevic A, Chauhan V, Arnott D, Eaton DL. B and T lymphocyte attenuator regulates B cell receptor signaling by targeting Syk and BLNK. *J Immunol.* (2009) 182:1509–17. doi: 10.4049/jimmunol.182.3.1509
39. Zhang M, Howard K, Winters A, Steavenson S, Anderson S, Smelt S, et al. Monoclonal antibodies to B and T lymphocyte attenuator (BTLA) have no effect on *in vitro* B cell proliferation and act to inhibit *in vitro* T cell proliferation when presented in a cis, but not trans, format relative to the activating stimulus. *Clin Exp Immunol.* (2011) 163:77–87. doi: 10.1111/j.1365-2249.2010.04259.x
40. Gan PY, Steinmetz OM, Tan DS, O'sullivan KM, Ooi JD, Iwakura Y, et al. Th17 cells promote autoimmune anti-myeloperoxidase glomerulonephritis.

J Am Soc Nephrol. (2010) 21:925–31. doi: 10.1681/ASN.2009070763

41. Truong W, Plester JC, Hancock WW, Merani S, Murphy TL, Murphy KM, et al. Combined coinhibitory and costimulatory modulation with Anti-BTLA and CTLA4Ig facilitates tolerance in murine islet allografts. *Am J Transpl.* (2007) 7:2663–74. doi: 10.1111/j.1600-6143.2007.01996.x
42. Uchiyama M, Jin X, Matsuda H, Bashuda H, Imazuru T, Shimokawa T, et al. An agonistic anti-BTLA mAb (3C10) induced generation of IL-10-dependent regulatory CD4+ T cells and prolongation of murine cardiac allograft. *Transplantation.* (2014) 97:301–9. doi: 10.1097/01.TP.0000438204.96723.8b

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.

frontiers in Immunology

ORIGINAL RESEARCH
published: 13 December 2019
doi: 10.3389/fimmu.2019.02877

Stem-Cell-Derived Circulating Progenitors Dysfunction in Behçet's Syndrome Patients Correlates With Oxidative Stress

Giacomo Emmi[1†], Amanda Mannucci[2†], Flavia Rita Argento[2], Elena Silvestri[1], Augusto Vaglio[2], Alessandra Bettiol[1], Alessandra Fanelli[3], Laura Stefani[4], Niccolò Taddei[2], Domenico Prisco[1], Claudia Fiorillo[2] and Matteo Becatti[2]*

[1] Department of Experimental and Clinical Medicine, University of Firenze, Firenze, Italy, [2] Department of Experimental and Clinical Biomedical Sciences "Mario Serio", University of Firenze, Firenze, Italy, [3] Central Laboratory, Azienda Ospedaliero Universitaria Careggi, Firenze, Italy, [4] Department of Clinical and Experimental Medicine, Center of Sports Medicine, University of Firenze, Firenze, Italy

OPEN ACCESS

Edited by:
Betty Diamond,
Feinstein Institute for Medical Research, United States

Reviewed by:
Haner Direskeneli,
Marmara University, Turkey
David Saadoun,
Assistance Publique Hopitaux De Paris (AP-HP), France

***Correspondence:**
Claudia Fiorillo
claudia.fiorillo@unifi.it

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Autoimmune and Autoinflammatory Disorders, a section of the journal Frontiers in Immunology

Received: 19 October 2019
Accepted: 22 November 2019
Published: 13 December 2019

Citation:
Emmi G, Mannucci A, Argento FR, Silvestri E, Vaglio A, Bettiol A, Fanelli A, Stefani L, Taddei N, Prisco D, Fiorillo C and Becatti M (2019) Stem-Cell-Derived Circulating Progenitors Dysfunction in Behçet's Syndrome Patients Correlates With Oxidative Stress. Front. Immunol. 10:2877. doi: 10.3389/fimmu.2019.02877

Behçet's syndrome (BS) is a systemic vasculitis considered as the prototype of a systemic inflammation-induced thrombotic condition whose pathogenesis cannot be explained just by coagulation abnormalities. Circulating hematopoietic progenitor cells (CPC), a population of rare, pre-differentiated adult stem cells originating in the bone marrow and capable of both self-renewal and multi-lineage differentiation, are mobilized in response to vascular injury and play a key role in tissue repair. In cardiovascular and thrombotic diseases, low circulating CPC number and reduced CPC function have been observed. Oxidative stress may be one of the relevant culprits that account for the dysfunctional and numerically reduced CPC in these conditions. However, the detailed mechanisms underlying CPC number reduction are unknown. On this background, the present study was designed to evaluate for the first time the possible relationship between CPC dysfunction and oxidative stress in BS patients. In BS patients, we found signs of plasma oxidative stress and significantly lower CD34+/CD45$^{-/dim}$ and CD34+/CD45$^{-/dim}$/CD133+ CPC levels. Importantly, in all the considered CPC subsets, significantly higher ROS levels with respect to controls were observed. Higher levels of caspase-3 activity in all the considered CPC population and a strong reduction in GSH content in CPC subpopulation from BS patients with respect to controls were also observed. Interestingly, in BS patients, ROS significantly correlated with CPC number and CPC caspase-3 activity and CPC GSH content significantly correlated with CPC number, in all CPC subsets. Collectively, these data demonstrate for the first time that CPC from BS patients show signs of oxidative stress and apoptosis and that a reduced CPC number is present in BS patients with respect to controls. Interestingly, we observed an inverse correlation between circulating CPC number and CPC ROS production, suggesting a possible toxic ROS effect on CPC in BS patients. The significant correlations between ROS production/GSH content and caspase-3 activity point out that oxidative

stress can represent a determinant in the onset of apoptosis in CPC. These data support the hypothesis that oxidative-stress-mediated CPC dysfunctioning may counteract their vascular repair actions, thereby contributing to the pathogenesis and the progression of vascular disease in BS.

Keywords: Behçet's syndrome, thrombosis, circulating progenitor cells, oxidation, apoptosis

INTRODUCTION

Behçet's syndrome (BS) is a systemic vasculitis of unknown etiology characterized by muco-cutaneous and ocular manifestations as well as articular, neurological, and gastrointestinal involvements (1). Vascular involvement is also present, and represents one of the more important manifestations in terms of morbidity and mortality (2). BS affects both veins and arteries of all sizes and is usually more frequent and severe in young males (3). To date, BS may be considered as the prototype of a systemic inflammation-induced thrombotic condition. Although some studies showed different hemostatic system defects in BS (3, 4), current data indicate that the pathogenesis of thrombosis in BS cannot be explained by coagulation abnormalities only (5). Indeed, neutrophils are pivotal in promoting thrombo-inflammation by producing high amounts of reactive oxygen species (ROS), mainly through NADPH oxidase. This mechanism ultimately leads to a modification of the fibrin clot that becomes less susceptible to plasmin-induced lysis (6). Moreover, in BS patients, endothelial injury plays a prominent role in the onset of thrombosis and inflammation leads to thrombosis also *via* endothelial damage and endothelial cell dysfunction (7). Altogether, these mechanisms may partly explain why immunosuppressive treatment is essential in the management of thrombosis occurring in BS, while anticoagulation generally displays limited effects (8).

Circulating hematopoietic progenitor cells (CPC) are a population of rare, pre-differentiated adult stem cells that originate in the bone marrow and are uniquely capable of both self-renewal and multi-lineage differentiation, including cardiomyocytes, smooth muscle cells, endothelial progenitor cells (EPC) and endothelial cells. CPC possess the ability to be mobilized in response to vascular injury and play a key role in tissue repair (9, 10). CPC replenish specialized somatic cells and maintain the normal turnover of regenerative tissues and organs, such as blood and skin. Interestingly, low circulating CPC number and reduced CPC function are associated with cardiovascular disease and mortality (11, 12).

Circulating CPC are involved in the regulation and repair of the endothelium and in vessel formation (13, 14). Indeed, enhanced mobilization of CPC into the blood has been associated with increased endothelial function and repair (11). However, circulating CPC number and function are dramatically altered when cardiovascular risk factors are present (14, 15). On the other hand, while acute inflammation increases CPC, a chronic inflammatory state might be accompanied by a progressive CPC reduction (16, 17). It has been demonstrated that oxidative stress represents one of the main determinant of CPC number reduction and dysfunction in cardiovascular diseases (18, 19). Upon ROS production inhibition, the observed CPC alterations have been reverted (18, 19). However, the underlying mechanisms of CPC reduction have not been well-understood.

To date, insufficient, and conflicting clinical data to document the CPC number/function in BS patients exist (20, 21). Therefore, the present study was designed to evaluate for the first time the possible relationship between CPC dysfunction and oxidative stress in BS patients.

MATERIALS AND METHODS

Study Population

This was a case–control study. Sixty-one consecutive patients with BS who attended the Behçet Center of the University Hospital of Firenze, Italy, were matched 1:1 for age and sex with healthy control subjects. Patients with other autoimmune diseases and active infectious or neoplastic conditions were excluded, as well as pregnant patients. Control subjects were excluded if they had a history of cerebro- and/or cardiovascular diseases, peripheral arteriopathy, venous thrombo-embolism events, or cancer. Both patients and control subjects were assessed for the presence of vascular risk factors and drug use.

The study protocol was approved by the local Ethical Committee and informed consent was obtained from all subject enrolled.

Blood Collection

Blood samples were obtained from an antecubital vein in the morning after an overnight fasting and were collected into evacuated plastic tubes (BD Vacutainer Systems, Plymouth, UK) containing ethylenediaminetetraacetate 0.17 mol/L for CPC evaluation.

Because inflammatory events are known to influence CPC number (16), blood was withdrawn after excluding the occurrence of infectious events, defined according to previously published criteria (22), in the previous 15 days.

Flow Cytometric Analysis of CPC Oxidative Stress and Apoptosis

CPC number was assessed by flow cytometry as previously described with minor modifications (16, 22, 23). Briefly, 200 μl of peripheral venous blood was incubated for 20 min in the dark with the appropriated monoclonal antibodies (PE anti-human CD34, BD Pharmigen, Becton Dickinson, San Jose, CA; APC anti-human CD133, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany; APC-Cy7 anti-human CD45 BD, Becton Dickinson, San Jose, CA). Then, 4 ml of BD FACS Lysing Solution (Becton

Dickinson Biosciences, San Jose, CA, USA) was added, gently mixed, and incubated at RT in the dark for 10 min, following the manufacturer's protocol. Then, the cells were centrifuged, the supernatant was discarded, and cells were washed twice in PBS. To determine the level of intracellular ROS generation, cells were incubated with H_2DCFDA (10 μM) (Invitrogen, CA, USA) in RPMI without serum and phenol red for 15 min at 37°C. After labeling, cells were washed and resuspended in PBS and immediately analyzed by FACS.

To determine the level of Caspase-3 activity, single-cell suspensions were incubated in RPMI without serum and phenol red with FAM-FLICA™ Caspase-3 solution (CaspaseFLICA kit FAM-DEVD-FMK) for 30 min at 37°C, following the manufacturer's protocol, and then washed twice with PBS and immediately analyzed by FACS.

To determine the level of intracellular GSH content, single-cell suspensions were incubated in RPMI without serum and phenol red with 5-chloromethylfluoresceindiacetate, CMFDA (10 μM), for 30 min at 37°C, washed twice with PBS, and analyzed immediately by FACS.

A total of 300,000 cells within the leukocyte gate were acquired using a FACSCanto analyzer (Becton Dickinson, San Jose, CA). Data were processed using BD FacsDiva software. By using a modification of the International Society of Hematotherapy and Graft Engineering guidelines (24), CPC were defined as cells forming a cluster with low side scatter, low-to-intermediate CD45 staining, positive for CD34, CD133, and CD34/CD133.

Protein Concentration Assay

Protein concentration in the samples was determined using the Bradford assay (25). A standard curve of bovine serum albumin (0–15 μg protein/200 μl volume) was used.

Protein Carbonyl Content (PC)

Oxidative modification on plasma proteins was assessed on the basis of carbonyl content using 2-4 dinitrophenylhydrazine, as described by Levine et al. (26).

Samples were diluted to obtain a protein concentration of 10 mg/ml, and 100 μl of each sample was aliquoted in Eppendorf tubes. For each sample, a blank measurement was prepared. Then, 400 μl of a DNPH solution (5 mM in 2.5 M HCl) was added to tubes. Blank tubes were also prepared, adding the HCl solution without DNPH. Then, all the tubes were incubated in the dark for an hour, vortexing every 15–20 min. After incubation, protein content was precipitated by adding 500 μl of a 20% trichloroacetic acid (TCA) solution, placing tubes on ice for 5 min, and centrifuging at 10,000 *g* for 5 min to pellet protein content. The supernatant was discarded and the pellet was washed once with 500 μl of 10%TCA, and then twice with 500 μl of a 1:1 solution of ethanol-ethyl acetate. Finally, the pellet was resuspended in guanidine hydrochloride at 37°C for 15 min and the absorbance of carbonyl-bound DNPH was read at 370 nm. The corrected absorbance was calculated subtracting the mean of blank values from raw DNPH values. Then, the concentration was determined using an extinction coefficient of 0.022 μM^{-1} cm^{-1}, and normalized with the total protein content.

TBARS (Thiobarbituric Acid Reactive Substances) Estimation

Plasma TBARS levels were measured using a TBARS assay kit (OXI-TEK, ENZO, USA) as previously reported (27). Briefly, the adduct generated by reacting malondialdehyde with thiobarbituric acid after 1 h at 95°C was measured spectrofluorimetrically, with excitation at 530 nm and emission at 550 nm. TBARS were expressed in terms of malondialdehyde equivalent (nmol/ml) and then normalized for protein concentration.

Total Antioxidant Capacity (TAC) Assay

The ORAC method (oxygen radical absorbance capacity) was performed as previously described on plasma samples (28). Briefly, fluorescein solution (6 nM) was prepared daily in 75 mM sodium phosphate buffer (pH 7.4) and Trolox (250 μM final concentration) was used as a standard. Seventy microliters of each sample with 100 μl of fluorescein was pre-incubated for 30 min at 37°C in each well, before rapidly adding AAPH solution (19 mM final concentration). Fluorescence was measured using Synergy H1 microplate reader (BioTek, Winooski, VT). Results were expressed as Trolox Equivalents (μM) and then normalized for protein concentration.

Statistical Analysis

To assess the statistical significance of differences in clinical data and progenitor cell numbers between patients with BS and control subjects, the χ^2 test for categorical variables and Mann–Whitney test for numeric variables were used. Logistic regression analysis, including age, drug use, and sex as variables possibly influencing the cell number, was performed to test the independency of associations. In this analysis, the logarithm of the cell number was used for a better evaluation of the OR. All analyses were performed using the SPSS (Statistical Package for Social Sciences, Chicago, IL) software for Windows (Version 15.0).

RESULTS

All the patients enrolled in the study fulfilled the International Criteria for Behçet Disease (ICBD) (29). At the beginning of the disease, almost all the patients presented oral ulcers (96.7%), followed by cutaneous and articular involvement (65.6 and 59%, respectively). More than one third of the patients also had ocular and intestinal manifestations, as well as genital ulcers and vascular involvement. HLA-B51 was present in 42.6% of the patients.

All the patients with a Behçet Disease Activity Form (BDCAF) with a score ≥ 1 were considered active, while BS patients with a BDCAF equal to 0 were defined inactive.

Only a minority of the patients had no treatment at the time of the enrollment or were on corticosteroid as the unique therapy (11.5 and 4.9%, respectively). The majority of the BS patients were on Disease Modifying Anti Rheumatic Drugs (DMARDs) (32.8%) or on biologic +/– traditional DMARDs (50.8%).

Demographic and clinical features of the population studied are summarized in detail in **Table 1**.

TABLE 1 | Main clinical and demographic features of the patients enrolled in the study.

	***N* (% out of 61)**
***N* obs**	61
Sex	
Male	32 (52.5)
Female	29 (47.5)
Age at diagnosis	
Median (IQR; range)	35 (26–42)
HLA-B51	
Positive	26 (42.6)
Manifestations at baseline (ICBD criteria)	
Oral aphthosis	59 (96.7)
Skin involvement	40 (65.6)
Articular involvement	36 (59.0)
Ocular involvement	23 (37.7)
Intestinal involvement	22 (36.1)
Genital aphthosis	21 (34.4)
Vascular involvement	20 (32.8)
Neurologic involvement	17 (27.9)
Positive pathergy test	4 (6.6)
Disease activity at time of sample collection	
Not active (BDCAF = 0)	21 (34.4)
Active (BDCAF ≥ 1)	40 (65.6)
Active manifestations at time of sample collection	
Oral aphthosis	22 (36.1)
Articular involvement	17 (27.9)
Intestinal involvement	11 (18.0)
Skin involvement	10 (16.4)
Ocular involvement	9 (14.8)
Neurologic involvement	5 (8.2)
Vascular involvement	4 (6.6)
Genital aphthosis	1 (1.6)
Ongoing pharmacological therapies	
No treatment	7 (11.5)
Only corticosteroids	3 (4.9)
Traditional DMARDs	20 (32.8)
Biologic (±traditional) DMARDs	31 (50.8)

Plasma Oxidative Stress

As reported in **Table 2**, patient plasma displayed significantly higher total PC and TBARS levels compared to healthy controls ($p < 0.0001$ vs. controls).

Levels of Circulating Progenitor Cells

Because several CPC may participate to vascular repair, different phenotypically defined subpopulations of CD34+ CPC were analyzed by FACS analysis, allowing one to determine the level of overall CD34+/CD45$^{-/dim}$ CPC, of CD34+/CD45$^{-/dim}$/CD133– CPC, and of CD34+/CD45$^{-/dim}$/CD133+, representative of more immature CPC. As summarized in **Figure 1A**, significantly lower CD34+/CD45$^{-/dim}$ and CD34+/CD45$^{-/dim}$/CD133+ CPC levels were observed in BS patients with respect to controls (245 ± 92 vs. 637 ± 96, $p < 0.0001$; 80 ± 28 vs. 536 ± 88, $p < 0.0001$, respectively). On the contrary, CD34+/CD45$^{-/dim}$/CD133– level was significantly higher ($p < 0.0001$) in BS patients with respect to controls (165 ± 70 vs. 101 ± 26).

TABLE 2 | Oxidative stress markers.

	Controls *n* = 61	**BS patients *n* = 61**	
Plasma PC (nmol/mg)	10.87 ± 3.08	17.75 ± 4.18	$p < 0.0001$
Plasma TBARS (nmol/ml)	0.66 ± 0.11	2.21 ± 0.82	$p < 0.0001$
Plasma TAC (nmol Trolox equivalent/mg of protein)	21.8 ± 3.9	15.2 ± 4.8	$p < 0.0001$

CPC Oxidative Stress and Apoptosis

As shown in **Figure 1B**, in all the considered CPC subsets, we observed significantly higher ($p < 0.0001$) ROS levels in BS patients with respect to controls (CD34+/CD45$^{-/dim}$: 14,333 ± 5104 vs. 2549 ± 794; CD34+/CD45$^{-/dim}$/CD133–: 16,941 ± 7444 vs. 4728 ± 2165; CD34+/CD45$^{-/dim}$/CD133+: 10,396 ± 3469 vs. 2169 ± 737). Likewise, as shown in **Figure 1C**, we observed significantly higher levels of caspase-3 activity ($p < 0.0001$) in all the considered CPC population in BS patients with respect to controls (CD34+/CD45$^{-/dim}$: 8704 ± 3158 vs. 323 ± 66; CD34+/CD45$^{-/dim}$/CD133–: 12,318 ± 5280 vs. 304 ± 73; CD34+/CD45$^{-/dim}$/CD133+: 2197 ± 1002 vs. 3274 ± 67). A strong reduction in GSH content (**Figure 1D**) in the CPC subpopulation from BS patients with respect to controls was observed (CD34+/CD45$^{-/dim}$: 8454 ± 1874 vs. 64,792 ± 7825; CD34+/CD45$^{-/dim}$/CD133–: 3993 ± 1407 vs. 48,943 ± 7764; CD34+/CD45$^{-/dim}$/CD133+: 17,598 ± 5101 vs. 67,828 ± 8206).

Correlation Between Investigated Parameters

As shown in **Figure 2A**, in all the considered CPC subsets, ROS significantly correlated with CPC number. At the same time, CPC caspase-3 activity (**Figure 2B**) and CPC GSH content (**Figure 2C**) significantly correlated with CPC number, in all CPC subsets.

DISCUSSION

BS is considered the prototype of systemic inflammatory disease causing thrombosis, but the mechanisms underlying the relationship between inflammation and vascular events are far to be elucidated.

In this study, we investigated in a cohort of Behçet's patients the role of CPC, a population of undifferentiated progenitor cells originated in the bone marrow with the ability to be mobilized in response to vascular injury and capable of multi-lineage differentiation including EPC and endothelial cells.

Both EPC and CPC are considered surrogate biomarkers of cardiovascular health since they appear to constitute a natural system for the maintenance of vascular function, improving endothelial repair and neovascularization (30–32). Notably,

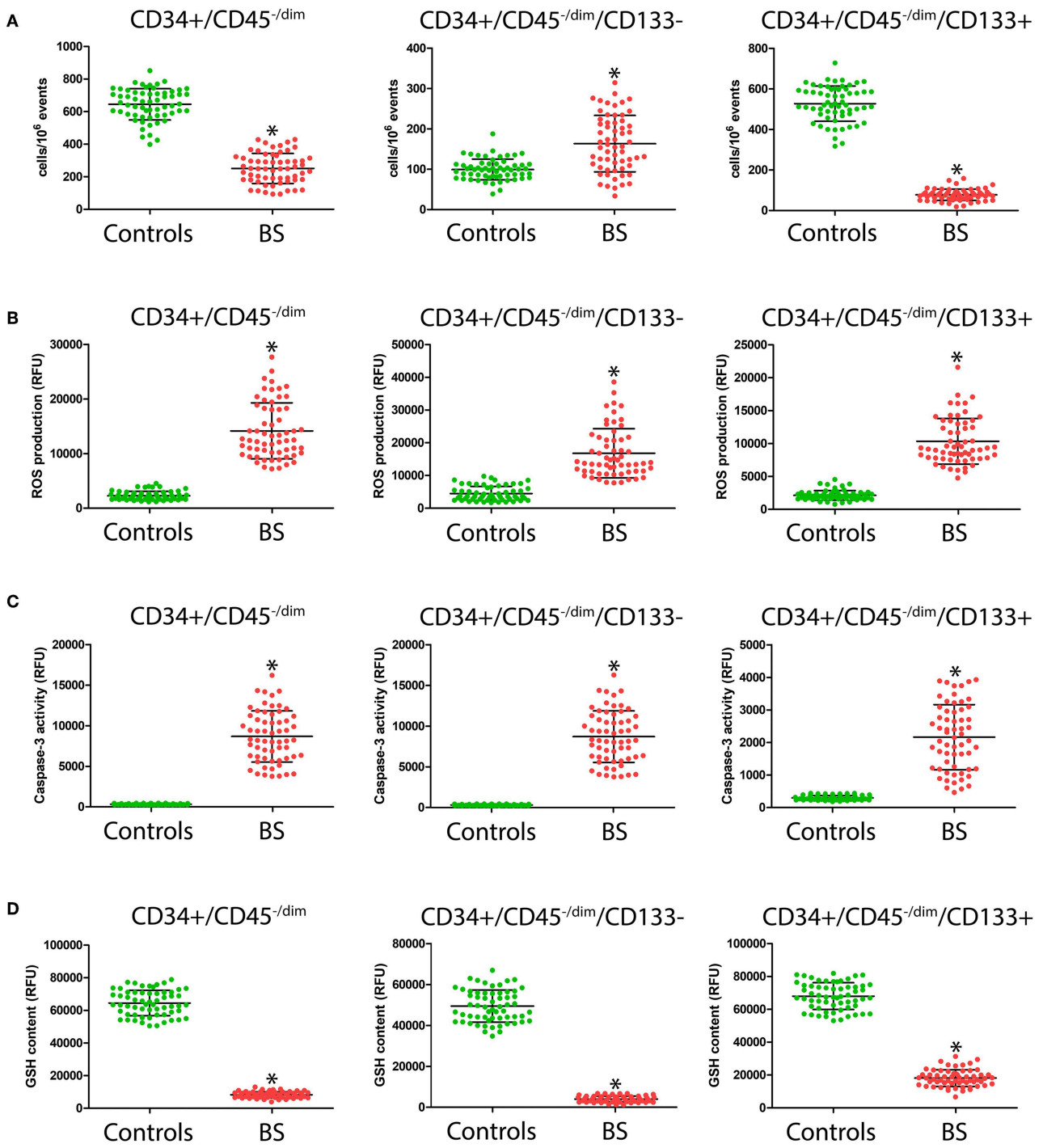


FIGURE 1 | Number **(A)**, intracellular ROS production **(B)**, caspase-3 activity **(C)**, and GSH content **(D)** in CD34+/CD45$^{-/dim}$, CD34+/CD45$^{-/dim}$/CD133– and of CD34+/CD45$^{-/dim}$/CD133+ CPC from patients and controls. *Significant difference vs. control at the $p < 0.0001$ level.

the restoration of blood supply to ischemic tissues is strictly dependent on endothelial regeneration and angiogenesis. Here, we demonstrate for the first time that CPC from BS patients, but not those from healthy subjects, show signs of oxidative stress and apoptosis. Another important finding emerging from our study is the reduced CPC number observed in BS patients with respect to control subjects. Importantly, the number and function of CPC may reflect the balance between endothelial integrity and repair

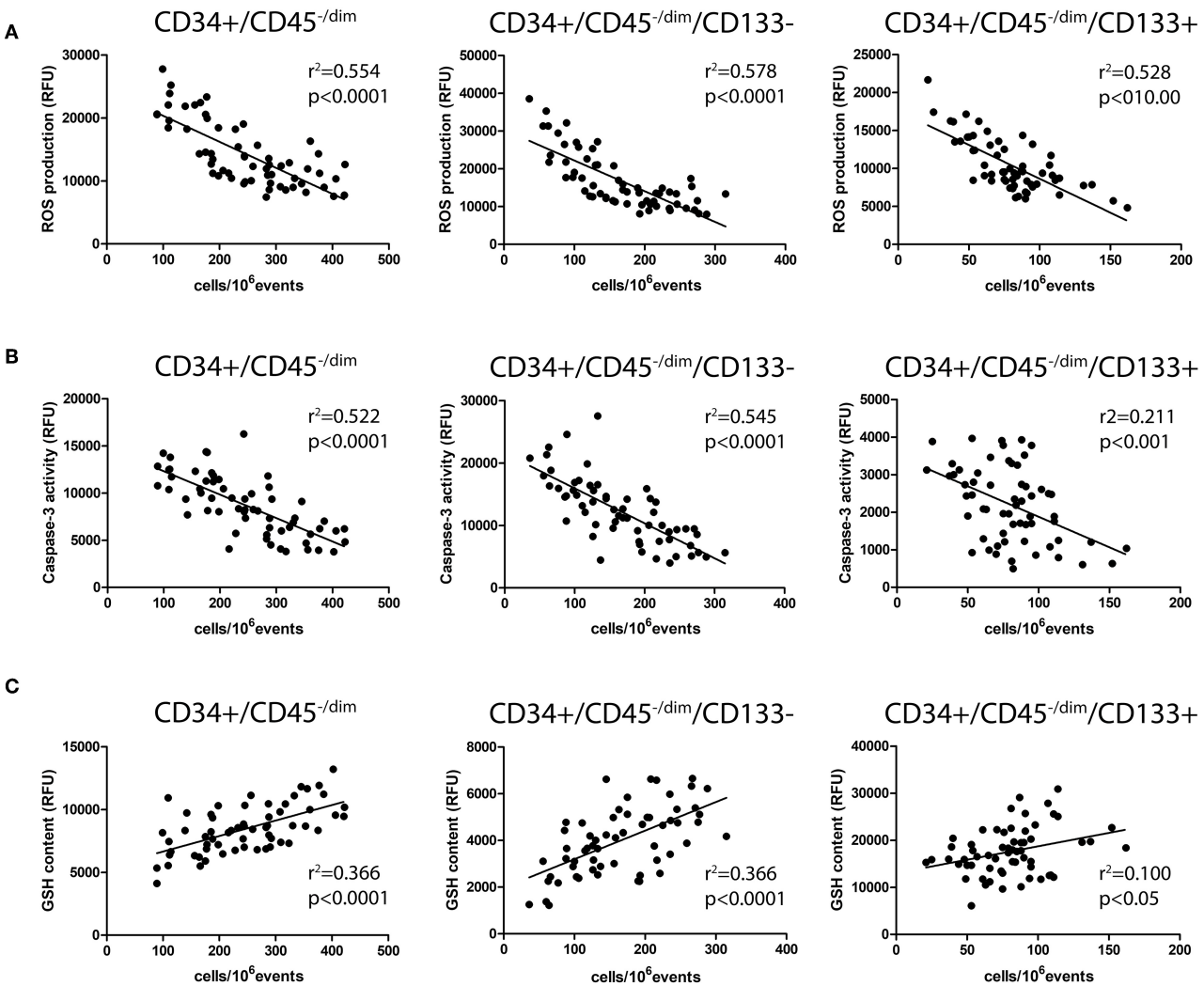


FIGURE 2 | Correlation analysis among CPC number and intracellular ROS production **(A)**, caspase-3 activity **(B)**, and GSH content **(C)**.

and can be used as a marker of endothelial function. Indeed, patients with hypertension, coronary artery disease, chronic renal failure, diabetes, sepsis, and rheumatoid arthritis exhibit decreased CPC number (33–36). Moreover, EPC isolated from patients with coronary artery disease and hypertension display an impaired migratory response (34, 35).

The decline in CPC number can be attributed to increased apoptosis, oxidative stress, inflammation, and senescence, in addition to reduced growth and migration from bone marrow (33). However, recent data suggest that increased CPC number may also represent a homoeostatic stress response contributing to vascular damage repair (36, 37). Indeed, in acute coronary syndromes, the early CPC mobilization from the bone marrow seems related to the extension of myocardial ischemia expressed as area at risk (38) and may contribute to the healing process by promoting neovascularization (39).

Moreover, we observed an inverse correlation between circulating CPC number and CPC ROS production, suggesting a possible toxic ROS effect on CPC in BS patients. Indeed, signs of oxidative stress (increased ROS production and reduced GSH content) and apoptosis in CPC from BS patients were observed, suggesting a functional impairment of these cells. Furthermore, the significant correlations between ROS production/GSH content and caspase-3 activity point out that oxidative stress can represent a determinant in the onset of apoptosis in CPC.

To date, few data are available about the possible pathogenetic role of CPC in systemic vasculitis. It was previously reported that the increased number of circulating inflammatory endothelial cells could represent an activity marker in patients with systemic necrotizing vasculitis (40). EPC were reported to be increased in number also in a patient with BS complicated with cerebral trombophlebitis (41). Recently, Bozkirli et al. demonstrated that EPC number was significantly higher in BS patients with thrombosis (42). On the other hand, it was also demonstrated that BS is associated with a progressive reduction in EPC number, which can be interpreted as a mechanism of induction and/or

progression of vascular injury in these patients (21). However, to date, there are no data on CPC function in BS patients.

In the case of EPC population, the univocal interpretation of data is limited by the extremely low frequency of the analyzed cell populations and by the lack of validation of the utilized markers. For this reason, in this study, we analyzed the most abundant CPC population instead of the rare EPC population (which accounts for about 0.01 – 0.0001% of nucleated cells). To our knowledge, this is the first study to detect ROS production, GSH content, and caspase-3 activation in CPC, defined as CD34+/CD45$^{-/low}$/CD133+ and CD34+/CD45$^{-/low}$/CD133–, in peripheral blood (not in isolated and cultured cells).

It is accepted that EPC mobilization can be stimulated by transient restricted inflammatory response, while high-grade inflammation results in decreased EPC number and EPC dysfunction (43). Considerable evidence also suggests that ROS play a key role in EPC mobilization/function (44). In particular, low ROS levels activate pro-angiogenic pathways in EPC, whereas high ROS levels impair EPC function. Therefore, oxidative stress is responsible not only for EPC circulating number reduction but also for an impairment EPC function with consequent harmful effects in vascular homeostasis. Indeed, during conditions such as diabetes mellitus, characterized by oxidative stress, the mobilization of dysfunctional EPC is observed (45). Indeed, increased superoxide generation reduces EPC levels and impairs EPC function (46). In addition, incubation of EPC with hydrogen peroxide has been shown to induce apoptosis (47), profoundly reducing EPC number (48). Furthermore, increased ROS production has been associated with reduced EPC levels in a rat model of myocardial infarction (49).

An overall imbalance in blood redox status has been proposed in BS (50). Recently, we demonstrated that neutrophils are responsible for an increased ROS production in BS patients, thus favoring thrombosis through a deep modification of fibrinogen secondary structure (51). Accordingly, in the present study, plasma protein carbonyls and TBARS were markedly and significantly increased in BS patients when compared with control subjects, thus confirming an altered oxidative status in BS patients.

In human vasculature, ROS production is counterbalanced by several antioxidant molecules aimed at ROS scavenging. Intracellular antioxidant enzymes, such as glutathione peroxidase, catalase, and manganese superoxide dismutase, were increased in EPC from healthy subjects with respect to differentiated, mature endothelial cells (52). This is in agreement with our data that show, for the first time, a marked increase (+62%) in ROS production in CD133— population with respect to the more immature CD133+ population, in human peripheral blood. In addition, our results indicate, in CPC from human peripheral blood, a significant reduction in GSH content compared with CPC from control subjects, suggesting that an impairment in antioxidant system can promote CPC sensitivity toward oxidative-stress-mediated apoptosis and consequently reduced CPC number in BS patients. Our observations were supported by the finding that glutathione peroxidase-1-deficient mice exhibited a reduced number and functional activity of progenitor cells (53).

The exact oxidative mechanisms underlying CPC dysfunction has not yet been understood. To date, no study has addressed the question whether redox balancing therapeutic strategies can modify CPC function and number. Only when antioxidant therapies will demonstrate to improve these parameters of CPC biology will a safe conclusion be drawn regarding ROS and CPC relationship in humans.

The results of the present study may have implications in the pathogenesis of thrombotic manifestations in BS. Indeed, CPC have not only been associated with coronary artery disease (54) and atherosclerosis (55). Different from other inflammatory immune-mediated conditions, BS is not associated with accelerated atherosclerosis, despite without having a clear pathogenetic explanation (56). Notably, CPC dysfunction has been also evoked as a potential mechanism in deep vein thrombosis occurrence (57) and aneurysm formation (58), typical clinical features of BS.

Future longitudinal studies on a larger BS population would be helpful in order to explore CPC dysfunction in specific subsets of BS patients. Moreover, functional analysis showing the impact of ROS production on vessel wall of BS patients would be of importance.

However, taking into account that oxidative stress plays an important role in the pathogenesis of all vascular diseases (59), our data support the hypothesis that oxidative-stress-mediated CPC dysfunctioning may counteract their vascular repair actions, thereby contributing to the pathogenesis and the progression of vascular disease in BS patients.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comitato Etico Università degli Studi di Firenze, Largo Brambilla 3, Firenze, Italy. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

GE, AM, FA, ES, AB, CF, and MB were responsible for data collection and analysis. MB, AM, and FA performed experiments. GE, ES, AV, and DP monitored patient inclusion. MB and CF were responsible for protocol development and study funding, and supervised the study. NT, AF, and LS gave critical guidance during the project. MB, GE, and CF designed the experiments and wrote the manuscript. All authors contributed substantially to the critical revision of the manuscript, and gave approval of the final draft.

FUNDING

This study was supported by Fondazione CR Firenze research funding no. 0849.2017 attributed to MB and by Fondi di Ateneo research funding from the University of Florence to GE. This study was also partly supported by the Associazione Italiana Sindrome e Malattia di Behçet (SIMBA).

REFERENCES

1. Leccese P, Alpsoy E. Behçet's disease: an overview of etiopathogenesis. *Front Immunol*. (2019) 10:1067. doi: 10.3389/fimmu.2019.01067
2. Emmi G, Bettiol A, Silvestri E, Di Scala G, Becatti M, Fiorillo C, et al. Vascular Behçet's syndrome: an update. *Intern Emerg Med*. (2019) 14:645–52. doi: 10.1007/s11739-018-1991-y
3. Emmi G, Silvestri E, Squatrito D, Amedei A, Niccolai E, D'Elios MM, et al. Thrombosis in vasculitis: from pathogenesis to treatment. *Thromb J*. (2015) 13:15. doi: 10.1186/s12959-015-0047-z
4. Espinosa G, Font J, Tàssies D, Vidaller A, Deulofeu R, López-Soto A, et al. Vascular involvement in Behçet's disease: relation with thrombophilic factors, coagulation activation, and thrombomodulin. *Am J Med*. (2002) 112:37–43. doi: 10.1016/S0002-9343(01)01048-8
5. Becatti M, Emmi G, Bettiol A, Silvestri E, Di Scala G, Taddei N, et al. Behçet's syndrome as a tool to dissect the mechanisms of thrombo-inflammation: clinical and pathogenetic aspects. *Clin Exp Immunol*. (2019) 195:322–33. doi: 10.1111/cei.13243
6. Emmi G, Becatti M, Bettiol A, Hatemi G, Prisco D, Fiorillo C. Behçet's syndrome as a model of thrombo-inflammation: the role of neutrophils. *Front Immunol*. (2019) 10:1085. doi: 10.3389/fimmu.2019.01085
7. Butta NV, Fernández-Bello I, López-Longo FJ, Jiménez-Yuste V. Endothelial dysfunction and altered coagulation as mediators of thromboembolism in behçet disease. *Semin Thromb Hemost*. (2015) 41:621–8. doi: 10.1055/s-0035-1556727
8. Hatemi G, Christensen R, Bang D, Bodaghi B, Celik AF, Fortune F, et al. 2018 update of the EULAR recommendations for the management of Behçet's syndrome. *Ann Rheum Dis*. (2018) 77:808–18. doi: 10.1136/annrheumdis-2018-213225
9. Weissman IL. Stem cells: units of development, units of regeneration, and units in evolution. *Cell*. (2000) 100:157–68. doi: 10.1016/S0092-8674(00)81692-X
10. Mazo IB, Massberg S, von Andrian UH. Hematopoietic stem and progenitor cell trafficking. *Trends Immunol*. (2011) 32:493–503. doi: 10.1016/j.it.2011.06.011
11. Foresta C, De Toni L, Ferlin A, Di Mambro A. Clinical implication of endothelial progenitor cells. *Expert Rev Mol Diagn*. (2010) 10:89–105. doi: 10.1586/erm.09.80
12. Sen S, McDonald SP, Coates PT, Bonder CS. Endothelial progenitor cells: novel biomarker and promising cell therapy for cardiovascular disease. *Clin Sci*. (2011) 120:263–83. doi: 10.1042/CS20100429
13. Lamping K. Endothelial progenitor cells: sowing the seeds for vascular repair. *Circ Res*. (2007) 100:1243–5. doi: 10.1161/01.RES.0000268193.46418.d1
14. Giannotti G, Doerries C, Mocharla PS, Mueller MF, Bahlmann FH, Horvàth T, et al. Impaired endothelial repair capacity of early endothelial progenitor cells in prehypertension: relation to endothelial dysfunction. *Hypertension*. (2010) 55:1389–97. doi: 10.1161/HYPERTENSIONAHA.109.141614
15. Marrotte EJ, Chen DD, Hakim JS, Chen AF. Manganese superoxide dismutase expression in endothelial progenitor cells accelerates wound healing in diabetic mice. *J Clin Invest*. (2010) 120:4207–19. doi: 10.1172/JCI36858
16. Cesari F, Caporale R, Marcucci R, Caciolli S, Stefano PL, Capalbo A, et al. NT-proBNP and the anti-inflammatory cytokines are correlated with endothelial progenitor cells' response to cardiac surgery. *Atherosclerosis*. (2008) 199:138–46. doi: 10.1016/j.atherosclerosis.2007.09.045
17. Fujii H, Li SH, Szmitko PE, Fedak PW, Verma S. C-reactive protein alters antioxidant defenses and promotes apoptosis in endothelial progenitor cells. *Arterioscler Thromb Vasc Biol*. (2006) 26:2476–82. doi: 10.1161/01.ATV.0000242794.65541.02
18. Chen DD, Dong YG, Yuan H, Chen AF. Endothelin 1 activation of endothelin A receptor/NADPH oxidase pathway and diminished antioxidants critically contribute to endothelial progenitor cell reduction and dysfunction in salt-sensitive hypertension. *Hypertension*. (2012) 59:1037–43. doi: 10.1161/HYPERTENSIONAHA.111.183368
19. Ceradini DJ, Yao D, Grogan RH, Callaghan MJ, Edelstein D, Brownlee M, et al. Decreasing intracellular superoxide corrects defective ischemia-induced new vessel formation in diabetic mice. *J Biol Chem*. (2008) 283:10930–8. doi: 10.1074/jbc.M707451200
20. Bozkirli ED, Keşkek SÖ, Kozanoglu I, Yücel AE. High levels of endothelial progenitor cells can be associated with thrombosis in patients with Behçet's disease. *Clin Exp Rheumatol*. (2014) 32(4 Suppl. 84):S49–53.
21. Fadini GP, Tognon S, Rodriguez L, Boscaro E, Baesso I, Avogaro A, et al. Low levels of endothelial progenitor cells correlate with disease duration and activity in patients with Behçet's disease. *Clin Exp Rheumatol*. (2009) 27:814–21.
22. Nencini P, Sarti C, Innocenti R, Pracucci G, Inzitari D. Acute inflammatory events and ischemic stroke subtypes. *Cerebrovasc Dis*. (2003) 15:215–21. doi: 10.1159/000068831
23. Cesari F, Sofi F, Caporale R, Capalbo A, Marcucci R, Macchi C, et al. Relationship between exercise capacity, endothelial progenitor cells and cytochemokines in patients undergoing cardiac rehabilitation. *Thromb Haemost*. (2009) 101:521–6. doi: 10.1160/TH08-10-0644
24. Sutherland DR, Anderson L, Keeney M, Nayar R, Chin-Yee I. The ISHAGE guidelines for CD34+ cell determination by flow cytometry. International society of hematotherapy and graft engineering. *J Hematother*. (1996) 5:213–26. doi: 10.1089/scd.1.1996.5.213
25. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. (1976) 72:248–54. doi: 10.1006/abio.1976.9999
26. Levine RL, Williams JA, Stadtman ER, Shacter E. Carbonyl assays for determination of oxidatively modified proteins. *Methods Enzymol*. (1994) 233:346–57. doi: 10.1016/S0076-6879(94)33040-9
27. Fiorillo C, Becatti M, Attanasio M, Lucarini L, Nassi N, Evangelisti L, et al. Evidence for oxidative stress in plasma of patients with Marfan syndrome. *Int J Cardiol*. (2010) 145:544–6. doi: 10.1016/j.ijcard.2010.04.077
28. Sofi F, Dinu M, Pagliai G, Cesari F, Gori AM, Sereni A, et al. Low-calorie vegetarian versus mediterranean diets for reducing body weight and improving cardiovascular risk profile: CARDIVEG study (Cardiovascular Prevention With Vegetarian Diet). *Circulation*. (2018) 137:1103–13. doi: 10.1161/CIRCULATIONAHA.117.030088
29. International Team for the Revision of the International Criteria for Behçet's Disease (ITR-ICBD). The international criteria for Behçet's disease (ICBD): a collaborative study of 27 countries on the sensitivity and specificity of the new criteria. *J Eur Acad Dermatol Venereol*. (2014) 28:338–47. doi: 10.1111/jdv.12107
30. Bianconi V, Sahebkar A, Kovanen P, Bagaglia F, Ricciuti B, Calabrò P, et al. Endothelial and cardiac progenitor cells for cardiovascular repair: a controversial paradigm in cell therapy. *Pharmacol Ther*. (2018) 181:156–68. doi: 10.1016/j.pharmthera.2017.08.004
31. Rigato M, Fadini GP. Circulating stem/progenitor cells as prognostic biomarkers in macro- and microvascular disease: a narrative review of prospective observational studies. *Curr Med Chem*. (2018) 25:4507–17. doi: 10.2174/0929867324666170920154020
32. Cesari F, Sofi F, Molino Lova R, Vannetti F, Pasquini G, Cecchi F, et al. Aging process, adherence to Mediterranean diet and nutritional status in a large cohort of nonagenarians: effects on endothelial progenitor cells. *Nutr Metab Cardiovasc Dis*. (2018) 28:84–90. doi: 10.1016/j.numecd.2017.09.003
33. Umemura T, Higashi Y. Endothelial progenitor cells: therapeutic target for cardiovascular diseases. *J Pharmacol Sci*. (2008) 108:1–6. doi: 10.1254/jphs.08R01CP

34. Vasa M, Fichtlscherer S, Aicher A, Adler K, Urbich C, Martin H, et al. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. *Circ Res*. (2001) 89:E1–7. doi: 10.1161/hh1301.093953
35. Vasa M, Fichtlscherer S, Adler K, Aicher A, Martin H, Zeiher AM, et al. Increase in circulating endothelial progenitor cells by statin therapy in patients with stable coronary artery disease. *Circulation*. (2001) 103:2885–90. doi: 10.1161/hc2401.092816
36. Grisar J, Aletaha D, Steiner CW, Kapral T, Steiner S, Seidinger D, et al. Depletion of endothelial progenitor cells in the peripheral blood of patients with rheumatoid arthritis. *Circulation*. (2005) 111:204–11. doi: 10.1161/01.CIR.0000151875.21836.AE
37. Valgimigli M, Rigolin GM, Fucili A, Porta MD, Soukhomovskaia O, Malagutti P, et al. CD34+ and endothelial progenitor cells in patients with various degrees of congestive heart failure. *Circulation*. (2004) 110:1209–12. doi: 10.1161/01.CIR.0000136813.89036.21
38. Porto I, Leone AM, De Maria GL, Hamilton Craig C, Tritarelli A, Camaioni C, et al. Are endothelial progenitor cells mobilized by myocardial ischemia or myocardial necrosis? A cardiac magnetic resonance study. *Atherosclerosis*. (2011) 216:355–8. doi: 10.1016/j.atherosclerosis.2011.02.014
39. Dubois C, Liu X, Claus P, Marsboom G, Pokreisz P, Vandenwijngaert S, et al. Differential effects of progenitor cell populations on left ventricular remodeling and myocardial neovascularization after myocardial infarction. *J Am Coll Cardiol*. (2010) 55:2232–43. doi: 10.1016/j.jacc.2009.10.081
40. Hergesell O, Andrassy K, Nawroth P. Elevated levels of markers of endothelial cell damage and markers of activated coagulation in patients with systemic necrotizing vasculitis. *Thromb Haemost*. (1996) 75:892–8. doi: 10.1055/s-0038-1650390
41. Camoin-Jau L, Kone-Paut I, Chabrol B, Sampol J, Dignat-George F. Circulating endothelial cells in Behçet's disease with cerebral thrombophlebitis. *Thromb Haemost*. (2000) 83:631–2. doi: 10.1055/s-0037-1613881
42. Bozkirli ED, Keşkek SÖ, Kozanoglu I, Yücel AE. High levels of endothelial progenitor cells can be associated with thrombosis in patients with Behçet's disease. *Clin Exp Rheumatol*. (2014) 32(4 Suppl. 84):S49–53.
43. Andreou I, Tousoulis D, Tentolouris C, Antoniades C, Stefanadis C. Potential role of endothelial progenitor cells in the pathophysiology of heart failure: clinical implications and perspectives. *Atherosclerosis*. (2006) 189:247–54. doi: 10.1016/j.atherosclerosis.2006.06.021
44. Yao EH, Yu Y, Fukuda N. Oxidative stress on progenitor and stem cells in cardiovascular diseases. *Curr Pharm Biotechnol*. (2006) 7:101–8. doi: 10.2174/138920106776597685
45. Schatteman GC, Hanlon HD, Jiao C, Dodds SG, Christy BA. Blood-derived angioblasts accelerate blood-flow restoration in diabetic mice. *J Clin Invest*. (2000) 106:571–8. doi: 10.1172/JCI9087
46. Thum T, Fraccarollo D, Schultheiss M, Froese S, Galuppo P, Widder JD, et al. Endothelial nitric oxide synthase uncoupling impairs endothelial progenitor cell mobilization and function in diabetes. *Diabetes*. (2007) 56:666–74. doi: 10.2337/db06-0699
47. Hung YC, Sava VM, Blagodarsky VA, Hong MY, Huang GS. Protection of tea melanin on hydrazine-induced liver injury. *Life Sci*. (2003) 72:1061–71. doi: 10.1016/S0024-3205(02)02348-2
48. Urbich C, Knau A, Fichtlscherer S, Walter DH, Brühl T, Potente M, et al. FOXO-dependent expression of the proapoptotic protein Bim: pivotal role for apoptosis signaling in endothelial progenitor cells. *FASEB J*. (2005) 19:974–6. doi: 10.1096/fj.04-2727fje
49. Thum T, Fraccarollo D, Galuppo P, Tsikas D, Frantz S, Ertl G, et al. Bone marrow molecular alterations after myocardial infarction: impact on endothelial progenitor cells. *Cardiovasc Res*. (2006) 70:50–60. doi: 10.1016/j.cardiores.2006.01.002
50. Ozyazgan S, Andican G, Erman H, Tuzcu A, Uzun H, Onal B, et al. Relation of protein oxidation parameters and disease activity in patients with Behçet's disease. *Clin Lab*. (2013) 59:819–25. doi: 10.7754/Clin.Lab.2012.120722
51. Becatti M, Emmi G, Silvestri E, Bruschi G, Ciucciarelli L, Squatrito D, et al. Neutrophil activation promotes fibrinogen oxidation and thrombus formation in Behçet disease. *Circulation*. (2016) 133:302–11. doi: 10.1161/CIRCULATIONAHA.115.017738
52. He T, Peterson TE, Holmuhamedov EL, Terzic A, Caplice NM, Oberley LW, et al. Human endothelial progenitor cells tolerate oxidative stress due to intrinsically high expression of manganese superoxide dismutase. *Arterioscler Thromb Vasc Biol*. (2004) 24:2021–7. doi: 10.1161/01.ATV.0000142810.27849.8f
53. Galasso G, Schiekofer S, Sato K, Shibata R, Handy DE, Ouchi N, et al. Impaired angiogenesis in glutathione peroxidase-1-deficient mice is associated with endothelial progenitor cell dysfunction. *Circ Res*. (2006) 98:254–61. doi: 10.1161/01.RES.0000200740.57764.52
54. Samman Tahhan A, Hammadah M, Raad M, Almuwaqqat Z, Alkhoder A, Sandesara PB, et al. Progenitor cells and clinical outcomes in patients with acute coronary syndromes. *Circ Res*. (2018) 122:1565–75. doi: 10.1161/CIRCRESAHA.118.312821
55. Xu Q. The impact of progenitor cells in atherosclerosis. *Nat Clin Pract Cardiovasc Med*. (2006) 3:94–101. doi: 10.1038/ncpcardio0396
56. Seyahi E, Ugurlu S, Cumali R, Balci H, Ozdemir O, Melikoglu M, et al. Atherosclerosis in Behçet's syndrome. *Semin Arthritis Rheum*. (2008) 38:1–12. doi: 10.1016/j.semarthrit.2007.09.009
57. Alessio AM, Beltrame MP, Nascimento MC, Vicente CP, de Godoy JA, Silva JC, et al. Circulating progenitor and mature endothelial cells in deep vein thrombosis. *Int J Med Sci*. (2013) 10:1746–54. doi: 10.7150/ijms.6887
58. Sung SH, Wu TC, Chen JS, Chen YH, Huang PH, Lin SJ, et al. Reduced number and impaired function of circulating endothelial progenitor cells in patients with abdominal aortic aneurysm. *Int J Cardiol*. (2013) 168:1070–7. doi: 10.1016/j.ijcard.2012.11.002
59. Kim YW, Byzova TV. Oxidative stress in angiogenesis and vascular disease. *Blood*. (2014) 123:625–31. doi: 10.1182/blood-2013-09-512749

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.

frontiers in Immunology

REVIEW
published: 17 December 2019
doi: 10.3389/fimmu.2019.02813

Current State of Precision Medicine in Primary Systemic Vasculitides

Erkan Demirkaya[1]*, *Zehra Serap Arici*[2], *Micol Romano*[1,3], *Roberta Audrey Berard*[1] and *Ivona Aksentijevich*[4]

[1] Division of Paediatric Rheumatology, Department of Paediatrics, Schulich School of Medicine & Dentistry, University of Western Ontario, London, ON, Canada, [2] Department of Paediatric Rheumatology, Sanliurfa Training and Research Hospital, Sanliurfa, Turkey, [3] Department of Pediatric Rheumatology, Istituto Ortopedico Gaetano Pini, Milan, Italy, [4] Inflammatory Disease Section, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD, United States

Precision medicine (PM) is an emerging data-driven health care approach that integrates phenotypic, genomic, epigenetic, and environmental factors unique to an individual. The goal of PM is to facilitate diagnosis, predict effective therapy, and avoid adverse reactions specific for each patient. The forefront of PM is in oncology; nonetheless, it is developing in other fields of medicine, including rheumatology. Recent studies on elucidating the genetic architecture of polygenic and monogenic rheumatological diseases have made PM possible by enabling physicians to customize medical treatment through the incorporation of clinical features and genetic data. For complex inflammatory disorders, the prevailing paradigm is that disease susceptibility is due to additive effects of common reduced-penetrance gene variants and environmental factors. Efforts have been made to calculate cumulative genetic risk score (GRS) and to relate specific susceptibility alleles for use of target therapies. The discovery of rare patients with single-gene high-penetrance mutations informed our understanding of pathways driving systemic inflammation. Here, we review the advances in practicing PM in patients with primary systemic vasculitides (PSVs). We summarize recent genetic studies and discuss current knowledge on the contribution of epigenetic factors and extracellular vesicles (EVs) in disease progression and treatment response. Implementation of PM in PSVs is a developing field that will require analysis of a large cohort of patients to validate data from genomics, transcriptomics, metabolomics, proteomics, and epigenomics studies for accurate disease profiling. This multi-omics approach to study disease pathogeneses should ultimately provide a powerful tool for stratification of patients to receive tailored optimal therapies and for monitoring their disease activity.

Keywords: precision medicine, vasculitis, vasculitides, genome-wide association studies, epigenetics, extracellular vesicles, monogenic systemic autoinflammatory diseases

OPEN ACCESS

Edited by:
Andreas Kronbichler, Innsbruck Medical University, Austria

Reviewed by:
Davide Martorana, University Hospital of Parma, Italy
Md Yuzaiful Md Yusof, University of Leeds, United Kingdom

***Correspondence:**
Erkan Demirkaya
erkan.demirkaya@lhsc.on.ca

Specialty section:
This article was submitted to Inflammation, a section of the journal Frontiers in Immunology

Received: *02 August 2019*
Accepted: *15 November 2019*
Published: *17 December 2019*

Citation:
Demirkaya E, Arici ZS, Romano M, Berard RA and Aksentijevich I (2019) Current State of Precision Medicine in Primary Systemic Vasculitides. Front. Immunol. 10:2813. doi: 10.3389/fimmu.2019.02813

INTRODUCTION

Precision medicine (PM) can be defined as tailored medical care that is primarily based upon understanding the molecular sequence of biologic events causal to disease and critical for diagnosis and therapy. The term precision medicine should be distinguished from the term personalized medicine whereby prevention and treatment are being developed uniquely for each patient, although these two terms are used interchangeably (1). Both communicate a concept of

individualized medicine, although in PM, the focus is on identifying health care approaches that will be effective for a group of patients who share similar genetic, environmental, and lifestyle disease susceptibility factors. Recognizing subgroups of patients with similar disease risk factors will ensure that they will receive optimal treatment to improve their quality of life and health outcomes.

Primary systemic vasculitides (PSVs) (**Table 1**) are a heterogeneous group of diseases in their etiology, clinical presentation, and response to therapy. Severity and location of symptoms vary greatly, and most classification schemes that attempt to advance clinicians the ability to diagnose and treat patients, are based on blood vessel size, autoantibodies profile [e.g., anti-neutrophil cytoplasmic antibody (ANCA)], and histopathological findings. In addition to genetic predisposition, there is a role for environmental factors, including exposure to drugs and infectious agents, in the pathogenesis, and prognosis of PSVs. The past decade has witnessed major advances in genetics research that have improved our understanding on molecular mechanisms in PSVs. New sequencing technologies and high-throughput genotyping arrays used for genome-wide association studies (GWAS) generated vast amount of data that will require validation by meta-analysis in large cohorts of patients. Discovery of monogenic diseases mimicking PSVs offered additional insights on biochemical pathways that are shared between rare and more common forms of vasculitis. Recent advances in epigenetic research support the role of extracellular vesicles (EVs) in the pathogenesis of PSVs, which could be used for developing novel biomarkers to monitor disease activity, prognosis, and treatment outcomes.

Herein, we discuss the up-to-date knowledge on the genetic predisposition to PSVs and review recent clinical research studies aimed to further our understanding of the pathogeneses and outcomes of vasculitides.

TABLE 1 | Chapel Hill Consensus Conference Nomenclature (CHCC 2012) of vasculitides (2).

- Large-vessel vasculitis (LVV)
  - Takayasu arteritis (TAK)
  - Giant cell arteritis (GCA)
- Medium-vessel vasculitis (MVV)
  - Polyarteritis nodosa (PAN)
  - Kawasaki disease (KD)
- Small-vessel vasculitis (SVV)
  - Antineutrophil cytoplasmic antibody (ANCA)–associated vasculitis (AAV)
    - Microscopic polyangiitis (MPA)
    - Granulomatosis with polyangiitis (Wegener's) (GPA)
    - Eosinophilic granulomatosis with polyangiitis (Churg-Strauss) (EGPA)
  - Immune complex SVV
    - Anti–glomerular basement membrane (anti-GBM) disease
    - Cryoglobulinemic vasculitis (CV)
    - IgA vasculitis (Henoch-Schönlein purpura) (IgAV/HSP)
    - Hypocomplementemic urticarial vasculitis (HUV) (anti-C1q vasculitis)
- Variable vessel vasculitis (VVV)
  - Behçet's disease (BD)
  - Cogan's syndrome (CS)
- Single-organ vasculitis (SOV)
  - Cutaneous leukocytoclastic angiitis
  - Cutaneous arteritis
  - Primary central nervous system vasculitis
  - Isolated aortitis
  - Others
- Vasculitis associated with systemic disease
  - Lupus vasculitis
  - Rheumatoid vasculitis
  - Sarcoid vasculitis
  - Others
- Vasculitis associated with probable etiology
  - Hepatitis C virus–associated cryoglobulinemic vasculitis
  - Hepatitis B virus–associated vasculitis
  - Syphilis-associated aortitis
  - Drug-associated immune complex vasculitis
  - Drug-associated ANCA-associated vasculitis
  - Cancer-associated vasculitis
  - Others (e.g., varicella zoster virus–associated vasculitis)

GWAS IN PSVs

GWAS have been used, following the completion of the Human Genome project in 2003 and the International HapMap Project in 2005, to identify association of common and reduced-penetrance variants, termed single nucleotide polymorphisms (SNPs), with human traits or diseases (3). The primary goal of GWAS has been to elucidate the biology of polygenic and complex human diseases, which can be translated toward the development of novel therapeutics. GWAS have also been used to study gene-environment interactions, response to therapies, and more recently for risk prediction. GWAS are based on the genotyping of large cohorts of patients and ancestry-matched controls for SNPs in the entire genome. These associations are tested with gene variants that have a minor allele frequency (MAF) higher than 5% in the general population and are considered significant at the *p*-value genome-wide threshold of 5×10^{-8} (4). For a number of autoimmune disorders, SNPs in the human leukocyte antigen (HLA) [also known as major histocompatibility complex (MHC)] and genes encoded within the locus have been shown to play a major role in susceptibility to disease (4). In primary vasculitides (PSVs), GWAS have been performed primarily in Kawasaki disease (KD) and Behçet's disease (BD) (**Table 2**) (5–42, 44–46).

Kawasaki Disease

KD is an acute, self-limited vasculitis that typically affects infants and children under the age of 5 years. Coronary artery aneurysms (CAAs) occur in 25% of untreated patients and may lead to ischemic heart disease, myocardial infarction, and sudden death at a young age. The pathogenesis of KD remains unknown; however, it is thought that host genetics play an important role in susceptibility and disease outcome. Interestingly, the incidence of KD is up to 50-fold higher in children of Asian descent. Epidemiologic and clinical features of KD also strongly support an infectious etiology in genetically predisposed children (47).

GWAS in KD have identified a number of susceptibility SNPs/genes that contribute to the risk of KD (*HLA*, *FCGR2A*, SLC8A1, ZFHX3, NMNAT2, *BLK*, *DAB1*, *HCP5*, *COPB2*, *ERAP1*,

TABLE 2 | Genome-wide association studies (GWAS) in primary systemic vasculitides (PSVs).

Related risk and references	Genes from GWAS	Therapeutic implications
Kawasaki Disease (KD)		
Susceptibility genes for KD (5–16)	*HLA*, *HCP5*, *FCGR2A*, *BLK*, SLC8A1, *CD40*, *NMNAT2*, *DAB1*, *COPB2*, *NAALADL2*, *IGHV, ZFHX3*, *NFKBIL1*, *ERAP1*, EBF2, CACNB2, *LTA,* and *LEF1*	SNP in SLC8A1 (calcium signaling pathway) can be proof for using calcineurin inhibitors in KD
Susceptibility genes for cardiovascular disease in KD (8, 12, 17–22)	*TIAM1*, *NEBL*, *PLCB4/PLCB1, TUBA3C*, SLC8A1, *PELI1*, *KCNN2, TIFAB*, and *AGT*	
Susceptibility genes for intravenous immunoglobulin (IVIG) resistance in KD (23, 24)	*BCL2L11* and *SAMD9L*	BCL2L11 and SAMD9L: prediction of IVIG resistance
Behçet's Syndrome (BS)		
Susceptibility genes for BS (25–34)	*HLA-B51, STAT4, IL10, GIMAP, IL23R-IL12RB2, CCR1, ERAP1, KLRC4, FUT2, IL12A, NAALADL2, YIPF7, CPVL, UBAC2, LOC100129342, UBASH3B,* and *KIAA1529*	Inhibition of IL-12/IL-23 pathway activation may be a treatment target
Antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV)		
Related with anti-MPO AAV (35)	*HLA-DQ*	
Related with anti-PR3 AAV (35, 36)	*HLA-DP, SERPINA1,* and *PRTN3*	
Related with AAV (36, 37)	PTPN22, *SEMA6A*	
Takayasu Arteritis (TAK)		
Susceptibility genes for TA (38–41)	*HLA-B/MICA, HLA-DQB1/HLA-DRB1, FCGR2A/FCGR3A, IL12B, RPS9/LILRB3, IL6, PTK2B, LILRA3/LILRB2, DUSP22, KLHL33, HSPA6/FCGR3A,* the intergenic locus on chromosome *chromosome21q.22, MICB,* and *HLA-B*52* (poor prognosis)	
Immunoglobulin A Vasculitis/Henoch-Schönlein Purpura (IgAV/HSP)		
Susceptibility locus for IgAV/HSP (42)	HLA-DRB1	
Giant cell arteritis (GCA)		
Susceptibilty genes for GCA (43)	HLA-DRB1*04, PLG, and P4HA2	

NAALADL2, *CD40, NFKBIL1, IGHV, EBF2, CACNB2, LTA*, and *LEF1*) (5–16) to the risk of cardiovascular disease in KD (*TIAM1*, *NEBL*, *PLCB4*/*PLCB1, TUBA3C*, *SLC8A1, PELI1*, *KCNN2, TIFAB*, and *AGT*) (8, 12, 17–22) and to the risk of intravenous immunoglobulin (IVIG) resistance (*BCL2L11* and *SAMD9L*) (23, 24). Involvement of the HLA region in susceptibility to KD has been controversial and has not been replicated across different ancestral groups.

Shimizu et al. (12) identified three functional SNPs in the solute carrier family 8, member 1 (*SLC8A1)* gene to be associated with susceptibility to KD in Japanese and European cohorts (meta analysis $p = 0.0001$). *SLC8A1* encodes NCX1 (a sodium/calcium exchanger) that functions as a bidirectional sodium/calcium channel. Patients homozygous for the risk allele (rs13017968) have higher rates of coronary artery abnormalities. Homozygosity for rs13017968 is associated with an increase in Ca^{2+} flux in EBV-transformed B cells of healthy individuals. The NCX1 protein expression was detected in the postmortem coronary artery tissue of a young KD patient. Another study by Onouchi et al. (48) found a coding SNP (rs3741596) in the ORAI Calcium Release-Activated Calcium Modulator 1 (*ORAI1*) gene to be significantly associated with KD in Japanese patients (meta analysis $p = 0.00041$). Interestingly, frequency of the risk allele is more than 20 times higher in Japanese compared to Europeans, which may account for higher prevalence of KD in the Japanese population. Together, these genetic and functional data provide evidence for the role of Ca^{2+}-mediated signaling pathways in the pathogenesis of KD and for the use of calcineurin inhibitors (49).

Lv et al. (46) used statistically significant candidate variants from multiple GWAS and other gene association studies for pathways analysis. This investigation showed that KD susceptibility genes are enriched in functional networks for calcium ion homeostasis and immune responses and highlighted the role of nuclear transcription factor of activated T cells (NF-AT) and nuclear factor (NF) kappa light chain enhancer of activated B cells (NF-κB) in the pathogenesis of KD.

Another indication from GWAS for the use of new therapies in KD has come from the study by Chang et al. (44). The promoter variant, rs2736340, in the B lymphoid tyrosine kinase (*BLK*) gene was significantly associated with susceptibility to KD in multiple Asian populations [odds ratio (OR) = 1.498, 1.354–1.657; $p = 4.74 \times 10(-31)$]. The transformed and primary B cells with the risk allele express significantly lower levels of BLK and have reduced signaling downstream of B cell receptors. These data suggest a role for humoral immunity in the pathogenesis of the acute stage of KD (44). Although B cells and autoantibodies have been found in blood samples of KD patients, their role, whether they are causal or bystanders of an activated immune system or specific to an infectious agent in the etiology of KD, is currently unknown (50).

Standard treatment for KD consists of a single infusion of high-dose IVIGs and high-dose aspirin (until the fever has

resolved); however, the mechanism of action remains elusive. Because IVIG therapy is the mainstay of treatment for KD, there has been interest to study the effect of SNPs and copy number variants (CNVs) on the function of receptors for IgG, the Fc-gamma receptors (FcγRs). There are five genes encoding the low-affinity FcγRs (*FCGR2A, FCGR2B, FCGR2C, FCGR3A*, and *FCGR3B)* that are clustered in the *FCGR2/3* locus. Recently, Nagelkerke et al. published a study that evaluated the previously reported genetic association with the rs1801274 variant in this gene locus. The *FCGR2A* coding SNP rs1801274 has been validated in a number of KD ancestry cohorts and was shown to cause a difference in the ability of FcγRIIa to bind the human IgG2 immunoglobulin isotype. This new study defined haplotype blocks (set of closely linked alleles/markers that are inherited together) across the *FCGR2/3* gene locus in over 4,000 individuals from different populations. The FCGR2C-ORF haplotype has stronger susceptibility to KD in the European cohort than the previously reported risk allele rs1801274. However, the FCGR2C-ORF haplotype is extremely rare in Asians and thus cannot explain the high prevalence of KD in Asian populations. This research points out the importance of proper interpretation of genetic association studies in the context of patients' ancestry (51).

Kuo et al. developed a genetic model to predict IVIG resistance in KD. They identified 11 candidate SNPs in *ADTRP, KLF6, EX0C2, ZNF438-ZEB1,* and *MIR548A3-ZPLD1* genes to calculate a weighted genetic risk score (wGRS) in 126 IVIG responders and 24 IVIG non-responders (52). Patients with IVIG resistance had higher wGRS scores than individuals who were IVIG responsive; however, the wide confident intervals (CIs) for the OR suggest that these findings should be replicated in larger cohorts of patients.

Kim et al. (24) recently identified a new gene locus, *SAMD9L*, that is associated with IVIG resistance in Korean and Japanese KD cohorts. The meta-analysis of IVIG non-responders ($n = 317$) and IVIG responders ($n = 1221$) showed the association of the common intronic variant (rs28662; MAF = 13% in gnomAD) with IVIG resistance [(OR) = 4.30, $p = 5.3 \times 10^{-6}$]. This SNP was predicted to change the motif for transcription factor binding site and was also associated with differential expression of the *SAMD9L* gene in lymphoblastoid cell lines. Previously, variants in *SAMD9L* have been associated with susceptibility for systemic lupus erythematosus, systemic sclerosis, and other immune-related diseases, such as juvenile myelomonocytic leukemia, acute myeloid leukemia, myelodysplastic syndrome, hepatitis-B-related hepatocellular carcinoma, and ataxia-pancytopenia syndrome. However, the role of SAMD9L in immune signaling pathway is unclear (24).

Behçet's Disease

BD is a chronic multisystem inflammatory disorder characterized by recurrent oral/genital ulcers, ocular involvement, skin lesions/pathergy, presenting with remissions and exacerbations. The disease is common in populations living along the ancient Silk Road: Turkish, Iranian, Chinese, Korean, and Japanese. Classic BD is a complex disease with a strong genetic predisposition. Although the etiology of BD remains unclear, recent immunogenetic findings from GWAS provide clues to its pathogenesis (53, 54).

*HLA-B*51* remains the most significant genetic susceptibility factor for BD in multiple populations; however, it accounts for <20% of the genetic risk (55, 56). The ongoing controversy is about whether the disease association with *HLA-B*51* is attributed to a role of this variant itself or is due to its linkage disequilibrium (LD) with another variant in the region. There are also other BD disease-associated amino acids variants in the HLA-B locus that are thought to reside within the antigen-binding grove of the molecule (57). The association between specific MHC class I alleles and certain disease manifestations have been reported (58–61). A meta-analysis based on 72 studies in 74 study populations revealed moderate association of *HLA-B*51/B5* with male gender and high prevalence of eye and mucocutaneous involvement (60).

GWAS have identified a number of population-specific associations in the following genes: *IL23R-IL12RB2, IL10, TNFAIP3, STAT4, CCR1-CCR3, KLRC4, ERAP1*, and *FUT2*. Variants in some genes, including *KIAA1529, UBASH3B, GIMAP, IL12A, NAALADL2, YIPF7, CPVL, LOC100129342*, and *UBAC2,* did not reach genome-wide significance ($p < 5.0 \times 10^{-8}$) (25–34). Most of these alleles act as susceptibility factors, while some variants appear to be protective. BD-risk alleles in the *IL10* susceptibility gene locus were the first variants identified outside of the HLA region by GWAS in Turkish and Japanese populations (29, 30). The SNP rs1518111 was subsequently replicated in Middle Eastern Arab, Greek, British, Korean, Iranian, and Han-Chinese samples and is today the only variant consistently associated with susceptibility to BD across all ancestries (57).

More recent studies investigated whether there is evidence for interaction (epistasis) among BD susceptibility genes. The endoplasmic reticulum (ER)-associated protease (ERAP1) haplotype tagged by the coding variant, rs17482078 (p.Arg725Gln), has a large effect with an OR of 3.78 in patient carriers for *HLA-B*51*. Interestingly, the same variant rs17482078 is protective for psoriasis and ankylosing spondylitis (AS). ERAP1 is an enzyme that clips proteasome-processed peptides in the ER before these peptides are loaded on class I HLA molecules (56). The epistasis observed between *HLA-B*51* and the risk haplotype of *ERAP1* suggests that an HLA class I-peptide presentation contributes to BD. The difference between risk and protection conferred by the *ERAP1* haplotype may be explained by altered binding affinities among specific peptides, trimmed or not trimmed by the ERAP1 variant, for different HLA class I molecules (62).

Dense genotyping of immune-related loci extended the list of susceptibility genes to include risk alleles in *IL1A-IL1B, IRF8, CEBPB-PTPN1*, and *ADO-EGR2* genes in the Turkish set of 1,900 cases and 1,779 controls. This study implicated the innate immune response to microbial exposure in susceptibility to BD (63). Bakir-Gungor et al. reported new BD-associated common variants in pathways, including focal adhesion, complement and coagulation cascades, mitogen-activated protein kinases (MAPK) signaling, proteasome pathways, extracellular matrix

(ECM)–receptor interaction, and transforming growth factor-β (TGFβ) signaling both in Japanese and Turkish populations (64).

Targeted sequencing of candidate immunological genes has revealed the contribution of rare non-synonymous variants of *NOD2, TLR4*, *IL23,* and *MEFV* in the BD pathogenesis. In particular, heterozygosity for a common high-penetrance familial Mediterranean fever (FMF)-associated mutation p.Met694Val is significantly associated with BD risk in the Turkish population, although not in Japanese patients due to its absence in the Asian populations (65). *MEFV* encodes pyrin, which regulates caspase-1-mediated interleukin (IL)-1β production.

Nakano et al. investigated the functional effect of GWAS-identified BD risk alleles in *IL-10* and *CCR1*. They observed a differential protein expression of IL10 and CCR1 in M1 vs. M2 macrophages (Mϕ) derived from BD patients. BD skin lesions showed predominance of M1 Mϕ. This study suggests that targeting pathways and genes that play a role in the Mϕ polarization should be explored in the treatment of BD (66).

Taken together, GWAS have implicated a number of genes of the innate and adaptive immunity with increased susceptibility to BD. Whether inhibition of these molecular pathways will be effective in the treatment of BD remains to be seen.

ANCA-Associated Vasculitis (AAV)

AAV is a heterogeneous group of disorders characterized by necrotizing inflammation of small vessels and by the presence of ANCA. The two most common ANCAs are the autoantibodies that target the proteins: myeloperoxidase (MPO) and proteinase 3 (PR3). AAV includes the clinical syndromes of microscopic polyangiitis (MPA), granulomatosis with polyangiitis (GPA), and eosinophilic granulomatosis with polyangiitis (EGPA). ANCA directed against PR3 are preferentially associated with GPA, and anti-MPO is associated mainly with MPA and EGPA. Although the role of ANCA in the pathogenesis of AAV is appreciated, the mechanism for ANCA-mediated development of AAV is not clear. Genetic predisposition and exposure to environmental factors, such as drugs and infectious agents, are possible triggers of excessive activation of neutrophils and injury to small blood vessels. The clinical signs vary and may affect several organs, such as the kidney, intestine, lung, and skin.

Currently, three GWAS have been performed in AAV. These studies identified risk alleles in *HLA-DQ, HLA-DP, HLA-DR, SERPINA1, PTPN22, PRTN3*, and *SEMA6A* genes as contributing to AAV (35–37). A meta-analysis of 140 reported genetic variants associated with AAV distinguished the most significant 33 alleles in 15 genes (67). Twenty of these 33 genetic variants were present in the HLA region, suggesting an important role for the antigen presentation in the disease pathogenesis. Two GWAS showed that the SNP association signal in the HLA region was fully accounted for by *HLA-DPB1*04* allele (35, 37).

Lyons et al. established that MPA and GPA are two distinct diseases based on their genetic profile (35). Anti-MPO positive AAV (MPA) is strongly associated with the HLA-DQ, whereas anti-PR3 AAV (GPA) is associated with the HLA-DP region and the genes encoding α(1)-antitrypsin (*SERPINA1*) and serine proteinase 3 (*PRTN3*) (35, 68). SERPINA1 is a major inhibitor of PR3, and lower levels of α (1)-antitrypsin can lead to higher levels of circulating PR3 and higher production of anti-PR3 autoantibodies. Population difference in HLA-class II genotype frequency plays a role in the epidemiology of this disease. Among AAVs, GPA and PR3-AAV are prevalent in European populations, whereas MPA and MPO-AAV are predominant in the Japanese population. In the North American population, the *HLA- DPB1*04* has the strongest association with GPA, whereas in the Japanese population, *HLA- DRB1*09:01* is strongly associated with MPA (68–70).

Other notable variants outside the HLA region include alleles in protein tyrosine phosphatase non-receptor type 2 (*PTPN22*) gene, which negatively regulates the production of an immunosuppressive cytokine interleukin (IL-10) (71). The gain-of-function risk allele, p.Arg260Trp, is linked to GPA-AAV in addition to a number of other autoimmune diseases (72, 73). A non-coding SNP in the vicinity of *SEMA6A* is linked to GPA in the GWAS study of Caucasian cohort of 528 patients and at a genome-wide signficance ($p = 2.09 \times 10^{-8}$). The *SEMA6A* gene codes for semaphorin 6A and is highly expressed in dendritic cells; however, its function is unclear (37).

Lower and higher copy number variants in the *FCGR3B* gene and a higher *DEFB4* gene copy number have been associated with risk for AAV (74–76). A possible relationship between the *FCGR3B* copy number and susceptibility to AAV needs to be clarified experimentally (77).

Lee et al. performed gene-ontology (GO) enrichment and protein–protein interaction (PPI) networks analysis of significant risk alleles to identify the key pathophysiological pathways in the pathogenesis of AAV. The most significant pathways include the processing of antigens *via* HLA class II, T-cell receptor signaling, and interferon (IFN)-γ mediated signaling pathways (78).

Takayasu Arteritis (TAK)

TAK is a large-vessel vasculitis that predominantly affects young women and can cause organ-threatening ischemia, such as pulseless limbs, aortic regurgitation, pulmonary infarct, and kidney failure by occluding the aorta and/or its branches. TAK is seen all over the world, but Eastern Asia, especially Japan, has the highest incidence. The etiology of TAK is elusive, but genetic predisposition is likely. Corticosteroids, anti-tumor necrosis factor (TNF)-α, anti-IL-6, and abatacept are used for induction of remission and with aim to prevent relapse.

GWAS have shown that *HLA-B/MICA, HLA-DQB1/HLA-DRB1, FCGR2A/FCGR3A, IL12B, RPS9/LILRB3*, *IL6, PTK2B, LILRA3/LILRB2, DUSP22, KLHL33,* and *HSPA6/FCGR3A* and the intergenic locus on chromosomes *21q.22, MICB,* and *HLA-B*52* are susceptibility gene loci in TAK (38–41). Risk alleles in *HLA-B/MICA* and *HLA-DQB1/HLA-DRB1* were identified in Turkish and European cohorts of patients. Other notable associations are in *PSMG1, IL12*, and *IL23* gene loci but not at the level of genome-wide significance (39). *HLA-B*52* allele is related to poor prognosis in TAK (79, 80). Risk alleles in the *RPS9/LILRB3* locus and *IL6* may have immunoregulatory effects (38). The disease risk variant in *RPS9/LILRB3*, rs11666543 ($p = 2.34 \times 10^{-8}$), correlates with reduced mRNA expression of the inhibitory leukocyte immunoglobulin-like receptor gene *LILRB3*. One study proposed that risk alleles in the *IL6* gene

and serum level of IL-6 can be used to stratify patients for anti-IL6 treatment (81). Another risk allele, rs6871626, in *IL12B* is shown to influence the expression of *IL-12p70* and *IL-12p40* genes, resulting in enhanced IL-12 signaling that might further contribute to the pathophysiology of TAK (82). Terao et al. showed that interaction of *HLA-B*52* and *LILRB1* may be responsible for activation of natural killer (NK) cells by a way of suppression of inhibitory pathways (41).

FCGR2A/FCGR3A is a common susceptibility gene locus for TAK and KD. The Fc-gamma receptors (FcγRs) are responsible for interactive relationship between adaptive and innate immune systems. All immune cells possess these receptors. FcγRs can be classified into one high-affinity receptor (FcγRI) and five low-affinity FcγRs (the different isoforms of FcγRII and FcγRIII) (83). Genetic variants of these receptors have been associated with susceptibility to autoimmune, autoinflammatory, and infectious diseases and response to immunotherapy in cancer patients (6, 84–91). Nagelkerke et al. (51) showed that activation of IgG receptors on monocytes and neutrophils plays a role in the pathophysiology of KD. Inhibition of this activation may be a targeted treatment for patients who carry the *FCGR2A/FCGR3A* risk alleles.

Immunoglobulin A Vasculitis/Henoch-Schönlein Purpura (IgAV/HSP)

IgAV/HSP is a small-vessel vasculitis of the skin, kidney, gastrointestinal tract, and joints. IgAV/HSP has typically a good prognosis because of the self-limiting disease course; however, renal and gastrointestinal involvement can cause morbidity. IgAV/HSP is the most common childhood vasculitis. Diagnosis of IgAV/HSP is based on clinical features; however, skin and kidney biopsy are useful for diagnosis in selected cases.

The first GWAS of IgAV/HSP revealed that the HLA class II region (mainly *HLA-DRB1*01* allele) in the European population is associated with the risk for IgAV/HSP, although this finding did not reach genome-wide significance (42). A meta-analysis confirmed that genetic susceptibility to IgAV/HSP is associated with *HLA-DRB1*01* and *HLA-DRB1*07* variants and suggested that IgAV may be an HLA class II disease (92). Immunoglobulin A vasculitis with nephritis (IgAVN) and IgA nephropathy (IgAN; also known as Berger's disease) are considered related diseases. The chromosome 1q32 locus that contains the complement factor H (CFH) was identified as an IgAN-susceptible locus. Jia et al. showed the contribution of rs6677604 allele in CFH to the regulation of the complement activation in both IgAN and IgAVN (93).

Giant Cell Arteritis (GCA)

GCA is a systemic vasculitis characterized by granulomatous inflammation of medium- and large-sized vessels and with particular predilection for the aorta and its extracranial branches.

GCA is the most common vasculitis in the elderly (mostly women) population from western countries (94). GCA can cause headaches, visual loss, jaw claudication, painful scalp, impairment of limb arteries, systemic inflammation, and polymyalgia rheumatica. GCA is a potentially devastating disease, with up to 25% of patients developing aneurysmal disease and stroke. First-line treatment of GCA is a high-dose glucocorticoid (GC), and it should be maintained until symptoms are resolved.

GC resistance and adverse effects are main concerns for treatment of patients with GCA (95). Other agents, such as methotrexate and TNF inhibitors, have been used with limited or no evidence of benefit. Recent studies implicated important roles for T_H1- and T_H17-driven inflammatory cascades. Results from new therapies with abatacept and ustekinumab have also been promising.

In 2017, the first GWAS in GCA identified the *HLA-DRB1∗04* as the strongest risk allele (43). This finding implies that GCA is a predominantly HLA class II disease and is distinct from the other large-vessel vasculitis, TAK, which is considered an HLA class I disease. Carmona et al. (43) reported susceptibility alleles in plasminogen (*PLG*) and prolyl 4-hydroxylase subunit alpha 2 (*P4HA2)* genes in 10 different populations of European ancestry at a genome-wide level of significance (rs4252134, $p = 1.23 \times 10^{-10}$, OR = 1.28; and rs128738, $p = 4.60 \times 10^{-9}$, OR = 1.32) (43). Both *PLG* and *P4HA2* play a role in the regulation of angiogenesis and vascular remodeling. The plasminogen system is known to regulate several pathways possibly related to the GCA pathogenesis, such as lymphocyte recruitment and production of TNF-α and IL-6 cytokines (96, 97). The *P4HA2* gene is a hypoxia-responsive gene with a role in collagen biosynthesis and composition of ECM. Hypoxia-inducible factor-1 (HIF-1) regulates the expression of *P4HA2* and other genes, such as *VEGF, IL6,* and *MMP9* (98).

EPIGENETICS AND EPIGENOME-WIDE ASSOCIATION STUDIES (EWAS) IN PRIMARY SYSTEMIC VASCULITIDES

Epigenetic mechanisms including DNA methylation, histone modifications, and non-coding RNAs regulate gene expression, cellular development, differentiation, and activity (99). Epigenomic alterations are not the result of genetic mutations, and they are reversible; however, they can act as integrators of genetic and environmental disease risk factors. Recent work has suggested that epigenome modifications, for example, histone modifications and micro RNA (miRNA) expression, may be used as a biomarker for disease activity and for monitoring disease progression (100). EWAS aim to identify epigenetic variations associated with disease, for example, differentially methylated CpG sites, by performing a hypothesis-free testing across the whole genome (101). Thus far, EWAS have mainly investigated DNA methylation and its association with human diseases. In general, hypermethylation of promoters typically acts to repress gene transcription.

Kawasaki Disease

FCGR2A (IgG receptor gene) encodes the low-affinity immunoglobulin gamma Fc region receptor II-a protein that is expressed on the surface of macrophages, neutrophils,

monocytes, and dendritic cells. This protein leads to increased phagocytosis and production of inflammatory mediators. Genetic variants in *FCGR2A* gene have been linked to a genetic risk for KD (100, 102). The first study to examine the DNA methylation array in KD showed that CpG sites within the promoter region of *FCGR2A* were hypomethylated in whole blood cells from KD patients compared with controls, and especially in patients resistant to IVIG treatment (103). Li et al. demonstrated that DNA methylation patterns changed after IVIG treatment in KD, including reversal of the disease-associated hypomethylation in *FCGR2A* (104). Furthermore, interaction of *FCGR2A* and Toll-like receptors (TLR) can induce a proinflammatory response (105, 106). DNA hypomethylation on Toll-like receptors (TLRs) was detected in KD patients, and the methylation level of the TLRs genes was rescued 3 weeks post-IVIG therapy (107).

FoxP3 acts as both transcriptional activator and repressor in regulatory T cells (Tregs), and *FoxP3*-dependent transcriptional program is epigenetically controlled by histone modifications at its binding site (108). *FoxP3* binds to ∼700 genes and a number of miRNAs, including *miRNA-155*, which upregulation likely contributes to proliferative potential of T_R cells. *FoxP3* expression in Tregs of patients with KD is affected by *miR-155* and *miR-31*. In patients with acute KD, decrease in $FoxP3^+$ Treg might be associated with reduced expression of miR-155 and overexpression of *miR-31*. These effects were partially reversed following the IVIG treatment (109).

Huang et al. (110) showed that the expression of a neutrophil surface molecule, CD177, is higher during acute stage of KD and correlates with epigenetic hypomethylation. They observed that the *CD177* mRNA level diminished sharply after IVIG treatment and were higher in patients with atypical presentation or KD or IVIG-resistant patients.

Chang et al. (111) recently published that gene expression of *PHLPP1* and *MAPK14* is significantly high in KD and is influenced by methylation. They proposed that hypomethylation status and upregulated expressions of *MAPK14* and *PHLPP1* could be candidate biomarkers for the diagnosis of KD.

Hepcidin, encoded by the *HAMP* gene, has a main role in the pathogenesis of inflammation-associated anemia. Hepcidin levels are high in KD patients, and they decrease after IVIG treatment. Huang et al. demonstrated that KD patients had a considerably higher epigenetic hypomethylation of HAMP promoter than controls, which was reversed following therapy with IVIG. They proposed that HAMP promoter hypomethylation and increased hepcidin levels may serve as a biomarker of KD (112). Huang et al. (113) performed the first genome-wide DNA methylation and gene expression assays in blood samples of KD patients. They found that specific CpG markers were hypomethylated in samples from patients with active disease compared to controls and hypermethylated in samples from patients in convalescent phase compared with samples from patients in acute phase. In particular, methylation changes of CpG markers correlated with the expression S100A genes. S100A8 and S100A9 are inflammatory biomarkers that are usually highly expressed in acute and chronic inflammation. Using *in vitro* model, they showed that S100A proteins enhanced leukocyte transendothelial migration of neutrophils. In summary, this study implicates the role of S100A proteins in the pathogenesis of KD (113).

Behçet's Disease

The first EWAS in BD showed that CD4+ T cells and monocytes extracted from the peripheral blood of BD patients were hypomethylated in genes associated with cytoskeletal remodeling, cell migration, and tissue invasion, such as *RAC1, RGS14,* and *FSCN2*. Some methylation deficiencies return to normal level during disease remission. These findings suggest that epigenetic modifications of cytoskeleton-related genes are important in the pathogenesis of BD (114). Hypermethylation of the IL-4, *TGF-β*, and GATA binding protein 3 (*GATA3*) in CD4+ cells was noted in patients with active BD, but this may not be disease-specific (115, 116). Conversely, treatment with corticosteroids and cyclosporine (CsA) has beneficial effects by decreasing the methylation level of *TGF-β* and *GATA3* (115). A systematic analysis of miRNA expression profiles and pathway analyses in BD identified a specific miRNA signatures in active disease that regulated the *IFNγ*, *TNF*, and *VEGF-VEGFR* signaling cascades (117). Woo et al. (118) observed differential expressions of miRNAs miR-638 and miR-4488 to be associated with elevated production of IL-6. Activation of the Notch pathway in active BD disease and its association with decreased expression of mir-23 and higher Th17 response have been observed (119). Two functional variants, rs2910164 (miR-146a) and rs11614913 (miR-196a2; Ets-1), confer the risk for BD in Han-Chinese by regulating production of proinflammatory cytokines (120, 121).

The differentially expressed genes identified by EWAS might be good candidate biomarkers for monitoring the disease activity and might represent promising candidates for design of novel therapeutic strategies in BD.

ANCA-Associated Vasculitis

Normally, the expression of *MPO* and *PRTN3* genes (encoding MPO and PR3 autoantigenes) in neutrophils occurs only in the early stages of cell maturation; however, *MPO* and *PRTN3* are found continuously expressed in neutrophils and monocytes of patients with AAV (122). This deregulation in gene expression is postulated to be related to epigenetic mechanisms (123–125). Jones et al. (124) showed that the gene-specific DNA methylation changes may regulate autoantigen expression, and they correlate with disease activity in AAV. In remission, DNA methylation is generally increased. Patients with active disease had hypomethylation and increased expression of *MPO* and *PRTN3*. The findings suggest that the reactivation of once-silenced genes can lead to increased antigen availability. Patients with decreased DNA methylation at the *PRTN3* promoter have a significantly greater risk of relapse. Therefore, fluctuations in the DNA methylation of the *PRTN3* promoter may be associated with stable remission. Methylation status may prove to be a potential biomarker for prognosis in AVV patients (124).

Immunoglobulin A Vasculitis/Henoch-Schönlein Purpura

Luo et al. (126) observed that global histone H3 acetylation and H3K4 methylation are increased in peripheral blood mononuclear cells (PBMCs) isolated from IgAV patients. These epigenetic modifications had positive correlations with disease activity and were more common in IgAV patients with renal involvement compared with IgAV patients without renal involvement and healthy controls. They observed increased mRNA expression of *GATA3, IL-4, IL-6*, and *IL-13* genes in IgAV patients. Serum protein levels of IL-4, IL-6, and IL-13 were significantly increased in HSP with kidney damage patients compared to healthy controls. Abnormal histone modifications of transcription factors such as GATA3 may lead to the Th1/Th2 cytokine imbalance in HSP and other immune diseases (126).

Giant Cell Arteritis

Only two epigenetic studies evaluated DNA methylation status and miRNA expression in temporal artery biopsies (TABs) of patients with GCA (127, 128). Coit et al. (127) identified 1,555 hypomethylated CpG sites in 853 genes by comparing 12 patients and 12 healthy age-, gender-, and ancrestry-matched controls. DNA was extracted from the affected temporal artery tissues in patients with GCA and from histologically confirmed normal arteries in controls.

Most of these genes have roles in T-cell activation and differentiation pathways, especially Th1 and Th17 cells. *TNF, LTA, LTB, CCR7, RUNX3, CD6, CD40LG, IL2, IL6, NLRP1, IL1B, IL18, IL21, IL23R*, and *IFNG* were proinflammatory hypomethylated genes that were found in this study. Interestingly, the significant hypomethylation of genes in the calcineurin (CaN)/NFAT pathway was shown within the temporal artery of GCA patients.

Increased activity in NFAT is one of the key factors in production of proinflammatory cytokines. This study provided evidence for use of NFAT inhibitors (e.g., dipyridamole) in the treatment of GCA (127, 129).

Croci et al. showed that miR-146b-5p,−146a,−21,−150,−155, and−299-5p are expressed at higher levels in affected vessels of TAB-positive GCA patients compared to non-inflamed TABs from GCA patients and to non-inflamed TABs from non-GCA patients. Interestingly, these miRNAs were mainly deregulated at the tissue level, whereas no difference in their expression was observed in peripheral blood mononuclear and polymorphonuclear cells from these three groups of patients and controls. Especially, miR-146b-5p expression was the most promising diagnostic biomarker for the presence of inflammation in TABs of GCA patients and the follow up of the disease activity. Further research is necessary to optimize their use in medical practice (128).

EXTRACELLULAR VESICLES IN PRIMARY SYSTEMIC VASCULITIDES

EVs were defined in 1967 as "dust" from platelets (130). More recently, it has been shown that EVs are membrane vesicles that are released by almost all eukaryotic cells during cell activation and programmed cell death (131–134). Heterogeneity of EVs is essential. They are classified into three groups with regard to biological features and their size: exosomes, microvesicles (MVs), and apoptotic bodies (132, 133, 135, 136). EVs are responsible for immune regulation, cell-to-cell interaction, and signal transmission by transporting bioactive molecules including proteins and lipids, DNA, and various RNAs, such as mRNAs, small-interfering RNAs (siRNAs), microRNAs (miRNAs), and long non-coding RNAs (lncRNAs) and other molecules produced by cells (132, 137–139). EVs can be detected in many organs, tissues, and all body fluids, such as urine, blood, and synovial fluid at low levels in physiological conditions (140, 141). The increased levels of EVs are noted in cardiovascular disease, cancer, and pathological conditions that are associated with vasculitis: inflammation, autoimmunity, endothelial damage, angiogenesis, procoagulation, and intimal hyperplasia (131–133, 136, 140–142). EVs are known to be released from injured endothelial cells and are found increased in many diseases associated with endothelial dysfunction. It is thought that EVs from platelets, lymphocytes, and monocyte/macrophages contribute to the pathogenesis of systemic vasculitis (143–170) (**Table 3**).

In 2004, Brogan et al. (143) reported that the endothelial microparticles expressing E-selectin-positive or CD105 and the platelet MVs expressing CD42a were significantly increased in patients with active vasculitis compared to controls or children with inactive vasculitis or other febrile illnesses. They found that endothelial microparticle levels correlate with the Birmingham Vasculitis Activity Score (BVAS) and acute-phase reactant levels (143).

Activation of the contact system has proinflammatory and vasoactive properties. The contact system triggers the kallikrein-kinin cascade releasing bradykinin from high-molecular-weight kininogen. Bradykinin concentrations were higher in the patients' plasma than in plasma from controls, and kinins were present in lesional biopsy tissues of kidney and skin of children with PSVs (173, 174). The kinin B1 receptor is induced by inflammatory stimuli, particularly in response to the cytokine IL-1β, and plays a major role in neutrophil recruitment. Kahn et al. (151) found that leukocyte-derived MVs transfer functional kinin B1-receptors and in this way may further promote kinin-mediated inflammation in vasculitis. Mossberg et al. (156) showed that patients with acute vasculitis had significantly higher levels of circulating endothelial MVs and more MVs carrying B1-receptors. These MVs can induce the inflammation by increasing neutrophil chemotaxis, whereas addition of a C1-inhibitor decreased the release of B1R+MVs. Kinin-B1 receptor activation of CXCL5 can further amplify neutrophil chemotaxis and chronic inflammation (175). Exploring inhibition of the kallikrein-kinin pathway is potentially a novel target for reducing neutrophil-mediated inflammation in PSVs.

Kawasaki Disease

Endothelial cell-derived MVs were detected in higher levels in children with KD (147–149, 163, 166) and HSP (144). These MVs may serve as biomarkers to predict coronary artery aneurysms in KD and to detect subclinical inflammation in HSP (144, 148).

TABLE 3 | The role of extracellular vesicles in vasculitis. This table is adapted from Wu et al. (133).

Author and references	Origin of EVs	Content of EVs	Related diseases and vessels	Possible pathogenic mechanism
Microvesicles				
Nakaoka et al. (147)	Endothelial cells, CD144	miR-145-5p, miR-320a	KD, medium vessels	Upregulation of proinflammatory cytokine
Raposo et al. (136)	Leukocytes CD45	B1-receptors	AAV, IgAV, small vessels	Kinin system
Raposo et al. (136)	Endothelial cells	B1-receptors	AAV, IgAV	Kinin system
Macey et al. (153)	Platelets CD42a/CD62P	–	BS	Inflammation
Dursun et al. (144)	Endothelial cells	–	IgAV/small vessels	Inflammation
Daniel et al. (167)	Neutrophils CD66b	–	AAV	Inflammation and procoagulation
Mendoza-Pinto et al. (171)	Neutrophils annexin	Tissue factor	AAV	Inflammation and thrombosis
Hong et al. (172)	Annexin V neutrophils	CD18/C, D11b, PR3, MPO	AAV	Inflammation and procoagulation
Huang et al. (161)	Annexin V	Tissue factor	AAV	Procoagulation
Mejia et al. (155)	Annexin V	–	BD	Procoagulation
Khan et al. (152)	Annexin V	Tissue factor	BD	Procoagulation
Eleftheriou et al. (145)	Annexin V, platelets CD41 endothelial cells CD62E	Tissue factor	MPA, GPA, PAN, KD, BS	Procoagulation
Martinez et al. (154)	Platelets CD6	–	BS	Procoagulation
Yahata et al. (170)	Platelets CD42b/CD42a	–	KD	Evaluation of platelets
Kim et al. (169)	Annexin V	–	KD	Evaluation of platelets
Hajj-Ali et al. (146)	Annexin endothelial cells CD105/CD144, platelets CD41, leukocytes CD18, neutrophils	–	GPA/small vessels	Platelets activation and endothelial damage
Tian et al. (166)	Annexin V V/CD62E/CD31	–	KD/medium vessels	Endothelial damage
Erdbruegger et al. (168)	Annexin V, endothelial cell CD105/CD6 2E	–	AAV/small vessels	Endothelial damage
Ding et al. (163)		–	KD/medium vessels	Endothelial dysfunction
Kumpers et al. (165)	Annexin V	–	CSS/small vessels	Endothelial damage
Clarke et al. (162)	Annexin V, endothelial cell	–	MPA, GPA, PAN, KD, BS	Endothelial damage
Guiducci et al. (160)	Platelets CD42, erythrocytes, T cells, endothelial cells	–	KD/medium vessels	Endothelial damage
Brogan et al. (143)	Annexin V, platelets CD42a/CD62	–	MPA, GPA, PAN, KD, BS	Endothelial activation
Tan et al. (149)	Endothelial cells CD31/CD146	–	KD/medium vessels	Endothelial damage
Shah et al. (148)	Endothelial cells CD105/CD62E	–	KD/medium vessels	Endothelial damage
Exosomes				
Jia et al. (150)	CD9/CD81/TS	miR-1246, miR44	KD/medium vessels	Diagnostic biomarker
Zhang et al. (159)	–	miR-328, miR-575, miR-134, miR-671- 5p	KD/medium vessels	Inflammation
Zhang et al. (158)	CD9/CD81/flotilin	38 different contents	KD/ medium vessels	Inflammation and procoagulation
Zhang et al. (157)	CD9/flotillin	69 different proteins	KD/medium vessels	Inflammation and procoagulation

The role of platelet activation dynamics in acute-phase KD patients was explored by assaying platelet-derived MVs (PDMVs). Prior to aspirin treatment, PDMV level was significantly higher in the acute-phase KD patients in comparison to patients with common febrile diseases. Guiducci et al. observed that MVs derived from platelets, endothelial cells, erythrocytes, and T cells are significantly elevated in plasma samples of patients with KD. Endothelial and T cells were the major source of MVs, and the levels were reduced by IVIG treatment (160). Platelet activation is important in the pathogenesis of KD, thus PDMVs may serve as a biomarker to evaluate the antiplatelet therapy response in KD (169, 170).

Measuring microRNAs (miR-4436b-5p, miR-1246, miR-671-5p, and miR-197-3p) in serum exosomes has been proposed as a diagnostic biomarker for the prediction of IVIG response in KD (149, 159). Serum exosomal microRNAs may have a

role in the pathogenesis of KD by regulating the expression of inflammatory genes (CX chemokine receptor types 1 and 2, IL-8, and its receptor) in mononuclear cells (159). Serum exosomes from patients with KD contain many proteins, such as apolipoprotein A-IV, insulin-like growth factor-binding protein complex, acid-labile subunit and complement C3, and their proteomic profile correlates with IVIG therapy (157). Zhang et al. performed proteomic analyses of serum exosomes from KD patients with coronary artery dilatation. They found 38 differentially expressed proteins, and the majority are involved in inflammatory and coagulation pathways (158). More recent proteomic analysis validated TN, APOA4, LRG1, and RBP4 proteins as differentially expressed in patients with coronary artery aneurysms (CAA) (176). These finding provide additional insights in the pathogenesis of CAA in patients with KD.

ANCA-Associated Vasculitis

Endothelium-derived MVs have been reported in active AAV patients (168). Plasma levels of MVs derived from platelet and neutrophil are high in acute-phase vasculitis (167). The neutrophil-derived MVs cause increased production of proinflammatory cytokines IL-6 and IL-8, increased expression of intercellular adhesion molecule-1, and production of reactive oxygen radicals by binding to endothelial cells in a CD18-dependent manner (172). Furthermore, endothelial MVs carry PR3 and MPO and may contribute to the extensive endothelial damage and inflammation seen in AAV (177, 178).

Thromboembolic disease complicating primary systemic vasculitis is associated with significant morbidity and mortality. The mechanisms of hypercoagulability in PSV remain poorly defined. Several studies attempted to identify risk factors of thrombosis to stratify patients who could benefit from prophylaxis with antiplatelet or anticoagulant agents. One study showed that stimulation with ANCAs causes C5a-primed neutrophils to release neutrophil tissue factor (TF)-expressing MVs and neutrophil extracellular traps (NETs) that might promote hypercoagulability in AAV (161). Eleftheriou at al. have demonstrated that thromboembolic disease in children with systemic vasculitis is linked to increased level of MVs-mediated thrombin (145).

Behçet's Disease

Thrombosis is common in BD patients, and there is a need for better assessment of risk factors. BD patients with a history of thrombosis have high serum levels of MVs expressing tissue factor (TF). The ratio of TF pathway inhibitor (TFPI)-positive MVs to TF-positive MVs was significantly lower in patients with thrombosis (152). In contrast, platelet-derived MVs and procoagulant MVs did not differentiate between BD patients with or without thrombosis (155). Macey et al. (153) showed that high-level platelet-derived CD62P+ MVs correlate with active disease in patients in a younger age group, whereas lower levels of MVs correlate with decreased disease activity in patients older than 50 years.

INSIGHTS FROM STUDIES OF MONOGENIC SYSTEMIC AUTOINFLAMMATORY DISEASES

Rare Mendelian diseases of systemic inflammation often present with severe-like phenotypes of polygenic diseases and can share underlying biochemical pathways with more common rheumatic diseases. Identification of patients with monogenic disease can point to genes and pathways that could be investigated in patients with polygenic disorders. For instance, polyarteritis nodosa (PAN) can present either as early-onset monogenic or late-onset polygenic disease. PAN is a systemic necrotizing vasculitis that predominantly affects medium-sized arteries, causing tissue ischemia and organ damage. The disease commonly affects the skin, gastrointestinal tract, and kidneys, and patients are at an increased risk of stroke. Most cases of PAN occur in the fourth or fifth decade, and men are more likely to be affected. A subset of patients with childhood-onset PAN are found to carry biallelic loss of function mutations in adenosine deaminase 2 gene. This disease is named deficiency of ADA2 (DADA2)(179, 180). Among many features of DADA2, subcortical ischemic strokes, hypertension, aneurysms, renal infarcts, and peripheral amputations have been reported in up to 50% of patients. DADA2 patients are highly responsive to treatment with TNF inhibitors (181). Although pathogenic variants in the *ADA2* gene may not account for a large number of sporadic adult-onset PAN patients, TNF inhibitors should be explored in the treatment of those patients as well (181).

Inflammasomopathies

Inflammasomopathies are rare monogenic autoinflammatory diseases caused by gain-of -function mutations in the multiprotein complexes termed the inflammasome. The inflammasome functions as cytosolic pathogen and danger-associated molecular patterns (PAMPs/DAMPs) recognition receptors (PRRs). The core of inflammasome is one of the nucleotide-binding domain leucine-rich repeat (NLR) proteins (NLRP1, NLRP3, AIM2, NLRC4) or pyrin. Upon stimulation, the inflammasome interacts with an inflammasome adaptor protein, apoptosis-associated speck-like protein with a caspase recruitment domain (ASC), or with pro-caspase 1 to form platform for caspase-1-mediated production of IL-1β and IL-18 cytokines. The best known inflammasomopathies are familial Mediterranean fever (FMF) and cryopyrinopathities (CAPS). FMF is caused by recessively inherited hypomorphic mutations in pyrin, whereas CAPS is linked to dominantly inherited gain-of-function mutations in NLRP3. The net effect of these pathogenic variants is increased production of IL-1β cytokine and systemic inflammation. FMF is a common disease in many Eastern Mediterranean countries where the frequency of pathogenic mutations is very high (182–205). Although vasculitis is not a primary feature of FMF, IgAV and PAN have been reported in about 3 and 1% of FMF patients, respectively (206). FMF-associated mutations predispose to the development of BD in the Turkish population (207–210). Small and medium vessel skin vasculitis and CNS vasculitis were

reported in other inflammasomopathies, including CAPS and other pyrin-mediated autoinflammatory diseases mevalonate kinase deficiency/hyperimmunoglobulin D (MKD/HIDS); pyogenic arthritis, pyoderma gangrenosum, and acne (PAPA); and pyoderma gangrenosum, acne, hidradenitis suppurativa (PASH) syndromes (211–221). NLRP3 inflammasome activation was demonstrated in two Kawasaki mouse models (222, 223). Underlying mechanisms of vasculitis in inflammasomopathies are unclear, although it is assumed that high systemic IL-1β production may cause endothelial cell inflammation and damage. Patients with BD may respond to colchicine and anti-IL1 therapies like patients with inflammasomopathies (224).

Relopathies

Relopathies are NF kappa-B (NF-κB)-mediated monogenic systemic autoinflammatory diseases driven by multiple cytokines including TNF and IL-6 (225). They result from pathogenic variants in proteins that regulate posttranslational ubiquitin modifications in the NF-κB pathway. Up to now, haploinsufficency of A20 (HA20) and OTULIN deficiency have been defined as relopathies (226). HA20 is a dominantly inherited disease caused by heterozygous loss-of-function mutations in *TNFAIP3*, which encodes the K-63 deubiquitinase protein A20 (227). Common variants in *TNFAIP3* have been linked to BD in the Chinese-Han population (228). Clinically, HA20 resembles early-onset BD. Two patients with HA20 were diagnosed with CNS vasculitis based on brain imaging and a frontal lobe punctate (229). OTULIN deficiency is caused by recessively inherited loss-of-function mutations in the enzyme that regulates linear deubiquitination. There are only few patients reported, and they present with early-onset severe systemic inflammation. One patient was described with vasculitis of small and medium vessels on skin biopsy (230). In most patients with HA20 and OTULIN deficiency, anti-cytokine therapies targeting TNF and IL-1 are efficient in suppressing the disease activity. Ubiquitin pathway has been implicated in the pathogenesis of BD by multiple association studies. The rs9517723 variant in the 3' region of ubiquitin-associated domain containing ubiquitin-associated domain containing 2 *(UBAC2)* gene is significantly associated with ocular and central nervous system (CNS) lesions under the recessive model (32, 231). *UBAC2* encodes a protein that plays a role in ubiquitination and proteasomal degradation. Homozygous risk allele (TT) of the rs9517723 correlates with increased *UBAC2* expression. BD has been also associated with other ubiquitination pathway-related genes, including ubiquitin associated and SH3 domain containing B (*UBASH3B*) and small ubiquitin-like modifier 4 (*SUMO4*) (232, 233).

Interferonopathies

Type I interferonopathies consist of Aicardi–Goutières syndrome (AGS), STING-associated vasculopathy with onset in infancy (SAVI), chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature (CANDLE) syndrome, and COPA syndrome (226). Intracerebral vasculitis is common in patients with AGS (234). High levels of IFN activities were demonstrated in the cerebrospinal fluid and serum of AGS patients (234). In SAVI, gain-of-function mutations in the *TMEM173* gene lead to a constitutively active STING protein, and a high expression of type I IFN-induced genes (235, 236). Clinically, the SAVI phenotype mimics the AAV with cutaneous rashes, interstitial lung disease, peripheral ulcerations/gangrene, and ANCA positivity in some patients (237). Patients with CANDLE have dysfunction in the protesome-mediated degradation pathway. Skin biopsies from CANDLE patients showed evidence for perivascular infiltration prominent for myeloid cells and leukocytoclasis but not for the typical vasculitis with fibrinoid necrosis of the vessel walls (238). JAK1/2 inhibition with baricitinib has been successful in treating clinical disease manifestations and in suppression of the IFN signature (239). The role of the IFN pathway in the pathogenesis of polygenic/complex vasculitides has been recently postulated by analysis of data from multiple GWAS (78).

Reports of vasculitis in other rare autoinflammatory diseases are scarce. One patient with the deficiency of IL-1 receptor antagonist (DIRA) had histopathological evidence of vasculitis in the connective tissue and cerebral vasculitis or vasculopathy was detected on magnetic resonance imaging (240). IL-1Ra-deficient mice frequently develop aortitis at the root of the aorta, which can be suppressed by a cross with TNF-α-deficient mice. Bone marrow transplantation of T cells from IL-1Ra$^{-/-}$ mice induced aortitis in recipient nu/nu mice or in irradiated wild-type recipient mice (241). This study demonstrates that IL-1Ra deficiency in T cells is responsible for the development of aortitis.

EXPERT COMMENTARY

i) The main limitations of current genetic datasets in PSVs are the lack of multiple meta-analyses of GWAS and inability to confirm functional significance of identified risk alleles. Validation of disease-associated variants in cohorts of patients of different ancestry would give more creditability to the reported findings; however, some genetic variants may not be present in all populations.
ii) As epigenetic modifications evolve and change over the life span, the data from EWAS will require validation in longitudinal cohort studies that are costly to maintain. Epigenomic investigations maybe accomplished in a shorter time period if they focus on studies in monozygotic twin pairs (MZ pairs) or if they would investigate differences in young vs. old MZ pairs. In addition, it would be interesting to explore cell-specific epigenomic modifications.
iii) Use of extracellular vesicles (MVs, PDMVs, EMVs) as potential biomarkers of disease activity in patients with primary systemic vasculitis is important for diagnostic and treatment monitoring. At present time, there are no standardized methods for the sampling process and detection of EVs.

5-YEAR FORWARD VIEW

Multicenter collaborations and studies in large cohorts of patients would increase the power of detection and confirmation of PSV disease-associated variants. These meta studies would also help

estimate a size effect of identified risk alleles, which is important for calculation of genetic risk score (GRS) and genetic counseling. Considering a significant decrease in the cost of whole exome and whole genome sequencing (WES/WGS), these new sequencing technologies should capture rare germ-line variants, structural variants, and mosaic mutations that may explain the missing heritability in these diseases. At this time, it is still unclear whether SNPs captured by GWAS are true disease-associated variants or in linkage disequilibrium with a risk allele. Performing WGS of patient cohorts would avoid a need for imputation studies. Effects of most common disease-associated variants on protein function are unclear, in particular for non-coding variants. Disease-associated common variants may have very small effect sizes but could lead to important and targetable pathways. Studies in cell culture systems and model organisms and experiments in primary patient cells are needed to answer this question.

CONCLUSION

Management of PSVs and other human diseases is beginning to be more directed with increased use of molecular data to improve diagnosis and to guide optimal treatment options. This new clinical paradigm has premise to apply knowledge of genomic, proteomic, and metabolic variants and epigenomic biomarkers to generate "omic" profiles. The translation into clinical practice can be achieved by integrating the "omic" information into a unique algorithm that will be able to make accurate diagnosis and to optimize therapeutic decisions to maximize benefit and minimize harm. Proper implementation of PM will require multidisciplinary teams of clinicians, bioinformaticians, geneticists, genetic counselors, and research scientists that can address multiple challenges in interpretation and integration of the multi-omics results. Along these lines, successful transition toward personalized care will also necessitate updating medical curriculums to facilitate training of a new generation of "omic"-literate physicians.

AUTHOR CONTRIBUTIONS

ED and ZA drafted the manuscript. MR and RB revised the manuscript and designed the tables. IA revised and finalized the manuscript.

ACKNOWLEDGMENTS

MR was the recipient of ARG-ITALIA Onlus (http://www.argitalia.it/) bursary for international research fellowship.

REFERENCES

1. Berman JJ. *Precision Medicine and the Reinvention of Human Disease*. London: Elsevier (2018). doi: 10.1016/B978-0-12-814393-3.00008-1
2. Jennette JC, Falk RJ, Bacon PA, Basu N, Cid MC, Ferrario F, et al. 2012 revised international chapel hill consensus conference nomenclature of vasculitides. *Arthr Rheumat*. (2013) 65:1–11. doi: 10.1002/art.37715
3. Institute. NNHGR. *Genome-Wide Association Studies Fact Sheet*. (2019). Available online at: https://www.genome.gov/about-genomics/fact-sheets/Genome-Wide-Association-Studies-Fact-Sheet (accessed May 01, 2019).
4. Gutierrez-Arcelus M, Rich SS, Raychaudhuri S. Autoimmune diseases - connecting risk alleles with molecular traits of the immune system. *Nat Rev Genet*. (2016) 17:160–74. doi: 10.1038/nrg.2015.33
5. Burgner D, Davila S, Breunis WB, Ng SB, Li Y, Bonnard C, et al. A genome-wide association study identifies novel and functionally related susceptibility Loci for Kawasaki disease. *PLoS Genet*. (2009) 5:e1000319. doi: 10.1371/journal.pgen.1000319
6. Khor CC, Davila S, Breunis WB, Lee YC, Shimizu C, Wright VJ, et al. Genome-wide association study identifies FCGR2A as a susceptibility locus for Kawasaki disease. *Nat Genet*. (2011) 43:1241–6. doi: 10.1038/ng.981
7. Kim JJ, Yun SW, Yu JJ, Yoon KL, Lee KY, Kil HR, et al. A genome-wide association analysis identifies NMNAT2 and HCP5 as susceptibility loci for Kawasaki disease. *J Hum Genet*. (2017) 62:1023–9. doi: 10.1038/jhg.2017.87
8. Kuo HC, Li SC, Guo MM, Huang YH, Yu HR, Huang FC, et al. Genome-wide association study identifies novel susceptibility genes associated with coronary artery aneurysm formation in Kawasaki disease. *PLoS ONE*. (2016) 11:e0154943. doi: 10.1371/journal.pone.0154943
9. Kwon YC, Kim JJ, Yun SW, Yu JJ, Yoon KL, Lee KY, et al. Male-specific association of the FCGR2A His167Arg polymorphism with Kawasaki disease. *PLoS ONE*. (2017) 12:e0184248. doi: 10.1371/journal.pone.0184248
10. Lee YC, Kuo HC, Chang JS, Chang LY, Huang LM, Chen MR, et al. Two new susceptibility loci for Kawasaki disease identified through genome-wide association analysis. *Nat Genet*. (2012) 44:522–5. doi: 10.1038/ng.2227
11. Onouchi Y, Ozaki K, Burns JC, Shimizu C, Terai M, Hamada H, et al. A genome-wide association study identifies three new risk loci for Kawasaki disease. *Nat Genet*. (2012) 44:517–21. doi: 10.1038/ng.2220
12. Shimizu C, Eleftherohorinou H, Wright VJ, Kim J, Alphonse MP, Perry JC, et al. Genetic variation in the SLC8A1 calcium signaling pathway is associated with susceptibility to Kawasaki disease and coronary artery abnormalities. *Circ Cardiovasc Genet*. (2016) 9:559–68. doi: 10.1161/CIRCGENETICS.116.001533
13. Tsai FJ, Lee YC, Chang JS, Huang LM, Huang FY, Chiu NC, et al. Identification of novel susceptibility Loci for Kawasaki disease in a Han chinese population by a genome-wide association study. *PLoS ONE*. (2011) 6:e16853. doi: 10.1371/journal.pone.0016853
14. Kim HJ, Yun SW, Yu JJ, Yoon KL, Lee KY, Kil HR, et al. Identification of LEF1 as a susceptibility locus for Kawasaki disease in patients younger than 6 months of age. *Genomics Inform*. (2018) 16:36–41. doi: 10.5808/GI.2018.16.2.36
15. Bae Y, Shin D, Nam J, Lee HR, Kim JS, Kim KY, et al. Variants in the gene EBF2 are associated with Kawasaki disease in a korean population. *Yonsei Med J*. (2018) 59:519–23. doi: 10.3349/ymj.2018.59.4.519
16. Oyamada J, Shimizu C, Kim J, Williams MR, Png E, Hibberd ML, et al. Bifid T waves on the ECG and genetic variation in calcium channel voltage-dependent beta 2 subunit gene (CACNB2) in acute Kawasaki disease. *Congenit Heart Dis*. (2019) 14:213–20. doi: 10.1111/chd.12696
17. Kim JJ, Hong YM, Sohn S, Jang GY, Ha KS, Yun SW, et al. A genome-wide association analysis reveals 1p31 and 2p13.3 as susceptibility loci for Kawasaki disease. *Hum Genet*. (2011) 129:487–95. doi: 10.1007/s00439-010-0937-x
18. Kim JJ, Park YM, Yoon D, Lee KY, Seob Song M, Doo Lee H, et al. Identification of KCNN2 as a susceptibility locus for coronary artery aneurysms in Kawasaki disease using genome-wide association analysis. *J Hum Genet*. (2013) 58:521–5. doi: 10.1038/jhg.2013.43
19. Lin MT, Hsu CL, Taiwan Pediatric CVG, Chen PL, Yang WS, Wang JK, et al. A genome-wide association analysis identifies novel susceptibility loci for coronary arterial lesions in patients with Kawasaki disease. *Transl Res*. (2013) 161:513–5. doi: 10.1016/j.trsl.2013.02.002

20. Lin YJ, Chang JS, Liu X, Tsang H, Chien WK, Chen JH, et al. Genetic variants in PLCB4/PLCB1 as susceptibility loci for coronary artery aneurysm formation in Kawasaki disease in Han Chinese in Taiwan. *Sci Rep*. (2015) 5:14762. doi: 10.1038/srep14762
21. Liu Y, Fu L, Pi L, Che D, Xu Y, Zheng H, et al. An angiotensinogen gene polymorphism (rs5050) is associated with the risk of coronary artery aneurysm in southern chinese children with Kawasaki disease. *Dis Markers*. (2019) 2019:2849695. doi: 10.1155/2019/2849695
22. Kwon YC, Kim JJ, Yu JJ, Yun SW, Yoon KL, Lee KY, et al. Identification of the TIFAB gene as a susceptibility locus for coronary artery aneurysm in patients with Kawasaki disease. *Pediatr Cardiol*. (2019) 40:483–8. doi: 10.1007/s00246-018-1992-7
23. Kwon YC, Kim JJ, Yun SW, Yu JJ, Yoon KL, Lee KY, et al. BCL2L11 is associated with Kawasaki disease in intravenous immunoglobulin responder patients. *Circ Genom Precis Med*. (2018) 11:e002020. doi: 10.1161/CIRCGEN.117.002020
24. Kim JJ, Yun SW, Yu JJ, Yoon KL, Lee KY, Kil HR, et al. Identification of SAMD9L as a susceptibility locus for intravenous immunoglobulin resistance in Kawasaki disease by genome-wide association analysis. *Pharmacogenomics J*. (2019). doi: 10.1038/s41397-019-0085-1. [Epub ahead of print].
25. Hou S, Yang Z, Du L, Jiang Z, Shu Q, Chen Y, et al. Identification of a susceptibility locus in STAT4 for Behcet's disease in Han Chinese in a genome-wide association study. *Arthritis Rheum*. (2012) 64:4104–13. doi: 10.1002/art.37708
26. Kappen JH, Medina-Gomez C, van Hagen PM, Stolk L, Estrada K, Rivadeneira F, et al. Genome-wide association study in an admixed case series reveals IL12A as a new candidate in Behcet disease. *PLoS ONE*. (2015) 10:e0119085. doi: 10.1371/journal.pone.0119085
27. Kim SW, Jung YS, Ahn JB, Shin ES, Jang HW, Lee HJ, et al. Identification of genetic susceptibility loci for intestinal Behcet's disease. *Sci Rep*. (2017) 7:39850. doi: 10.1038/srep39850
28. Kirino Y, Bertsias G, Ishigatsubo Y, Mizuki N, Tugal-Tutkun I, Seyahi E, et al. Genome-wide association analysis identifies new susceptibility loci for Behcet's disease and epistasis between HLA-B*51 and ERAP1. *Nat Genet*. (2013) 45:202–7. doi: 10.1038/ng.2520
29. Mizuki N, Meguro A, Ota M, Ohno S, Shiota T, Kawagoe T, et al. Genome-wide association studies identify IL23R-IL12RB2 and IL10 as Behcet's disease susceptibility loci. *Nat Genet*. (2010) 42:703–6. doi: 10.1038/ng.624
30. Remmers EF, Cosan F, Kirino Y, Ombrello MJ, Abaci N, Satorius C, et al. Genome-wide association study identifies variants in the MHC class I, IL10, and IL23R-IL12RB2 regions associated with Behcet's disease. *Nature genetics*. (2010) 42:698–702. doi: 10.1038/ng.625
31. Xavier JM, Shahram F, Sousa I, Davatchi F, Matos M, Abdollahi BS, et al. FUT2: filling the gap between genes and environment in Behcet's disease? *Ann Rheum Dis*. (2015) 74:618–24. doi: 10.1136/annrheumdis-2013-204475
32. Fei Y, Webb R, Cobb BL, Direskeneli H, Saruhan-Direskeneli G, Sawalha AH. Identification of novel genetic susceptibility loci for Behcet's disease using a genome-wide association study. *Arthritis Res Ther*. (2009) 11:R66. doi: 10.1186/ar2695
33. Lee YJ, Horie Y, Wallace GR, Choi YS, Park JA, Choi JY, et al. Genome-wide association study identifies GIMAP as a novel susceptibility locus for Behcet's disease. *Ann Rheum Dis*. (2013) 72:1510–6. doi: 10.1136/annrheumdis-2011-200288
34. Yu H, Zheng M, Zhang L, Li H, Zhu Y, Cheng L, et al. Identification of susceptibility SNPs in IL10 and IL23R-IL12RB2 for Behcet's disease in Han Chinese. *J Allergy Clin Immunol*. (2017) 139:621–7. doi: 10.1016/j.jaci.2016.05.024
35. Lyons PA, Rayner TF, Trivedi S, Holle JU, Watts RA, Jayne DR, et al. Genetically distinct subsets within ANCA-associated vasculitis. *N Engl J Med*. (2012) 367:214–23.
36. Merkel PA, Xie G, Monach PA, Ji X, Ciavatta DJ, Byun J, et al. Identification of functional and expression polymorphisms associated with risk for antineutrophil cytoplasmic autoantibody-associated vasculitis. *Arthritis Rheumatol*. (2017) 69:1054–66. doi: 10.1002/art.40034
37. Xie G, Roshandel D, Sherva R, Monach PA, Lu EY, Kung T, et al. Association of granulomatosis with polyangiitis (Wegener's) with HLA-DPB1*04 and SEMA6A gene variants: evidence from genome-wide analysis. *Arthritis Rheum*. (2013) 65:2457–68. doi: 10.1002/art.38036
38. Renauer PA, Saruhan-Direskeneli G, Coit P, Adler A, Aksu K, Keser G, et al. Identification of susceptibility loci in IL6, RPS9/LILRB3, and an intergenic locus on chromosome 21q22 in takayasu arteritis in a genome-wide association study. *Arthritis Rheumatol*. (2015) 67:1361–8. doi: 10.1002/art.39035
39. Saruhan-Direskeneli G, Hughes T, Aksu K, Keser G, Coit P, Aydin SZ, et al. Identification of multiple genetic susceptibility loci in Takayasu arteritis. *Am J Hum Genet*. (2013) 93:298–305. 10.1016/j.ajhg.2013.05.026
40. Terao C, Yoshifuji H, Kimura A, Matsumura T, Ohmura K, Takahashi M, et al. Two susceptibility loci to Takayasu arteritis reveal a synergistic role of the IL12B and HLA-B regions in a Japanese population. *Am J Hum Genet*. (2013) 93:289–97. doi: 10.1016/j.ajhg.2013.05.024
41. Terao C, Yoshifuji H, Matsumura T, Naruse TK, Ishii T, Nakaoka Y, et al. Genetic determinants and an epistasis of LILRA3 and HLA-B*52 in Takayasu arteritis. *Proc Natl Acad Sci USA*. (2018) 115:13045–50. doi: 10.1073/pnas.1808850115
42. Lopez-Mejias R, Carmona FD, Castaneda S, Genre F, Remuzgo-Martinez S, Sevilla-Perez B, et al. A genome-wide association study suggests the HLA Class II region as the major susceptibility locus for IgA vasculitis. *Sci Rep*. (2017) 7:5088. doi: 10.1038/s41598-017-03915-2
43. Carmona FD, Vaglio A, Mackie SL, Hernandez-Rodriguez J, Monach PA, Castaneda S, et al. A Genome-wide association study identifies risk alleles in plasminogen and P4HA2 associated with giant cell arteritis. *Am J Hum Genet*. (2017) 100:64–74. doi: 10.1016/j.ajhg.2016.11.013
44. Chang CJ, Kuo HC, Chang JS, Lee JK, Tsai FJ, Khor CC, et al. Replication and meta-analysis of GWAS identified susceptibility loci in Kawasaki disease confirm the importance of B lymphoid tyrosine kinase (BLK) in disease susceptibility. *PLoS ONE*. (2013) 8:e72037. doi: 10.1371/journal.pone.0072037
45. Kim JJ, Yun SW, Yu JJ, Yoon KL, Lee KY, Kil HR, et al. Common variants in the CRP promoter are associated with a high C-reactive protein level in Kawasaki disease. *Pediatr Cardiol*. (2015) 36:438–44. doi: 10.1007/s00246-014-1032-1
46. Lv YW, Wang J, Sun L, Zhang JM, Cao L, Ding YY, et al. Understanding the pathogenesis of Kawasaki disease by network and pathway analysis. *Comput Math Methods Med*. (2013) 2013:989307. doi: 10.1155/2013/989307
47. Rowley AH, Shulman ST. The Epidemiology and pathogenesis of Kawasaki disease. *Front Pediatr*. (2018) 6:374. doi: 10.3389/fped.2018.00374
48. Onouchi Y, Fukazawa R, Yamamura K, Suzuki H, Kakimoto N, Suenaga T, et al. Variations in ORAI1 gene associated with Kawasaki disease. *PLoS ONE*. (2016) 11:e0145486. doi: 10.1371/journal.pone.0145486
49. Hamada H, Suzuki H, Onouchi Y, Ebata R, Terai M, Fuse S, et al. Efficacy of primary treatment with immunoglobulin plus ciclosporin for prevention of coronary artery abnormalities in patients with Kawasaki disease predicted to be at increased risk of non-response to intravenous immunoglobulin (KAICA): a randomised controlled, open-label, blinded-endpoints, phase 3 trial. *Lancet*. (2019) 393:1128–37. doi: 10.1093/rheumatology/kez063.031
50. Lindquist ME, Hicar MD. B cells and antibodies in Kawasaki disease. *Int J Mol Sci*. (2019) 20:E1834. doi: 10.3390/ijms20081834
51. Nagelkerke SQ, Tacke CE, Breunis WB, Tanck MWT, Geissler J, Png E, et al. Extensive ethnic variation and linkage disequilibrium at the FCGR2/3 locus: different genetic associations revealed in Kawasaki disease. *Front Immunol*. (2019) 10:185. doi: 10.3389/fimmu.2019.00185
52. Kuo HC, Wong HS, Chang WP, Chen BK, Wu MS, Yang KD, et al. Prediction for intravenous immunoglobulin resistance by using weighted genetic risk score identified from genome-wide association study in Kawasaki disease. *Circ Cardiovasc Genet*. (2017) 10:e001625. doi: 10.1161/CIRCGENETICS.116.001625
53. Gul A. Genetics of Behcet's disease: lessons learned from genomewide association studies. *Curr Opin Rheumatol*. (2014) 26:56–63. doi: 10.1097/BOR.0000000000000003
54. Deng Y, Zhu W, Zhou X. Immune regulatory genes are major genetic factors to behcet disease: systematic review. *Open Rheumatol J*. (2018) 12:70–85. doi: 10.2174/1874312901812010070
55. Hatemi G, Karatemiz G, Yazici H. Behcet's disease: an MHC-I-opathy? *Clin Exp Rheumatol*. (2017) 35 Suppl 104:5.

56. Takeuchi M, Ombrello MJ, Kirino Y, Erer B, Tugal-Tutkun I, Seyahi E, et al. A single endoplasmic reticulum aminopeptidase-1 protein allotype is a strong risk factor for Behcet's disease in HLA-B*51 carriers. *Ann Rheum Dis*. (2016) 75:2208–11. doi: 10.1136/annrheumdis-2015-209059
57. Takeuchi M, Kastner DL, Remmers EF. The immunogenetics of Behcet's disease: A comprehensive review. *J Autoimmun*. (2015) 64:137–48. doi: 10.1016/j.jaut.2015.08.013
58. Chang HK, Kim JU, Cheon KS, Chung HR, Lee KW, Lee IH. HLA-B51 and its allelic types in association with Behcet's disease and recurrent aphthous stomatitis in Korea. *Clin Exp Rheumatol*. (2001) 19(5 Suppl. 24):S31–5.
59. Hamzaoui A, Houman MH, Massouadia M, Ben Salem T, Khanfir MS, Ben Ghorbel I, et al. Contribution of Hla-B51 in the susceptibility and specific clinical features of Behcet's disease in Tunisian patients. *Eur J Intern Med*. (2012) 23:347–9. doi: 10.1016/j.ejim.2011.12.011
60. Maldini C, Lavalley MP, Cheminant M, de Menthon M, Mahr A. Relationships of HLA-B51 or B5 genotype with Behcet's disease clinical characteristics: systematic review and meta-analyses of observational studies. *Rheumatology*. (2012) 51:887–900. doi: 10.1093/rheumatology/ker428
61. Wallace GR. HLA-B*51 the primary risk in Behcet disease. *Proc Natl Acad Sci USA*. (2014) 111:8706–7. doi: 10.1073/pnas.1407307111
62. Pepelyayeva Y, Amalfitano A. The role of ERAP1 in autoinflammation and autoimmunity. *Hum Immunol*. (2019) 80:302–9. doi: 10.1016/j.humimm.2019.02.013
63. Takeuchi M, Mizuki N, Meguro A, Ombrello MJ, Kirino Y, Satorius C, et al. Dense genotyping of immune-related loci implicates host responses to microbial exposure in Behcet's disease susceptibility. *Nat Genet*. (2017) 49:438–43. doi: 10.1038/ng.3786
64. Bakir-Gungor B, Remmers EF, Meguro A, Mizuki N, Kastner DL, Gul A, et al. Identification of possible pathogenic pathways in Behcet's disease using genome-wide association study data from two different populations. *Eur J Hum Genet*. (2015) 23:678–87. doi: 10.1038/ejhg.2014.158
65. Kirino Y, Zhou Q, Ishigatsubo Y, Mizuki N, Tugal-Tutkun I, Seyahi E, et al. Targeted resequencing implicates the familial Mediterranean fever gene MEFV and the toll-like receptor 4 gene TLR4 in Behcet disease. *Proc Natl Acad Sci USA*. (2013) 110:8134–9. doi: 10.1073/pnas.1306352110
66. Nakano H, Kirino Y, Takeno M, Higashitani K, Nagai H, Yoshimi R, et al. GWAS-identified CCR1 and IL10 loci contribute to M1 macrophage-predominant inflammation in Behcet's disease. *Arthritis Res Ther*. (2018) 20:124. doi: 10.1186/s13075-018-1613-0
67. Rahmattulla C, Mooyaart AL, van Hooven D, Schoones JW, Bruijn JA, Dekkers OM, et al. Genetic variants in ANCA-associated vasculitis: a meta-analysis. *Ann Rheum Dis*. (2016) 75:1687–92. doi: 10.1136/annrheumdis-2015-207601
68. Nakazawa D, Masuda S, Tomaru U, Ishizu A. Pathogenesis and therapeutic interventions for ANCA-associated vasculitis. *Nat Rev Rheumatol*. (2019) 15:91–101. doi: 10.1038/s41584-018-0145-y
69. Fujimoto S, Watts RA, Kobayashi S, Suzuki K, Jayne DR, Scott DG, et al. Comparison of the epidemiology of anti-neutrophil cytoplasmic antibody-associated vasculitis between Japan and the U.K. *Rheumatology*. (2011) 50:1916–20. doi: 10.1093/rheumatology/ker205
70. Kawasaki A, Hasebe N, Hidaka M, Hirano F, Sada KE, Kobayashi S, et al. Protective role of HLA-DRB1*13:02 against Microscopic Polyangiitis and MPO-ANCA-Positive vasculitides in a japanese population: a case-control study. *PLoS ONE*. (2016) 11:e0154393. doi: 10.1371/journal.pone.0154393
71. Cao Y, Yang J, Colby K, Hogan SL, Hu Y, Jennette CE, et al. High basal activity of the PTPN22 gain-of-function variant blunts leukocyte responsiveness negatively affecting IL-10 production in ANCA vasculitis. *PLoS ONE*. (2012) 7:e42783. doi: 10.1371/journal.pone.0042783
72. Carr EJ, Niederer HA, Williams J, Harper L, Watts RA, Lyons PA, et al. Confirmation of the genetic association of CTLA4 and PTPN22 with ANCA-associated vasculitis. *BMC Med Genet*. (2009) 10:121. doi: 10.1186/1471-2350-10-121
73. Jagiello P, Aries P, Arning L, Wagenleiter SE, Csernok E, Hellmich B, et al. The PTPN22 620W allele is a risk factor for Wegener's granulomatosis. *Arthritis Rheum*. (2005) 52:4039–43. doi: 10.1002/art.21487
74. Alberici F, Martorana D, Bonatti F, Gioffredi A, Lyons PA, Vaglio A. Genetics of ANCA-associated vasculitides: HLA and beyond. *Clin Exp Rheumatol*. (2014) 32(3 Suppl. 82):S90–7.
75. Fanciulli M, Norsworthy PJ, Petretto E, Dong R, Harper L, Kamesh L, et al. FCGR3B copy number variation is associated with susceptibility to systemic, but not organ-specific, autoimmunity. *Nat Genet*. (2007) 39:721–3. doi: 10.1038/ng2046
76. Zhou XJ, Cheng FJ, Lv JC, Luo H, Yu F, Chen M, et al. Higher DEFB4 genomic copy number in SLE and ANCA-associated small vasculitis. *Rheumatology*. (2012) 51:992–5. doi: 10.1093/rheumatology/ker419
77. Qi Y, Zhou X, Bu D, Hou P, Lv J, Zhang H. Low copy numbers of FCGR3A and FCGR3B associated with Chinese patients with SLE and AASV. *Lupus*. (2017) 26:1383–9. doi: 10.1177/0961203317700485
78. Lee KS, Kronbichler A, Pereira Vasconcelos DF, Pereira da Silva FR, Ko Y, Oh YS, et al. Genetic variants in antineutrophil cytoplasmic antibody-associated vasculitis: a bayesian approach and systematic review. *J Clin Med*. (2019) 8:E266. doi: 10.3390/jcm8020266
79. Harky A, Fok M, Balmforth D, Bashir M. Pathogenesis of large vessel vasculitis: Implications for disease classification and future therapies. *Vasc Med*. (2019) 24:79–88. doi: 10.1177/1358863X18802989
80. Sahin Z, Bicakcigil M, Aksu K, Kamali S, Akar S, Onen F, et al. Takayasu's arteritis is associated with HLA-B*52, but not with HLA-B*51, in Turkey. *Arthritis Res Ther*. (2012) 14:R27. doi: 10.1186/ar3730
81. Ozen S, Batu ED. Vasculitis pathogenesis: can we talk about precision medicine? *Front Immunol*. (2018) 9:1892. doi: 10.3389/fimmu.2018.01892
82. Nakajima T, Yoshifuji H, Shimizu M, Kitagori K, Murakami K, Nakashima R, et al. A novel susceptibility locus in the IL12B region is associated with the pathophysiology of Takayasu arteritis through IL-12p40 and IL-12p70 production. *Arthritis Res Ther*. (2017) 19:197. doi: 10.1186/s13075-017-1408-8
83. Nagelkerke SQ, Kuijpers TW. Immunomodulation by IVIg and the role of Fc-gamma receptors: classic mechanisms of action after all? *Front Immunol*. (2014) 5:674. doi: 10.3389/fimmu.2014.00674
84. Adu B, Dodoo D, Adukpo S, Hedley PL, Arthur FK, Gerds TA, et al. Fc gamma receptor IIIB (FcgammaRIIIB) polymorphisms are associated with clinical malaria in Ghanaian children. *PLoS ONE*. (2012) 7:e46197. doi: 10.1371/journal.pone.0046197
85. Asano K, Matsushita T, Umeno J, Hosono N, Takahashi A, Kawaguchi T, et al. A genome-wide association study identifies three new susceptibility loci for ulcerative colitis in the Japanese population. *Nat Genet*. (2009) 41:1325–9. doi: 10.1038/ng.482
86. Breunis WB, van Mirre E, Bruin M, Geissler J, de Boer M, Peters M, et al. Copy number variation of the activating FCGR2C gene predisposes to idiopathic thrombocytopenic purpura. *Blood*. (2008) 111:1029–38. doi: 10.1182/blood-2007-03-079913
87. Cartron G, Dacheux L, Salles G, Solal-Celigny P, Bardos P, Colombat P, et al. Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG Fc receptor FcgammaRIIIa gene. *Blood*. (2002) 99:754–8. doi: 10.1182/blood.V99.3.754
88. Lee YH, Ji JD, Song GG. Associations between FCGR3A polymorphisms and susceptibility to rheumatoid arthritis: a metaanalysis. *J Rheumatol*. (2008) 35:2129–35. doi: 10.3899/jrheum.080186
89. Lee YH, Ji JD, Song GG. Fcgamma receptor IIB and IIIB polymorphisms and susceptibility to systemic lupus erythematosus and lupus nephritis: a meta-analysis. *Lupus*. (2009) 18:727–34. doi: 10.1177/0961203309104020
90. Tamura K, Shimizu C, Hojo T, Akashi-Tanaka S, Kinoshita T, Yonemori K, et al. FcgammaR2A and 3A polymorphisms predict clinical outcome of trastuzumab in both neoadjuvant and metastatic settings in patients with HER2-positive breast cancer. *Ann Oncol*. (2011) 22:1302–7. doi: 10.1093/annonc/mdq585
91. Treon SP, Yang G, Hanzis C, Ioakimidis L, Verselis SJ, Fox EA, et al. Attainment of complete/very good partial response following rituximab-based therapy is an important determinant to progression-free survival, and is impacted by polymorphisms in FCGR3A in Waldenstrom macroglobulinaemia. *Br J Haematol*. (2011) 154:223–8. doi: 10.1111/j.1365-2141.2011.08726.x
92. Carmona FD, Martin J, Gonzalez-Gay MA. Genetics of vasculitis. *Curr Opin Rheumatol*. (2015) 27:10–7. doi: 10.1097/BOR.0000000000000124
93. Jia M, Zhu L, Zhai YL, Chen P, Xu BY, Guo WY, et al. Variation in complement factor H affects complement activation in immunoglobulin A

vasculitis with nephritis. *Nephrology*. (2019). doi: 10.1111/nep.13580. [Epub ahead of print].
94. Watts RA, Scott DG. Epidemiology of the vasculitides. *Semin Respir Crit Care Med*. (2004) 25:455–64. doi: 10.1055/s-2004-836139
95. Smutny T, Barvik I, Veleta T, Pavek P, Soukup T. Genetic predispositions of glucocorticoid resistance and therapeutic outcomes in polymyalgia rheumatica and giant cell arteritis. *J Clin Med*. (2019) 8:582. doi: 10.3390/jcm8050582
96. Acosta-Herrera M, Gonzalez-Gay MA, Martin J, Marquez A. Leveraging genetic findings for precision medicine in vasculitis. *Front Immunol*. (2019) 10:1796. doi: 10.3389/fimmu.2019.01796
97. Ly KH, Regent A, Tamby MC, Mouthon L. Pathogenesis of giant cell arteritis: more than just an inflammatory condition? *Autoimmun Rev*. (2010) 9:635–45. doi: 10.1016/j.autrev.2010.05.002
98. Gilkes DM, Bajpai S, Chaturvedi P, Wirtz D, Semenza GL. Hypoxia-inducible factor 1 (HIF-1) promotes extracellular matrix remodeling under hypoxic conditions by inducing P4HA1, P4HA2, and PLOD2 expression in fibroblasts. *J Biol Chem*. (2013) 288:10819–29. doi: 10.1074/jbc.M112.442939
99. Allis CD, Jenuwein T. The molecular hallmarks of epigenetic control. *Nat Rev Genet*. (2016) 17:487–500. doi: 10.1038/nrg.2016.59
100. Coit P, Direskeneli H, Sawalha AH. An update on the role of epigenetics in systemic vasculitis. *Curr Opin Rheumatol*. (2018) 30:4–15. doi: 10.1097/BOR.0000000000000451
101. Carnero-Montoro E, Alarcon-Riquelme ME. Epigenome-wide association studies for systemic autoimmune diseases: the road behind and the road ahead. *Clin Immunol*. (2018) 196:21–33. doi: 10.1016/j.clim.2018.03.014
102. Duan J, Lou J, Zhang Q, Ke J, Qi Y, Shen N, et al. A genetic variant rs1801274 in FCGR2A as a potential risk marker for Kawasaki disease: a case-control study and meta-analysis. *PLoS ONE*. (2014) 9:e103329. doi: 10.1371/journal.pone.0103329
103. Kuo HC, Chang JC, Kuo HC, Yu HR, Wang CL, Lee CP, et al. Identification of an association between genomic hypomethylation of FCGR2A and susceptibility to Kawasaki disease and intravenous immunoglobulin resistance by DNA methylation array. *Arthritis Rheumatol*. (2015) 67:828–36. doi: 10.1002/art.38976
104. Li SC, Chan WC, Huang YH, Guo MM, Yu HR, Huang FC, et al. Major methylation alterations on the CpG markers of inflammatory immune associated genes after IVIG treatment in Kawasaki disease. *BMC Med Genomics*. (2016) 9 (Suppl. 1):37. doi: 10.1186/s12920-016-0197-2
105. Ellerman JE, Brown CK, de Vera M, Zeh HJ, Billiar T, Rubartelli A, et al. Masquerader: high mobility group box-1 and cancer. *Clin Cancer Res*. (2007) 13:2836–48. doi: 10.1158/1078-0432.CCR-06-1953
106. Vogelpoel LT, Hansen IS, Visser MW, Nagelkerke SQ, Kuijpers TW, Kapsenberg ML, et al. FcgammaRIIa cross-talk with TLRs, IL-1R, and IFNgammaR selectively modulates cytokine production in human myeloid cells. *Immunobiology*. (2015) 220:193–9. doi: 10.1016/j.imbio.2014.07.016
107. Huang YH, Li SC, Huang LH, Chen PC, Lin YY, Lin CC, et al. Identifying genetic hypomethylation and upregulation of Toll-like receptors in Kawasaki disease. *Oncotarget*. (2017) 8:11249–58. doi: 10.18632/oncotarget.14497
108. Rudensky AY. Regulatory T cells and Foxp3. *Immunol Rev*. (2011) 241:260–8. doi: 10.1111/j.1600-065X.2011.01018.x
109. Ni FF, Li CR, Li Q, Xia Y, Wang GB, Yang J. Regulatory T cell microRNA expression changes in children with acute Kawasaki disease. *Clin Exp Immunol*. (2014) 178:384–93. doi: 10.1111/cei.12418
110. Huang YH, Lo MH, Cai XY, Liu SF, Kuo HC. Increase expression of CD177 in Kawasaki disease. *Pediatr Rheumatol Online J*. (2019) 17:13. doi: 10.1186/s12969-019-0315-8
111. Chang D, Qian C, Li H, Feng H. Comprehensive analyses of DNA methylation and gene expression profiles of Kawasaki disease. *J Cell Biochem*. (2019) 120:13001–11. doi: 10.1002/jcb.28571
112. Huang YH, Kuo HC, Li SC, Cai XY, Liu SF, Kuo HC. HAMP promoter hypomethylation and increased hepcidin levels as biomarkers for Kawasaki disease. *J Mol Cell Cardiol*. (2018) 117:82–7. doi: 10.1016/j.yjmcc.2018.02.017
113. Huang LH, Kuo HC, Pan CT, Lin YS, Huang YH, Li SC. Multiomics analyses identified epigenetic modulation of the S100A gene family in Kawasaki disease and their significant involvement in neutrophil transendothelial migration. *Clin Epigenetics*. (2018) 10:135. doi: 10.1186/s13148-018-0557-1
114. Hughes T, Ture-Ozdemir F, Alibaz-Oner F, Coit P, Direskeneli H, Sawalha AH. Epigenome-wide scan identifies a treatment-responsive pattern of altered DNA methylation among cytoskeletal remodeling genes in monocytes and CD4+ T cells from patients with Behcet's disease. *Arthritis Rheumatol*. (2014) 66:1648–58. doi: 10.1002/art.38409
115. Zhu Y, Qiu Y, Yu H, Yi S, Su W, Kijlstra A, et al. Aberrant DNA methylation of GATA binding protein 3 (GATA3), interleukin-4 (IL-4), and transforming growth factor-beta (TGF-beta) promoters in Behcet's disease. *Oncotarget*. (2017) 8:64263–72. doi: 10.18632/oncotarget.19500
116. Zhu Y, Yu H, Qiu Y, Ye Z, Su W, Deng J, et al. Promoter hypermethylation of GATA3, IL-4, and TGF-beta confers susceptibility to vogt-koyanagi-harada disease in Han Chinese. *Invest Ophthalmol Vis Sci*. (2017) 58:1529–36. doi: 10.1167/iovs.16-21188
117. Puccetti A, Pelosi A, Fiore PF, Patuzzo G, Lunardi C, Dolcino M. MicroRNA expression profiling in Behcet's disease. *J Immunol Res*. (2018) 2018:2405150. doi: 10.1155/2018/2405150
118. Woo MY, Yun SJ, Cho O, Kim K, Lee ES, Park S. MicroRNAs differentially expressed in Behcet disease are involved in interleukin-6 production. *J Inflamm*. (2016) 13:22. doi: 10.1186/s12950-016-0130-7
119. Qi J, Yang Y, Hou S, Qiao Y, Wang Q, Yu H, et al. Increased Notch pathway activation in Behcet's disease. *Rheumatology*. (2014) 53:810–20. doi: 10.1093/rheumatology/ket438
120. Qi J, Hou S, Zhang Q, Liao D, Wei L, Fang J, et al. A functional variant of pre-miRNA-196a2 confers risk for Behcet's disease but not for Vogt-Koyanagi-Harada syndrome or AAU in ankylosing spondylitis. *Hum Genet*. (2013) 132:1395–404. doi: 10.1007/s00439-013-1346-8
121. Zhou Q, Hou S, Liang L, Li X, Tan X, Wei L, et al. MicroRNA-146a and Ets-1 gene polymorphisms in ocular Behcet's disease and Vogt-Koyanagi-Harada syndrome. *Ann Rheum Dis*. (2014) 73:170–6. doi: 10.1136/annrheumdis-2012-201627
122. Yang JJ, Pendergraft WF, Alcorta DA, Nachman PH, Hogan SL, Thomas RP, et al. Circumvention of normal constraints on granule protein gene expression in peripheral blood neutrophils and monocytes of patients with antineutrophil cytoplasmic autoantibody-associated glomerulonephritis. *J Am Soc Nephrol*. (2004) 15:2103–14. doi: 10.1097/01.ASN.0000135058.46193.72
123. Ciavatta DJ, Yang J, Preston GA, Badhwar AK, Xiao H, Hewins P, et al. Epigenetic basis for aberrant upregulation of autoantigen genes in humans with ANCA vasculitis. *J Clin Invest*. (2010) 120:3209–19. doi: 10.1172/JCI40034
124. Jones BE, Yang J, Muthigi A, Hogan SL, Hu Y, Starmer J, et al. Gene-specific DNA methylation changes predict remission in patients with ANCA-associated vasculitis. *J Am Soc Nephrol*. (2017) 28:1175–87. doi: 10.1681/ASN.2016050548
125. Yang J, Ge H, Poulton CJ, Hogan SL, Hu Y, Jones BE, et al. Histone modification signature at myeloperoxidase and proteinase 3 in patients with anti-neutrophil cytoplasmic autoantibody-associated vasculitis. *Clin Epigenetics*. (2016) 8:85. doi: 10.1186/s13148-016-0251-0
126. Luo S, Liang G, Zhang P, Zhao M, Lu Q. Aberrant histone modifications in peripheral blood mononuclear cells from patients with Henoch-Schonlein purpura. *Clin Immunol*. (2013) 146:165–75. doi: 10.1016/j.clim.2012.12.009
127. Coit P, De Lott LB, Nan B, Elner VM, Sawalha AH. DNA methylation analysis of the temporal artery microenvironment in giant cell arteritis. *Ann Rheum Dis*. (2016) 75:1196–202. doi: 10.1136/annrheumdis-2014-207116
128. Croci S, Zerbini A, Boiardi L, Muratore F, Bisagni A, Nicoli D, et al. MicroRNA markers of inflammation and remodelling in temporal arteries from patients with giant cell arteritis. *Ann Rheum Dis*. (2016) 75:1527–33. doi: 10.1136/annrheumdis-2015-207846
129. Kuret T, Burja B, Feichtinger J, Thallinger GG, Frank-Bertoncelj M, Lakota K, et al. Gene and miRNA expression in giant cell arteritis-a concise systematic review of significantly modified studies. *Clin Rheumatol*. (2019) 38:307–16. doi: 10.1007/s10067-018-4231-y
130. Wolf P. The nature and significance of platelet products in human plasma. *Br J Haematol*. (1967) 13:269–88. doi: 10.1111/j.1365-2141.1967.tb08741.x
131. Chen Y, Li G, Liu ML. Microvesicles as emerging biomarkers and therapeutic targets in cardiometabolic diseases. *Genom Proteom Bioinform*. (2018) 16:50–62. doi: 10.1016/j.gpb.2017.03.006

132. Liu ML, Williams KJ, Werth VP. Microvesicles in autoimmune diseases. *Adv Clin Chem*. (2016) 77:125–75. doi: 10.1016/bs.acc.2016.06.005
133. Wu X, Liu Y, Wei W, Liu ML. Extracellular vesicles in autoimmune vasculitis - Little dirts light the fire in blood vessels. *Autoimmun Rev*. (2019) 18:593–606. doi: 10.1016/j.autrev.2018.12.007.
134. Yoon S, Kovalenko A, Bogdanov K, Wallach D. MLKL, the Protein that mediates necroptosis, also regulates endosomal trafficking and extracellular vesicle generation. *Immunity*. (2017) 47:51–65 e7. doi: 10.1016/j.immuni.2017.06.001
135. Colombo M, Raposo G, Thery C. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. *Annu Rev Cell Dev Biol*. (2014) 30:255–89. doi: 10.1146/annurev-cellbio-101512-122326
136. Raposo G, Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. *J Cell Biol*. (2013) 200:373–83. doi: 10.1083/jcb.201211138
137. Chaput N, Thery C. Exosomes: immune properties and potential clinical implementations. *Semin Immunopathol*. (2011) 33:419–40. doi: 10.1007/s00281-010-0233-9
138. Record M, Subra C, Silvente-Poirot S, Poirot M. Exosomes as intercellular signalosomes and pharmacological effectors. *Biochem Pharmacol*. (2011) 81:1171–82. doi: 10.1016/j.bcp.2011.02.011
139. Sluijter JP, Verhage V, Deddens JC, van den Akker F, Doevendans PA. Microvesicles and exosomes for intracardiac communication. *Cardiovasc Res*. (2014) 102:302–11. doi: 10.1093/cvr/cvu022
140. Park KS, Lee J, Lee C, Park HT, Kim JW, Kim OY, et al. Sepsis-like systemic inflammation induced by nano-sized extracellular vesicles from feces. *Front Microbiol*. (2018) 9:1735. doi: 10.3389/fmicb.2018.01735
141. van der Pol E, Boing AN, Harrison P, Sturk A, Nieuwland R. Classification, functions, and clinical relevance of extracellular vesicles. *Pharmacol Rev*. (2012) 64:676–705. doi: 10.1124/pr.112.005983
142. Buzas EI, Gyorgy B, Nagy G, Falus A, Gay S. Emerging role of extracellular vesicles in inflammatory diseases. *Nat Rev Rheumatol*. (2014) 10:356–64. doi: 10.1038/nrrheum.2014.19
143. Brogan PA, Shah V, Brachet C, Harnden A, Mant D, Klein N, et al. Endothelial and platelet microparticles in vasculitis of the young. *Arthritis Rheum*. (2004) 50:927–36. doi: 10.1002/art.20199
144. Dursun I, Dusunsel R, Poyrazoglu HM, Gunduz Z, Patiroglu T, Ulger H, et al. Circulating endothelial microparticles in children with Henoch-Schonlein purpura; preliminary results. *Rheumatol Int*. (2011) 31:1595–600. doi: 10.1007/s00296-010-1528-9
145. Eleftheriou D, Hong Y, Klein NJ, Brogan PA. Thromboembolic disease in systemic vasculitis is associated with enhanced microparticle-mediated thrombin generation. *J Thromb Haemost*. (2011) 9:1864–7. doi: 10.1111/j.1538-7836.2011.04434.x
146. Hajj-Ali RA, Major J, Langford C, Hoffman GS, Clark T, Zhang L, et al. The interface of inflammation and subclinical atherosclerosis in granulomatosis with polyangiitis (Wegener's): a preliminary study. *Transl Res*. (2015) 166:366–74. doi: 10.1016/j.trsl.2015.04.001
147. Nakaoka H, Hirono K, Yamamoto S, Takasaki I, Takahashi K, Kinoshita K, et al. MicroRNA-145-5p and microRNA-320a encapsulated in endothelial microparticles contribute to the progression of vasculitis in acute Kawasaki Disease. *Sci Rep*. (2018) 8:1016. doi: 10.1038/s41598-018-19310-4
148. Shah V, Christov G, Mukasa T, Brogan KS, Wade A, Eleftheriou D, et al. Cardiovascular status after Kawasaki disease in the UK. *Heart*. (2015) 101:1646–55. doi: 10.1136/heartjnl-2015-307734
149. Tan Z, Yuan Y, Chen S, Chen Y, Chen TX. Plasma endothelial microparticles, TNF-a and IL-6 in Kawasaki disease. *Indian Pediatr*. (2013) 50:501–3. doi: 10.1007/s13312-013-0152-7
150. Jia HL, Liu CW, Zhang L, Xu WJ, Gao XJ, Bai J, et al. Sets of serum exosomal microRNAs as candidate diagnostic biomarkers for Kawasaki disease. *Sci Rep*. (2017) 7:44706. doi: 10.1038/srep44706
151. Kahn R, Mossberg M, Stahl AL, Johansson K, Lopatko Lindman I, Heijl C, et al. Microvesicle transfer of kinin B1-receptors is a novel inflammatory mechanism in vasculitis. *Kidney Int*. (2017) 91:96–105. doi: 10.1016/j.kint.2016.09.023
152. Khan E, Ambrose NL, Ahnstrom J, Kiprianos AP, Stanford MR, Eleftheriou D, et al. A low balance between microparticles expressing tissue factor pathway inhibitor and tissue factor is associated with thrombosis in Behcet's syndrome. *Sci Rep*. (2016) 6:38104. doi: 10.1038/srep38104
153. Macey M, Hagi-Pavli E, Stewart J, Wallace GR, Stanford M, Shirlaw P, et al. Age, gender and disease-related platelet and neutrophil activation *ex vivo* in whole blood samples from patients with Behcet's disease. *Rheumatology*. (2011) 50:1849–59. doi: 10.1093/rheumatology/ker177
154. Martinez M, Ricart JM, Ruiz-Aja S, Rus A, Todoli J, Calvo J, et al. Platelet activation and red blood cell phosphatidylserine exposure evaluated by flow cytometry in patients with Behcet's disease: are they related to thrombotic events? *Pathophysiol Haemost Thromb*. (2007) 36:18–22. doi: 10.1159/000112635
155. Mejia JC, Ortiz T, Tassies D, Solanich X, Vidaller A, Cervera R, et al. Procoagulant microparticles are increased in patients with Behcet's disease but do not define a specific subset of clinical manifestations. *Clin Rheumatol*. (2016) 35:695–9. doi: 10.1007/s10067-015-2903-4
156. Mossberg M, Stahl AL, Kahn R, Kristoffersson AC, Tati R, Heijl C, et al. C1-inhibitor decreases the release of vasculitis-like chemotactic endothelial microvesicles. *J Am Soc Nephrol*. (2017) 28:2472–81. doi: 10.1681/ASN.2016060637
157. Zhang L, Song QF, Jin JJ, Huang P, Wang ZP, Xie XF, et al. Differential protein analysis of serum exosomes post-intravenous immunoglobulin therapy in patients with Kawasaki disease. *Cardiol Young*. (2017) 27:1786–96. doi: 10.1017/S1047951117001433
158. Zhang L, Wang W, Bai J, Xu YF, Li LQ, Hua L, et al. Proteomic analysis associated with coronary artery dilatation caused by Kawasaki disease using serum exosomes. *Rev Port Cardiol*. (2016) 35:265–73. doi: 10.1016/j.repce.2015.11.024
159. Zhang X, Xin G, Sun D. Serum exosomal miR-328, miR-575, miR-134 and miR-671-5p as potential biomarkers for the diagnosis of Kawasaki disease and the prediction of therapeutic outcomes of intravenous immunoglobulin therapy. *Exp Ther Med*. (2018) 16:2420–32. doi: 10.3892/etm.2018.6458
160. Guiducci S, Ricci L, Romano E, Ceccarelli C, Distler JH, Miniati I, et al. Microparticles and Kawasaki disease: a marker of vascular damage? *Clin Exp Rheumatol*. (2011) 29(1 Suppl. 64):S121–5.
161. Huang YM, Wang H, Wang C, Chen M, Zhao MH. Promotion of hypercoagulability in antineutrophil cytoplasmic antibody-associated vasculitis by C5a-induced tissue factor-expressing microparticles and neutrophil extracellular traps. *Arthritis Rheumatol*. (2015) 67:2780–90. doi: 10.1002/art.39239
162. Clarke LA, Hong Y, Eleftheriou D, Shah V, Arrigoni F, Klein NJ, et al. Endothelial injury and repair in systemic vasculitis of the young. *Arthritis Rheum*. (2010) 62:1770–80. doi: 10.1002/art.27418
163. Ding YY, Ren Y, Feng X, Xu QQ, Sun L, Zhang JM, et al. Correlation between brachial artery flow-mediated dilation and endothelial microparticle levels for identifying endothelial dysfunction in children with Kawasaki disease. *Pediatr Res*. (2014) 75:453–8. doi: 10.1038/pr.2013.240
164. Kambas K, Chrysanthopoulou A, Vassilopoulos D, Apostolidou E, Skendros P, Girod A, et al. Tissue factor expression in neutrophil extracellular traps and neutrophil derived microparticles in antineutrophil cytoplasmic antibody associated vasculitis may promote thromboinflammation and the thrombophilic state associated with the disease. *Ann Rheum Dis*. (2014) 73:1854–63. doi: 10.1136/annrheumdis-2013-203430
165. Kumpers P, Erdbrugger U, Grossheim M, Meyer GP, Hiss M, Gwinner W, et al. Endothelial microparticles as a diagnostic aid in Churg-Strauss vasculitis-induced cardiomyopathy. *Clin Exp Rheumatol*. (2008) 26(3. Suppl 49):S86–9.
166. Tian J, Lv HT, An XJ, Ling N, Xu F. Endothelial microparticles induce vascular endothelial cell injury in children with Kawasaki disease. *Eur Rev Med Pharmacol Sci*. (2016) 20:1814–8.
167. Daniel L, Fakhouri F, Joly D, Mouthon L, Nusbaum P, Grunfeld JP, et al. Increase of circulating neutrophil and platelet microparticles during acute vasculitis and hemodialysis. *Kidney Int*. (2006) 69:1416–23. doi: 10.1038/sj.ki.5000306
168. Erdbruegger U, Grossheim M, Hertel B, Wyss K, Kirsch T, Woywodt A, et al. Diagnostic role of endothelial microparticles in vasculitis. *Rheumatology*. (2008) 47:1820–5. doi: 10.1093/rheumatology/ken373

169. Kim HJ, Choi EH, Lim YJ, Kil HR. The usefulness of platelet-derived microparticle as biomarker of antiplatelet therapy in Kawasaki disease. *J Korean Med Sci*. (2017) 32:1147–53. doi: 10.3346/jkms.2017.32.7.1147
170. Yahata T, Suzuki C, Yoshioka A, Hamaoka A, Ikeda K. Platelet activation dynamics evaluated using platelet-derived microparticles in Kawasaki disease. *Circ J*. (2014) 78:188–93. doi: 10.1253/circj.CJ-12-1037
171. Mendoza-Pinto C, Garcia-Carrasco M, Jimenez-Hernandez M, Jimenez Hernandez C, Riebeling-Navarro C, Nava Zavala A, et al. Etiopathogenesis of Behcet's disease. *Autoimmun Rev*. (2010) 9:241–5. doi: 10.1016/j.autrev.2009.10.005
172. Hong Y, Eleftheriou D, Hussain AA, Price-Kuehne FE, Savage CO, Jayne D, et al. Anti-neutrophil cytoplasmic antibodies stimulate release of neutrophil microparticles. *J Am Soc Nephrol*. (2012) 23:49–62. doi: 10.1681/ASN.2011030298
173. Kahn R, Hellmark T, Leeb-Lundberg LM, Akbari N, Todiras M, Olofsson T, et al. Neutrophil-derived proteinase 3 induces kallikrein-independent release of a novel vasoactive kinin. *J Immunol*. (2009) 182:7906–15. doi: 10.4049/jimmunol.0803624
174. Kahn R, Herwald H, Muller-Esterl W, Schmitt R, Sjogren AC, Truedsson L, et al. Contact-system activation in children with vasculitis. *Lancet*. (2002) 360:535–41. doi: 10.1016/S0140-6736(02)09743-X
175. Duchene J, Lecomte F, Ahmed S, Cayla C, Pesquero J, Bader M, et al. A novel inflammatory pathway involved in leukocyte recruitment: role for the kinin B1 receptor and the chemokine CXCL5. *J Immunol*. (2007) 179:4849–56. doi: 10.4049/jimmunol.179.7.4849
176. Xie XF, Chu HJ, Xu YF, Hua L, Wang ZP, Huang P, et al. Proteomics study of serum exosomes in Kawasaki disease patients with coronary artery aneurysms. *Cardiol J*. (2018) 26:584–93. doi: 10.5603/CJ.a2018.0032
177. Pitanga TN, de Aragao Franca L, Rocha VC, Meirelles T, Borges VM, Goncalves MS, et al. Neutrophil-derived microparticles induce myeloperoxidase-mediated damage of vascular endothelial cells. *BMC Cell Biol*. (2014) 15:21. doi: 10.1186/1471-2121-15-21
178. Slater TW, Finkielsztein A, Mascarenhas LA, Mehl LC, Butin-Israeli V, Sumagin R. Neutrophil microparticles deliver active myeloperoxidase to injured mucosa to inhibit epithelial wound healing. *J Immunol*. (2017) 198:2886–97. doi: 10.4049/jimmunol.1601810
179. Kastner DL, Zhou Q, Aksentijevich I. Mutant ADA2 in vasculopathies. *N Engl J Med*. (2014) 371:480–1. doi: 10.1056/NEJMc1405506
180. Zhou Q, Yang D, Ombrello AK, Zavialov AV, Toro C, Zavialov AV, et al. Early-onset stroke and vasculopathy associated with mutations in ADA2. *N Engl J Med*. (2014) 370:911–20. doi: 10.1056/NEJMoa1307361
181. Ombrello AK, Qin J, Hoffmann PM, Kumar P, Stone D, Jones A, et al. Treatment strategies for deficiency of adenosine deaminase 2. *N Engl J Med*. (2019) 380:1582–4. doi: 10.1056/NEJMc1801927
182. Glikson M, Galun E, Schlesinger M, Cohen D, Haskell L, Rubinow A, et al. Polyarteritis nodosa and familial Mediterranean fever: a report of 2 cases and review of the literature. *J Rheumatol*. (1989) 16:536–9.
183. Henckes M, Roskams T, Vanneste S, Van Damme B, Vanrenterghem Y. Polyarteritis nodosa type vasculitis in a patient with familial Mediterranean fever treated with cyclosporin A. *Transpl Int*. (1994) 7:292–6. doi: 10.1111/j.1432-2277.1994.tb01577.x
184. Kocak H, Cakar N, Hekimoglu B, Atakan C, Akkok N, Unal S. The coexistence of familial Mediterranean fever and polyarteritis nodosa; report of a case. *Pediatr Nephrol*. (1996) 10:631–3. doi: 10.1007/s004670050176
185. Ozdogan H, Arisoy N, Kasapcapur O, Sever L, Caliskan S, Tuzuner N, et al. Vasculitis in familial Mediterranean fever. *J Rheumatol*. (1997) 24:323–7.
186. Sachs D, Langevitz P, Morag B, Pras M. Polyarteritis nodosa and familial Mediterranean fever. *Br J Rheumatol*. (1987) 26:139–41. doi: 10.1093/rheumatology/26.2.139
187. Schlesinger M, Kopolovic J, Viskoper RJ, Ron N. A case of familial Mediterranean fever with cutaneous vasculitis and immune complex nephritis: light, electron, and immunofluorescent study of renal biopsy. *Am J Clin Pathol*. (1983) 80:511–4. doi: 10.1093/ajcp/80.4.511
188. Serrano R, Martinez MA, Andres A, Morales JM, Samartin R. Familial mediterranean fever and acute myocardial infarction secondary to coronary vasculitis. *Histopathology*. (1998) 33:163–7. doi: 10.1046/j.1365-2559.1998.00462.x
189. Sohar E, Gafni J, Pras M, Heller H. Familial Mediterranean fever. A survey of 470 cases and review of the literature. *Am J Med*. (1967) 43:227–53. doi: 10.1016/0002-9343(67)90167-2
190. Tekin M, Yalcinkaya F, Tumer N, Akar N, Misirlioglu M, Cakar N. Clinical, laboratory and molecular characteristics of children with Familial Mediterranean Fever-associated vasculitis. *Acta Paediatr*. (2000) 89:177–82. doi: 10.1080/080352500750028799
191. Balbir-Gurman A, Nahir AM, Braun-Moscovici Y. Vasculitis in siblings with familial Mediterranean fever: a report of three cases and review of the literature. *Clin Rheumatol*. (2007) 26:1183–5. doi: 10.1007/s10067-006-0323-1
192. Bosacki C, Richard O, Freycon F, Mosnier JF, Cathebras P. [The association of polyarteritis nodosa and familial Mediterranean fever]. *Presse Med*. (2003) 32:24–6.
193. Braun E, Schapira D, Guralnik L, Azzam ZS. Acute vasculitis with multiorgan involvement in a patient with familial Mediterranean fever. *Am J Med Sci*. (2003) 325:363–4. doi: 10.1097/00000441-200306000-00007
194. Hatemi G, Masatlioglu S, Gogus F, Ozdogan H. Necrotizing vasculitis associated with familial Mediterranean fever. *Am J Med*. (2004) 117:516–9. doi: 10.1016/j.amjmed.2004.02.050
195. Kone-Paut I, Hentgen V, Guillaume-Czitrom S, Compeyrot-Lacassagne S, Tran TA, Touitou I. The clinical spectrum of 94 patients carrying a single mutated MEFV allele. *Rheumatology*. (2009) 48:840–2. doi: 10.1093/rheumatology/kep121
196. Luger S, Harter PN, Mittelbronn M, Wagner M, Foerch C. Brain stem infarction associated with familial Mediterranean fever and central nervous system vasculitis. *Clin Exp Rheumatol*. (2013) 31(3 Suppl. 77):93–5.
197. Ozen S, Bakkaloglu A, Yilmaz E, Duzova A, Balci B, Topaloglu R, et al. Mutations in the gene for familial Mediterranean fever: do they predispose to inflammation? *J Rheumatol*. (2003) 30:2014–8.
198. Ozkaya O, Bek K, Alaca N, Ceyhan M, Acikgoz Y, Tasdemir HA. Cerebral vasculitis in a child with Henoch-Schonlein purpura and familial Mediterranean fever. *Clin Rheumatol*. (2007) 26:1729–32. doi: 10.1007/s10067-006-0485-x
199. Topaloglu R, Ozaltin F, Yilmaz E, Ozen S, Balci B, Besbas N, et al. E148Q is a disease-causing MEFV mutation: a phenotypic evaluation in patients with familial Mediterranean fever. *Ann. Rheumat. Dis*. (2005) 64:750–2. doi: 10.1136/ard.2004.026963
200. Zihni FY, Kalfa M, Ocakci PT, Tarhan F, Parildar M, Keser G, et al. Coexistence of Takayasu's arteritis with familial Mediterranean fever. *Rheumatol Int*. (2012) 32:1675–8. doi: 10.1007/s00296-011-1853-7
201. Jarjour RA, Al-Berrawi S. Familial Mediterranean fever in Syrian children: phenotype-genotype correlation. *Rheumatol Int*. (2015) 35:629–34. doi: 10.1007/s00296-014-3116-x
202. Komatsu S, Honma M, Igawa S, Tsuji H, Ishida-Yamamoto A, Migita K, et al. Cutaneous necrotizing vasculitis as a manifestation of familial Mediterranean fever. *J Dermatol*. (2014) 41:827–9. doi: 10.1111/1346-8138.12588
203. Taylan A, Yildiz Y, Sari I, Ozkok G. Vasculitis and long standing ankylosing spondylitis in a patient with familial Mediterranean fever. *J Res Med Sci*. (2014) 19:1009–11.
204. Ozcakar ZB, Cakar N, Uncu N, Celikel BA, Yalcinkaya F. Familial Mediterranean fever-associated diseases in children. *QJM*. (2017) 110:287–90. doi: 10.1093/qjmed/hcw230
205. Ugan Y, Dogru A, Sencan H, Sahin M, Ercan Tunc S. Sacroiliitis and polyarteritis nodosa in a patient with familial mediterranean fever. *Case Rep Med*. (2016) 2016:5134546. doi: 10.1155/2016/5134546
206. Jain A, Misra DP, Sharma A, Wakhlu A, Agarwal V, Negi VS. Vasculitis and vasculitis-like manifestations in monogenic autoinflammatory syndromes. *Rheumatol Int*. (2018) 38:13–24. doi: 10.1007/s00296-017-3839-6
207. Imirzalioglu N, Dursun A, Tastan B, Soysal Y, Yakicier MC. MEFV gene is a probable susceptibility gene for Behcet's disease. *Scand J Rheumatol*. (2005) 34:56–8. doi: 10.1080/03009740510017931
208. Schwartz T, Langevitz P, Zemer D, Gazit E, Pras M, Livneh A. Behcet's disease in Familial Mediterranean fever: characterization of the association between the two diseases. *Semin Arthritis Rheum*. (2000) 29:286–95. doi: 10.1016/S0049-0172(00)80015-3

209. Touitou I, Magne X, Molinari N, Navarro A, Quellec AL, Picco P, et al. MEFV mutations in Behcet's disease. *Hum Mutat*. (2000) 16:271–2. doi: 10.1002/1098-1004(200009)16:3<271::AID-HUMU16>3.0.CO;2-A
210. Wu Z, Zhang S, Li J, Chen S, Li P, Sun F, et al. Association between MEFV mutations M694V and M680I and behcet's disease: A Meta-Analysis. *PLoS ONE*. (2015) 10:e0132704. doi: 10.1371/journal.pone.0132704
211. Ebrahimi-Fakhari D, Wahlster L, Mackensen F, Blank N. Clinical manifestations and longterm followup of a patient with CINCA/NOMID syndrome. *J Rheumatol*. (2010) 37:2196–7. doi: 10.3899/jrheum.100290
212. Kolivras A, Theunis A, Ferster A, Lipsker D, Sass U, Dussart A, et al. Cryopyrin-associated periodic syndrome: an autoinflammatory disease manifested as neutrophilic urticarial dermatosis with additional perieccrine involvement. *J Cutan Pathol*. (2011) 38:202–8. doi: 10.1111/j.1600-0560.2010.01638.x
213. Russo RA, Katsicas MM. Chronic infantile neurological cutaneous and articular syndrome: two new cases with rare manifestations. *Acta Paediatr*. (2001) 90:1076–9. doi: 10.1080/080352501316978192
214. Khemani C, Khubchandani R. CINCA Syndrome. *Indian Pediatr*. (2007) 44:933–6.
215. Boom BW, Daha MR, Vermeer BJ, van der Meer JW. IgD immune complex vasculitis in a patient with hyperimmunoglobulinemia D and periodic fever. *Arch Dermatol*. (1990) 126:1621–4. doi: 10.1001/archderm.126.12.1621
216. Drenth JP, Boom BW, Toonstra J, Van der Meer JW. Cutaneous manifestations and histologic findings in the hyperimmunoglobulinemia D syndrome. International Hyper IgD Study Group. *Arch Dermatol*. (1994) 130:59–65. doi: 10.1001/archderm.130.1.59
217. Topaloglu R, Saatci U. Hyperimmunoglobulinaemia D and periodic fever mimicking familial Mediterranean fever in the Mediterranean. *Postgrad Med J*. (1991) 67:490–1. doi: 10.1136/pgmj.67.787.490-a
218. van der Meer JW, Vossen JM, Radl J, van Nieuwkoop JA, Meyer CJ, Lobatto S, et al. Hyperimmunoglobulinaemia D and periodic fever: a new syndrome. *Lancet*. (1984) 1:1087–90. doi: 10.1016/S0140-6736(84)92505-4
219. Toro JR, Aksentijevich I, Hull K, Dean J, Kastner DL. Tumor necrosis factor receptor-associated periodic syndrome: a novel syndrome with cutaneous manifestations. *Arch Dermatol*. (2000) 136:1487–94. doi: 10.1001/archderm.136.12.1487
220. Khatibi K, Heit JJ, Telischak NA, Elbers JM, Do HM. Cerebral vascular findings in PAPA syndrome: cerebral arterial vasculopathy or vasculitis and a posterior cerebral artery dissecting aneurysm. *J Neurointerv Surg*. (2016) 8:e29. doi: 10.1136/neurintsurg-2015-011753.rep
221. Niv D, Ramirez JA, Fivenson DP. Pyoderma gangrenosum, acne, and hidradenitis suppurativa (PASH) syndrome with recurrent vasculitis. *JAAD Case Rep*. (2017) 3:70–3. doi: 10.1016/j.jdcr.2016.11.006
222. Chen Y, Li X, Boini KM, Pitzer AL, Gulbins E, Zhang Y, et al. Endothelial Nlrp3 inflammasome activation associated with lysosomal destabilization during coronary arteritis. *Biochim Biophys Acta*. (2015) 1853:396–408. doi: 10.1016/j.bbamcr.2014.11.012
223. Xia M, Boini KM, Abais JM, Xu M, Zhang Y, Li PL. Endothelial NLRP3 inflammasome activation and enhanced neointima formation in mice by adipokine visfatin. *Am J Pathol*. (2014) 184:1617–28. doi: 10.1016/j.ajpath.2014.01.032
224. Bettiol A, Silvestri E, Di Scala G, Amedei A, Becatti M, Fiorillo C, et al. The right place of interleukin-1 inhibitors in the treatment of Behcet's syndrome: a systematic review. *Rheumatol Int*. (2019) 39:971–90. doi: 10.1007/s00296-019-04259-y
225. Moghaddas F, Masters SL. Monogenic autoinflammatory diseases: cytokinopathies. *Cytokine*. (2015) 74:237–46. doi: 10.1016/j.cyto.2015.02.012
226. Demir S, Sag E, Dedeoglu F, Ozen S. Vasculitis in systemic autoinflammatory diseases. *Front Pediatr*. (2018) 6:377. doi: 10.3389/fped.2018.00377
227. Zhou Q, Wang H, Schwartz DM, Stoffels M, Park YH, Zhang Y, et al. Loss-of-function mutations in TNFAIP3 leading to A20 haploinsufficiency cause an early-onset autoinflammatory disease. *Nat Genet*. (2016) 48:67–73. doi: 10.1038/ng.3459
228. Li H, Liu Q, Hou S, Du L, Zhou Q, Zhou Y, et al. TNFAIP3 gene polymorphisms confer risk for Behcet's disease in a Chinese Han population. *Hum Genet*. (2013) 132:293–300. doi: 10.1007/s00439-012-1250-7
229. Aeschlimann FA, Batu ED, Canna SW, Go E, Gul A, Hoffmann P, et al. A20 haploinsufficiency (HA20): clinical phenotypes and disease course of patients with a newly recognised NF-kB-mediated autoinflammatory disease. *Ann Rheumat Dis*. (2018) 77:728–35. doi: 10.1136/annrheumdis-2017-212403
230. Zhou Q, Yu X, Demirkaya E, Deuitch N, Stone D, Tsai WL, et al. Biallelic hypomorphic mutations in a linear deubiquitinase define otulipenia, an early-onset autoinflammatory disease. *Proc Natl Acad Sci USA*. (2016) 113:10127–32. doi: 10.1073/pnas.1612594113
231. Hou S, Shu Q, Jiang Z, Chen Y, Li F, Chen F, et al. Replication study confirms the association between UBAC2 and Behcet's disease in two independent Chinese sets of patients and controls. *Arthritis Res Ther*. (2012) 14:R70. doi: 10.1186/ar3789
232. Hou S, Kijlstra A, Yang P. The genetics of Behcet's disease in a Chinese population. *Front Med*. (2012) 6:354–9. doi: 10.1007/s11684-012-0234-2
233. Park G, Kim HS, Choe JY, Kim SK. SUMO4 C438T polymorphism is associated with papulopustular skin lesion in Korean patients with Behcet's disease. *Rheumatol Int*. (2012) 32:3031–7. doi: 10.1007/s00296-011-2086-5
234. Crow YJ, Chase DS, Lowenstein Schmidt J, Szynkiewicz M, Forte GM, Gornall HL, et al. Characterization of human disease phenotypes associated with mutations in TREX1, RNASEH2A, RNASEH2B, RNASEH2C, SAMHD1, ADAR, and IFIH1. *Am J Med Genet A*. (2015) 167A:296–312. doi: 10.1055/s-0036-1592307
235. Liu Y, Jesus AA, Marrero B, Yang D, Ramsey SE, Sanchez GAM, et al. Activated STING in a vascular and pulmonary syndrome. *N Engl J Med*. (2014) 371:507–18. doi: 10.1056/NEJMoa1312625
236. Schneider WM, Chevillotte MD, Rice CM. Interferon-stimulated genes: a complex web of host defenses. *Annu Rev Immunol*. (2014) 32:513–45. doi: 10.1146/annurev-immunol-032713-120231
237. Munoz J, Rodiere M, Jeremiah N, Rieux-Laucat F, Oojageer A, Rice GI, et al. Stimulator of interferon genes-associated vasculopathy with onset in infancy: a mimic of childhood granulomatosis with polyangiitis. *JAMA Dermatol*. (2015) 151:872–7. doi: 10.1001/jamadermatol.2015.0251
238. Torrelo A, Colmenero I, Requena L, Paller AS, Ramot Y, Richard Lee CC, et al. Histologic and Immunohistochemical Features of the Skin Lesions in CANDLE Syndrome. *Am J Dermatopathol*. (2015) 37:517–22. doi: 10.1097/DAD.0000000000000340
239. Sanchez GAM, Reinhardt A, Ramsey S, Wittkowski H, Hashkes PJ, Berkun Y, et al. JAK1/2 inhibition with baricitinib in the treatment of autoinflammatory interferonopathies. *J Clin Invest*. (2018) 128:3041–52. doi: 10.1172/JCI98814
240. Aksentijevich I, Masters SL, Ferguson PJ, Dancey P, Frenkel J, van Royen-Kerkhoff A, et al. An autoinflammatory disease with deficiency of the interleukin-1-receptor antagonist. *N Engl J Med*. (2009) 360:2426–37. doi: 10.1056/NEJMoa0807865
241. Matsuki T, Isoda K, Horai R, Nakajima A, Aizawa Y, Suzuki K, et al. Involvement of tumor necrosis factor-alpha in the development of T cell-dependent aortitis in interleukin-1 receptor antagonist-deficient mice. *Circulation*. (2005) 112:1323–31. doi: 10.1161/CIRCULATIONAHA.105.564658

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.

frontiers in Immunology

ORIGINAL RESEARCH
published: 10 March 2020
doi: 10.3389/fimmu.2020.00358

Cytokine Profiling in Aqueous Humor Samples From Patients With Non-Infectious Uveitis Associated With Systemic Inflammatory Diseases

Martina Bonacini[1†], Alessandra Soriano[2†], Luca Cimino[3], Luca De Simone[3], Elena Bolletta[3], Fabrizio Gozzi[3], Francesco Muratore[2], Maria Nicastro[1], Lucia Belloni[1], Alessandro Zerbini[1], Luigi Fontana[4], Carlo Salvarani[2,5‡] and Stefania Croci[1‡]*

[1] Clinical Immunology, Allergy and Advanced Biotechnologies Unit, Azienda Unità Sanitaria Locale-IRCCS di Reggio Emilia, Reggio Emilia, Italy, [2] Rheumatology Unit, Azienda Unità Sanitaria Locale-IRCCS di Reggio Emilia, Reggio Emilia, Italy, [3] Ocular Immunology Unit, Azienda Unità Sanitaria Locale-IRCCS di Reggio Emilia, Reggio Emilia, Italy, [4] Ophthalmology Unit, Azienda Unità Sanitaria Locale-IRCCS di Reggio Emilia, Reggio Emilia, Italy, [5] Department of Surgery, Medicine, Dentistry and Morphological Sciences, With Interest in Transplants, Oncology and Regenerative Medicine, University of Modena and Reggio Emilia, Modena, Italy

OPEN ACCESS

Edited by:
Rudolf Lucas, Augusta University, United States

Reviewed by:
Alfredo Adan, Hospital Clínic de Barcelona, Spain
Svetlana Khaiboullina, University of Nevada, Reno, United States
Justus G. Garweg, Lindenhof Hospital, Switzerland

***Correspondence:**
Stefania Croci
stefania.croci@ausl.re.it

[†] These authors have contributed equally to this work

[‡] These authors share senior authorship

Specialty section:
This article was submitted to Inflammation, a section of the journal Frontiers in Immunology

Received: *24 November 2019*
Accepted: *14 February 2020*
Published: *10 March 2020*

Citation:
Bonacini M, Soriano A, Cimino L, De Simone L, Bolletta E, Gozzi F, Muratore F, Nicastro M, Belloni L, Zerbini A, Fontana L, Salvarani C and Croci S (2020) Cytokine Profiling in Aqueous Humor Samples From Patients With Non-Infectious Uveitis Associated With Systemic Inflammatory Diseases. Front. Immunol. 11:358. doi: 10.3389/fimmu.2020.00358

Non-infectious uveitis are intraocular inflammatory conditions caused by dysregulated activation of the immune response without any detectable infectious agents. The aim of this study was to explore potential markers and therapeutic targets for two distinct types of non-infectious uveitis associated with Behçet's disease (BD) and Vogt Koyanagi Harada (VKH) disease. Concentrations of 27 cytokines were investigated in aqueous humor (AH) samples from patients with active uveitis vs. healthy controls (HC) ($n = 10$ patients with BD-associated uveitis; $n = 10$ patients with VKH-associated uveitis; $n = 10$ HC) using the Bio-Plex Pro^{TM} human cytokine group I panel. Additionally, leukocytes in AH samples were counted with hemocytometers and characterized by flow cytometry. Eleven cytokines were differentially expressed between patients with uveitis and HC with a median concentration greater than 10 pg/ml. IL-6, IP-10, G-CSF, and IFNγ showed higher concentrations in AH samples from both BD and VKH patients while IL-2, IL-8, IL-13, TNFα, eotaxin, IL-1ra showed statistically significant higher concentrations only in AH samples from BD patients. GM-CSF was the sole cytokine with an opposite profile showing decreased levels in AH samples from BD patients. IL-1ra and IL-6 were detected at higher frequencies in AH samples from BD and VKH patients compared with those from HC while IFNγ and TNFα were not detected in HC. The concentrations of IL-6, IL-8, IP-10, G-CSF, IFNγ, TNFα, eotaxin, IL-1ra positively correlated with the concentrations of leukocytes in AH, suggesting that such cytokines can be produced by immune cells and/or attract and/or promote proliferation and survival of immune cells in these types of uveitis. The correlation matrix of cytokine concentrations in AH samples revealed that IFNγ, TNFα, eotaxin, IL-6, G-CSF highly correlated each other. The ratios of cytokine concentrations between AH and plasma intra-individuals showed that IL-2, IL-6, IP-10, GM-CSF were increased intraocularly. In conclusion, AH sampling followed by multiplex analysis of cytokines should be fostered in non-infectious uveitis to identify cytokines dysregulated intraocularly in each individual laying the groundwork for precision medicine.

Keywords: uveitis, cytokines, vasculitis, precision medicine, Behçet's disease, Vogt Koyanagi Harada disease, aqueous humor

INTRODUCTION

Uveitis are fearsome forms of intraocular inflammation, potentially causing visual impairment and blindness without a prompt diagnosis and therapy. The incidence is low, estimated at 17–52/100,000 people per year. Nevertheless, they account for ∼10% of blindness worldwide (1). Uveitis can derive either from infectious agents (infectious uveitis) or from aberrant, deregulated activation of immune system (e.g., against self-proteins, environmental triggers, tissue damage) in absence of any detectable infectious agents (non-infectious uveitis). An etiological diagnosis of non-infectious uveitis is possible in most cases, from 47 to 75% depending on the studies and they are frequently associated with systemic immune-mediated diseases (2–4).

The management of infectious uveitis requires specific treatments against the infectious agents. On the contrary, the management of non-infectious uveitis represents a challenge for clinicians due to disease heterogeneity, still scarce knowledge on disease pathogenesis, and paucity of randomized controlled trials assessing the real efficacy of available drugs (5). Current guidelines for non-infectious uveitis are based on non-specific immunosuppression. Corticosteroids are recommended as first-line therapy, followed by immunosuppressive drugs in case of relapses, and with steroid-sparing purposes (6). Traditional immunosuppressive agents such as azathioprine, cyclosporine A, or mycophenolate mofetil are usually preferred in the case of posterior uveitis (7). Biological agents (e.g., those inhibiting the TNFα signaling and recombinant IFNα) are currently part of the therapeutic armamentarium (5, 8–10). In the incoming era of precision medicine, tailored treatment of uveitis remains to be defined (11).

Successful therapeutic strategies in non-infectious uveitis require to act on patients' immune responses. The identification of specific immune effectors associated to and/or responsible for the different types of uveitis is necessary in order to select the most promising among the available targeted-therapies or design new targeted-therapies on an individual basis. Immune profiling of patients with different non-infectious uveitis has indeed highlighted some immune effectors (e.g., cytokines, chemokines, immune cell subsets) in aqueous humor (AH) and peripheral blood possibly involved in uveitis pathogenesis and that may allow to differentiate the various types of uveitis (12, 13). These immune effectors could be exploited to implement precision medicine for treatment of non-infectious uveitis. However, further drivers of uveitis pathogenesis need to be discovered and data from single-center studies should be confirmed by other independent studies.

In this study we investigated the concentrations of 27 cytokines in AH from patients affected by two distinct types of non-infectious uveitis, associated with systemic inflammatory diseases: Behçet's disease (BD) and Vogt Koyanagi Harada (VKH) disease, both in active phase, to provide potential markers and therapeutic targets.

BD is a chronic systemic inflammatory vasculitis of unknown etiology characterized by recurrent episodes of oral aphthous ulcers, genital ulcers, non-granulomatous uveitis, retinal vasculitis, skin lesions, and other manifestations (14). VKH is a systemic autoimmune disease characterized by bilateral granulomatous panuveitis with or without auditory, neurological, and cutaneous manifestations, partly attributed to immune responses directed against one or more antigens expressed by melanocytes and retinal pigment epithelium (15). Both BD and VKH are rare diseases.

MATERIALS AND METHODS

Cohorts of Patients and Healthy Controls

This is an exploratory, monocentric, independent study performed at the Azienda Unità Sanitaria Locale-IRCCS, Reggio Emilia, Italy, one of the national reference centers for BD. A cohort of 10 patients with BD-related uveitis and a cohort of 10 patients with VKH-related uveitis, both in active disease phase were enrolled. A cohort of 10 Caucasian subjects who underwent phacoemulsification intervention for cataract ($n = 6$) and cornea surgery ($n = 4$), not affected by any other concomitant inflammatory and/or infectious diseases nor with prior history of uveitis were recruited as healthy controls (HC). All BD patients satisfied the 1990 criteria for diagnosis of Behçet's disease (16). Diagnosis of VKH was based on the revised international diagnostic criteria (17). The median age for the BD cohort was 30 years (InterQuartile Range; IQR: 25–43) and gender distribution was: 8/10 males and 2/10 females. The median age for the VKH cohort was 47 years (IQR: 36–63) and gender distribution was 1/10 male and 9/10 females. The median age for the HC cohort was 64 years (IQR: 40–79) and gender distribution was: 6/10 males and 4/10 females.

Patients were examined with slit lamp biomicroscopy, indirect ophthalmoscopy, retinography, optical coherence tomography, fluorescein and indocyanine angiography. Patients with BD were considered as having active uveitis at ophthalmologic evaluation in case of non-granulomatous panuveitis with vitritis, or macular oedema, or occlusive retinal vasculitis, or retinal ischemia, or retinal and/or optic nerve neovascularization. Patients with VKH were considered as having active uveitis at ophthalmologic evaluation in case of granulomatous panuveitis with bilateral papillitis and exudative retinal neuroepithelium detachment with mild or absent vitreitis and choroidal granulomas. The features of uveitis for each patient are shown in **Table 1**. 5/10 BD patients and 2/10 VKH patients were receiving therapies at the moment of sample collection. The study was approved by the local ethics committee (Reggio Emilia, Italy, protocol number 2015/0024354) in compliance with the declaration of Helsinki and written informed consent was obtained from all patients and HC.

Biological Sample Collection

Samples of AH (100–200 μl) were obtained by anterior chamber paracentesis (18) conducted under surgical microscope. Ethylenediaminetetraacetic acid (EDTA) was added at 2 mM to prevent cell aggregation. Cell concentrations were determined by manual counting in Neubauer hemocytometers. AH samples were then centrifuged at 400 × g for 8 min. Cell pellets were analyzed by flow cytometry while cell-free supernatants were stored frozen at −80°C until cytokine profiling. Six milliliter of venous blood were collected from BD and VKH patients

TABLE 1 | Clinical characteristics of patients with BD and VKH-associated uveitis.

Patients	Therapy	Anatomic location	Type of uveitis	Macular edema	Keratic precipitates	Vitreitis	Papillitis	Diffuse capillaritis	Exudative retinal detachment	Choroidal lesions	Vessel sheathing
BD#1	No	Bilateral Panuveitis	Non-granulomatous	Yes	Yes	2	No	Yes	No	No	Yes
BD#2	Yes	Bilateral Panuveitis	Non-granulomatous	Yes	Yes	1	Yes	Yes	No	No	Yes
BD#3	Yes	Bilateral Panuveitis	Non-granulomatous	Yes	Yes	1	Yes	Yes	No	No	No
BD#4	Yes	Unilateral Panuveitis	Non-granulomatous	Yes	Yes	1	Yes	Yes	No	No	No
BD#5	Yes	Bilateral Panuveitis	Non-granulomatous	Yes	No	1	No	Yes	No	No	No
BD#6	No	Bilateral Panuveitis	Non-granulomatous	Yes	Yes	3	Yes	Yes	No	No	No
BD#7	No	Bilateral Panuveitis	Non-granulomatous	No	Yes	1	Yes	Yes	Yes	No	Yes
BD#8	Yes	Bilateral Panuveitis	Non-granulomatous	No	Yes	1	No	Yes	No	No	No
BD#9	No	Unilateral Panuveitis	Non-granulomatous	No	Yes	0,5	No	Yes	No	No	No
BD#10	No	Bilateral Panuveitis	Non-granulomatous	No	Yes	1	Yes	Yes	No	No	No
VKH#1	No	Bilateral Panuveitis	Granulomatous	No	No	No	Yes	No	Yes	Yes (5)	No
VKH#2	Yes	Bilateral Panuveitis	Granulomatous	No	No	No	Yes	No	Yes	Yes (5)	No
VKH#3	No	Bilateral Panuveitis	Granulomatous	No	No	No	Yes	No	Yes	Yes (5)	No
VKH#4	No	Bilateral Panuveitis	Granulomatous	No	No	No	Yes	No	Yes	Yes (5)	No
VKH#5	No	Bilateral Panuveitis	Granulomatous	Yes	No	No	Yes	No	Yes	No	No
VKH#6	No	Bilateral Panuveitis	Granulomatous	Yes	No	No	Yes	No	Yes	Yes (5)	No
VKH#7	No	Bilateral Panuveitis	Granulomatous	No	No	No	Yes	No	Yes	Yes (5)	No
VKH#8	No	Bilateral Panuveitis	Granulomatous	No	No	No	Yes	No	Yes	No	No
VKH#9	No	Bilateral Panuveitis	Granulomatous	No	No	No	Yes	No	Yes	Yes (5)	No
VKH#10	Yes	Bilateral Panuveitis	Granulomatous	No	Yes	No	Yes	No	Yes	Yes (5)	No

Hypopyon was present only in BD#6. Retinitis was present only in BD#7 and BD#8. Snowbank was present only in BD#2. Snowballs were not present.

into EDTA coated tubes. Plasma samples were obtained by centrifugation at 1,300 × g for 20 min and stored at −80°C until use. Biosafety level 2 procedures were applied when working with patients' samples.

Flow Cytometry

AH cells were suspended in 300 μL phosphate-buffered saline (PBS, Euroclone) + 1% heat inactivated fetal bovine serum (FBS, Thermo Fisher) and acquired with the FACSCanto II flow cytometer (BD Biosciences), equipped with two lasers for excitation at 488 and 633 nm. Data were analyzed with FACSDiva 8.0.1. software. Lymphocytes, monocytes, and granulocytes were identified according to the forward scatter (FSC) and side scatter (SSC) as shown in **Figure S1**.

Cytokine Profiling

Concentrations of IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17A, eotaxin, basic FGF, G-CSF, GM-CSF, IFNγ, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF-BB, RANTES, TNFα, and VEGF were determined in AH samples using the Bio-Plex Pro™ human cytokine group I panel, 27-Plex (Bio-Rad) following the manufacturer's instruction. Cell-free AH samples were centrifuged at 10,000 × g for 10 min at 4°C and then diluted 4-fold in Bio-Plex sample diluent adding bovine serum albumin (BSA) at 0.5% as recommended for samples with low content of albumin. Plasma samples were centrifuged at 10,000 × g for 10 min at 4°C then were diluted 4-fold in Bio-Plex sample diluent. Eight serial dilutions of cytokine standards plus blanks (diluent) were included in each assay. Data were obtained with Bio-Plex MAGPIX™ multiplex reader instrument and analyzed with Bio-Plex Manager™ software. Standard curves were calculated with the five-parameter logistic equation regression method. Values extrapolated from the standard curves were considered not reliable and concentrations = 0.01 pg/ml were arbitrarily assigned to be able to draw graphs with logarithmic axes. The lower limits of cytokine detection are reported in **Table S1**.

Clustering and Pathway Analysis

Clustering was performed using the ClustVis software (19) consisting of the following data pre-processing: (1) logarithmic transformation [ln (x+1)]; (2) row centering; (3) no scaling and applying the Euclidean complete distance for rows and columns. Pathway analysis was performed using REACTOME and PANTHER analysis software (reactome.org; pantherdb.org).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6 software. In order to compare two groups, non-parametric Mann-Whitney U-test was used for quantitative variables, while Fisher's exact test was used for qualitative variables. The Kruskal-Wallis test with Dunn's correction for multiple comparisons was used to compare cytokine concentrations in AH samples. Spearman's correlation was chosen to determine correlations between quantitative variables followed by the Bonferroni

TABLE 2 | Concentrations of cytokines in AH from BD patients, VKH patients and HC.

	Concentration (pg/mL)			*P*-value		
	BD $n = 10$	**VKH** $n = 10$	**HC** $n = 10$	**BD vs. HC**	**VKH vs. HC**	**BD vs. VKH**
IL-1β	0.01 (0.01–12.63)	0.01 (0.01–0.85)	n.d.	n.s.	n.s.	n.s.
IL-1ra	185.80 (31.64–534)	46.99 (24.83–74.54)	0.01 (0.01–39.96)	0.0053	n.s.	n.s.
IL-2	13.32 (9.16–33.60)	9.88 (7.96–11.69)	8.32 (7.13–9.83)	0.0384	n.s.	n.s.
IL-4	1.24 (0.01–6.97)	0.60 (0.01–1.53)	n.d.	0.0118	n.s.	n.s.
IL-5	0.01 (0.01–8.42)	0.01 (0.01–1.18)	n.d.	n.s.	n.s.	n.s.
IL-6	812.2 (107.5–32478)	333.70 (33.13–2303)	6.79 (0.01–27.66)	0.0004	0.0153	n.s.
IL-7	14.43 (0.01–28.99)	5.00 (0.01–15.46)	0.01 (0.01–4.67)	n.s.	n.s.	n.s.
IL-8	69.46 (15.48–532.3)	24.15 (9.18–97.47)	6.53 (0.01–33.14)	0.0088	n.s.	n.s.
IL-9	8.36 (2.85–10.65)	2.85 (0.01–8.50)	1.23 (0.01–4.92)	n.s.	n.s.	n.s.
IL-10	14.41 (10.51–23.84)	10.43 (8.75–14.57)	10.39 (8.71–12.69)	n.s.	n.s.	n.s.
IL-12 (p70)	27.36 (13.82–54.45)	15.40 (8.80–27.85)	18.15 (0.01–32.33)	n.s.	n.s.	n.s.
IL-13	10.43 (2.66–14.04)	5.06 (2.08–9.46)	1.87 (1.11–3.05)	0.0263	n.s.	n.s.
IL-15	0.01 (0.01–108.9)	12.12 (0.01–70.30)	47.45 (0.01–65.57)	n.s.	n.s.	n.s.
IL-17A	17.00 (13.16–69.00)	20.83 (11.57–28.16)	22.03 (13.45–27.81)	n.s.	n.s.	n.s.
Eotaxin	16.27 (6.63–70.58)	13.83 (0.01–24.22)	0.01 (0.01–9.13)	0.0260	n.s.	n.s.
Basic FGF	85.24 (57.83–127.80)	65.29 (34.83–88.79)	84.37 (70.57–106.40)	n.s.	n.s.	n.s.
G-CSF	85.19 (0.01–1638)	46.17 (6.48–393.40)	0.01 (0.01–3.31)	0.0176	0.0305	n.s.
GM-CSF	209.30 (175.40–244.10)	230.80 (133.30–683.40)	438.40 (299.10–709.50)	0.0072	n.s.	n.s.
IFNγ	54.82 (9.26–574.70)	41.46 (0.01–66.48)	n.d.	0.0011	0.0321	n.s.
IP-10	10,311 (3,172–155,581)	2,400 (724.3–10,990)	123.20 (31.59–214.80)	0.0001	0.0063	n.s.
MCP-1	397.6 (128.9–2,306)	323.6 (167.60–626.40)	235.20 (114.40–420.30)	n.s.	n.s.	n.s.
MIP-1α	2.04 (0.01–6.81)	1.69 (0.01–2.56)	0.01 (0.01–0.87)	0.0407	n.s.	n.s.
MIP-1β	41.63 (9.13–124.90)	19.06 (14.5–29.78)	35.23 (8.16–61.61)	n.s.	n.s.	n.s.
PDGF-BB	8.54 (0.01–26.34)	2.24 (0.01–10.22)	0.01 (0.01–2.81)	n.s.	n.s.	n.s.
RANTES	7.63 (0.01–48.72)	0.01 (0.01–0.01)	0.01 (0.01–17.24)	n.s.	n.s.	n.s.
TNFα	20.48 (0.01–268.80)	17.74 (0.01–46.59)	n.d.	0.0055	n.s.	n.s.
VEGF	58.44 (34.66–116.70)	49.90 (26.39–85.33)	65.62 (28.95–108.60)	n.s.	n.s.	n.s.

n.d., non detectable; n.s., not significant. Cytokines with concentrations below the lower limits of detection were considered as non-detected and concentrations = 0.01 pg/ml were arbitrarily assigned. Data were analyzed with the Kruskal-Wallis test with Dunn's correction for multiple comparisons. Median concentrations with interquartile ranges (in brackets) are shown.

correction for multiple testing. $P < 0.05$ (two-tailed) were considered statistically significant.

RESULTS

Cytokine Concentrations in AH Samples From BD and VKH Patients Compared to HC

To better understand ocular inflammatory *milieu* of patients with non-infectious uveitis, concentrations of 27 cytokines were determined in AH samples from patients with BD and VKH-associated uveitis in comparison with HC.

IL-6, IP-10, G-CSF, and IFNγ showed higher concentrations in AH samples from both BD and VKH patients (**Table 2** and **Figure 1A**). IL-6 and IP-10 were detected in all the patients' samples: IL-6 had 116-fold difference between BD and HC ($p = 0.0004$) and 48-fold difference between VKH and HC ($p = 0.0153$); IP-10 had 83 fold difference between BD and HC ($p = 0.0001$) and 20-fold difference between VKH and HC ($p = 0.0063$). Moreover, IL-2, IL-4, IL-8, IL-13, TNFα, MIP-1α, eotaxin, IL-1ra showed statistically significant higher concentrations in AH samples from BD patients compared to HC (**Table 2** and **Figure 1B**). GM-CSF was the unique cytokine with an opposite profile, revealing lower concentrations in AH samples from BD patients compared to HC (**Table 2**). AH samples from VKH patients showed a bimodal distribution regarding GM-CSF and 6/10 patients had concentrations lower than HC (**Figure 1C**).

IL-1β, IL-5, IL-7, IL-9, IL-10, IL-12p70, IL-15, IL-17, MCP-1, MIP-1β, PDGF-BB, RANTES, VEGF, basic FGF showed comparable levels among groups (**Table 2** and **Figure S2**). Moreover, no differences in AH cytokine levels were found between BD and VKH patients (**Table 2**).

It must be underlined that some patients were receiving immunosuppressive therapies at the moment of AH collection despite the presence of active uveitis. To investigate if therapies affected cytokine concentrations, we compared AH samples

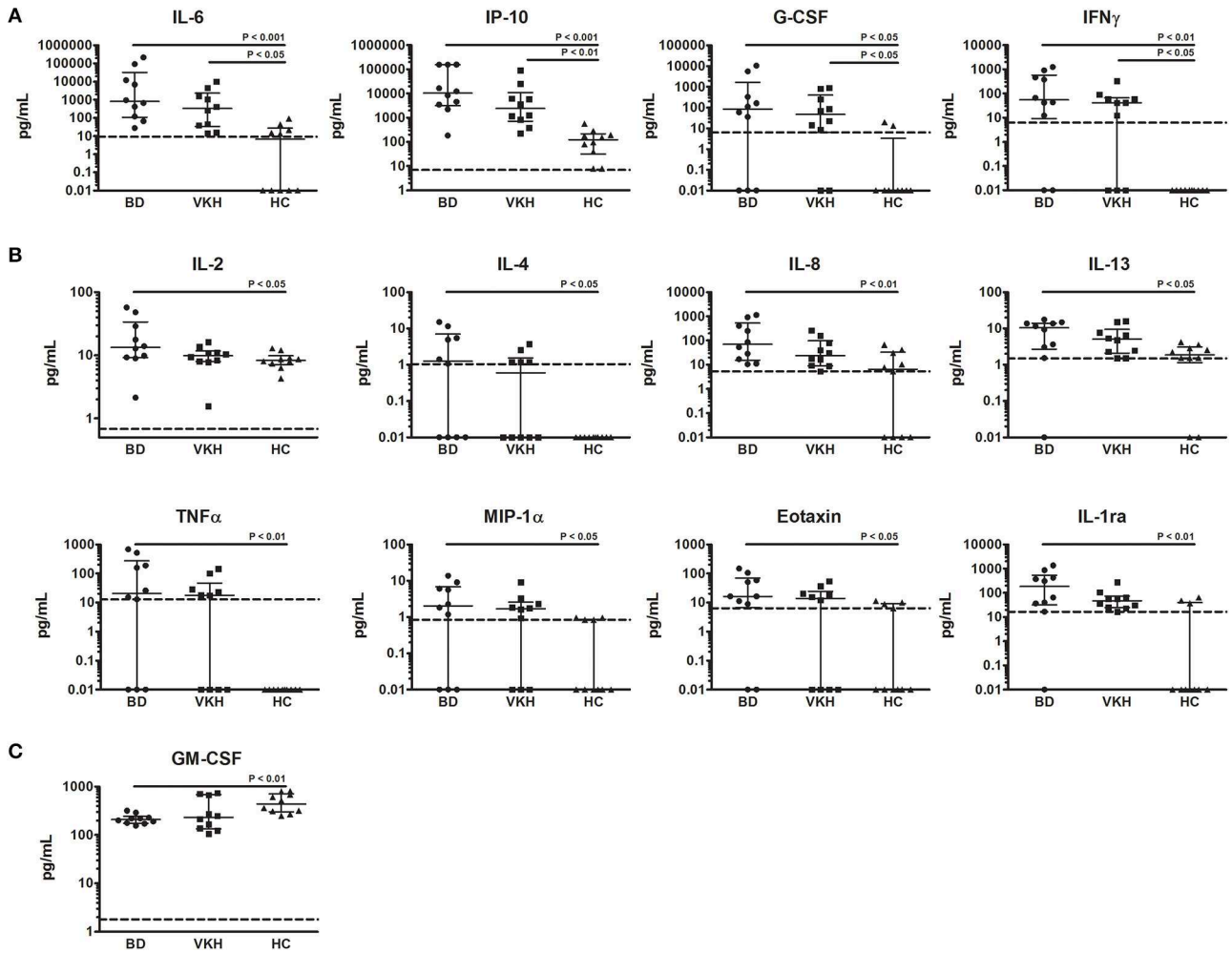


FIGURE 1 | Cytokines differentially expressed in AH from patients with BD- and VKH-associated uveitis compared to HC. Dot plot visualization of cytokine concentrations (pg/ml) in AH from patients with BD ($n = 10$), VKH ($n = 10$), and HC ($n = 10$). **(A)** Cytokines expressed at higher levels both in BD and VKH. **(B)** Cytokines expressed at higher levels only in BD. **(C)** Cytokines expressed at lower levels in BD. Horizontal lines show the median ± IQR. Dotted lines indicate the lower limits of cytokine detection. Data were analyzed by the Kruskal-Wallis test with Dunn's correction for multiple comparisons.

from patients with and without therapies. This analysis was feasible only in the cohort of BD patients which included 5 patients under therapies and 5 patients naïve from therapies (**Table 1**). MCP-1, IL-7, IL-8, G-CSF, MIP-1α, MIP-1α, TNFα, IFNγ showed higher concentrations and GM-CSF showed lower concentrations in AH from patients without therapies (**Figure S3**) but the statistical significances were not maintained after correction for multiple testing.

To investigate if some cytokines were detected more frequently in AH from patients than from HC, the percentages of AH samples with detectable levels of cytokines were compared among the cohorts. IL-1ra, IL-4, IL-6, IFNγ, and TNFα were detected at higher frequencies in AH samples from BD and VKH patients compared with those from HC (**Table 3**). In particular, IL-4, IFNγ and TNFα were not detected in any of the samples from HC.

No differences in terms of frequencies of cytokine detection in AH samples were found between BD and VKH patients (**Table 3**).

Subsequent analyses were focused on the 11 cytokines which resulted differentially expressed between patients with uveitis and HC and, to have a higher degree of confidence, which showed median concentrations greater than 10 pg/ml. To identify cytokines with similar profiles, we calculated the correlation matrix of cytokine concentrations in the AH samples. IFNγ, TNFα, eotaxin, IL-6, G-CSF highly correlated each other ($p < 10^{-6}$) (**Table 4**).

To investigate which cytokines had higher levels in the ocular environment, cytokine concentrations in AH were compared to those in plasma intra-individuals. GM-CSF and IL-6 showed higher levels in AH from all the patients, IL-2 from 19/20 patients and IP-10 from 16/20 patients. Specifically, IL-2 was detected only in AH samples from 18 patients and IL-6 was detected only in AH samples from 12 patients. On the contrary, eotaxin showed lower levels in AH from 16/20 patients and was detected only in plasma from 6 patients. The other cytokines showed heterogeneous patterns (**Table 5**). Looking at the AH

over plasma cytokine profiles across patients, BD patient#9 displayed higher concentrations in AH of all the cytokines while BD patient#2, BD patient#7 and VKH patient#7 showed higher concentrations in AH of 10/11 cytokines with the exception of eotaxin (**Table 5**).

TABLE 3 | Presence of cytokines in AH from BD patients, VKH patients and HC.

	Positive sample (fraction)			Fisher test (*p*-value)		
	BD $n = 10$	VKH $n = 10$	HC $n = 10$	BD vs. HC	VKH vs. HC	BD vs. VKH
IL-1β	4/10	2/10	0/10	n.s.	n.s.	n.s.
IL-1ra	9/10	10/10	3/10	0.0198	0.0031	n.s.
IL-2	10/10	10/10	10/10	n.s.	n.s.	n.s.
IL-4	6/10	5/10	0/10	0.0108	0.0325	n.s.
IL-5	4/10	2/10	0/10	n.s.	n.s.	n.s.
IL-6	10/10	10/10	5/10	0.0325	0.0325	n.a.
IL-7	7/10	6/10	3/10	n.s.	n.s.	n.s.
IL-8	10/10	10/10	6/10	n.s.	n.s.	n.a.
IL-9	9/10	7/10	5/10	n.s.	n.s.	n.s.
IL-10	9/10	10/10	9/10	n.s.	n.s.	n.s.
IL-12 (p70)	8/10	9/10	7/10	n.s.	n.s.	n.s.
IL-13	9/10	10/10	8/10	n.s.	n.s.	n.s.
IL-15	4/10	5/10	7/10	n.s.	n.s.	n.s.
IL-17A	10/10	9/10	10/10	n.a.	n.s.	n.s.
Eotaxin	8/10	6/10	4/10	n.s.	n.s.	n.s.
Basic FGF	10/10	10/10	10/10	n.a.	n.a.	n.a.
G-CSF	7/10	8/10	2/10	n.s.	0.0230	n.s.
GM-CSF	10/10	10/10	10/10	n.a.	n.a.	n.a.
IFNγ	8/10	7/10	0/10	0.0007	0.0031	n.s.
IP-10	10/10	10/10	10/10	n.a.	n.a.	n.a.
MCP-1	10/10	10/10	10/10	n.a.	n.a.	n.a.
MIP-1α	7/10	7/10	4/10	n.s.	n.s.	n.s.
MIP-1β	10/10	10/10	10/10	n.a.	n.a.	n.a.
PDGF-BB	6/10	5/10	3/10	n.s.	n.s.	n.s.
RANTES	5/10	1/10	4/10	n.s.	n.s.	n.s.
TNFα	7/10	6/10	0/10	0.0031	0.0108	n.s.
VEGF	9/10	9/10	9/10	n.s.	n.s.	n.s.

n.a., not applicable; n.s., not significant. The numbers of samples in which cytokine concentrations were equal to or higher than the respective lower limits of detection are shown.

Correlation Between Cytokine Concentrations and Loads of Inflammatory Cells in AH Samples

Concentrations of leukocytes in AH samples were assessed by manual counting with hemocytometers while concentrations of lymphocytes, monocytes and granulocytes (neutrophils) were semi-quantitatively estimated applying their percentages obtained by flow cytometry (**Figure S1**). Samples were heterogeneous in terms of concentrations of infiltrating leukocytes and two samples did not show any cells (**Table 6**). The median concentration of leukocytes in AH from BD patients did not differ from that of VKH patients (24,700 cells/ml, IQR: 470–63,375 vs. 3,750 cells/ml, IQR: 1,004–16,050). Concentrations of IL-6, IL-8, IP-10, G-CSF, IFNγ, TNFα, eotaxin, IL-1ra positively correlated with concentrations of leukocytes. Among them, IP-10 and IL-8 showed the best correlations ($p = 0.0011$, $r = 0.82$ and $r = 0.76$). Instead IL-2, IL-13, and GM-CSF levels did not correlate with leukocyte concentrations (**Figure 2**).

Correlation analyses between cytokine concentrations and leukocyte subsets (**Table 6**) showed that IL-6, IL-8, IP-10, G-CSF, IFNγ, TNFα, eotaxin, IL-1ra concentrations positively correlated also with the semi-quantitative degrees of lymphocytes/ml, monocytes/ml and neutrophils/ml in AH. Higher correlations (lower *p*-values and higher *r* values) were found with the degrees of monocytes and neutrophils compared to those of lymphocytes (**Figures S4–S6**). In particular, the best correlations ($p = 0.0011$, $r = 0.75$) were shown by IL-8 and IFNγ with the semi-quantitative densities of neutrophils/ml.

Finally, cytokine concentrations did not correlate with the percentages of lymphocytes, monocytes and neutrophils in AH (data not shown).

Unsupervised Cluster Analysis and Pathway Analysis

To explore possible clustering of the subjects based on the 27 cytokine profiles in AH samples, unsupervised cluster analysis was performed (**Figure 3**). Subjects were clustered in 2 major groups: the first one composed of all HC plus 7 BD and VKH patients; the second one composed only of BD and VKH patients ($n = 13$). Within such two groups other two clusters appeared. In particular, cluster II contained 9/10 HCs and BD#8 whose

TABLE 4 | Correlation matrix of cytokine concentrations in AH samples from patients with BD- and VKH-associated uveitis.

	IL-1ra	IL-2	IL-6	IL-8	IL-13	Eotaxin	G-CSF	GM-CSF	IFNγ	IP-10
IL-2	0.424308									
IL-6	0.001086	0.568329								
IL-8	0.002796	1.000000	0.000662							
IL-13	1.000000	0.212626	0.896414	1.000000						
Eotaxin	0.008220	0.663621	0.000000	0.005417	0.252160					
G-CSF	0.000338	0.445325	0.000000	0.015374	0.314126	0.000000				
GM-CSF	1.000000	1.000000	0.311010	0.176605	0.019136	0.052681	0.192300			
IFNγ	0.000008	0.184949	0.000000	0.000399	0.252101	0.000000	0.000000	0.122415		
IP-10	0.015567	1.000000	0.079025	0.000099	1.000000	0.020413	0.184789	0.097607	0.006462	
TNFα	0.001325	0.348987	0.000000	0.001020	0.258832	0.000000	0.000000	0.093605	0.000000	0.014286

Spearman's correlation was calculated for every pair of data set. P-values are shown after the Bonferroni correction.

TABLE 5 | Ratios between cytokine concentrations in AH and plasma for each patient.

	IL-6	IP-10	G-CSF	IFNγ	IL-2	IL-8	IL-13	TNFα	Eotaxin	IL-1ra	GM-CSF
BD#1	Only AH	14.6	5.8	2.4	Only AH	10.7	1.3	1.7	−2.6	1.5	6.7
BD#2	Only AH	794.7	34.9	Only AH	Only AH	Only AH	1.8	Only AH	−1.5	18.3	4.3
BD#3	Only AH	11.9	Non detected	Only AH	Only AH	Only AH	−2.9	Non detected	Only plasma	Only AH	4.3
BD#4	Only AH	11.1	1.6	−1.8	Only AH	2.4	−3.3	−2.2	−2.7	−1.3	5.9
BD#5	7.7	−3.2	Only plasma	Only plasma	Only AH	−1.1	−3.4	Only plasma	Only plasma	−9.0	7.0
BD#6	7,360.6	151.0	92.1	2.2	3.6	27.1	−1.1	4.3	1.2	3.1	2.4
BD#7	Only AH	184.1	Only AH	Only AH	Only AH	Only AH	1.9	Only AH	1.3	Only AH	3.2
BD#8	1.4	3.8	Only plasma	Only plasma	−1.5	−2.5	−7.8	Only plasma	−27.0	Only plasma	6.6
BD#9	Only AH	5.0	713.9	Only AH	Only AH	Only AH	3.9	Only AH	2.8	20.3	Only AH
BD#10	Only AH	43.8	Only AH	Only AH	Only AH	Only AH	Non detected	Only AH	−3.8	Only AH	Only AH
VKH#1	Only AH	13.0	14.2	1.0	Only AH	5.7	−7.6	1.1	−3.2	−1.2	4.4
VKH#2	3.9	10.6	Only plasma	Only plasma	Only AH	1.2	−5.1	Only plasma	Only plasma	−29.3	46.2
VKH#3	Only AH	2.5	−1.9	−11.2	Only AH	1.1	1.1	Only plasma	Only plasma	−1.2	5.9
VKH#4	Only AH	39.7	2.0	1.0	Only AH	25.0	1.8	Only AH	−6.2	−2.1	5.9
VKH#5	Only AH	5.0	Only AH	Only AH	Only AH	Only AH	Only AH	Only AH	−2.2	Only AH	3.3
VKH#6	34.3	−1.1	2.3	−1.9	Only AH	2.3	3.8	−1.2	−4.3	−3.7	Only AH
VKH#7	Only AH	59.6	Only AH	Only AH	Only AH	Only AH	Only AH	Only AH	−1.1	Only AH	Only AH
VKH#8	612.9	−2.0	8.9	−2.3	Only AH	1.2	−7.0	−1.2	−2.4	−2.4	16.4
VKH#9	1.7	8.8	Only plasma	Only plasma	Only AH	−3.1	−3.4	Only plasma	Only plasma	−3.5	Only AH
VKH#10	3.0	−1.3	−8.7	Only plasma	Only AH	−2.6	−6.0	Only plasma	Only plasma	−4.1	33.3

Ratios between cytokine concentrations in AH and plasma are reported. In case the ratios were below 1 the following formula was applied:−−1/ratio. Therefore, negative values indicate a higher expression of the cytokines in plasma, positive values indicate a higher expression in AH. "Only AH," cytokines detected only in AH samples. "Only plasma," cytokines detected only in plasma samples. "Non detected," cytokines detected neither in AH nor in plasma samples.

TABLE 6 | Characteristics of the immune cells present in the AH samples.

Patients	Leukocytes/ml	Lymphocytes (%)	Monocytes (%)	Granulocytes (%)	Lymphocytes/ml§	Monocytes/ml#	Granulocytes/ml**
BD#1	25,000	84	13	2	+++	++	++
BD#2	47,500	62	33	4	+++	+++	+++
BD#3	470	64	25	11	+	+	+
BD#4	28,100	80	18	1	+++	+++	++
BD#5	0	0	0	0	0	0	0
BD#6	220,000	24	11	65	+++	+++	+++
BD#7	24,400	46	16	38	++	+++	+++
BD#8	470	70	5	24	+	+	+
BD#9	12,500	50	8	42	++	++	+++
BD#10	111,000	52	17	31	+++	+++	+++
VKH#1	15,000	82	15	3	++	++	++
VKH#2	630	72	11	17	+	+	+
VKH#3	0	0	0	0	0	0	0
VKH#4	19,200	80	18	2	++	++	++
VKH#5	5,000	68	26	6	++	++	+
VKH#6	1,129	34	39	27	+	++	+
VKH#7	166,250	93	6	1	+++	+++	+++
VKH#8	2,500	38	14	48	++	++	++
VKH#9	3,750	85	7	9	++	+	++
VKH#10	3,750	57	27	17	++	++	++

Leukocytes/ml were determined by counting with Neubauer hemocytometers. Percentages of lymphocytes, monocytes and granulocytes were determined by flow cytometry setting gates based on FSC and SSC. Such percentages were applied to the leukocytes/ml to estimate lymphocytes/ml, monocytes/ml and granulocytes/ml. Concentrations of such immune cell subsets were divided in three classes: ≤33% percentile; 33–67% percentile; ≥67% percentile to semi-quantitatively assess the immune cell loads.

§: +, ≤900 lymphocytes/ml; ++, 900–16,000 lymphocytes/ml; +++, ≥16,000 lymphocytes/ml.

#: +, ≤350 monocytes/ml; ++, 350–3,500 monocytes/ml; +++, ≥3,500 monocytes/ml.

***: +, ≤300 granulocytes/ml; ++, 300–1,200 granulocytes/ml; +++, ≥1,200 granulocytes/ml.*

Samples of AH from HC did not contain any leukocytes.

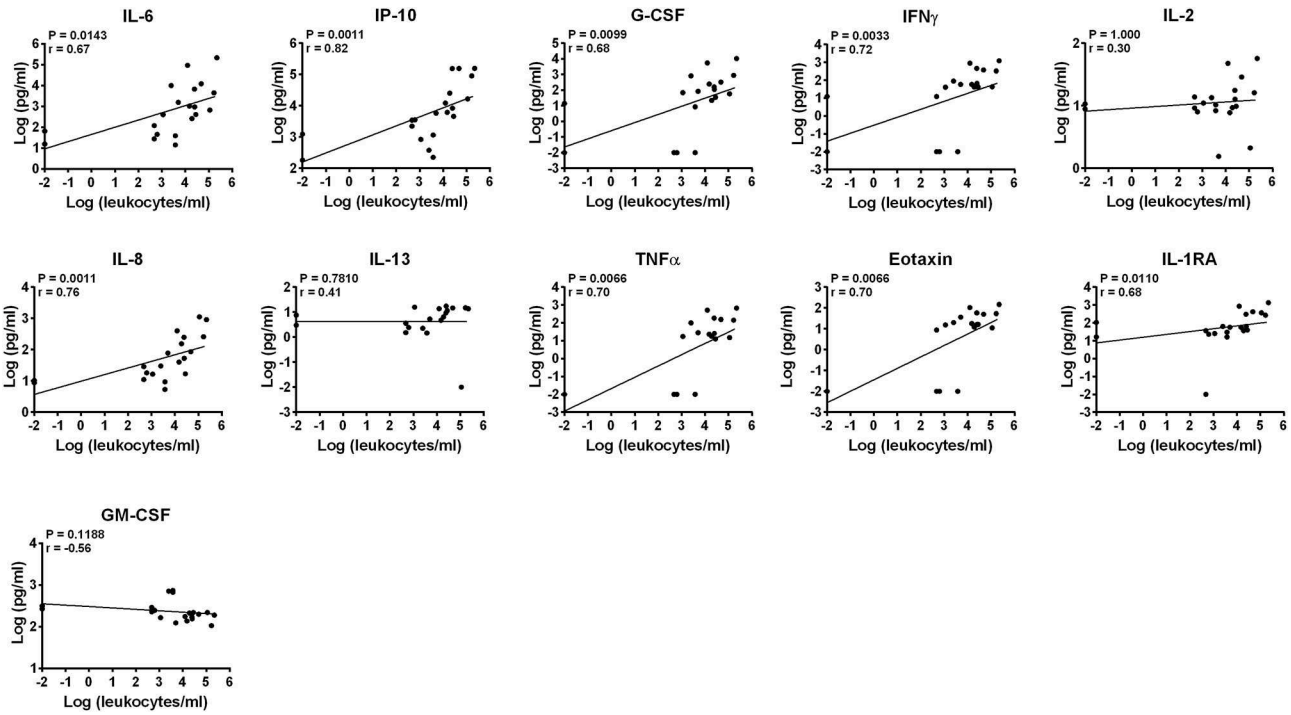


FIGURE 2 | Correlation of cytokine concentrations with the load of leukocytes in AH. Correlations between log-scale cytokine concentrations and leukocyte concentrations in AH samples are depicted ($n = 20$). Spearman's correlation was determined. *P*-values after the Bonferroni correction for multiple testing are shown.

AH sample derived from a blinded eye with few leukocytes/ml (**Table 6**). 5/7 patients in cluster I-II vs. 2/13 patients in cluster III-IV were receiving therapies at the moment of AH sampling ($p = 0.0223$, Fisher's exact test). Patients in cluster I-II had lower leukocytes/ml than patients in cluster III-IV (470 cells/ml, IQR: 0–3,750 vs. 24,400 cells/ml, IQR: 8,750–79,250; $p = 0.0011$, Mann-Whitney U-test). Finally, cluster III included 5/6 patients with higher neutrophils/ml (score = + + +, **Table 6**). We were unable to find other common clinical characteristics (**Table 1**) in the clustered patients.

Cytokines were clustered in two major groups: the first one included cytokines with the highest fold difference in concentrations between patients with uveitis and controls: IL-6, IL-8, IFNγ, TNFα, IP-10, G-CSF, IL-1ra (**Figure 3**).

To appreciate cytokine profiles in the clinical diagnostic clusters, a heatmap displaying cytokine concentrations in AH from each subject classified as BD, VKH and HC was drawn (**Figure S7**).

Loading the list of the 11 dysregulated cytokines in REACTOME and PANTHER software produced respectively the "interleukin-10 signaling pathway" as top over-represented pathway and "granulocyte and leukocyte chemotaxis" as biological process.

DISCUSSION

The present study proposes multiplex analysis of cytokine concentrations in AH from patients with non-infectious uveitis vs. HC, as a tool to identify cytokines deregulated intraocularly in each individual, in order to gain insight in uveitis pathogenesis and explore potential targets for the development of tailored treatments.

Concentrations of the deregulated cytokines: IL-6, IL-8, IP-10, G-CSF, IFNγ, TNFα, eotaxin, IL-1ra positively correlated with the levels of inflammatory cells in AH. This was expected supposing that cytokines in AH from patients with uveitis are produced by leukocytes and/or attract leukocytes and/or sustain leukocyte survival and proliferation. Moreover it is in line with findings by other authors regarding positive correlations between cytokine levels in AH and disease activity in patients with BD- and VKH-associated uveitis, graded according to the criteria of the Standardization of Uveitis Nomenclature Working Group (20–23). Such cytokines can be produced by and/or attract monocytes/macrophages and neutrophils. This is consistent with the stronger correlations found between cytokine levels and the degrees of monocytes and granulocytes in AH, which points to a possible role of the innate immune system in BD- and VHK-associated uveitis.

Concentrations of IL-2 and IL-13 did not correlate with the loads of inflammatory cells. That was unexpected because CD4+ and CD8+ T cells, NK cells, and dendritic cells are the major sources of IL-2, while CD4+ T cells, NKT cells, mast cells, basophils and eosinophils are the major sources of IL-13. However, these findings could be explained by production of IL-2 and IL-13 also by ocular cells, supported by the fact that IL-2 and IL-13 were detected also in AH from HC that normally

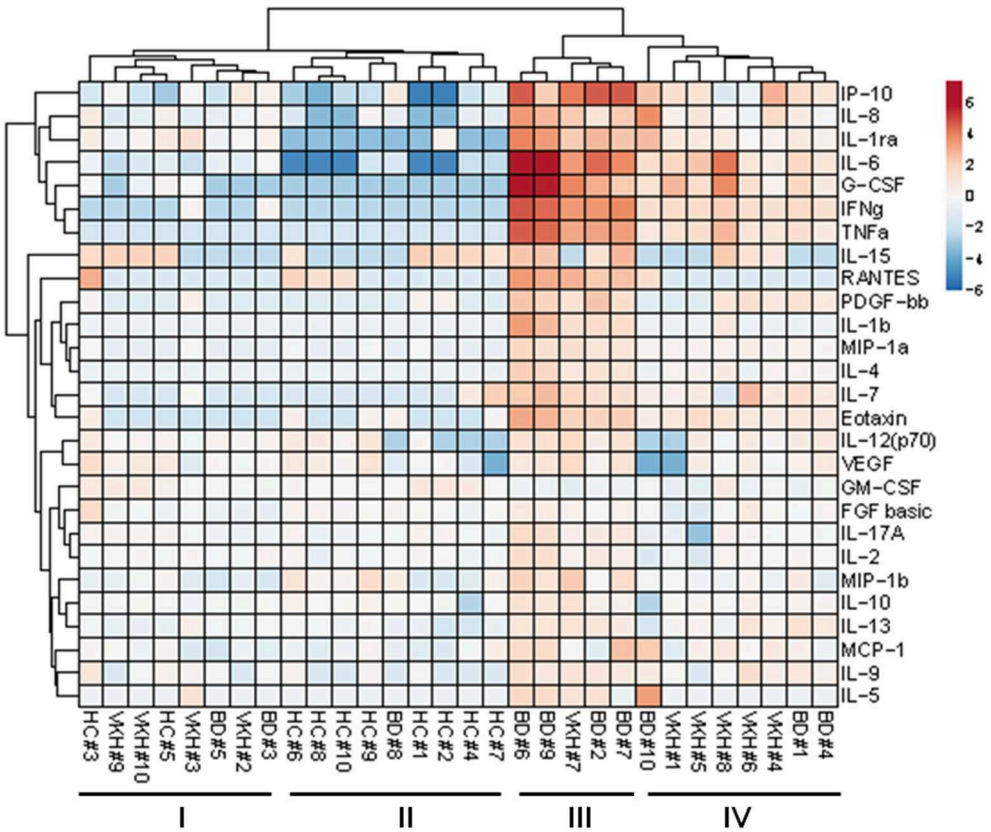


FIGURE 3 | Unsupervised clustering. Clustering was performed with ClustVis software.

did not contain leukocytes. Thus, we might speculate that IL-2 and IL-13 play a role not only in inflammation but also in ocular homeostasis.

Another cytokine which could be produced also by ocular cells is GM-CSF, being highly detected in AH from HC. The decreased levels of GM-CSF in AH from patients with non-infectious uveitis may suggest eye-protective, anti-inflammatory effects of such a cytokine. GM-CSF can be secreted by both immune cells (e.g., macrophages, T lymphocytes, NK cells) and stromal cells (e.g., endothelial cells and fibroblasts). It is generally thought as a pro-inflammatory cytokine playing as a growth and differentiation factor for granulocytes and macrophages. However, it can also promote immunological tolerance (24, 25) and induce the expression of the immune checkpoint molecule PD-L1 dampening immune responses (26). We may suppose that GM-CSF is involved in the maintenance of ocular *immune privilege*, a phenomenon restraining local immune and inflammatory responses in order to preserve vision through physical barriers (i.e., blood–ocular barrier), soluble and surface-bound molecules and modulation of systemic immune responses (27).

The detection of a cytokine does not imply that it plays a role in disease pathogenesis. However the identification of which cytokines are deregulated in each patients can provide a rationale to select targeted therapies, in particular the exclusion of the drugs whose targets are not deregulated or detected in individual patients.

The specific presence of IFNγ and TNFα in AH samples only from patients with uveitis renders these cytokines promising targets in a therapeutic perspective. Indeed, biological drugs anti-TNFα have been proven to be effective in non-infectious uveitis and have been approved by FDA and EMEA for therapy (6, 10, 28–32). Inhibitors of IFNγ (e.g., AMG811; Fontolizumab, Emapalumab) are being evaluated in clinical trials on immune mediated diseases. Our results suggest that they might be also evaluated for treatment of patients with non-infectious uveitis.

To be noted, administration of recombinant IFNα which belongs to the type I interferons, has been proved to be effective in patients with BD-related uveitis refractory to conventional immunosuppressive treatment (33, 34). IFNα has anti-viral, anti-proliferative, anti-angiogenic and anti-tumor activities and can modulate the immune system. Although there are not evidences that BD results from direct infection by viruses or bacteria, many data suggest an important role for infective agents as triggers of the immune-responses observed in BD (35). Administration of IFNα seems to (1) shape polarization of CD4+ lymphocytes toward Th2; (2) decrease Th17 lymphocytes, γδ T lymphocytes and NK cells; (3) reduce the expression of Toll-like receptors on CD4+ T lymphocytes and monocytes; (4)

inhibit neovascularization; (5) enhance functions of blood-ocular barrier (36–39).

The great differences in the concentrations of IP-10, IL-6, and G-CSF between AH from patients with uveitis and HC (at least 20-fold), in the majority of the patients, make them top candidate therapeutic targets as well. Therefore it could be interesting to test whether biological drugs such as anti-IP10 (e.g., eldelumab BMS-936557, MDX-1100), anti-IL-6 (e.g., tocilizumab, sarilumab, siltuximab, sirukumab, olokizumab, clazakizumab), anti-G-CSF (e.g., CSL324) might be beneficial in non-infectious uveitis. In support of such hypothesis, the anti-IL-6 receptor monoclonal antibody tocilizumab has already shown some efficacy in patients with non-infectious uveitis (40–42).

To the best of our knowledge, this is the first report about an increased production of IL-1ra in BD-associated uveitis. IL-1ra is a natural endogenous inhibitor of the pro-inflammatory effect of IL-1β through the binding IL-1 receptor. IL-1ra is mainly produced by monocytes, neutrophils, epithelial cells, and keratinocytes. An increased production of IL-1ra has been documented also in AH from patients with HLA-B27-associated anterior uveitis (43). The increased production of an inhibitor of inflammation could represent a feedback loop to dampen the inflammatory responses. The biological drug Anakinra is a recombinant, slightly modified version of IL-1ra and its efficacy has been reported in the management of BD-related uveitis (44). Whether Anakinra efficacy might depend on the baseline levels of IL-1ra in AH is unknown. Since AH cytokine levels were heterogeneous among patients, clinical trials are strongly needed to verify the hypothesis of beneficial effects of different class of biological therapies in patients with non-infectious uveitis based on the levels of the respective targets in AH.

To identify which cytokines can be consistently dysregulated in AH from patients with uveitis associated with BD and VKH, we compared our data with literature data (**Table 7**). IL-1ra, G-CSF, IL-9, PDGF, and basic FGF were investigated for the first time in the present work, whereas the other cytokines have been previously investigated by other groups. Increased levels of IL-6, IL-8, IFNγ, IP-10 are confirmed respectively by four, nine, six and three independent studies (20–22, 45, 46, 48–56). Three studies confirm higher levels of TNFα in AH (20, 47, 48), while three studies are discordant with our results (45, 46, 49).

The profile of the other cytokines is heterogeneous in the literature and not consistently in line with our results (**Table 7**). Differences in the results can derive from technical aspects (e.g., types of assays used to quantify the cytokines; processing of AH samples: cell-free AH samples vs. whole AH samples) and from differences in the clinical characteristics of the cohorts of patients (e.g., therapies at the moment of sample collection; degree of uveitis). Efforts are needed (1) to standardize the protocols inter-laboratories using common cytokine standards to have data which can be compared among laboratories; (2) to define the range of cytokine concentrations in AH from HC to have references of non-inflammatory conditions; (3) to promote multicentric studies.

Merging our data with literature data (**Table 7**) pointed out that IL-8 could be an additional promising target for therapies in non-infectious uveitis. IL-8 can induce chemotaxis in neutrophils and other granulocytes, stimulate phagocytosis and promote angiogenesis. Inhibitors of IL-8 (e.g., BMS-986253), currently in development for treatment of some solid tumors, might be also tested in non-infectious uveitis.

TABLE 7 | Literature data about the expression of the investigated cytokines in AH from patients with BD- and VKH- associated uveitis compared to HC.

Cytokine	Our data	Literature data	References
IL-1β	=*	nd/↑/=/=	(22, 45–47)
IL-1ra	↑	no data	
IL-2	↑	nd/nd/=/=/↑	(45, 46, 48–50)
IL-4	↑**	nd/nd/↓/=/=	(45, 46, 48–50)
IL-5	=*	nd/nd/=	(45, 46, 49)
IL-6	↑	↑/↑/↑/↑	(22, 45, 46, 51)
IL-7	=	=	(46)
IL-8/CXCL8	↑	↑/↑/↑/↑/↑/↑/↑/↑/↑	(21, 45, 46, 51–56)
IL-9	=**	no data	
IL-10	=	↓/=/=/=/nd/nd/↑/↑	(20, 45–50, 52)
IL-12p70	=	=/=/=/=	(23, 45, 46, 48)
IL-13	↑	=	(46)
IL-15	=*	↑/↑/=	(20, 46, 48)
IL-17	=	↑	(20)
Eotaxin/CCL11	↑	=/↑	(46, 57)
FGF basic	=	no data	
G-CSF	↑	no data	
GM-CSF	↓	↑/=	(23, 46)
IFNγ	↑	↑/↑/↑/↑/↑/↑	(20, 45, 46, 48–50)
IP-10/CXCL10	↑	↑/↑/↑	(21, 54, 55)
TNFα	↑	↑/↑/↑/nd/nd/=	(20, 45–49)
MCP-1/CCL2	=	=/↑/↑/↑	(46, 51, 53, 57)
MIP-1α/CCL3	↑**	=	(53)
MIP-1β/CCL4	=	=	(53)
PDGF	=**	no data	
RANTES/CCL5	= *	=/=/=	(46, 53, 55)
VEGF	=	=/↑/↑	(51, 52, 56)

Only studies including BD-associated uveitis and VKH-associated uveitis compared with HC are reported. ↑, increased production; ↓, decreased production; nd, not detected; =, unchanged.

**cytokines with median concentrations lower than the limits of detection.*

***cytokines with median concentrations lower than 10 pg/ml.*

Limits of the present study are (1) the small number of patients (although that is in line with the other studies on cytokine profiling in AH from patients with non-infectious uveitis); (2) the heterogeneity in terms of treatment schedule and degree of ocular inflammation at the moment of AH sample collection.

On the other hand, the strengths of the study mainly consist in the accurate clinical evaluation performed by expert rheumatologists and immune-ophthalmologists for defining BD activity and uveitis activity respectively, apart from simultaneous profiling of several cytokines and the characterization of the immune cells in AH by manual counting and flow cytometry, which has been rarely performed in other studies.

To resume, AH sampling followed by cytokine profiling allows identifying potential therapeutic targets for non-infectious uveitis and could help stratify patients for tailored treatments. IP-10, IFNγ, IL-6, G-CSF, TNFα IL-8, IL-1ra, and GM-CSF emerged

as the most promising cytokines to be further investigated for treatment of BD- and VKH-associated uveitis.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the local ethics committee (Reggio Emilia, Italy, protocol number 402 2015/0024354). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AS, LC, and SC designed the experimental protocol. AS, LC, LD, EB, FG, FM, LF, and CS recruited patients. MB, SC, and MN performed the experiments. MB, LB, AZ, CS, and SC interpreted the data. SC wrote the manuscript. All authors drafted the manuscript or revised it critically for important intellectual content and approved the final version to be published.

FUNDING

This study has been supported by the Italian Society of Rheumatology (SIR, 2015/2016 Grant for Young Researchers appointed to AS), and by the National Association of Patients Affected by Behçet's Disease (S.I.M.B.A.).

ACKNOWLEDGMENTS

We thank the National Association of Patients Affected With Behçet's Disease (S.I.M.B.A.) for supporting the project.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00358/full#supplementary-material

REFERENCES

1. Jabs DA. Epidemiology of uveitis. *Ophthal Epidemiol*. (2008) 15:283–4. doi: 10.1080/09286580802478724
2. Barisani-Asenbauer T, Maca SM, Mejdoubi L, Emminger W, Machold K, Auer H. Uveitis- a rare disease often associated with systemic diseases and infections- a systematic review of 2619 patients. *Orphanet J Rare Dis*. (2012) 7:57. doi: 10.1186/1750-1172-7-57
3. Bertrand PJ, Jamilloux Y, Ecochard R, Richard-Colmant G, Gerfaud-Valentin M, Guillaud M, et al. Uveitis: autoimmunity and beyond. *Autoimmun Rev*. (2019) 18:102351. doi: 10.1016/j.autrev.2019.102351
4. Prete M, Dammacco R, Fatone MC, Racanelli V. Autoimmune uveitis: clinical, pathogenetic, and therapeutic features. *Clin Exp Med*. (2016) 16:125–36. doi: 10.1007/s10238-015-0345-6
5. Shahab MA, Mir TA, Zafar S. Optimising drug therapy for non-infectious uveitis. *Int Ophthalmol*. (2019) 39:1633–50. doi: 10.1007/s10792-018-0984-1
6. Dick AD, Rosenbaum JT, Al-Dhibi HA, Belfort R Jr, Brezin AP, Chee SP, et al. Guidance on noncorticosteroid systemic immunomodulatory therapy in noninfectious uveitis: Fundamentals Of Care for UveitiS (FOCUS) initiative. *Ophthalmology*. (2018) 125:757–73. doi: 10.1016/j.ophtha.2018.03.005
7. Hatemi G, Christensen R, Bang D, Bodaghi B, Celik AF, Fortune F, et al. 2018 update of the EULAR recommendations for the management of Behcet's syndrome. *Ann Rheum Dis*. (2018) 77:808–18. doi: 10.1136/annrheumdis-2018-213225
8. Bodaghi B, Gendron G, Wechsler B, Terrada C, Cassoux N, Huong du LT, et al. Efficacy of interferon alpha in the treatment of refractory and sight threatening uveitis: a retrospective monocentric study of 45 patients. *Br J Ophthalmol*. (2007) 91:335–9. doi: 10.1136/bjo.2006.101550
9. Hassan M, Karkhur S, Bae JH, Halim MS, Ormaechea MS, Onghanseng N, et al. New therapies in development for the management of non-infectious uveitis: a review. *Clin Exp Ophthalmol*. (2019) 47:396–417. doi: 10.1111/ceo.13511
10. Touhami S, Diwo E, Seve P, Trad S, Bielefeld P, Sene D, et al. Expert opinion on the use of biological therapy in non-infectious uveitis. *Expert Opin Biol Ther*. (2019) 19:477–90. doi: 10.1080/14712598.2019.1595578
11. Airody A, Heath G, Lightman S, Gale R. Non-infectious uveitis: optimising the therapeutic response. *Drugs*. (2016) 76:27–39. doi: 10.1007/s40265-015-0502-y
12. Park UC, Kim TW, Yu HG. Immunopathogenesis of ocular Behcet's disease. *J Immunol Res*. (2014) 2014:653539. doi: 10.1155/2014/653539
13. Agrawal R, Iyer J, Connolly J, Iwata D, Teoh S. Cytokines and biologics in non-infectious autoimmune uveitis: bench to bedside. *Indian J Ophthalmol*. (2014) 62:74–81. doi: 10.4103/0301-4738.126187
14. Davatchi F, Chams-Davatchi C, Shams H, Shahram F, Nadji A, Akhlaghi M, et al. Behcet's disease: epidemiology, clinical manifestations, and diagnosis. *Expert Rev Clin Immunol*. (2017) 13:57–65. doi: 10.1080/1744666X.2016.1205486
15. Lavezzo MM, Sakata VM, Morita C, Rodriguez EE, Abdallah SF, da Silva FT, et al. Vogt-Koyanagi-Harada disease: review of a rare autoimmune disease targeting antigens of melanocytes. *Orphanet J Rare Dis*. (2016) 11:29. doi: 10.1186/s13023-016-0412-4
16. International Study Group for Behcet's Disease. Criteria for diagnosis of Behcet's disease. *Lancet*. (1990) 335:1078–80.
17. Read RW, Holland GN, Rao NA, Tabbara KF, Ohno S, Arellanes-Garcia L, et al. Revised diagnostic criteria for Vogt-Koyanagi-Harada disease: report of an international committee on nomenclature. *Am J Ophthalmol*. (2001) 131:647–52. doi: 10.1016/S0002-9394(01)00925-4
18. Cimino L, Aldigeri R, Parmeggiani M, Belloni L, Zotti CA, Fontana L, et al. Searching for viral antibodies and genome in intraocular fluids of patients with Fuchs uveitis and non-infectious uveitis. *Graefes Arch Clin Exp Ophthalmol*. (2013) 251:1607–12. doi: 10.1007/s00417-013-2287-6
19. Metsalu T, Vilo J. ClustVis: a web tool for visualizing clustering of multivariate data using Principal Component Analysis and heatmap. *Nucleic Acids Res*. (2015) 43:W566–70. doi: 10.1093/nar/gkv468
20. El-Asrar AM, Struyf S, Kangave D, Al-Obeidan SS, Opdenakker G, Geboes K, et al. Cytokine profiles in aqueous humor of patients with different clinical entities of endogenous uveitis. *Clin Immunol*. (2011) 139:177–84. doi: 10.1016/j.clim.2011.01.014
21. El-Asrar AM, Al-Obeidan SS, Kangave D, Geboes K, Opdenakker G, Van DJ, et al. CXC chemokine expression profiles in aqueous humor of patients with different clinical entities of endogenous uveitis. *Immunobiology*. (2011) 216:1004–9. doi: 10.1016/j.imbio.2011.03.007
22. Abu El-Asrar AM, Berghmans N, Al-Obeidan SA, Mousa A, Opdenakker G, Van DJ, et al. The cytokine interleukin-6 and the chemokines CCL20 and CXCL13 are novel biomarkers of specific endogenous uveitic entities. *Invest Ophthalmol Vis Sci*. (2016) 57:4606–13. doi: 10.1167/iovs.16-19758
23. Abu El-Asrar AM, Berghmans N, Al-Obeidan SA, Gikandi PW, Opdenakker G, Van DJ, et al. Local cytokine expression profiling in patients with specific autoimmune uveitic entities. *Ocul Immunol Inflamm*. (2019). doi: 10.1080/09273948.2019.1604974. [Epub ahead of print].

24. Bhattacharya P, Thiruppathi M, Elshabrawy HA, Alharshawi K, Kumar P, Prabhakar BS. GM-CSF: an immune modulatory cytokine that can suppress autoimmunity. *Cytokine*. (2015) 75:261–71. doi: 10.1016/j.cyto.2015.05.030
25. Bhattacharya P, Budnick I, Singh M, Thiruppathi M, Alharshawi K, Elshabrawy H, et al. Dual role of GM-CSF as a pro-inflammatory and a regulatory cytokine: implications for immune therapy. *J Interferon Cytokine Res*. (2015) 35:585–99. doi: 10.1089/jir.2014.0149
26. Wang TT, Zhao YL, Peng LS, Chen N, Chen W, Lv YP, et al. Tumour-activated neutrophils in gastric cancer foster immune suppression and disease progression through GM-CSF-PD-L1 pathway. *Gut*. (2017) 66:1900–11. doi: 10.1136/gutjnl-2016-313075
27. Taylor AW. Ocular immune privilege and transplantation. *Front Immunol*. (2016) 7:37. doi: 10.3389/fimmu.2016.00037
28. Fabiani C, Vitale A, Lopalco G, Iannone F, Frediani B, Cantarini L. Different roles of TNF inhibitors in acute anterior uveitis associated with ankylosing spondylitis: state of the art. *Clin Rheumatol*. (2016) 35:2631–8. doi: 10.1007/s10067-016-3426-3
29. Fabiani C, Vitale A, Emmi G, Vannozzi L, Lopalco G, Guerriero S, et al. Efficacy and safety of adalimumab in Behcet's disease-related uveitis: a multicenter retrospective observational study. *Clin Rheumatol*. (2017) 36:183–9. doi: 10.1007/s10067-016-3480-x
30. Schwartzman S, Schwartzman M. The use of biologic therapies in uveitis. *Clin Rev Allergy Immunol*. (2015) 49:307–16. doi: 10.1007/s12016-014-8455-6
31. Niccoli L, Nannini C, Benucci M, Chindamo D, Cassara E, Salvarani C, et al. Long-term efficacy of infliximab in refractory posterior uveitis of Behcet's disease: a 24-month follow-up study. *Rheumatology*. (2007) 46:1161–4. doi: 10.1093/rheumatology/kem101
32. Fabiani C, Vitale A, Rigante D, Emmi G, Bitossi A, Lopalco G, et al. Comparative efficacy between adalimumab and infliximab in the treatment of non-infectious intermediate uveitis, posterior uveitis, and panuveitis: a retrospective observational study of 107 patients. *Clin Rheumatol*. (2019) 38:407–15. doi: 10.1007/s10067-018-4228-6
33. Eser-Ozturk H, Sullu Y. The results of interferon-alpha treatment in Behcet uveitis. *Ocul Immunol Inflamm*. (2019). doi: 10.1080/09273948.2019.1587473. [Epub ahead of print].
34. Hasanreisoglu M, Cubuk MO, Ozdek S, Gurelik G, Aktas Z, Hasanreisoglu B. Interferon alpha-2a therapy in patients with refractory Behcet uveitis. *Ocul Immunol Inflamm*. (2017) 25:71–5. doi: 10.3109/09273948.2015.1133835
35. Leccese P, Alpsoy E. Behcet's disease: an overview of etiopathogenesis. *Front Immunol*. (2019) 10:1067. doi: 10.3389/fimmu.2019.01067
36. Albayrak O, Oray M, Can F, Uludag KG, Gul A, Tugal-Tutkun I, et al. Effect of interferon alfa-2a treatment on adaptive and innate immune systems in patients with Behcet disease uveitis. *Invest Ophthalmol Vis Sci*. (2019) 60:52–63. doi: 10.1167/iovs.18-25548
37. Aydinoglu-Candan O, Araz-Ersan B, Gul A, Badur S, Tugal-Tutkun I. Anti-interferon alpha antibodies and autoantibodies in patients with Behcet's disease uveitis treated with recombinant human interferon alpha-2a. *Graefes Arch Clin Exp Ophthalmol*. (2015) 253:457–65. doi: 10.1007/s00417-014-2856-3
38. Treusch M, Vonthein R, Baur M, Gunaydin I, Koch S, Stubiger N, et al. Influence of human recombinant interferon-alpha2a (rhIFN-alpha2a) on altered lymphocyte subpopulations and monocytes in Behcet's disease. *Rheumatology*. (2004) 43:1275–82. doi: 10.1093/rheumatology/keh311
39. Gillies MC, Su T. Interferon-alpha 2b enhances barrier function of bovine retinal microvascular endothelium *in vitro*. *Microvasc Res*. (1995) 49:277–88. doi: 10.1006/mvre.1995.1024
40. Thomas AS. Biologics for the treatment of noninfectious uveitis: current concepts and emerging therapeutics. *Curr Opin Ophthalmol*. (2019) 30:138–50. doi: 10.1097/ICU.0000000000000562
41. Sepah YJ, Sadiq MA, Chu DS, Dacey M, Gallemore R, Dayani P, et al. Primary (Month-6) outcomes of the STOP-uveitis study: evaluating the safety, tolerability, and efficacy of tocilizumab in patients with noninfectious uveitis. *Am J Ophthalmol*. (2017) 183:71–80. doi: 10.1016/j.ajo.2017.08.019
42. Lopalco G, Fabiani C, Sota J, Lucherini OM, Tosi GM, Frediani B, et al. IL-6 blockade in the management of non-infectious uveitis. *Clin Rheumatol*. (2017) 36:1459–69. doi: 10.1007/s10067-017-3672-z
43. Zhao B, Chen W, Jiang R, Zhang R, Wang Y, Wang L, et al. Expression profile of IL-1 family cytokines in aqueous humor and sera of patients with HLA-B27 associated anterior uveitis and idiopathic anterior uveitis. *Exp Eye Res*. (2015) 138:80–6. doi: 10.1016/j.exer.2015.06.018
44. Fabiani C, Vitale A, Emmi G, Lopalco G, Vannozzi L, Guerriero S, et al. Interleukin (IL)-1 inhibition with anakinra and canakinumab in Behcet's disease-related uveitis: a multicenter retrospective observational study. *Clin Rheumatol*. (2017) 36:191–7. doi: 10.1007/s10067-016-3506-4
45. Priya CG, Rathinam SR, Muthukkaruppan V. Evidence for endotoxin as a causative factor for leptospiral uveitis in humans. *Invest Ophthalmol Vis Sci*. (2008) 49:5419–24. doi: 10.1167/iovs.08-2174
46. Curnow SJ, Falciani F, Durrani OM, Cheung CM, Ross EJ, Wloka K, et al. Multiplex bead immunoassay analysis of aqueous humor reveals distinct cytokine profiles in uveitis. *Invest Ophthalmol Vis Sci*. (2005) 46:4251–9. doi: 10.1167/iovs.05-0444
47. Abu El-Asrar AM, Berghmans N, Al-Obeidan SA, Gikandi PW, Opdenakker G, Van DJ, et al. Expression of interleukin (IL)-10 family cytokines in aqueous humour of patients with specific endogenous uveitic entities: elevated levels of IL-19 in human leukocyte antigen-B27-associated uveitis. *Acta Ophthalmol*. (2019) 97:e780–4. doi: 10.1111/aos.14039
48. Ahn JK, Yu HG, Chung H, Park YG. Intraocular cytokine environment in active Behcet uveitis. *Am J Ophthalmol*. (2006) 142:429–34. doi: 10.1016/j.ajo.2006.04.016
49. Takase H, Futagami Y, Yoshida T, Kamoi K, Sugita S, Imai Y, et al. Cytokine profile in aqueous humor and sera of patients with infectious or noninfectious uveitis. *Invest Ophthalmol Vis Sci*. (2006) 47:1557–61. doi: 10.1167/iovs.05-0836
50. Lacomba MS, Martin CM, Chamond RR, Galera JM, Omar M, Estevez EC. Aqueous and serum interferon gamma, interleukin (IL) 2, IL-4, and IL-10 in patients with uveitis. *Arch Ophthalmol*. (2000) 118:768–72. doi: 10.1001/archopht.118.6.768
51. Bae JH, Lee SC. Effect of intravitreal methotrexate and aqueous humor cytokine levels in refractory retinal vasculitis in Behcet disease. *Retina*. (2012) 32:1395–402. doi: 10.1097/IAE.0b013e31823496a3
52. Simsek M, Cakar OP, Akbiyik F, Citirik M, Berker N, Ozdamar EY, et al. Aqueous humor IL-8, IL-10, and VEGF levels in Fuchs' uveitis syndrome and Behcet's uveitis. *Int Ophthalmol*. (2019) 39:2629–36. doi: 10.1007/s10792-019-01112-w
53. Pei M, Liu X, Zhao C, Gao F, Tao Y, Zhang M. Chemokine and adhesion molecule profiles in aqueous humor of clinically quiescent uveitic cataracts. *Curr Eye Res*. (2019) 44:194–9. doi: 10.1080/02713683.2018.1532012
54. El-Asrar AMA, Berghmans N, Al-Obeidan SA, Gikandi PW, Opdenakker G, Van DJ, et al. Differential CXC and CX3C chemokine expression profiles in aqueous humor of patients with specific endogenous uveitic entities. *Invest Ophthalmol Vis Sci*. (2018) 59:2222. doi: 10.1167/iovs.17-23225
55. Kim TW, Chung H, Yu HG. Chemokine expression of intraocular lymphocytes in patients with Behcet uveitis. *Ophthalmic Res*. (2011) 45:5–14. doi: 10.1159/000313546
56. Paroli MP, Teodori C, D'Alessandro M, Mariani P, Iannucci G, Paroli M. Increased vascular endothelial growth factor levels in aqueous humor and serum of patients with quiescent uveitis. *Eur J Ophthalmol*. (2007) 17:938–42. doi: 10.1177/112067210701700611
57. El-Asrar AM, Berghmans N, Al-Obeidan SA, Gikandi PW, Opdenakker G, Van DJ, et al. The CC chemokines CCL8, CCL13 and CCL20 are local inflammatory biomarkers of HLA-B27-associated uveitis. *Acta Ophthalmol*. (2019) 97:e122–8. doi: 10.1111/aos.13835

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.

frontiers in Immunology

REVIEW
published: 09 April 2020
doi: 10.3389/fimmu.2020.00525

Animal Models of ANCA Associated Vasculitis

Lani Shochet[1,2], *Stephen Holdsworth*[1,2,3] *and A. Richard Kitching*[1,2,4]*

[1] Centre for Inflammatory Diseases, Monash University Department of Medicine, Monash Medical Centre, Clayton, VIC, Australia, [2] Department of Nephrology, Monash Health, Clayton, VIC, Australia, [3] Department of Immunology, Monash Health, Clayton, VIC, Australia, [4] Department of Pediatric Nephrology, Monash Health, Clayton, VIC, Australia

Anti-neutrophil cytoplasmic antibody (ANCA) associated vasculitis (AAV) is a rare and severe autoimmune multisystemic disease. Its pathogenesis involves multiple arms of the immune system, as well as complex interactions between immune cells and target organs. Experimental animal models of disease can provide the crucial link from human disease to translational research into new therapies. This is particularly true in AAV, due to low disease incidence and substantial disease heterogeneity. Animal models allow for controlled environments in which disease mechanisms can be defined, without the clinical confounders of environmental and lifestyle factors. To date, multiple animal models have been developed, each of which shed light on different disease pathways. Results from animal studies of AAV have played a crucial role in enhancing our understanding of disease mechanisms, and have provided direction toward newer targeted therapies. This review will summarize our understanding of AAV pathogenesis as has been gleaned from currently available animal models, as well as address their strengths and limitations. We will also discuss the potential for current and new animal models to further our understanding of this important condition.

Keywords: autoantibodies, antineutrophil cytoplasmic, animal models, autoimmunity, glomerulonephritis, myeloperoxidase, proteinase 3, translational medical research

OPEN ACCESS

Edited by:
Andreas Kronbichler, Innsbruck Medical University, Austria

Reviewed by:
Benjamin Wilde, University of Duisburg-Essen, Germany
Akihiro Ishizu, Hokkaido University, Japan

***Correspondence:**
A. Richard Kitching
richard.kitching@monash.edu

Specialty section:
This article was submitted to Autoimmune and Autoinflammatory Disorders, a section of the journal Frontiers in Immunology

Received: *03 December 2019*
Accepted: *09 March 2020*
Published: *09 April 2020*

Citation:
Shochet L, Holdsworth S and Kitching AR (2020) Animal Models of ANCA Associated Vasculitis. Front. Immunol. 11:525. doi: 10.3389/fimmu.2020.00525

INTRODUCTION

The anti-neutrophil cytoplasmic antibody (ANCA) associated vasculitides (AAV) are autoimmune diseases characterized by systemic inflammation and subsequent destruction of small to medium blood vessels within target organs, particularly the kidneys and respiratory tract. It is a rare but life-threatening condition, with an incidence of 13–20 people per million per year worldwide, and a peak age of onset of 65–74 years (1). Syndromically, AAV can present as granulomatosis with polyangiitis (GPA; formerly known as Wegener's granulomatosis), microscopic polyangiitis (MPA) or eosinophilic granulomatosis with polyangiitis (EGPA). If untreated, mortality of AAV may be as high as 80% within 1 year of diagnosis (2). Treatment involves potent immunosuppressive agents that may have significant associated adverse effects, including infection and malignancy. Infection accounts for almost half of the deaths in treated patients in the first year (3). AAV-related glomerulonephritis (GN) is an important cause of end stage kidney disease and commonly defines outcomes in AAV.

AAV is a largely heterogeneous condition, with substantial variation in clinical presentation and sequelae. This variability presents significant challenges for patients and their doctors, as well as for recruitment and categorization in clinical studies. The hallmark of disease is the presence of auto-antibodies targeting proteins within azurophilic (primary) granules of neutrophils, with the two

most clinically relevant autoantigens being proteinase 3 (PR3) and myeloperoxidase (MPO). These proteins are important players in the antimicrobial activity of neutrophils. AAV can be classified based on syndromic features or on the auto-antigen involved, specifically MPO-AAV or PR3-AAV. The Chapel Hill Consensus guidelines divide AAV into syndromic categories: GPA, MPA, and EGPA (4). The majority of people with GPA or MPA are ANCA positive at diagnosis, but in around 10% of patients ANCA are not detected in sera by conventional assays. Dual positive PR3-ANCA and MPO-ANCA serology is uncommon. Whilst most patients with GPA are PR3-ANCA positive, and similarly for MPA patients with MPO-ANCA, overlap between the clinical syndrome and ANCA specificity is incomplete. Observational studies have suggested that serological classification may better predict clinical features such as relapse rate (5), renal survival and mortality (6). The concept that PR3-AAV and MPO-AAV are different but related conditions is further supported by the identification of different genetic and epidemiological backgrounds between PR3-AAV and MPO-AAV [reviewed by Cornec et al. (7)].

The pathophysiology of AAV is complex and remains incompletely understood. First, T and B cell tolerance to MPO or PR3 is lost, via mechanisms that remain incompletely described (**Figure 1**). Subsequently, with T cell help, autoreactive

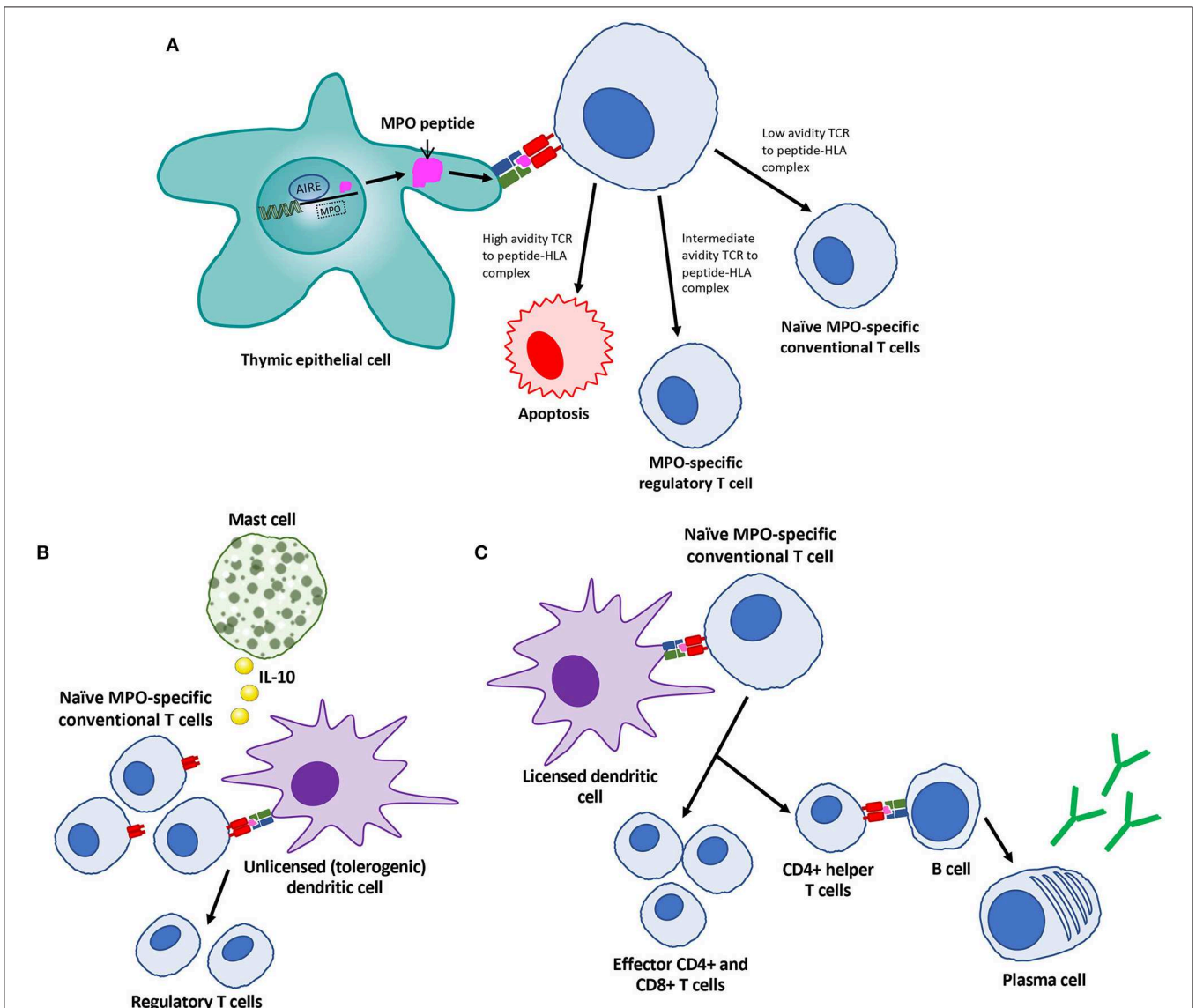


FIGURE 1 | Features of central and peripheral mechanisms of tolerance to MPO as defined by animal models. **(A)** Central tolerance in the thymus is regulated by the transcription factor autoimmune regulator (AIRE) within the nucleus of thymic epithelial cells. This regulates autoantigen presentation to T cells on human leukocyte antigen (HLA) class II molecules, with subsequent T cell selection. **(B)** Peripherally, tolerance to MPO is maintained through MPO presentation on HLA class II by unlicensed dendritic cells to naïve T cells under the influence of IL-10 producing mast cells, promoting the development of regulatory T cells. **(C)** In certain situations, tolerance to MPO is lost, prompting expansion of T cells, and subsequent help for B cells to produce ANCA.

B cells or plasma cells produce ANCA. ANCA activate neutrophils (**Figure 2**) and induce their adherence to vulnerable microvascular beds, such as the glomerulus, where they degranulate and undergo NETosis, inducing endothelial injury (**Figure 3**). In this process, ANCA antigens are also deposited in the glomerulus, with the ability to be recognized by effector T cells, further contributing to injury [reviewed in Hutton et al. (8)]. It is hypothesized that monocyte/macrophages play a role later in disease (9); they themselves can be activated by ANCA and also have the capacity to present antigens to effector T cells (10).

Much of our understanding of the pathophysiology of AAV comes from animal models of disease, coupled with observations in human disease and *in vitro* studies. For example, since the discovery of ANCA in humans in 1982 (11), *in vitro* studies during the 1990s demonstrated that ANCA could activate human neutrophils (12–14), with animal studies later confirming the pathogenicity of ANCA *in vivo* with passive transfer of ANCA into mice (15). Similar advances have been made in understanding the role of effector T cells (16), complement (17), and the nature of T and B cell epitopes (18–20) in the pathogenesis of AAV. Furthermore, the judicious use of animal models has allowed pre-clinical investigation of new targeted therapies, exemplified by work on complement in a model involving the passive transfer of anti-MPO antibodies (21, 22). Whilst clinical and *in vitro* research into PR3-AAV is plentiful, no consistent PR3-AAV animal models currently exist, meaning that the *in vivo* understanding of AAV pathogenesis is based largely on models of anti-MPO disease. Whilst PR3-AAV and MPO-AAV share many pathological and clinical similarities, the differences between them span epidemiology, genetic predisposition, clinical features and histopathology [reviewed by Hilhorst et al. (23)]. Given these differences, it is important that animal models of PR3-AAV are developed, to further our understanding of the complexities of PR3-AAV, the differences between MPO-AAV and PR3-AAV, and to more accurately target treatments.

AAV is a unique autoimmune disease. Its pathogenesis involves all aspects of the immune system, with complex interplay between innate and adaptive immunity. It is one of only a few autoimmune diseases in which a single pathogenic autoantibody is measured. Furthermore, ANCA is pathogenic by binding to neutrophils and monocytes and inducing cellular activation, with resultant microvascular endothelial injury. However, depletion of the autoantibody alone may not be effective in disease control, and disease can be quiescent while the antibody remains detectable, suggesting redundancy in injurious autoimmune pathways.

Animal models of disease allow for a controlled environment, with the consequent ability to thoroughly interrogate human clinical observations and test hypotheses derived from these observations. AAV is a rare disease and consequently human studies often have limited numbers of patients. Patients are often heterogeneous and difficult to compare, due to confounders such as autoantigen specificity and potential epitope spreading throughout the course of disease, diverse clinical manifestations and immunosuppressive treatments. Animal models are also necessary for pre-clinical development of more effective, targeted treatments, before their translation into clinical experimentation. Ultimately though, models are just that: models. Whilst their use is invaluable in scientific research, they are only part of the puzzle of comprehensive understanding of a uniquely human disease.

This review will outline existing models which have contributed to the field of AAV. While many of these models have illuminated the biology of AAV, no single animal model presented here is able to replicate every stage of AAV, from loss of tolerance through to the development of end-organ fibrosis. Furthermore, these models still leave us with significant gaps in our disease understanding, including loss of tolerance,

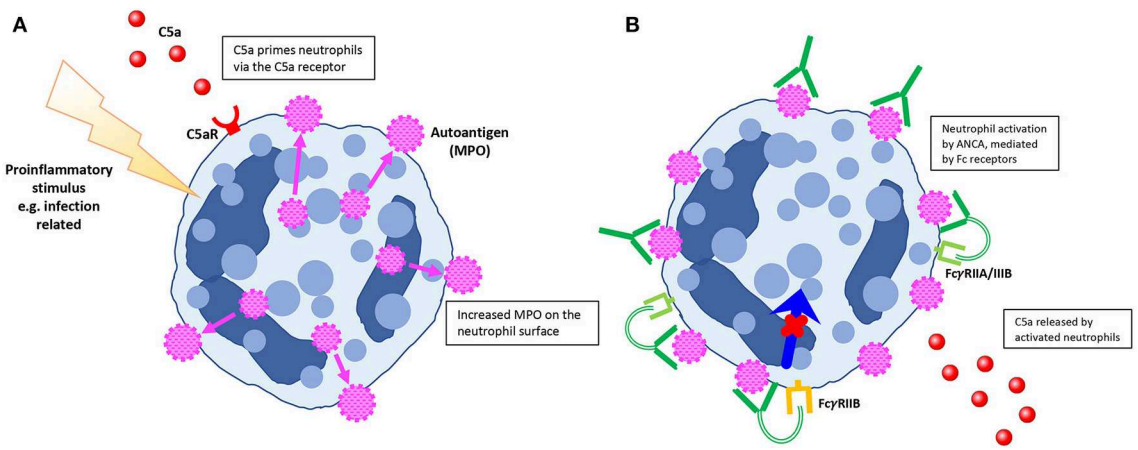


FIGURE 2 | Features of neutrophil priming and activation by ANCA as defined by animal models. **(A)** Pro-inflammatory stimuli (including lipopolysaccharide and complement factor C5a) cause neutrophil priming, with increased expression of ANCA antigens on the neutrophil surface. **(B)** Mediated by regulatory Fcγ receptors, ANCA have the capacity to activate neutrophils. Neutrophil activation causes release of C5a, with subsequent complement pathway activation as well as further neutrophil priming.

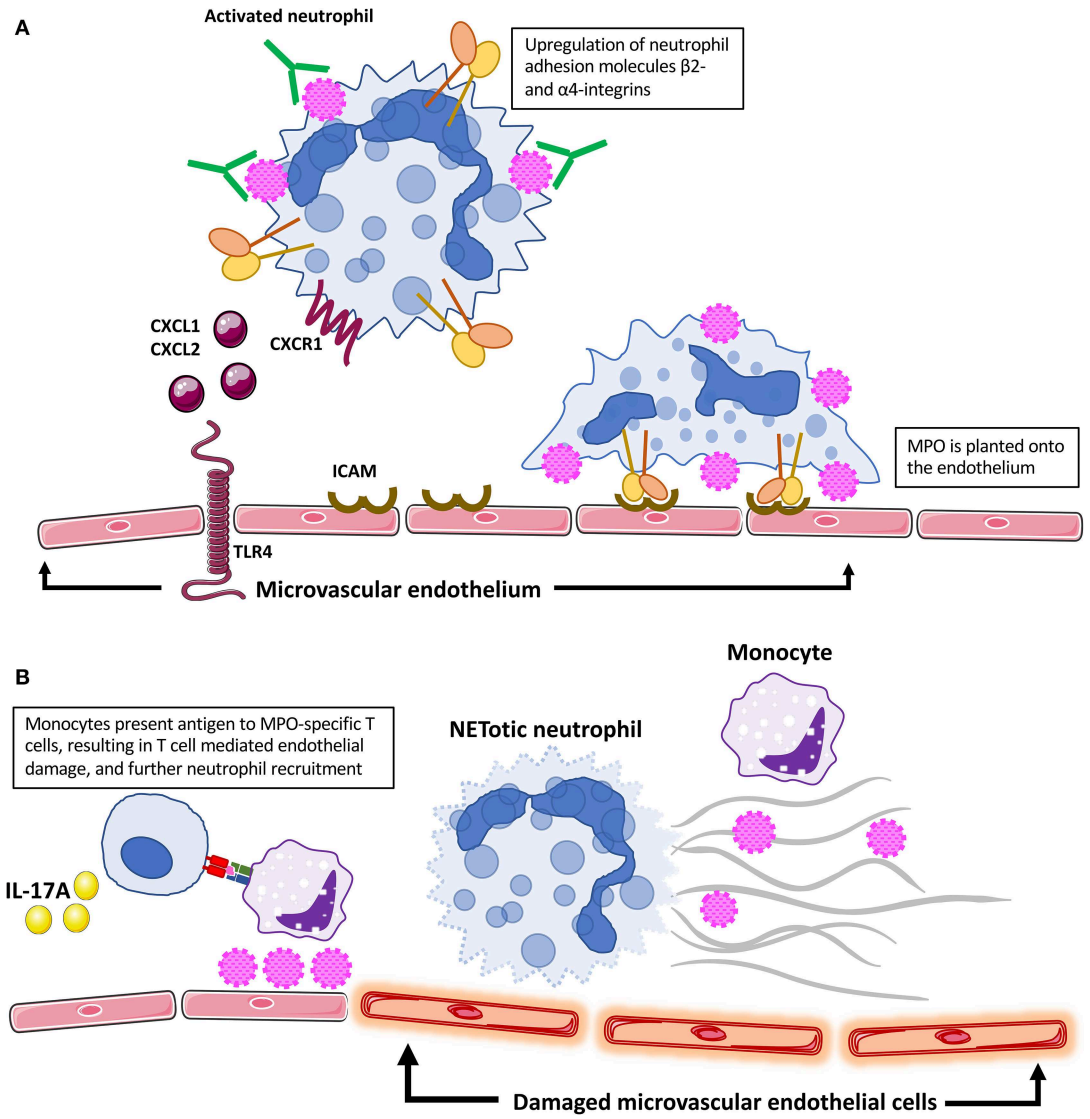


FIGURE 3 | Features of neutrophil migration and adhesion, and endothelial damage as defined by animal models. **(A)** Activated neutrophils migrate to vulnerable vascular beds, including the glomerulus. The presence of TNF is associated with TLR4 upregulation on glomerular endothelial cells, which contributes to neutrophil migration through production of chemoattractants CXCL1 and CXCL2. After activation by ANCA, neutrophils express β2-integrins (LFA-1 and MAC-1), which enhance neutrophil adhesion to the glomerular endothelium. Neutrophil retention within the glomerular capillaries is moderated by the C5a receptor. **(B)** MPO is planted onto the glomerular endothelium, allowing local recognition by MPO-specific effector T cells and subsequent injury. Circulating monocytes have been shown experimentally to present antigens within glomeruli; however, microvascular endothelial cells and dendritic cells may also be involved in antigen recognition by effector T cells. Release of interleukin-17A (IL-17A) by T cells further encourages neutrophil migration. After localization to vulnerable vascular beds, neutrophils undergo necroptosis, and form neutrophil extracellular traps (NETs). This process promotes complement activation, and subsequent endothelial damage. Furthermore, NETs facilitate MPO presentation and propagation of the autoimmune response.

phenotypic heterogeneity and relapse prediction. Despite their critical roles in advancing our understanding of diseases, models have also been limited by their lack of consistency between laboratories, making research collaboration and conducting replication studies a significant challenge.

Of note, most animal models of AAV assess the impact of disease on the kidneys. Although renal disease is responsible for a major part of disease burden, other common organ manifestations are largely unstudied.

Different models have each shed light on different aspects of disease pathogenesis. Inspired by the identification of ANCA and its ability to activate neutrophils *in vitro*, earlier studies confirmed the pathogenicity of ANCA. The roles of priming and activating neutrophils, neutrophil migration to target organs and neutrophil degradation and extracellular trap formation have been investigated. The complex interplay between the adaptive and innate immune systems continues to be explored, including the role of T cells, complement and mast cells.

ANIMAL MODELS OF MPO-AAV

In particular, animal models of MPO-AAV have been invaluable, in part due to significant homology with the human equivalent. The autoantigen itself is highly homologous, the pattern of ANCA binding to neutrophils is similar and the effects in the kidney are comparable, with a pauci-immune focal segmental crescentic GN. **Table 1** summarizes selected models of MPO-ANCA associated renal vasculitis.

Passive Transfer of Anti-MPO Antibodies, Splenocytes, or MPO-Specific T Cells

Transfer of Anti-MPO Antibodies

Models centered on passive transfer of MPO-ANCA-like antibodies have been used in several laboratories to elucidate the complex pathogenesis of disease (15, 25, 31). Antibodies are usually generated by immunizing MPO deficient mice. While these antibodies are similar to MPO-ANCA, they are generated in mice that are not tolerant to MPO (15, 32) and therefore are most accurately described as anti-MPO antibodies. Models based on these principles have been valuable in explaining several areas of the effector response in AAV. These include the role of ANCA, neutrophil priming, activation, migration and adhesion, followed by endothelial injury. Furthermore, targets for potential treatments have been identified and trialed (21).

ANCA were initially identified in patients with segmental necrotising GN in the 1980s (11). While subsequent *in vitro* studies showed that ANCA can cause neutrophil activation and degranulation (12, 14, 33, 34), the first *in vivo* animal model data supporting the pathogenicity of ANCA were not published until many years later, when Kobayashi et al. showed enhancement of glomerular injury caused by anti-glomerular basement membrane (GBM) antibodies when co-administered with anti-MPO serum (24). Mouse models of anti-MPO GN involving passive transfer of anti-MPO antibodies were subsequently developed. Transfer of anti-MPO IgG, from MPO-immunized $Mpo^{-/-}$ mice, into C57BL/6 mice and $Rag2^{-/-}$ mice (that lack T and B lymphocytes) caused focal necrotising and crescentic pauci-immune GN, demonstrating the pathogenicity of ANCA *in vivo* and its role in acute glomerular injury (15). A key role for neutrophils as effectors in this model was demonstrated by neutrophil depletion that completely protected mice from glomerular histological injury, suggesting that ANCA induced glomerular injury was neutrophil mediated and that neutrophils were a major ANCA target (35).

Whilst passive transfer of anti-MPO antibodies without neutrophil priming has been shown to cause GN (15), disease is usually more severe when pro-inflammatory signals, such as lipopolysaccharide (LPS), are administered around the time of antibody transfer. Administration of LPS shortly after anti-MPO antibody transfer, with subsequent elevation in tumor necrosis factor (TNF) and circulating MPO levels, was shown to result in a significantly greater proportion of glomerular crescents and glomerular necrosis. This effect could be attenuated by TNF blockade (32). Neutrophil numbers, in addition to priming, were shown to be important in later experiments. Daily granulocyte colony stimulating factor (G-CSF) injections, causing an increase in circulating neutrophils, were administered in addition to LPS in the passive transfer model, leading to more severe disease (31). Based on *in vitro* experiments, the ability for ANCA to activate neutrophils is thought to be dependent on Fcγ receptors, in particular FcγRIIA (36–38). *In vivo* data supports a regulatory role for FcγRIIB in ANCA mediated injury, with $Fcgr2b^{-/-}$ mice pre-treated with LPS and anti-MPO antibodies developing increased glomerular injury compared with FcγRIIB intact mice (39).

Given the role of complement as a potent mediator in vascular inflammation in other diseases, Xiao et al. hypothesized that despite the paucity of complement deposition in renal biopsies in AAV, complement activation may well be an important player. Xiao et al. showed *in vitro* that complement is activated after stimulation of human neutrophils with ANCA, via C3a detection (17), with subsequent experiments confirming that this effect was through activation of the C5a receptor (C5aR) (40). C5 deficient mice or mice treated with a neutralizing anti-C5a antibody were protected from glomerular damage after passive transfer of anti-MPO IgG (41). In addition to neutrophil priming, activation of the C5aR on dendritic cells promotes autoimmunity to MPO (22). A C5aR antagonist CCX168 (avacopan) protected mice expressing human C5aR from glomerular injury (21), with subsequent translation into human clinical trials of this compound in the treatment of AAV (42). The membrane attack complex does not seem to play a significant role, showing that C5a's effects are via the C5aR (21).

Further work has explored the relative contributions of other complement factors. C3 depletion with Cobra Venom Factor (CVF) prevented GN after passive transfer of anti-MPO IgG or anti-MPO splenocytes (17), though C3aR deficient mice are not protected from glomerular injury after passive transfer of anti-MPO IgG (43), and C3aR is not required for neutrophil priming (40). As such, it is conceivable that since C3 is upstream in the complement cascade, its role in neutrophil activation is as a precursor for C5a generation.

The alternative pathway of complement appears to be the dominant pathway in neutrophil activation in experimental AAV. Deficiency of factor B, which is specifically involved in alternate pathway activation, protected mice from development of disease. In contrast, mice deficient in C4, required for activation of the classical and lectin pathways, were not protected (44). Initiation of complement activation appears to be independent of both properdin, which is released by activated neutrophils and can initiate the alternative pathway, and MBL-associated serine protease 2 (MASP-2), which can activate the lectin pathway (41). Properdin deficient mice were not protected from disease after anti-MPO antibody transfer. As such, the initiator of the alternative pathway in AAV remains unknown. Recent *in vitro* data suggests that NETs may provide a scaffold for complement activation (45), including allowing MPO interaction with Factor H, an important alternative complement pathway regulator (46).

After activation by ANCA, neutrophils are attracted to vulnerable vascular beds, where they degranulate and subsequently cause endothelial damage and disease. Inhibiting neutrophil migration may serve as a promising target for treatment of AAV. Experiments by Summers et al. explored

TABLE 1 | Selected models of MPO-ANCA associated renal vasculitis.

	Animal	Severity (+ to + + +)[a]	Duration (effector phase)	Contribution to knowledge of pathogenesis[b]	Limitations
EXPERIMENTAL MPO-AAV					
Passive transfer					No model studies active autoimmunity to MPO
Anti-MPO serum with anti-GBM Ab (24)	Wistar rats	++ to + + +	3 or 15 h, or 14 days	Neutrophil activation is a prerequisite	Dual hit required Strong linear IgG deposition
Transfer of anti-MPO Ab (15)	C57BL/6 or *Rag2*$^{-/-}$ mice	++	6 days	Proof of pathogenic role of anti-MPO Ab and neutrophils	Not strictly autoimmune (anti-MPO Ab raised in *Mpo*$^{-/-}$ mice)
Transfer of splenocytes from MPO-immunized *Mpo*$^{-/-}$ mice (15)	*Rag2*$^{-/-}$ mice	++	13 days	Injury mediated by MPO specific cells	Some immune complex deposition Not strictly autoimmune Immunodeficient recipients
Transfer of MPO intact bone marrow to MPO-immunized *Mpo*$^{-/-}$ mice (25)	*Mpo*$^{-/-}$ mice	++	8 weeks	MPO expression by leukocytes is required for anti-MPO Ab effects	Not strictly autoimmune in the induction of immunity Requires bone marrow transplantation
Transfer of effector MPO-specific CD4+(18, 26) or CD8+ (20) T cells/T cell clones	*Rag1*$^{-/-}$ mice	++	14 days	MPO-specific CD4+/CD8+ T cells recognize MPO planted in the glomerulus, then effect injury	Anti-MPO Ab have been used for antigen deposition/recognition, but often uses sheep anti-mouse GBM Immunodeficient recipients
Active autoimmunity					
Active autoimmunity, with disease trigger: neutrophil lysosomal enzyme extract with H_2O_2 (27), ischemia/reperfusion (28), low-dose anti-GBM Ab (29)	Brown Norway rats	++ to + + +	10 days	MPO-ANCA alone may not be sufficient for disease; trigger required	Significant IgG and C3 deposition Some versions technically challenging
Active autoimmunity in GN-susceptible rats (30)	WKY rats	++	6 weeks	Loss of tolerance to MPO after immunization	Rat strain specific No clear demarcation between induction of immunity and effector responses
Active autoimmunity, with disease trigger (16)	C57BL/6 mice	+ to ++	4–5 days	Understanding of steps in antigen recognition and role of T cells as effectors	Requires trigger Short term effector phase due to the development of active immune responses to foreign globulin

[a] *+, mild; ++, moderate; + + + severe.*

[b] *Only initial contribution listed due to space limitations.*

Ab, antibody; ANCA, anti-neutrophil cytoplasmic antibodies; GBM, glomerular basement membrane; GN, glomerulonephritis; MPO, myeloperoxidase; Rag, recombination activating gene; WKY, Wistar Kyoto.

the drivers of neutrophil recruitment to target organs (47). Highly purified LPS, which specifically engages toll-like receptor 4 (TLR4), was associated with increased neutrophil recruitment and functional renal injury when injected with anti-MPO antibodies. TLR4 was predominantly upregulated in glomerular endothelial cells in mice and human cell lines, leading to production of functionally important neutrophil chemoattractants. These effects were mediated by TLR4 expressed both by immune cells and by endothelial cells secreting CXCL1 and CXCL2, the murine homologs of CXCL8 interleukin-8 (IL-8). Subsequent studies in human MPO-ANCA associated GN demonstrated that intrarenal TLR4 expression correlates with the extent of renal injury (48).

Intravital microscopy in both rats and mice after passive transfer of anti-MPO antibodies has enabled a more detailed exploration of interactions between neutrophils and the vascular endothelium. Initial work in this area studied the rat mesenteric post capillary venule (49). CXCL1 was applied topically to the rat mesentery, and rat MPO-ANCA or control antibodies were injected. Topical CXCL1 alone significantly increased endothelial leukocyte adhesion, as well as transmigration that was further enhanced in rats receiving MPO-ANCA after CXCL1. Similar findings were demonstrated in an active immunization model. In both models, changes were not seen in rats with MPO-ANCA that did not receive CXCL1, highlighting the requirement for chemokine involvement in endothelial injury.

Nolan et al. imaged post capillary venules in the cremaster muscle to assess the response to administration of anti-MPO IgG after pre-treatment with several different cytokines (50). Within minutes of anti-MPO IgG administration, there was reduced leukocyte rolling, enhanced adhesion and increased transmigration across the endothelium. These interactions were Fcγ receptor and β2 integrin dependent, and were mediated by cytokines, in particular TNF. Neutrophil migration and adhesion is partly dependent on activation of the kinin system, with genetic deficiency or pharmacological blockade of bradykinin receptor 1 associated with reduced neutrophil surface expression of adhesion molecules and attenuated GN (51).

Post capillary venules are the primary sites of physiological and pathological leukocyte recruitment, but in AAV leukocytes are recruited to capillary beds, particularly glomerular capillaries. The glomerular microvasculature is not only an important target in AAV, but also is unique in the manner by which leukocytes interact with the endothelium (52, 53); as such, the effects of anti-MPO antibodies in promoting leukocyte adhesion within the glomerulus itself were examined. In a murine model, anti-MPO antibodies were shown to bind to circulating neutrophils, altering adhesion molecules and inducing glomerular leukocyte adhesion via multiple pathways. Mechanisms of adhesion to the glomerular endothelium were affected by the dose of anti-MPO antibodies, as well as pre-treatment with LPS to model a pre-existing inflammatory state (53). In the presence of LPS, low dose anti-MPO antibodies induced CD11a/CD18 dependent glomerular neutrophil adhesion, while higher dose antibodies induced α4 integrin dependent adhesion. In these experiments, the same stimuli did not induce leukocyte recruitment to cremasteric post capillary venules, consistent with clinical observations of preferential and often selective renal involvement of glomeruli in AAV. *In vivo* multiphoton microscopy, with the capacity to image glomerular leukocyte behaviors over time without the risk of endothelial photoactivation, has demonstrated that neutrophils are retained in glomeruli in inflammation, are activated and crawl bidirectionally within the glomerular microvasculature. A single activated neutrophil can crawl at 10 μm per minute, a finding that may explain the acute segmental glomerular necrotic lesions seen in AAV (52). This system has also furthered the understanding of the role of complement in neutrophil migration and endothelial injury. C5a was shown to play an important role in MPO-ANCA induced neutrophil retention and activation within the glomerulus (22).

After activation by ANCA and localization to vulnerable vascular beds, neutrophils degranulate but they can also undergo cell death via multiple mechanisms, including neutrophil extracellular trap (NET) formation (known as NETosis) (54). These web-like histone containing structures contain MPO and PR3, and many pro-inflammatory proteins and peptides, some of which are endogenous TLR agonists (55). NETs are released within the microvasulature, where they not only contribute to endothelial injury but also may activate TLRs expressed on resident tissue cells (48, 56) and may promote local alternate complement pathway activation (57). They also deposit the autoantigens MPO and PR3 in target tissues, making them potentially able to be presented to effector antigen-specific T cells (58). While mechanistic understanding of NETs has largely been gleaned from *in vitro* studies (54, 59), the functional role of NETs as effectors of injury has also been translated to animal models of AAV. After anti-MPO antibody transfer into mice, enhanced degradation of NETs by administration of DNase I was protective against development of anti-MPO antibody GN (45).

Endothelial cell activation and injury in AAV is mediated by signal transduction pathways, including NF-κB signaling. Activation of NF-κB by external stimuli allows for migration of NF-κB into the nucleus, to promote transcription of pro-inflammatory signals. Choi et al. identified that release of TNF from ANCA-activated neutrophils upregulated NF-κB in endothelial cells, with subsequent IL-8 production. Prophylactic application of immunoliposomes which downregulated endothelial NF-κB was associated with reduced glomerular necrosis (60).

Whilst the role of neutrophils is well-established, monocyte/macrophages have context driven pro-inflammatory roles in immune diseases and play a pathogenic role in AAV. In addition to their general pro-inflammatory properties, primed monocytes also express MPO and PR3 on the cell surface and so plausibly may also play a role in disease (10, 61, 62). Anti-MPO antibody transfer experiments showed that selective monocyte depletion limited histological but not functional kidney injury (9). Further evidence of the potential role of macrophages in AAV is gleaned from clinical data. Macrophages comprise a significant proportion of leukocytes in kidneys of people with AAV, especially in early disease (63–65). They generate macrophage extracellular traps (METs) and ∼25% of CD68+ macrophages are positive for MPO protein by immunostaining (55). Furthermore elevated urinary soluble CD163, shed by monocytes and macrophages, is strongly associated with active renal vasculitis and has potential as a biomarker to detect renal relapses of AAV (66).

The demonstration of the pathogenicity of ANCA in this model has prompted strategies to alter interactions between ANCA and effector leukocytes. Modification of ANCA IgG glycosylation via IgG hydrolysis limited the clinical and pathological features of GN (67). Interrupting leukocyte signaling has been examined in this and other models (68). A specific inhibitor of p38 mitogen-activated protein kinase (MAPK) administered either before or after transfer of anti-MPO antibodies limited glomerular crescent formation without reducing haematuria or proteinuria (69). Dooley et al. trialed EDO-S101, a drug combining the alkylating agent bendamustine with the histone deacetylase inhibitor, vorinostat. Whilst pretreatment with this drug reduced circulating leukocytes, it did not prevent development of GN in the passive transfer model (70).

The effects of factors such as environmental exposures on AAV are unclear. However, age is a risk factor for AAV development and severity (6). To evaluate the effect of MPO-ANCA in aged animals, anti-MPO antibodies were passively transferred into recipient aged mice. Aged mice developed more severe GN, with increased circulating and glomerular neutrophils and increased gene expression of pro-inflammatory

cytokines (71). Although costly, aged mice may better model aged humans and in the future may contribute significantly to the understanding of disease pathogenesis and to studies of new treatments for AAV. Similarly, mice exposed to infections may have an immune system more analogous to adult humans (72, 73), and although these systems currently come with several drawbacks, they may contribute to our understanding in the future.

Transfer of Splenocytes From MPO-Immunized Mice

As well as transfer of antibodies, splenocytes from MPO-immunized *Mpo*$^{-/-}$ mice can induce nephritis. Transfer of splenocytes (including both T and B cells) from *Mpo*$^{-/-}$ mice hyperimmunized with either MPO (or BSA as a control protein) into immunodeficient *Rag2*$^{-/-}$ mice resulted in detectable serum anti-MPO antibodies within 3 days with dose-dependent necrotising and crescentic GN and renal impairment by day 13, as well as variable involvement of other organs. There was significant glomerular immune complex deposition both in mice receiving anti-MPO splenocytes and control mice which received anti-BSA splenocytes (15).

Transfer of MPO Intact Bone Marrow to MPO-Immunized Mpo$^{-/-}$ Mice

The pathogenicity of anti-MPO antibodies and the requirement for MPO expression by innate leukocytes was further explored in a bone marrow transfer model where *Mpo*$^{-/-}$ mice were immunized with MPO, irradiated and reconstituted with bone marrow from MPO intact mice (25). Mice that received wild type bone marrow developed GN with crescent formation in ∼30% of glomeruli. MPO-immunized mice that received *Mpo*$^{-/-}$ bone marrow remained disease free. Disease could be induced by transfer of anti-MPO antibodies into non-immunized *Mpo*$^{-/-}$ mice reconstituted with bone marrow from MPO-intact mice. Collectively, this model confirms the requirement for MPO to be present on leukocytes, most likely neutrophils. Further evidence of the pathogenicity of ANCA was provided in this model by Bontscho et al., with a reduction in renal disease, fewer glomerular neutrophils and lower anti-MPO antibody titres after administration of the proteasome inhibitor bortezomib (or corticosteroids and cyclophosphamide) post bone marrow transplantation (74).

This model has also been used to enhance our understanding of the pathways involved in anti-MPO antibody-neutrophil interactions and injury. One such pathway involves phosphoinositide 3-kinase (PI3K), which controls neutrophil respiratory burst and migration. Bone marrow cells from PI3Kγ-deficient (but MPO intact) mice resulted in only mild glomerular abnormalities. Further, a small molecule inhibitor of PI3Kγ (AS605250) protected mice from development of GN after transfer of wild type bone marrow (68). Neutrophil serine proteases (NSPs: cathepsin G, neutrophil elastase and proteinase 3) mediate inflammation and injury. To determine their roles, bone marrow transfer studies were undertaken. Mice receiving NSP deficient bone marrow or marrow from mice lacking dipeptidyl peptidase I (DPPI, required for activation of NSPs) were protected from developing crescentic GN, possibly due to disrupted signaling via IL-1β (75). In addition to the passive anti-MPO antibody studies outlined above, this bone marrow transplant model has helped established a role for C5a-C5aR interactions using C5aR deficient mice (40), as well as a role for NETs in the effector phase of MPO-AAV using Receptor-interacting protein kinase-3 (RIPK3) deficient mice (45).

Transfer of Effector CD4+ or CD8+ T Cells

Passive T cell transfer studies have defined a role for CD4+ and CD8+ cells in AAV. Autoreactive CD4+ and CD8+ cells are present in people with AAV (76–79). The obligatory and well-documented presence of the autoantigen in inflamed tissues in these conditions (due to local myeloid cell release of MPO and PR3) (55) implies a role for T cells as effectors of injury, assuming these antigens can be processed and presented to antigen-specific T cells locally. Model antigens have been shown to recruit T cells to glomeruli to cause injury (80), and at least for MPO, a similar process operates. Experimentally, transfer of antigen-specific CD4+ or CD8+ T cells (as clones) induces necrotising GN when MPO is planted in glomeruli (18, 20).

Experimentally, MPO or MPO peptides can be deposited in glomeruli in several ways. Early experiments perfused renal arteries with MPO (27). In the context of T cell transfer studies, MPO has been deposited in glomeruli in three ways. Firstly, injection of low dose heterologous anti-GBM antibodies induces transient neutrophil recruitment with MPO deposition in glomeruli. Secondly passive transfer of anti-MPO antibodies with LPS results in similar deposition of MPO (18). Thirdly, immunogenic MPO peptides can be coupled to a "carrier" monoclonal mouse anti-GBM monoclonal IgG1 antibody (18, 81). The nephritogenicity of the antibody itself is negligible as mouse IgG1 does not fix complement and has low affinity for Fc receptors. The transfer of clones specific for MPO, either effector Th1 CD4+ T cells or CD8+ cells, to *Rag1*$^{-/-}$ mice results in severe necrotising GN after MPO is planted in glomeruli. Glomerular injury is mild to minimal after transfer of cells specific to an irrelevant specificity (ovalbumin) (18, 20). Antigen can be presented intravascularly by patrolling monocytes (58) and may also be presented locally by glomerular endothelial cells, while later in disease dendritic cells infiltrate diseased glomeruli in human AAV (82).

Experimental Autoimmune Anti-MPO Disease Induced by Active Immunization With MPO

Whilst passive transfer experiments have contributed greatly to our understanding of effector mechanisms in AAV, these systems do not include loss of tolerance to MPO (or PR3). In most passive transfer studies, recipients receive fixed doses of antibodies or cells from donor MPO-immunized animals. Thus, tolerance is not broken in recipients and they do not develop active autoimmunity, meaning that fundamental questions relating to loss of tolerance and potential therapies to re-establish tolerance cannot be addressed. As such, active immunization models of disease have been developed in both rats and mice.

Active Experimental Anti-MPO Disease in Rats

Immunization of Brown Norway rats with MPO, with subsequent development of MPO-ANCA, is not sufficient for development of disease. However, necrotising crescentic GN in the presence of ANCA has been initiated by causing glomerular endothelial damage either through perfusion with lysosomal extract containing MPO (27), ischemia/reperfusion (28), or a sub-nephritogenic dose of anti-GBM antibodies (also known as "nephrotoxic serum") (29). There are several possible explanations for the increased injury observed by these additional triggers. However, subsequent studies have demonstrated that this enhanced injury is largely due to planting of MPO, as an antigen, within glomerular capillaries and subsequent recognition by effector T cells (16, 18, 20). This occurs either by direct localization of MPO (or antigenic MPO peptides), or by transient recruitment of neutrophils that deposit MPO, allowing local recognition of MPO by MPO-specific effector T cells. A variation of these models in rats investigated the role of products released from activated neutrophils, including MPO, in the pathogenicity of MPO-ANCA, especially in the development of ANCA-associated pulmonary disease (83, 84).

Active MPO immunization of the GN-susceptible Wistar Kyoto (WKY) rat strain results in loss of tolerance to MPO with ANCA, mild GN and at times pulmonary disease (30). The role of TNF has been explored in this model. Treatment with anti-TNF antibodies 4 weeks after immunization significantly curtailed active AAV, both functionally and pathologically, without affecting MPO-ANCA titres (85). However, administration of TNF did not enhance disease and TNF levels were not different in rats immunized with MPO compared with controls (86). Unfortunately, a human trial of anti-TNF therapy using etanercept were not successful (87), despite anecdotal reporting of its benefit in humans (88). Although not effective in a mouse passive transfer model of disease, treatment with EDO-S101, that combines an alkylating agent with a histone deacetylase inhibitor, limited renal and lung pathology in MPO-immunized WKY rats even when given after disease establishment, suggesting significant effects on active anti-MPO autoimmunity (70). Co-administration of a sub-nephritogenic dose of anti-GBM antibody to WKY rats enhances glomerular crescent formation and albuminuria. This is associated with overexpression of glomerular chemoattractants including CXCL1 and CXCL2, and enhanced neutrophil activation and adherence to endothelial cells (86).

Active Anti-MPO Glomerulonephritis in Mice

Based on studies in rats in the 1990s (29), an active model of AAV was developed in mice (16). In these models, autoimmunity to MPO is initiated in genetically intact mice, but the MPO-ANCA that develops is not sufficient in itself to induce disease. GN is triggered by injection of a low dose of sheep anti-mouse glomerular basement membrane (anti-GBM) antibodies that, as in the T cell transfer models described above, transiently recruits neutrophils to glomeruli (89) with deposition of MPO. When mice are immunized with MPO, moderate injury develops, but immunization with an irrelevant antigen (usually ovalbumin) results in minimal injury mediated only by the anti-GBM antibodies. Initial studies in this model demonstrated that it is dependent on MPO, as $Mpo^{-/-}$ mice did not develop disease despite mounting an immune response to MPO. This outcome confirms that the use of low-dose anti-GBM antibodies can achieve neutrophil influx and MPO deposition with minimal potential confounding injury. CD4+ T cell depletion in the effector phase markedly attenuated injury, demonstrating the role of effector CD4+ T cells. Furthermore, B cell deficient mice developed similar disease to mice with intact B cells thereby proving the antibody-independent role of T cells in this model of anti-MPO associated GN. Later experiments further highlighted the role of T-cell mediated injury. GN caused by passive transfer of anti-MPO antibodies into B cell deficient mice was enhanced by pre-immunization with MPO to induce MPO-specific CD4+ T cells. These effects could be prevented with T cell depletion (26). Effectively, this active model demonstrated that in the presence of MPO locally in human AAV, most likely via ANCA-activated neutrophil adhesion, effector T cells mediate injury. In the model, ANCA is bypassed by the use of low dose anti-GBM antibodies to deposit MPO in glomeruli, resulting in an effector response that is akin to a delayed type hypersensitivity reaction.

This model has led to further investigation into the role of T cells in AAV. CD4+ effector T cells, in particular upon differentiation to Th17 cells, mediate production of neutrophil chemoattractants by tissue cells via release of IL-17A (80). After MPO immunization, IL-17A deficient mice were protected from disease, via effects on both neutrophils and macrophages (90). Studies using mice deficient in Th1 or Th17 defining cytokines have shown an initial Th17 dominant lesion followed later by a Th1 dominant outcome, where Th17 defining cytokines were redundant (81). Other types of T cells have also been implicated, including CD8+ T cells (20). Unconventional γδ T cells, also a source of IL-17A, play a role in glomerular T cell and neutrophil recruitment. Mice genetically deficient in γδ T cells developed less severe disease compared to wild type mice (91).

The role of FcγRIIB beyond its involvement in neutrophil activation by ANCA was assessed in this T cell dependent model of disease. Whilst not expressed on T cells, FcγRIIB was shown to inhibit T cell responses via a tonic inhibitory effect on professional antigen presenting cells, with FcγRIIB deficient mice having increased CD4+ T cells, macrophage and neutrophil recruitment to glomeruli, resulting in increased glomerular injury (39). In addition to its effects on T and B cell responses, FcγRIIB is also likely to directly limit the activity of effector macrophages. In an analogous manner, complement plays an important role in disease beyond neutrophil stimulation. C5aR1 modulates development of autoimmunity to MPO, with $C5ar1^{-/-}$ mice relatively protected from T cell mediated disease, as dendritic cells lacking the C5aR1 are not able to fully activate anti-MPO T cells (22). In contrast, the absence of C3aR did not affect the development of disease in this active model (43).

Given the induction of autoimmunity in this model, it can be used to interrogate mechanisms of loss of tolerance to MPO. Tan et al. determined that the transcription factor autoimmune regulator (AIRE) promotes the expression of MPO in the thymus,

enabling the deletion of autoreactive anti-MPO T cells in the thymus (**Figure 1A**). This work also found that depletion of regulatory T cells led to more anti-MPO-specific T cells, higher ANCA titres and more severe GN (92). Mast cells contribute to peripheral tolerance to MPO, through IL-10 mediated effects on regulatory T cells (**Figure 1B**) (93). Furthermore, autoimmunity and GN can be attenuated by disodium cromoglicate, a mast cell stabilizer (94).

Animal models have been used to discover and define immunodominant MPO T and B cell epitopes. Specifically, three papers have collectively defined an MPO epitope hot spot in a similar region of the MPO heavy chain, with pathogenic CD4+ T cells (mouse studies across several MHC II allomorphs) (18), B cells (human studies with functional murine models) (95) and CD8+ T cells (a pathogenic MPO peptide in mice that is likely to also bind to common HLA Class I alleles) (20). The CD4+ T cell and B cell epitopes have been validated in human studies (96). As antigen-specific tolerogenic therapies have potential as curative therapies in autoimmune disease (97, 98), knowledge of these epitopes has been used in combination with several tolerogenic platforms in AAV, including nasal tolerance (99), injection of MPO peptide loaded apoptotic cells (81), and injection of tolerogenic dendritic cells (100). While the exact type of regulatory cell varies with different strategies, the mechanism of action in each of these three studies is via the generation of MPO-specific T cells that regulate and suppress established anti-MPO autoimmunity. Collectively these studies show proof of concept that using the previous defined immunodominant MPO T cell epitope can be used in tolerogenic studies, with antigen-specific effects.

Clinical observations have suggested a correlation between infection and AAV (101, 102). There are several potential mechanistic explanations for this association that are not mutually exclusive. One potential explanation involves the engagement of TLRs, expressed on leukocytes or on intrinsic tissue cells, which stimulate immunity and can alter the strength and direction of the immune response. When mice were co-immunized with MPO and TLR ligand, Summers et al. observed enhanced cellular and humoral autoimmunity, compared with mice immunized with MPO alone (103). TLR2 ligands directed Th17 anti-MPO autoimmunity while TLR9 ligands supported Th1 immunity. Infection may also be associated with loss of tolerance to MPO through molecular mimicry. Immunization of mice with a plasmid-encoded peptide with some sequence homology with the T cell immunodominant MPO epitope, but found only in some strains of *Staphylococcus aureus*, induced anti-MPO autoimmunity and vasculitis (104).

A variation of models that immunize with MPO was published by Yumura et al. (105) BSA administration induced anti-BSA antibodies, which themselves activate neutrophils causing release of MPO, promoting loss of tolerance to MPO and the development of anti-MPO antibodies. Mice developed features of pulmonary disease and severe crescentic GN. Despite pulmonary disease being a common clinical feature in AAV, most animal models are limited to renal manifestations of AAV.

ANIMAL MODELS OF PR3-AAV

Compared with experimental models of MPO-AAV, consistent animal models of PR3-AAV (summarized in **Table 2**) have been difficult to establish. There are several possible reasons for this. Compared with MPO, human and mouse PR3 have limited homology of 68%, and the antigenic determinants of human PR3 may not be preserved in mouse PR3 (106). Peripheral blood neutrophil numbers are lower in mice (107) and total white blood cell numbers may be lower compared to humans, depending on mouse strain (108). In contrast to humans, where neutrophils account for up to 70% of total leukocytes, in commonly used inbred mouse strains (C57BL/6 and 129/Sv) neutrophils account for 7.7 and 14% of total white blood cells ($9–9.4 \times 10^3/\mu L$), respectively (109). Under resting conditions, PR3 is expressed on the plasma membrane in humans through its co-expression with CD177, via a hydrophobic patch on PR3 (110). Murine PR3 lacks this hydrophobic patch, possibly accounting for why PR3 is not strongly expressed on the mouse neutrophil surface, especially in the resting state (111). This may be significant in the development of murine models of PR3-AAV, as it is believed that expression of PR3 on the neutrophil surface is critical to the pathogenesis of AAV (112). Furthermore, *in vitro* evidence suggests that PR3-ANCA causes neutrophil activation via FcγRIIA expressed on human neutrophils (37). Importantly, this Fc receptor does not have a murine ortholog (113), which may contribute to the inability of PR3-ANCA to activate mouse neutrophils.

In contrast to MPO-AAV, it has been difficult to use PR3-AAV models of disease to confirm unequivocally the pathogenicity of PR3-ANCA *in vivo*. Passive transfer of human PR3-ANCA from patients with active vasculitis into BALB/c wild type mice led to development of mouse ANCA. After some months many mice developed respiratory inflammation, and diffuse immunoglobulin deposition in the glomeruli, as opposed to the classical pauci-immune GN found in humans with PR3-AAV (114, 115). Subsequent studies passively transferred mouse anti-PR3 antibodies. PR3 antibodies raised in mPR3/neutrophil elastase (mNE) double-deficient mice and passively transferred to wild type mice were not able to induce vasculitis (116). Similarly, anti-PR3 antibodies from rats immunized with chimeric human/mouse PR3 were transferred into wild type mice. Despite high titres of PR3-ANCA, the mice did not develop clinical or histological features of vasculitis (117).

The presence of PR3-ANCA in itself did not cause disease in non-obese diabetic (NOD) mice, which develop spontaneous autoimmune disease. However, splenocyte transfer from these mice into NOD-severe combined immunodeficiency (NOD-SCID) mice caused crescentic GN and death. In contrast, splenocytes transferred into *Rag1*$^{-/-}$ mice did not develop vasculitis. These experiments imply a significant role for the regulatory immune response in maintaining tolerance and limiting effector responses in PR3-AAV (118).

To overcome species-specific differences in PR3 structure and of Fc receptors, Little et al. transferred PR3-ANCA into mice with a humanized immune system. Irradiated NOD-SCID-*Il2rγ*$^{-/-}$ mice were immune reconstituted with human

hematopoietic stem cells. Passive transfer of human PR3-ANCA caused pauci-immune proliferative GN, and histological evidence of pulmonary vasculitis (119), providing the strongest *in vivo* experimental evidence to date for the pathogenicity of PR3-ANCA.

The latest attempts to overcome species differences and develop a representative murine model of PR3-AAV have involved mice that express human PR3. Mice with podocytes that express human PR3 (under a podocin promoter) did not develop disease after injection with anti-PR3 antibodies (120), perhaps due to a lack of access of the antibodies to the antigen that was expressed extravascularly on podocytes. A double-transgenic approach produced mice with human PR3 in neutrophils along with its co-receptor CD177. Vasculitis could not be induced through passive transfer of anti-PR3 antibodies, possibly because the mice may have been unable to process human pro-PR3 into mature PR3 (121). A third transgenic approach by Martin et al. generated mice expressing the mature form of human PR3, which appeared to be enzymatically active. In a model of zymosan-induced peritonitis, the presence of the hPR3 transgene increased neutrophil accumulation and enhanced neutrophil survival compared to PR3 wild type controls (122). Studies in experimental AAV using this mouse are yet to be reported.

OTHER MODELS OF AAV

These models are summarized in **Table 2**.

Models of ANCA-Associated Pulmonary Vasculitis

Most animal models of AAV use glomerular disease as the primary endpoint. However, several animal models of pulmonary disease have been designed. Focal pulmonary hemorrhage and granuloma formation were identified in MPO-immunized Brown Norway rats 7 and 14 days after intravenous infusion of human neutrophil lysosomal extract and hydrogen peroxide (84). These changes were accompanied by haemorrhagic lesions within the intestines. Of note, granuloma formation is more closely associated with PR3-AAV, rather than MPO-AAV. A variation of this model explored the role of ANCA in the lung (83). Isolated left lungs of MPO-immunized rats were perfused with a neutrophil lysosomal extract. The isolated left lung displayed inflammatory lesions in both immunized and non-immunized mice, though more extensive in the immunized group; in contrast, there were inflammatory infiltrates also in the right lung only in the presence of anti-MPO antibodies.

Other systems have modeled PR3-AAV associated pulmonary involvement. C-ANCA from patients with GPA was transferred to Wistar rats (123). All animals displayed marked pulmonary vasculitis 24 h after antibody transfer in a dose-dependent manner [despite the limited homology between human and mouse PR3 (106)], with no disease observed in mice treated with control IgG. Co-perfusion of isolated rat lungs with primed human neutrophils along with murine monoclonal PR3 antibody rapidly caused oedema formation and increased microvascular permeability (124). This was not observed after perfusion of primed neutrophils alone or anti-PR3 antibodies alone, suggesting synergistic roles for anti-PR3 antibodies and neutrophils in pulmonary pathology in AAV.

Pulmonary findings have been described in models that otherwise focus on renal disease. Pulmonary capillaritis developed in 5 of 16 mice after passive transfer of high doses of anti-MPO splenocytes into *Rag2*$^{-/-}$ mice, and in 2 of 6 wild type mice after transfer of anti-MPO antibodies (15). A similar result was also seen in MPO-immunized *Mpo*$^{-/-}$ mice which were irradiated and reconstituted with bone marrow from wild type mice (25), and after transfer of human PR3-ANCA into irradiated NOD-SCID-*Il2rγ*$^{-/-}$ mice reconstituted with human hematopoietic stem cells (119). Pulmonary lesions were occasionally and inconsistently detected after transfer of MPO peptide-specific CD4+ T cell clones, despite all mice developing glomerulonephritis (18).

Experimental AAV Induced by Autoimmunity to LAMP-2

Whilst MPO and PR3 are the most common antigenic targets of ANCA, other neutrophil proteins have been identified, including lysosome-associated membrane protein-2 (LAMP-2). Autoantibodies to LAMP-2 have been detected in humans, and were detectable in WKY rats after immunization with LAMP-2; these antibodies induced acute focal necrotising GN on transfer (125). The bacterial adhesin FimH contains a sequence with strong homology to the immunogenic epitope of LAMP-2 (with eight of the nine amino acids identical). Immunization with FimH induced anti-LAMP-2 antibodies and pauci-immune GN. It was proposed that autoimmunity to LAMP-2 was due to molecular mimicry, potentially explaining the temporal relationship between bacterial infection and AAV. The authors hypothesized that antibodies to LAMP-2 may alter presentation by neutrophils of cytoplasmic antigens, facilitating generation of autoantibodies to MPO or PR3. The results of these animal experiments were not replicated by another research group (129), though anti-LAMP-2 antibodies were found in European AAV cohorts (130, 131).

ANCA and Disease in Lupus Prone Mice

Clinically, ANCA can be detected in some patients with systemic lupus erythematosus (SLE) (132, 133), and recent observations suggest that this is associated with a more vasculitic renal phenotype (134). Similarly, some mouse strains that develop systemic autoimmunity also have perinuclear ANCA formation and crescentic GN. A proportion of lupus-prone MRL/*lpr*^{fas} mice develop MPO-ANCA that bind to MPO on the neutrophil surface. These mice exhibit a phenotype with some characteristics of AAV (135, 136). MRL/*lpr* mice have been further genetically modified to knockout *Nos3*, in order to investigate the potential for endothelial nitric oxide synthase (eNOS, or NOS3) to inhibit vascular inflammation. Paradoxically, NOS3 deficient mice had accelerated onset and increased incidence of renal vasculitis compared with control MRL/*lpr* mice (137).

Derived from two lupus prone strains, MRP/*lpr* and BXSB, and selectively bred for crescent formation, spontaneous crescentic GN forming/Kinjoh (SCG/Kj) mice have more

TABLE 2 | Selected models of ANCA associated vasculitis (see **Table 1** for animal models of MPO-ANCA associated renal vasculitis).

	Animal	Severity (+ to + + +)[a]	Duration (effector phase)	Contribution to knowledge of pathogenesis[b]	Limitations
EXPERIMENTAL PR3-AAV					
Passive transfer					
PR3-specific splenocyte transfer (118)	NOD-SCID mice	++ to + + +	20-40 days	Anti-PR3 B and T cells mediate injury; role for regulatory immune response	Poor homology between human and mouse PR3
Passive transfer of human PR3-ANCA to mice reconstituted with human stem cells (119)	Irradiated NOD-SCID-*Il2rγ*$^{-/-}$ mice	+	6 days	*In vivo* evidence for human PR3-ANCA pathogenicity	Human PR3 present on chimeric neutrophils and monocytes required Challenging immune reconstitution of the mice
PULMONARY DISEASE					
Active anti-MPO autoimmunity with human neutrophil lysosomal extract infusion (84)	Brown Norway rats	++ to + + +	14 days	Chronic inflammation and fibrosis seen at 14 days	Granuloma formation unusual in MPO-AAV
Active anti-MPO autoimmunity with localized single lung human neutrophil lysosomal extract infusion (83)	Brown Norway rats	++	10 days	Local and systemic effects of neutrophil degranulation	Infusion caused pulmonary damage in the absence of MPO-ANCA
Passive transfer of human PR3-ANCA into rats (123)	Wistar rats	++ to + + +	24 h	*In vivo* evidence of pathogenicity of PR3-ANCA	Not strictly autoimmune
Perfusion of isolated rat lungs with primed human neutrophils and monoclonal PR3 Ab (124)	CD (SD) rats	++	3 h	Acute lung injury caused by neutrophil degranulation and free oxygen radicals	*Ex vivo* model Does not model the process of neutrophil migration to the lungs *in vivo*
OTHER MODELS					
Passive transfer of LAMP-2 Ab (125)	WKY rats	+ to ++	5 days	LAMP-2 is an additional target of ANCA	Not all Ab preparations are pathogenic
Immunization with FimH (125)	WKY rats	++	39 days	Molecular mimicry may underpin loss of tolerance to LAMP-2	Antigen processing not taken into account No clear demarcation between induction of immunity and effector responses
Spontaneous crescent formation in autoimmune-prone mice (126)	SCG/Kj mice	+ + +	Life span 120–135 days	Early onset severe disease	Derived from lupus prone strains Other auto-Ab present Significant immune complex deposition in glomeruli
Passive transfer of NET-loaded DC (59)	BALB/c and C57BL/6 mice	++ to + + +	3 months	NETs may be involved in development of autoimmunity to MPO and PR3	Production of other auto-Ab in addition to ANCA Long model, requires multiple DC infusions
Passive transfer of PTU-induced abnormal NETs, PTU-induced MPO-ANCA production (127)	WKY rats	+	30 days	Prolonged MPO exposure via NETs may participate in loss of tolerance	Mild disease
Nephrotoxic serum nephritis (128)	C57BL/6 mice	+ + +	7–21 days	Mechanisms of severe nephritis	Mechanistically different effectors No induction of responses to ANCA antigens or transfer of specific cells or Ab

[a] *+, mild; ++, moderate; + + + severe.*

[b] *Only initial contribution listed due to space limitations.*

Ab, antibody; ANCA, anti-neutrophil cytoplasmic antibodies; CD (SD) Cesarean derived (Sprague-Dawley); DC, dendritic cells; LAMP-2, lysosome-associated membrane protein 2; MPO, myeloperoxidase; NETs, neutrophil extracellular traps; NOD, non-obese diabetic; PR3, proteinase 3; PTU, propylthiouracil; Rag, recombination activating gene; SCG/Kj, spontaneous crescentic glomerulonephritis-forming/Kinjoh; SCID, severe combined immunodeficiency; WKY, Wistar Kyoto.

extensive glomerular crescents in all mice (126), though the renal disease may be largely immune complex mediated (138), with development of disease and progression to renal failure significantly delayed by calorie restriction (139). Initial suggestions that neutrophils are required for ANCA-associated GN development were based on experiments in these mice, in

which increased peripheral neutrophil numbers and glomerular neutrophil infiltration correlated with disease (140). Multiple genetic associations with MPO-ANCA development in SCG/Kj mice have been defined (141). This model has also been used to investigate possible treatments for ANCA-associated GN, including 15-deoxyspergualin (142, 143) and omega-3 fatty acid eicosapentaenoic acid (EPA) (144).

Dendritic cell transfer studies have implicated NETs in the development of autoimmunity to MPO and PR3 (59). NETosis-prone neutrophils from naïve mice were co-cultured with myeloid dendritic cells (mDC), resulting in mDC uploading with NET components including MPO. Wild type mice subsequently immunized with NET-loaded dendritic cells (but not dendritic cells exposed to apoptotic or DNAse1 treated neutrophils), lost tolerance to MPO and PR3 and produced ANCA. Furthermore, induction of ANCA was associated with moderate to severe renal injury. However, antibodies to other targets were also produced, including single-stranded and double-stranded DNA, implying a more generalized loss of tolerance consistent with SLE or an SLE-like syndrome.

It is difficult to isolate the effects of anti-MPO and anti-PR3 autoreactivity in these models. While ANCA are present, so are other autoantibodies, including anti-double stranded DNA (dsDNA) antibodies. These models are potentially useful as models of vasculitis occurring in the context of SLE with concurrent MPO-ANCA, where in humans segmental necrosis is more likely and in which ANCA may play a role (134).

Drug-Induced Experimental AAV

The development of MPO-ANCA and clinical features of AAV has been reported in association with many different drugs [review in Gao and Zhao (145)]. The anti-thyroid drug propylthiouracil (PTU) is associated with production of MPO-ANCA in up to 30% of patients, with some patients developing MPO-AAV (146). *In vitro*, incubation of human neutrophils with PTU in the presence of phorbol myristate acetate (PMA) induces NETs. These PTU induced NETs have an abnormal conformation and are more resistant to breakdown by DNase I. It is thought that prolonged MPO exposure on NETs participates in loss of tolerance to MPO, and subsequent ANCA production. Passive transfer of these abnormal NETs into WKY rats caused development of MPO-ANCA and pulmonary capillaritis. In an active model of disease, MPO-ANCA and features of vasculitis developed in rats given PTU in addition to intraperitoneal PMA, with evidence of DNase1-resistant abnormal NET formation. In contrast, mice given only intraperitoneal PMA developed NETs without development of MPO-AAV (127). In a similar model of PTU-induced MPO-ANCA associated vasculitis, BALB/c mice were given PTU; NET formation was attenuated by peptidylarginine deiminase (PAD) inhibition, with lower MPO-ANCA titres (147).

Autologous Phase Anti-GBM GN (Nephrotoxic Serum Nephritis)

The most commonly used model of severe and rapidly progressive GN is the autologous phase (accelerated or non-accelerated) "anti-GBM" model, also known as nephrotoxic serum nephritis (128). This model has been used as proxy for ANCA-associated GN (148). However, while glomerular injury may appear similar histologically, the pathogenesis of this model is substantially different to that of AAV. Glomerular injury in this model is not due to autoimmunity and autoreactivity to MPO is not present (149, 150).

The relative contributions of cellular and humoral effectors in this model depend on the strain and species of rodent used, and the nature, timing and dose of the foreign globulin. The initial phase of injury is neutrophil mediated, though unlike ANCA-associated vasculitis, here the heterologous globulin binds to the glomerulus and neutrophils are retained in glomeruli via this *in situ* immune complex mediated disease (89, 151). Subsequently, in non-accelerated iterations of this model, immunity to the foreign globulin (usually raised in sheep or rabbits) as a foreign antigen (not as an autoantigen) develops. Anti-sheep (rabbit) antibodies and/or anti-sheep (rabbit) T cells localize to glomeruli as the antigen (the heterologous globulin) is bound to the glomerulus (152, 153). In the accelerated model, immunity to sheep (rabbit) globulin has been induced by priming with the foreign antigen in adjuvant, but injury does not occur until the antigen localizes to the glomerular basement membrane.

Thus, while there may be some similarities in cellular effectors, critical differences in the induction of immunity (including regulatory T cells), and effector mechanisms mean that this model does not represent ANCA-associated GN. It should not be described as autoimmune and care should be taken in extrapolating results in these models to autoimmune ANCA-associated GN.

DISCUSSION AND CONCLUDING REMARKS

The use of animal models of AAV, especially combined with careful observational and *in vitro* human studies has been instrumental in the major advances in our understanding of the pathogenesis of AAV, ranging from the pathogenicity of ANCA, through to elements of loss of tolerance, the role of infection and the participation of cellular immunity. Animal studies on the functional role of complement have led to human trials of new therapies based on complement inhibition. Each of the models used in this search and described in this review has informed us about different aspects of pathogenesis. **Table 3** summarizes insights into the pathogenesis of AAV obtained by using animal models and their translational potential.

However, the development of a single, accurate and translatable animal model of ANCA-associated vasculitis has challenged researchers for decades. This disease, and its pathogenesis, is unique in multiple respects. Firstly, it is a systemic autoimmune disease, though there is only one autoantibody clinically detected. ANCAs themselves are unusual in that they cause activation, rather than destruction of the target cell. Effector responses are complex. The autoantigens themselves are both interesting and unusual; whilst MPO and PR3 are present systemically, the disease manifestations are in organs where the autoantigen is not expressed. Clinically,

TABLE 3 | Influence of animal models on selected elements of the pathogenesis of ANCA-associated vasculitis.

Observation from humans and *in vitro*	*In vivo* animal models	Future directions
RISK FACTORS		
Incidence increases with age (1)	Anti-MPO antibodies transferred into aged mice associated with more severe disease (71)	PR3 models of disease
PR3-AAV significantly associated with HLA-DP4 (154) and HLA-DR15 (155)		Understanding mechanism of risk
LOSS OF TOLERANCE		
Association between AAV and infection (102) Autoimmunity to PR3 may be triggered by exposure to complementary proteins (156)	Anti-MPO immunity triggered by exposure to bacterial peptide with MPO sequence homology (104) Autoimmunity to LAMP-2 developed after immunization with a homologous peptide from FimH (125)	Re-induction of tolerance
PATHOGENICITY OF ANCA		
Presence of ANCA in patients with AAV (11) *In vitro*, capacity of ANCA to induce neutrophil stimulation and degranulation (12, 34) Treatment response to autoantibody and B cell depletion	Passive transfer anti anti-MPO antibodies caused development of GN (15) Binding of ANCA to neutrophils induces glomerular leukocyte adhesion (53)	Clarify the role of autoantibody depletion in induction of disease remission Role for treatments which alter the antibody itself
NEUTROPHILS AS EFFECTORS		
Paucity of immunoglobulin in renal biopsies suggests antibody-independent mechanisms Number of activated neutrophils in glomeruli is associated with severity of renal disease (13) *In vitro*, neutrophil degranulation after stimulation with ANCA causes endothelial damage (157)	Presence of ANCA-like antibodies without neutrophil activation is insufficient to cause disease (24) Increased peripheral neutrophil numbers and glomerular neutrophil infiltration correlated with disease (140) Neutrophil depletion protects from disease (35) G-CSF administration exacerbates renal injury (31)	Potential therapies for AAV that de-activate neutrophils
EFFECT OF CYTOKINES AND CHEMOKINES ON NEUTROPHIL PRIMING, MIGRATION AND ADHESION		
Association between AAV and infection (102, 158) *In vitro*, priming of neutrophils is required for optimum activation by ANCA (159)	Passive transfer of anti-MPO antibodies in conjunction with LPS (neutrophil priming) causes more severe disease (32) Engagement of TLR4 on glomerular endothelial cells with highly-purified LPS associated with production of CXCL1 and CXCL2, leading to increased neutrophil migration, adhesion and transmigration (47) Upregulation of β2 integrins, mediated by TNF, is associated with decreased leukocyte rolling and enhanced adhesion (50)	Further studies of anti-TNF therapies as potential treatment Studies of inhibiting neutrophil adhesion in AAV
COMPLEMENT IN AAV		
Role of complement first elucidated in animal models, and then confirmed in humans Patients with AAV have increase plasma levels of alternative pathway activation markers (160)	C3 depletion prevents GN in mice in passive transfer model (17) Plays a role in neutrophil retention within the glomerulus and subsequent glomerular injury (22)	Role of complement inhibition in disease management e.g. CCX168/avacopan
NEUTROPHIL EXTRACELLULAR TRAPS (NETs)		
NETs at sites of vascular injury (55)	After activation by ANCA, neutrophils undergo cell death and develop NETs, which promotes autoimmunity to MPO and propagates glomerular endothelial damage (161)	Role for enhanced NET degeneration for disease treatment
ROLE OF T CELLS		
Tubulointerstitial and intraglomerular T cells associated with worse renal injury (55)	Induction of T cell autoimmunity to MPO results in NCGN, even in the absence of B cells and ANCA (16) T cells involved in neutrophil chemoattraction through production of IL-17A (80) Depletion of peripheral regulatory T cells associated with more severe disease (93)	Understanding the mechanism of CD8 T cell mediated end-organ damage Regulatory T cell based therapies for AAV
MONOCYTES		
Activated by ANCA (10) Presence of monocytes and macrophages in renal biopsies Soluble CD163 in urine, which is shed by monocytes, is strongly associated with active renal vasculitis (66)	Monocyte depletion reduces glomerular necrosis and crescent formation after passive transfer (9)	Pre-clinical evaluation of monocyte-related biomarkers and therapies

AAV, ANCA-associated vasculitis; ANCA, anti-neutrophil cytoplasmic antibodies; G-CSF, granulocyte colony stimulating factor; GN, glomerulonephritis; HLA, human leukocyte antigen; LAMP-2, lysosome-associated membrane protein-2; LPS, lipopolysaccharide; MPO, myeloperoxidase; NCGN, necrotizing crescentic glomerulonephritis; NETs, neutrophil extracellular traps; PR3, proteinase 3; TLR4, toll-like receptor 4; TNF, tumor necrosis factor.

the disease is heterogeneous, with significant variability in genetic predisposition, environmental risk factors, severity, organ involvement, and risk of relapse.

Given the multi-faceted pathophysiology of AAV, it is not surprising that no single model can recapitulate all aspects of disease. Several disease models are required to comprehensively model and study AAV. Thus far, passive transfer models have proven valuable in studying early effector responses and have resulted in the translation of anti-C5aR therapies into Phase 2 and Phase 3 clinical trials (21, 42, 162). Further work is necessary to refine and establish animal models that reflect human disease as accurately as possible. Furthermore, the models should be reliably reproducible, tractable and transferrable between laboratories, to promote collaborative research and treatment development. Establishment of such models will facilitate a human-rodent-human iterative approach which may accelerate understanding, discovery and research translation.

Despite the extensive advancement in knowledge over the past decades, treatment of ANCA-associated vasculitis remains non-specific and toxic. With the likely exception of rituximab, new therapies have not been more efficacious than standard of care, itself associated with significant risks of infection and malignancy. Given the complexity of the pathophysiology, treatment may need to be multi-targeted, requiring collaborative research for development and testing.

Given the emerging knowledge of the differences between PR3-AAV and MPO-AAV, there is a growing need for a model of PR3-AAV. Much can be learnt from the experiences of previous attempts to develop an animal model. The ideal model would likely require human mature PR3 expression in the neutrophil through genetic mutation, manipulation of neutrophil numbers and PR3 membrane expression, as well as consider the importance of Fcγ receptors in the ability of ANCA to activate neutrophils. Furthermore, mice transgenic for human immune genes, such as HLA, may be used for understanding the strong genetic associations identified with PR3 (154, 155, 163, 164) and model key pathways in loss of tolerance and effector responses. Currently there are no published models of eosinophilic granulomatosis with polyangiitis (EGPA). A significant proportion of people with EGPA have MPO-ANCA antibodies and recent GWAS studies suggest a combination of genes relevant both to autoimmunity and to allergy/eosinophil function may be involved in EGPA (165).

In the future, the current animal models need to continue to evolve to address key clinical questions at hand. Examples of how these questions might be addressed are outlined below. ANCA associated vasculitis is largely a disease of older people, and older age is associated with worse renal outcomes and increased mortality, with more complications of treatment (166). The use of aged mice in translational research is increasing, and allows a unique opportunity to more closely mimic human disease (167). Multiple genetic associations with AAV have been identified, especially with regards to antigen presentation (154, 155, 168), which could be mechanistically explored in HLA transgenic mice. The role of concurrent infections, including but not limited to latent cytomegalovirus infection, in loss of tolerance to ANCA antigens, as well as disease outcomes, needs to be considered (169, 170).

At this stage, animal models of MPO-AAV are unable to meaningfully mimic chronic end-organ disease. Clinically, how to best manage AAV in the medium to long term, given its chronic relapsing autoimmunity with tissue injury and damage is a major challenge, and can lead to use of long-term immunosuppression that may or may not be required. Precision medicine would ideally include the capacity to recognize patients at risk of relapse, and reliably identify relapses before end-organ damage ensues. Furthermore, as in many chronic inflammatory diseases, treatments that prevent progressive fibrosis are needed to preserve function after tissue damage mediated by anti-PR3 and anti-MPO autoimmunity.

AUTHOR CONTRIBUTIONS

LS and AK conducted literature searches, selected relevant articles, planned the format of the article, and wrote the article. LS, SH, and AK reviewed, edited, and finalized the article for submission.

FUNDING

The authors' work in this area was funded by National Health and Medical Research Council (NHMRC) project grants (1128267 and 1104422 to AK and SH) and an NHMRC European Union collaborative research grant (1115805 to AK) as part of the EU Horizon 20/20 RELapses prevENTion in chronic autoimmune disease (RELENT) Consortium. LS was supported by an NHMRC Postgraduate Research Scholarship (1151380) and the Royal Australasian College of Physicians Jacquot NHMRC Award.

REFERENCES

1. Watts RA, Mahr A, Mohammad AJ, Gatenby P, Basu N, Flores-Suarez LF. Classification, epidemiology and clinical subgrouping of antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis. *Nephrol Dial Transplant.* (2015) 30(Suppl. 1):i14–22. doi: 10.1093/ndt/gfv022
2. Walton EW. Giant-cell granuloma of the respiratory tract (Wegener's granulomatosis). *Br Med J.* (1958) 2:265–70. doi: 10.1136/bmj.2.5091.265
3. 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
4. Jennette JC. Overview of the 2012 revised international Chapel Hill consensus conference nomenclature of vasculitides. *Clin Exp Nephrol.* (2013) 17:603–6. doi: 10.1007/s10157-013-0869-6
5. Cordova-Sanchez BM, Mejia-Vilet JM, Morales-Buenrostro LE, Loyola-Rodriguez G, Uribe-Uribe NO, Correa-Rotter R. Clinical presentation and outcome prediction of clinical, serological, and histopathological classification schemes in ANCA-associated vasculitis with renal involvement. *Clin Rheumatol.* (2016) 35:1805–16. doi: 10.1007/s10067-016-3195-z
6. Salmela A, Tornroth T, Poussa T, Ekstrand A. Prognostic factors for survival and relapse in ANCA-associated vasculitis with renal involvement:

a clinical long-term follow-up study. *Int J Nephrol.* (2018) 2018:6369814. doi: 10.1155/2018/6369814
7. Cornec D, Cornec-Le Gall E, Fervenza FC, Specks U. ANCA-associated vasculitis - clinical utility of using ANCA specificity to classify patients. *Nat Rev Rheumatol.* (2016) 12:570–9. doi: 10.1038/nrrheum.2016.123
8. Hutton HL, Holdsworth SR, Kitching AR. ANCA-associated vasculitis: pathogenesis, models, and preclinical testing. *Semin Nephrol.* (2017) 37:418–35. doi: 10.1016/j.semnephrol.2017.05.016
9. Rousselle A, Kettritz R, Schreiber A. Monocytes promote crescent formation in anti-myeloperoxidase antibody-induced glomerulonephritis. *Am J Pathol.* (2017) 187:1908–15. doi: 10.1016/j.ajpath.2017.05.003
10. Weidner S, Neupert W, Goppelt-Struebe M, Rupprecht HD. Antineutrophil cytoplasmic antibodies induce human monocytes to produce oxygen radicals *in vitro*. *Arthritis Rheum.* (2001) 44:1698–706. doi: 10.1002/1529-0131(200107)44:7<1698::AID-ART294>3.0.CO;2-J
11. Davies DJ, Moran JE, Niall JF, Ryan GB. Segmental necrotising glomerulonephritis with antineutrophil antibody: possible arbovirus aetiology? *Br Med J.* (1982) 285:606. doi: 10.1136/bmj.285.6342.606
12. Falk RJ, Terrell RS, Charles LA, Jennette JC. Anti-neutrophil cytoplasmic autoantibodies induce neutrophils to degranulate and produce oxygen radicals *in vitro*. *Proc Natl Acad Sci USA.* (1990) 87:4115–9. doi: 10.1073/pnas.87.11.4115
13. Brouwer E, Huitema MG, Mulder AH, Heeringa P, van Goor H, Tervaert JW, et al. Neutrophil activation *in vitro* and *in vivo* in Wegener's granulomatosis. *Kidney Int.* (1994) 45:1120–31. doi: 10.1038/ki.1994.149
14. Keogan MT, Esnault VL, Green AJ, Lockwood CM, Brown DL. Activation of normal neutrophils by anti-neutrophil cytoplasm antibodies. *Clin Exp Immunol.* (1992) 90:228–34. doi: 10.1111/j.1365-2249.1992.tb07934.x
15. 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/JCI0215918
16. Ruth AJ, Kitching AR, Kwan RY, Odobasic D, Ooi JD, Timoshanko JR, et al. Anti-neutrophil cytoplasmic antibodies and effector CD4+ cells play nonredundant roles in anti-myeloperoxidase crescentic glomerulonephritis. *J Am Soc Nephrol.* (2006) 17:1940–9. doi: 10.1681/ASN.2006020108
17. Xiao H, Schreiber A, Heeringa P, Falk RJ, Jennette JC. Alternative complement pathway in the pathogenesis of disease mediated by anti-neutrophil cytoplasmic autoantibodies. *Am J Pathol.* (2007) 170:52–64. doi: 10.2353/ajpath.2007.060573
18. Ooi JD, Chang J, Hickey MJ, Borza DB, Fugger L, Holdsworth SR, et al. The immunodominant myeloperoxidase T-cell epitope induces local cell-mediated injury in antimyeloperoxidase glomerulonephritis. *Proc Natl Acad Sci USA.* (2012) 109:E2615–24. doi: 10.1073/pnas.1210147109
19. Roth A, Ooi J, Hess J, van Timmeren M, Berg E, Poulton C, et al. ANCA epitope specificity determines pathogenicity, detectability and clinical predictive value. *Presse Med.* (2013) 42:664. doi: 10.1016/j.lpm.2013.02.033
20. Chang J, Eggenhuizen P, O'Sullivan KM, Alikhan MA, Holdsworth SR, Ooi JD, et al. CD8+ T cells effect glomerular injury in experimental anti-myeloperoxidase GN. *J Am Soc Nephrol.* (2017) 28:47–55. doi: 10.1681/ASN.2015121356
21. Xiao H, Dairaghi DJ, Powers JP, Ertl LS, Baumgart T, Wang Y, et al. C5a receptor (CD88) blockade protects against MPO-ANCA GN. *J Am Soc Nephrol.* (2014) 25:225–31. doi: 10.1681/ASN.2013020143
22. Dick J, Gan PY, Ford SL, Odobasic D, Alikhan MA, Loosen SH, et al. C5a receptor 1 promotes autoimmunity, neutrophil dysfunction and injury in experimental anti-myeloperoxidase glomerulonephritis. *Kidney Int.* (2018) 93:615–25. doi: 10.1016/j.kint.2017.09.018
23. Hilhorst M, van Paassen P, Tervaert JW, Limburg Renal R. Proteinase 3-ANCA vasculitis versus myeloperoxidase-ANCA vasculitis. *J Am Soc Nephrol.* (2015) 26:2314–27. doi: 10.1681/ASN.2014090903
24. Kobayashi K, Shibata T, Sugisaki T. Aggravation of rat nephrotoxic serum nephritis by anti-myeloperoxidase antibodies. *Kidney Int.* (1995) 47:454–63. doi: 10.1038/ki.1995.58
25. Schreiber A, Xiao H, Falk RJ, Jennette JC. Bone marrow-derived cells are sufficient and necessary targets to mediate glomerulonephritis and vasculitis induced by anti-myeloperoxidase antibodies. *J Am Soc Nephrol.* (2006) 17:3355–64. doi: 10.1681/ASN.2006070718
26. Gan PY, Holdsworth SR, Kitching AR, Ooi JD. Myeloperoxidase (MPO)-specific CD4+ T cells contribute to MPO-anti-neutrophil cytoplasmic antibody (ANCA) associated glomerulonephritis. *Cell Immunol.* (2013) 282:21–7. doi: 10.1016/j.cellimm.2013.04.007
27. Brouwer E, Huitema MG, Klok PA, de Weerd H, Tervaert JW, Weening JJ, et al. Antimyeloperoxidase-associated proliferative glomerulonephritis: an animal model. *J Exp Med.* (1993) 177:905–14. doi: 10.1084/jem.177.4.905
28. Brouwer E, Klok PA, Huitema MG, Weening JJ, Kallenberg CG. Renal ischemia/reperfusion injury contributes to renal damage in experimental anti-myeloperoxidase-associated proliferative glomerulonephritis. *Kidney Int.* (1995) 47:1121–9. doi: 10.1038/ki.1995.160
29. Heeringa P, Brouwer E, Klok PA, Huitema MG, van den Born J, Weening JJ, et al. Autoantibodies to myeloperoxidase aggravate mild anti-glomerular-basement-membrane-mediated glomerular injury in the rat. *Am J Pathol.* (1996) 149:1695–706.
30. Little MA, Smyth L, Salama AD, Mukherjee S, Smith J, Haskard D, et al. Experimental autoimmune vasculitis: an animal model of anti-neutrophil cytoplasmic autoantibody-associated systemic vasculitis. *Am J Pathol.* (2009) 174:1212–20. doi: 10.2353/ajpath.2009.080458
31. Freeley SJ, Coughlan AM, Popat RJ, Dunn-Walters DK, Robson MG. Granulocyte colony stimulating factor exacerbates antineutrophil cytoplasmic antibody vasculitis. *Ann Rheum Dis.* (2013) 72:1053–8. doi: 10.1136/annrheumdis-2012-202160
32. Huugen D, Xiao H, van Esch A, Falk RJ, Peutz-Kootstra CJ, Buurman WA, et al. Aggravation of anti-myeloperoxidase antibody-induced glomerulonephritis by bacterial lipopolysaccharide: role of tumor necrosis factor-alpha. *Am J Pathol.* (2005) 167:47–58. doi: 10.1016/S0002-9440(10)62952-5
33. Franssen CF, Huitema MG, Muller Kobold AC, Oost-Kort WW, Limburg PC, Tiebosch A, et al. *In vitro* neutrophil activation by antibodies to proteinase 3 and myeloperoxidase from patients with crescentic glomerulonephritis. *J Am Soc Nephrol.* (1999) 10:1506–15.
34. Charles LA, Caldas ML, Falk RJ, Terrell RS, Jennette JC. Antibodies against granule proteins activate neutrophils *in vitro*. *J Leukoc Biol.* (1991) 50:539–46. doi: 10.1002/jlb.50.6.539
35. Xiao H, Heeringa P, Liu Z, Huugen D, Hu P, Maeda N, et al. The role of neutrophils in the induction of glomerulonephritis by anti-myeloperoxidase antibodies. *Am J Pathol.* (2005) 167:39–45. doi: 10.1016/S0002-9440(10)62951-3
36. Mulder AH, Heeringa P, Brouwer E, Limburg PC, Kallenberg CG. Activation of granulocytes by anti-neutrophil cytoplasmic antibodies (ANCA): a Fc gamma RII-dependent process. *Clin Exp Immunol.* (1994) 98:270–8. doi: 10.1111/j.1365-2249.1994.tb06137.x
37. Porges AJ, Redecha PB, Kimberly WT, Csernok E, Gross WL, Kimberly RP. Anti-neutrophil cytoplasmic antibodies engage and activate human neutrophils via Fc gamma RIIa. *J Immunol.* (1994) 153:1271–80.
38. Kettritz R, Jennette JC, Falk RJ. Crosslinking of ANCA-antigens stimulates superoxide release by human neutrophils. *J Am Soc Nephrol.* (1997) 8:386–94.
39. Ooi JD, Gan PY, Chen T, Eggenhuizen PJ, Chang J, Alikhan MA, et al. FcgammaRIIB regulates T-cell autoreactivity, ANCA production, and neutrophil activation to suppress anti-myeloperoxidase glomerulonephritis. *Kidney Int.* (2014) 86:1140–9. doi: 10.1038/ki.2014.189
40. Schreiber A, Xiao H, Jennette JC, Schneider W, Luft FC, Kettritz R. C5a receptor mediates neutrophil activation and ANCA-induced glomerulonephritis. *J Am Soc Nephrol.* (2009) 20:289–98. doi: 10.1681/ASN.2008050497
41. Freeley SJ, Popat RJ, Parmar K, Kolev M, Hunt BJ, Stover CM, et al. Experimentally-induced anti-myeloperoxidase vasculitis does not require properdin, MASP-2 or bone marrow-derived C5. *J Pathol.* (2016) 240:61–71. doi: 10.1002/path.4754
42. Jayne DRW, Bruchfeld AN, Harper L, Schaier M, Venning MC, Hamilton P, et al. Randomized trial of c5a receptor inhibitor avacopan in ANCA-associated vasculitis. *J Am Soc Nephrol.* (2017) 28:2756–67. doi: 10.1681/ASN.2016111179
43. Dick J, Gan PY, Kitching AR, Holdsworth SR. The C3aR promotes macrophage infiltration and regulates ANCA production

but does not affect glomerular injury in experimental anti-myeloperoxidase glomerulonephritis. *PLoS ONE.* (2018) 13:e0190655. doi: 10.1371/journal.pone.0190655
44. Huugen D, van Esch A, Xiao H, Peutz-Kootstra CJ, Buurman WA, Tervaert JW, et al. Inhibition of complement factor C5 protects against anti-myeloperoxidase antibody-mediated glomerulonephritis in mice. *Kidney Int.* (2007) 71:646–54. doi: 10.1038/sj.ki.5002103
45. Schreiber A, Rousselle A, Becker JU, von Massenhausen A, Linkermann A, Kettritz R. Necroptosis controls NET generation and mediates complement activation, endothelial damage, and autoimmune vasculitis. *Proc Natl Acad Sci U S A.* (2017) 114:E9618–25. doi: 10.1073/pnas.1708247114
46. Chen SF, Wang FM, Li ZY, Yu F, Chen M, Zhao MH. Myeloperoxidase influences the complement regulatory activity of complement factor H. *Rheumatology.* (2018) 57:2213–24. doi: 10.1093/rheumatology/kex529
47. Summers SA, van der Veen BS, O'Sullivan KM, Gan PY, Ooi JD, Heeringa P, et al. Intrinsic renal cell and leukocyte-derived TLR4 aggravate experimental anti-MPO glomerulonephritis. *Kidney Int.* (2010) 78:1263–74. doi: 10.1038/ki.2010.327
48. O'Sullivan KM, Ford SL, Longano A, Kitching AR, Holdsworth SR. Intrarenal Toll-like receptor 4 and Toll-like receptor 2 expression correlates with injury in antineutrophil cytoplasmic antibody-associated vasculitis. *Am J Physiol Renal Physiol.* (2018) 315:F1283–94. doi: 10.1152/ajprenal.00040.2018
49. Little MA, Smyth CL, Yadav R, Ambrose L, Cook HT, Nourshargh S, et al. Antineutrophil cytoplasm antibodies directed against myeloperoxidase augment leukocyte-microvascular interactions *in vivo*. *Blood.* (2005) 106:2050–8. doi: 10.1182/blood-2005-03-0921
50. Nolan SL, Kalia N, Nash GB, Kamel D, Heeringa P, Savage CO. Mechanisms of ANCA-mediated leukocyte-endothelial cell interactions *in vivo*. *J Am Soc Nephrol.* (2008) 19:973–84. doi: 10.1681/ASN.2007111166
51. Hu P, Su H, Xiao H, Gou SJ, Herrera CA, Alba MA, et al. Kinin B1 receptor is important in the pathogenesis of myeloperoxidase-specific ANCA GN. *J Am Soc Nephrol.* (2019). doi: 10.1681/ASN.2019010032
52. Devi S, Li A, Westhorpe CL, Lo CY, Abeynaike LD, Snelgrove SL, et al. Multiphoton imaging reveals a new leukocyte recruitment paradigm in the glomerulus. *Nat Med.* (2013) 19:107–12. doi: 10.1038/nm.3024
53. Kuligowski MP, Kwan RY, Lo C, Wong C, James WG, Bourges D, et al. Antimyeloperoxidase antibodies rapidly induce alpha-4-integrin-dependent glomerular neutrophil adhesion. *Blood.* (2009) 113:6485–94. doi: 10.1182/blood-2008-12-192617
54. Kessenbrock K, Krumbholz M, Schonermarck 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
55. O'Sullivan KM, Lo CY, Summers SA, Elgass KD, McMillan PJ, Longano A, et al. Renal participation of myeloperoxidase in antineutrophil cytoplasmic antibody (ANCA)-associated glomerulonephritis. *Kidney Int.* (2015) 88:1030–46. doi: 10.1038/ki.2015.202
56. Wang H, Gou SJ, Zhao MH, Chen M. The expression of Toll-like receptors 2, 4 and 9 in kidneys of patients with anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis. *Clin Exp Immunol.* (2014) 177:603–10. doi: 10.1111/cei.12365
57. Wang H, Wang C, Zhao MH, Chen M. Neutrophil extracellular traps can activate alternative complement pathways. *Clin Exp Immunol.* (2015) 181:518–27. doi: 10.1111/cei.12654
58. Westhorpe CLV, Norman MU, Hall P, Snelgrove SL, Finsterbusch M, Li A, et al. Effector CD4(+) T cells recognize intravascular antigen presented by patrolling monocytes. *Nat Commun.* (2018) 9:747. doi: 10.1038/s41467-018-03181-4
59. 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
60. Choi M, Schreiber A, Eulenberg-Gustavus C, Scheidereit C, Kamps J, Kettritz R. Endothelial NF-kappaB blockade abrogates ANCA-Induced GN. *J Am Soc Nephrol.* (2017) 28:3191–204. doi: 10.1681/ASN.2016060690
61. Ralston DR, Marsh CB, Lowe MP, Wewers MD. Antineutrophil cytoplasmic antibodies induce monocyte IL-8 release. Role of surface proteinase-3, α1-antitrypsin, and Fcγ receptors. *J Clin Invest.* (1997) 100:1416–24. doi: 10.1172/JCI119662
62. Charles LA, Falk RJ, Jennette JC. Reactivity of antineutrophil cytoplasmic autoantibodies with mononuclear phagocytes. *J Leukoc Biol.* (1992) 51:65–8. doi: 10.1002/jlb.51.1.65
63. Zhao L, David MZ, Hyjek E, Chang A, Meehan SM. M2 macrophage infiltrates in the early stages of ANCA-associated pauci-immune necrotizing GN. *Clin J Am Soc Nephrol.* (2015) 10:54–62. doi: 10.2215/CJN.03230314
64. Weidner S, Carl M, Riess R, Rupprecht HD. Histologic analysis of renal leukocyte infiltration in antineutrophil cytoplasmic antibody-associated vasculitis: importance of monocyte and neutrophil infiltration in tissue damage. *Arthritis Rheum.* (2004) 50:3651–7. doi: 10.1002/art.20607
65. Cunningham MA, Huang XR, Dowling JP, Tipping PG, Holdsworth SR. Prominence of cell-mediated immunity effectors in "pauci-immune" glomerulonephritis. *J Am Soc Nephrol.* (1999) 10:499–506.
66. O'Reilly VP, Wong L, Kennedy C, Elliot LA, O'Meachair S, Coughlan AM, et al. Urinary soluble CD163 in active renal vasculitis. *J Am Soc Nephrol.* (2016) 27:2906–16. doi: 10.1681/ASN.2015050511
67. van Timmeren MM, van der Veen BS, Stegeman CA, Petersen AH, Hellmark T, Collin M, et al. IgG glycan hydrolysis attenuates ANCA-mediated glomerulonephritis. *J Am Soc Nephrol.* (2010) 21:1103–14. doi: 10.1681/ASN.2009090984
68. Schreiber A, Rolle S, Peripelittchenko L, Rademann J, Schneider W, Luft FC, et al. Phosphoinositol 3-kinase-gamma mediates antineutrophil cytoplasmic autoantibody-induced glomerulonephritis. *Kidney Int.* (2010) 77:118–28. doi: 10.1038/ki.2009.420
69. van der Veen BS, Chen M, Muller R, van Timmeren MM, Petersen AH, Lee PA, et al. Effects of p38 mitogen-activated protein kinase inhibition on anti-neutrophil cytoplasmic autoantibody pathogenicity *in vitro* and *in vivo*. *Ann Rheum Dis.* (2011) 70:356–65. doi: 10.1136/ard.2010.129106
70. Dooley D, van Timmeren MM, O'Reilly VP, Brady G, O'Brien EC, Fazekas B, et al. Alkylating histone deacetylase inhibitors may have therapeutic value in experimental myeloperoxidase-ANCA vasculitis. *Kidney Int.* (2018) 94:926–36. doi: 10.1016/j.kint.2018.05.028
71. Wang Q, van Timmeren MM, Petersen AH, Yuan J, Moser J, Brouwer E, et al. Age-determined severity of anti-myeloperoxidase autoantibody-mediated glomerulonephritis in mice. *Nephrol Dial Transplant.* (2017) 32:254–64. doi: 10.1093/ndt/gfw202
72. Beura LK, Hamilton SE, Bi K, Schenkel JM, Odumade OA, Casey KA, et al. Normalizing the environment recapitulates adult human immune traits in laboratory mice. *Nature.* (2016) 532:512–6. doi: 10.1038/nature17655
73. Kitching AR, Ooi JD. From bench to pet shop to bedside? The environment and immune function in mice. *Kidney Int.* (2016) 90:1142–3. doi: 10.1016/j.kint.2016.08.017
74. Bontscho J, Schreiber A, Manz RA, Schneider W, Luft FC, Kettritz R. Myeloperoxidase-specific plasma cell depletion by bortezomib protects from anti-neutrophil cytoplasmic autoantibodies-induced glomerulonephritis. *J Am Soc Nephrol.* (2011) 22:336–48. doi: 10.1681/ASN.2010010034
75. Schreiber A, Pham CT, Hu Y, Schneider W, Luft FC, Kettritz R. Neutrophil serine proteases promote IL-1beta generation and injury in necrotizing crescentic glomerulonephritis. *J Am Soc Nephrol.* (2012) 23:470–82. doi: 10.1681/ASN.2010080892
76. Seta N, Tajima M, Kobayashi S, Kawakami Y, Hashimoto H, Kuwana M. Autoreactive T-cell responses to myeloperoxidase in patients with antineutrophil cytoplasmic antibody-associated vasculitis and in healthy individuals. *Mod Rheumatol.* (2008) 18:593–600. doi: 10.3109/s10165-008-0109-1
77. Griffith ME, Coulthart A, Pusey CD. T cell responses to myeloperoxidase (MPO) and proteinase 3 (PR3) in patients with systemic vasculitis. *Clin Exp Immunol.* (1996) 103:253–8. doi: 10.1046/j.1365-2249.1996.d01-629.x
78. Nogueira E, Hamour S, Sawant D, Henderson S, Mansfield N, Chavele KM, et al. Serum IL-17 and IL-23 levels and autoantigen-specific Th17 cells are elevated in patients with ANCA-associated vasculitis. *Nephrol Dial Transplant.* (2010) 25:2209–17. doi: 10.1093/ndt/gfp783
79. Brouwer E, Stegeman CA, Huitema MG, Limburg PC, Kallenberg CG. T cell reactivity to proteinase 3 and myeloperoxidase in patients with Wegener's granulomatosis (WG). *Clin Exp Immunol.* (1994) 98:448–53. doi: 10.1111/j.1365-2249.1994.tb05511.x

80. Summers SA, Steinmetz OM, Li M, Kausman JY, Semple T, Edgtton KL, et al. Th1 and Th17 cells induce proliferative glomerulonephritis. *J Am Soc Nephrol.* (2009) 20:2518–24. doi: 10.1681/ASN.2009030337
81. Gan PY, Chan A, Ooi JD, Dick J, Nagai K, O'Sullivan KM, et al. Biologicals targeting T helper cell subset differentiating cytokines are effective in the treatment of murine anti-myeloperoxidase glomerulonephritis. *Kidney Int.* (2019) 96:1121–33. doi: 10.1016/j.kint.2019.05.012
82. Wilde B, van Paassen P, Damoiseaux J, Heerings-Rewinkel P, van Rie H, Witzke O, et al. Dendritic cells in renal biopsies of patients with ANCA-associated vasculitis. *Nephrol Dial Transplant.* (2009) 24:2151–6. doi: 10.1093/ndt/gfp019
83. Foucher P, Heeringa P, Petersen AH, Huitema MG, Brouwer E, Tervaert JW, et al. Antimyeloperoxidase-associated lung disease. An experimental model. *Am J Respir Crit Care Med.* (1999) 160:987–94. doi: 10.1164/ajrccm.160.3.9807139
84. Heeringa P, Foucher P, Klok PA, Huitema MG, Tervaert JW, Weening JJ, et al. Systemic injection of products of activated neutrophils and H2O2 in myeloperoxidase-immunized rats leads to necrotizing vasculitis in the lungs and gut. *Am J Pathol.* (1997) 151:131–40.
85. Little MA, Bhangal G, Smyth CL, Nakada MT, Cook HT, Nourshargh S, et al. Therapeutic effect of anti-TNF-alpha antibodies in an experimental model of anti-neutrophil cytoplasm antibody-associated systemic vasculitis. *J Am Soc Nephrol.* (2006) 17:160–9. doi: 10.1681/ASN.2005060616
86. Kanzaki G, Nagasaka S, Higo S, Kajimoto Y, Kanemitsu T, Aoki M, et al. Impact of anti-glomerular basement membrane antibodies and glomerular neutrophil activation on glomerulonephritis in experimental myeloperoxidase-antineutrophil cytoplasmic antibody vasculitis. *Nephrol Dial Transplant.* (2016) 31:574–85. doi: 10.1093/ndt/gfv384
87. Wegener's Granulomatosis Etanercept Trial (WGET) Research Group. Etanercept plus standard therapy for Wegener's granulomatosis. *N Engl J Med.* (2005) 352:351–61. doi: 10.1056/NEJMoa041884
88. McAdoo SP, Pusey CD. Is there a role for TNFα blockade in ANCA-associated vasculitis and glomerulonephritis? *Nephrol Dial Transplant.* (2017) 32(Suppl. 1):i80–8. doi: 10.1093/ndt/gfw361
89. Kitching AR, Ru Huang X, Turner AL, Tipping PG, Dunn AR, Holdsworth SR. The requirement for granulocyte-macrophage colony-stimulating factor and granulocyte colony-stimulating factor in leukocyte-mediated immune glomerular injury. *J Am Soc Nephrol.* (2002) 13:350–8.
90. Gan PY, Steinmetz OM, Tan DS, O'Sullivan KM, Ooi JD, Iwakura Y, et al. Th17 cells promote autoimmune anti-myeloperoxidase glomerulonephritis. *J Am Soc Nephrol.* (2010) 21:925–31. doi: 10.1681/ASN.2009070763
91. Gan PY, Fujita T, Ooi JD, Alikhan MA, Dick J, Shim R, et al. Pathogenic role for gammadelta T Cells in autoimmune anti-myeloperoxidase glomerulonephritis. *J Immunol.* (2017) 199:3042–50. doi: 10.4049/jimmunol.1602025
92. Tan DS, Gan PY, O'Sullivan KM, Hammett MV, Summers SA, Ooi JD, et al. Thymic deletion and regulatory T cells prevent antimyeloperoxidase GN. *J Am Soc Nephrol.* (2013) 24:573–85. doi: 10.1681/ASN.2012090898
93. Gan PY, Summers SA, Ooi JD, O'Sullivan KM, Tan DS, Muljadi RC, et al. Mast cells contribute to peripheral tolerance and attenuate autoimmune vasculitis. *J Am Soc Nephrol.* (2012) 23:1955–66. doi: 10.1681/ASN.2012060572
94. Gan PY, O'Sullivan KM, Ooi JD, Alikhan MA, Odobasic D, Summers SA, et al. Mast cell stabilization ameliorates autoimmune anti-myeloperoxidase glomerulonephritis. *J Am Soc Nephrol.* (2016) 27:1321–33. doi: 10.1681/ASN.2014090906
95. Roth AJ, Ooi JD, Hess JJ, van Timmeren MM, Berg EA, Poulton CE, et al. Epitope specificity determines pathogenicity and detectability in ANCA-associated vasculitis. *J Clin Invest.* (2013) 123:1773–83. doi: 10.1172/JCI65292
96. Free ME, Stember KG, Hess JJ, McInnis EA, Lardinois O, Hogan SL, et al. Restricted myeloperoxidase epitopes drive the adaptive immune response in MPO-ANCA vasculitis. *J Autoimmun.* (2019) 106:102306. doi: 10.1016/j.jaut.2019.102306
97. Benham H, Nel HJ, Law SC, Mehdi AM, Street S, Ramnoruth N, et al. Citrullinated peptide dendritic cell immunotherapy in HLA risk genotype–positive rheumatoid arthritis patients. *Sci Transl Med.* (2015) 7:290ra87. doi: 10.1126/scitranslmed.aaa9301
98. Capini C, Jaturanpinyo M, Chang HI, Mutalik S, McNally A, Street S, et al. Antigen-specific suppression of inflammatory arthritis using liposomes. *J Immunol.* (2009) 182:3556–65. doi: 10.4049/jimmunol.0802972
99. Gan PY, Tan DS, Ooi JD, Alikhan MA, Kitching AR, Holdsworth SR. Myeloperoxidase peptide-based nasal tolerance in experimental ANCA-associated GN. *J Am Soc Nephrol.* (2016) 27:385–91. doi: 10.1681/ASN.2015010089
100. Odobasic D, Oudin V, Ito K, Gan PY, Kitching AR, Holdsworth SR. Tolerogenic dendritic cells attenuate experimental autoimmune antimyeloperoxidase glomerulonephritis. *J Am Soc Nephrol.* (2019) 30:2140–57. doi: 10.1681/ASN.2019030236
101. Arimura Y, Minoshima S, Kamiya Y, Tanaka U, Nakabayashi K, Kitamoto K, et al. Serum myeloperoxidase and serum cytokines in anti-myeloperoxidase antibody-associated glomerulonephritis. *Clin Nephrol.* (1993) 40:256–64.
102. Stegeman CA, Tervaert JW, Sluiter WJ, Manson WL, de Jong PE, Kallenberg CG. Association of chronic nasal carriage of *Staphylococcus aureus* and higher relapse rates in Wegener granulomatosis. *Ann Intern Med.* (1994) 120:12–7. doi: 10.7326/0003-4819-120-1-199401010-00003
103. Summers SA, Steinmetz OM, Gan PY, Ooi JD, Odobasic D, Kitching AR, et al. Toll-like receptor 2 induces Th17 myeloperoxidase autoimmunity while Toll-like receptor 9 drives Th1 autoimmunity in murine vasculitis. *Arthritis Rheum.* (2011) 63:1124–35. doi: 10.1002/art.30208
104. Ooi JD, Jiang JH, Eggenhuizen PJ, Chua LL, van Timmeren M, Loh KL, et al. A plasmid-encoded peptide from *Staphylococcus aureus* induces anti-myeloperoxidase nephritogenic autoimmunity. *Nat Commun.* (2019) 10:3392. doi: 10.1038/s41467-019-11255-0
105. Yumura W, Itabashi M, Ishida-Okawara A, Tomizawa K, Yamashita J, Kaneshiro Y, et al. A novel mouse model for MPO-ANCA-associated glomerulonephritis. *Microbiol Immunol.* (2006) 50:149–57. doi: 10.1111/j.1348-0421.2006.tb03780.x
106. Jenne DE, Fröhlich L, Hummel AM, Specks U. Cloning and functional expression of the murine homologue of proteinase 3: implications for the design of murine models of vasculitis. *FEBS Lett.* (1997) 408:187–90. doi: 10.1016/S0014-5793(97)00418-3
107. Nagarajan P, Shailendra A, Venkatesan R, Kumar MJM, Majumdar SS, Juyal RC. Sex and strain-related differences in the peripheral blood cell values of mutant mouse strains. *Comp Clin Path.* (2011) 21:1577–85. doi: 10.1007/s00580-011-1330-4
108. O'Connell KE, Mikkola AM, Stepanek AM, Vernet A, Hall CD, Sun CC, et al. Practical murine hematopathology: a comparative review and implications for research. *Comp Med.* (2015) 65:96–113.
109. Kile BT, Mason-Garrison CL, Justice MJ. Sex and strain-related differences in the peripheral blood cell values of inbred mouse strains. *Mamm Genome.* (2003) 14:81–5. doi: 10.1007/s00335-002-2160-0
110. von Vietinghoff S, Tunnemann G, Eulenberg C, Wellner M, Cristina Cardoso M, Luft FC, et al. NB1 mediates surface expression of the ANCA antigen proteinase 3 on human neutrophils. *Blood.* (2007) 109:4487–93. doi: 10.1182/blood-2006-10-055327
111. Hu N, Westra J, Huitema MG, Bijl M, Brouwer E, Stegeman CA, et al. Coexpression of CD177 and membrane proteinase 3 on neutrophils in antineutrophil cytoplasmic autoantibody-associated systemic vasculitis: anti-proteinase 3-mediated neutrophil activation is independent of the role of CD177-expressing neutrophils. *Arthritis Rheum.* (2009) 60:1548–57. doi: 10.1002/art.24442
112. Rarok AA, Stegeman CA, Limburg PC, Kallenberg CG. Neutrophil membrane expression of proteinase 3 (PR3) is related to relapse in PR3-ANCA-associated vasculitis. *J Am Soc Nephrol.* (2002) 13:2232–8. doi: 10.1097/01.ASN.0000028642.26222.00
113. Bruhns P. Properties of mouse and human IgG receptors and their contribution to disease models. *Blood.* (2012) 119:5640–9. doi: 10.1182/blood-2012-01-380121
114. Blank M, Tomer Y, Stein M, Kopolovic J, Wiik A, Meroni PL, et al. Immunization with anti-neutrophil cytoplasmic antibody (ANCA) induces the production of mouse ANCA and perivascular lymphocyte infiltration. *Clin Exp Immunol.* (1995) 102:120–30. doi: 10.1111/j.1365-2249.1995.tb06645.x
115. Tomer Y, Gilburd B, Blank M, Lider O, Hershkoviz R, Fishman P, et al. Characterization of biologically active antineutrophil cytoplasmic antibodies

induced in mice. Pathogenetic role in experimental vasculitis. *Arthritis Rheum.* (1995) 38:1375–81. doi: 10.1002/art.1780381004
116. Pfister H, Ollert M, Frohlich LF, Quintanilla-Martinez L, Colby TV, Specks U, et al. Antineutrophil cytoplasmic autoantibodies against the murine homolog of proteinase 3 (Wegener autoantigen) are pathogenic *in vivo*. *Blood.* (2004) 104:1411–8. doi: 10.1182/blood-2004-01-0267
117. van der Geld YM, Hellmark T, Selga D, Heeringa P, Huitema MG, Limburg PC, et al. Rats and mice immunised with chimeric human/mouse proteinase 3 produce autoantibodies to mouse Pr3 and rat granulocytes. *Ann Rheum Dis.* (2007) 66:1679–82. doi: 10.1136/ard.2006.064626
118. Primo VC, Marusic S, Franklin CC, Goldmann WH, Achaval CG, Smith RN, et al. Anti-PR3 immune responses induce segmental and necrotizing glomerulonephritis. *Clin Exp Immunol.* (2010) 159:327–37. doi: 10.1111/j.1365-2249.2009.04072.x
119. Little MA, Al-Ani B, Ren S, Al-Nuaimi H, Leite M Jr, Alpers CE, et al. Anti-proteinase 3 anti-neutrophil cytoplasm autoantibodies recapitulate systemic vasculitis in mice with a humanized immune system. *PLoS ONE.* (2012) 7:e28626. doi: 10.1371/journal.pone.0028626
120. Relle M, Cash H, Schommers N, Reifenberg K, Galle PR, Schwarting A. PR3 antibodies do not induce renal pathology in a novel PR3-humanized mouse model for Wegener's granulomatosis. *Rheumatol Int.* (2013) 33:613–22. doi: 10.1007/s00296-012-2406-4
121. Schreiber A, Eulenberg-Gustavus C, Bergmann A, Jerke U, Kettritz R. Lessons from a double-transgenic neutrophil approach to induce antiproteinase 3 antibody-mediated vasculitis in mice. *J Leukoc Biol.* (2016) 100:1443–52. doi: 10.1189/jlb.5A0116-037R
122. Martin KR, Pederzoli-Ribeil M, Pacreau E, Burgener SS, Dahdah A, Candalh C, et al. Transgenic mice expressing human proteinase 3 exhibit sustained neutrophil-associated peritonitis. *J Immunol.* (2017) 199:3914–24. doi: 10.4049/jimmunol.1601522
123. Weidebach W, Viana VS, Leon EP, Bueno C, Leme AS, Arantes-Costa FM, et al. C-ANCA-positive IgG fraction from patients with Wegener's granulomatosis induces lung vasculitis in rats. *Clin Exp Immunol.* (2002) 129:54–60. doi: 10.1046/j.1365-2249.2002.01888.x
124. Hattar K, Oppermann S, Ankele C, Weissmann N, Schermuly RT, Bohle RM, et al. c-ANCA-induced neutrophil-mediated lung injury: a model of acute Wegener's granulomatosis. *Eur Respir J.* (2010) 36:187–95. doi: 10.1183/09031936.00143308
125. Kain R, Exner M, Brandes R, Ziebermayr R, Cunningham D, Alderson CA, et al. Molecular mimicry in pauci-immune focal necrotizing glomerulonephritis. *Nat Med.* (2008) 14:1088–96. doi: 10.1038/nm.1874
126. Kinjoh K, Kyogoku M, Good RA. Genetic selection for crescent formation yields mouse strain with rapidly progressive glomerulonephritis and small vessel vasculitis. *Proc Natl Acad Sci USA.* (1993) 90:3413–7. doi: 10.1073/pnas.90.8.3413
127. Nakazawa D, Tomaru U, Suzuki A, Masuda S, Hasegawa R, Kobayashi T, et al. Abnormal conformation and impaired degradation of propylthiouracil-induced neutrophil extracellular traps: implications of disordered neutrophil extracellular traps in a rat model of myeloperoxidase antineutrophil cytoplasmic antibody-associated vasculitis. *Arthritis Rheum.* (2012) 64:3779–87. doi: 10.1002/art.34619
128. Odobasic D, Ghali JR, O'Sullivan KM, Holdsworth SR, Kitching AR. Glomerulonephritis induced by heterologous anti-GBM globulin as a planted foreign antigen. *Curr Protoc Immunol.* (2014) 106:15.26.1–20. doi: 10.1002/0471142735.im1526s106
129. Roth AJ, Brown MC, Smith RN, Badhwar AK, Parente O, Chung H, et al. Anti-LAMP-2 antibodies are not prevalent in patients with antineutrophil cytoplasmic autoantibody glomerulonephritis. *J Am Soc Nephrol.* (2012) 23:545–55. doi: 10.1681/ASN.2011030273
130. Peschel A, Basu N, Benharkou A, Brandes R, Brown M, Rees AJ, et al. Autoantibodies to hLAMP-2 in ANCA-negative pauci-immune focal necrotizing GN. *J Am Soc Nephrol.* (2014) 25:455–63. doi: 10.1681/ASN.2013030320
131. Kain R, Tadema H, McKinney EF, Benharkou A, Brandes R, Peschel A, et al. High prevalence of autoantibodies to hLAMP-2 in anti-neutrophil cytoplasmic antibody-associated vasculitis. *J Am Soc Nephrol.* (2012) 23:556–66. doi: 10.1681/ASN.2011090920
132. Chin HJ, Ahn C, Lim CS, Chung HK, Lee JG, Song YW, et al. Clinical implications of antineutrophil cytoplasmic antibody test in lupus nephritis. *Am J Nephrol.* (2000) 20:57–63. doi: 10.1159/000013557
133. Galeazzi M, Morozzi G, Sebastiani GD, Bellisai F, Marcolongo R, Cervera R, et al. Anti-neutrophil cytoplasmic antibodies in 566 European patients with systemic lupus erythematosus: prevalence, clinical associations and correlation with other autoantibodies. European concerted action on the immunogenetics of SLE. *Clin Exp Rheumatol.* (1998) 16:541–6.
134. Turner-Stokes T, Wilson HR, Morreale M, Nunes A, Cairns T, Cook HT, et al. Positive antineutrophil cytoplasmic antibody serology in patients with lupus nephritis is associated with distinct histopathologic features on renal biopsy. *Kidney Int.* (2017) 92:1223–31. doi: 10.1016/j.kint.2017.04.029
135. Harper JM, Thiru S, Lockwood CM, Cooke A. Myeloperoxidase autoantibodies distinguish vasculitis mediated by anti-neutrophil cytoplasm antibodies from immune complex disease in MRL/Mp-lpr/lpr mice: a spontaneous model for human microscopic angiitis. *Eur J Immunol.* (1998) 28:2217–26.
136. Harper JM, Healey DG, Thiru S, Gordon C, Cook A. Factors involved in the pathogenesis of neutrophilic vasculitis in MRL/Mp-lpr/lpr mice: a model for human microscopic angiitis. *Autoimmunity.* (1999) 31:133–45. doi: 10.3109/08916939908994057
137. Schoeb TR, Jarmi T, Hicks MJ, Henke S, Zarjou A, Suzuki H, et al. Endothelial nitric oxide synthase inhibits the development of autoimmune-mediated vasculitis in mice. *Arthritis Rheum.* (2012) 64:4114–24. doi: 10.1002/art.37683
138. Neumann I, Birck R, Newman M, Schnulle P, Kriz W, Nemoto K, et al. SCG/Kinjoh mice: a model of ANCA-associated crescentic glomerulonephritis with immune deposits. *Kidney Int.* (2003) 64:140–8. doi: 10.1046/j.1523-1755.2003.00061.x
139. Cherry, Engelman RW, Wang BY, Kinjoh K, El-Badri NS, Good RA. Calorie restriction delays the crescentic glomerulonephritis of SCG/Kj mice. *Proc Soc Exp Biol Med.* (1998) 218:218–22. doi: 10.3181/00379727-218-44289
140. Ishida-Okawara A, Ito-Ihara T, Muso E, Ono T, Saiga K, Nemoto K, et al. Neutrophil contribution to the crescentic glomerulonephritis in SCG/Kj mice. *Nephrol Dial Transplant.* (2004) 19:1708–15. doi: 10.1093/ndt/gfh275
141. Hamano Y, Tsukamoto K, Abe M, Sun GD, Zhang D, Fujii H, et al. Genetic dissection of vasculitis, myeloperoxidase-specific antineutrophil cytoplasmic autoantibody production, and related traits in spontaneous crescentic glomerulonephritis-forming/Kinjoh mice. *J Immunol.* (2006) 176:3662–73. doi: 10.4049/jimmunol.176.6.3662
142. Birck R, Newman M, Braun C, Neumann I, Nemoto K, Yard B, et al. 15-Deoxyspergualin and cyclophosphamide, but not mycophenolate mofetil, prolong survival and attenuate renal disease in a murine model of ANCA-associated crescentic nephritis. *Nephrol Dial Transplant.* (2006) 21:58–63. doi: 10.1093/ndt/gfi070
143. Tomizawa K, Nagao T, Kusunoki R, Saiga K, Oshima M, Kobayashi K, et al. Reduction of MPO-ANCA epitopes in SCG/Kj mice by 15-deoxyspergualin treatment restricted by IgG2b associated with crescentic glomerulonephritis. *Rheumatology.* (2010) 49:1245–56. doi: 10.1093/rheumatology/keq087
144. Hirahashi J, Kawahata K, Arita M, Iwamoto R, Hishikawa K, Honda M, et al. Immunomodulation with eicosapentaenoic acid supports the treatment of autoimmune small-vessel vasculitis. *Sci Rep.* (2014) 4:6406. doi: 10.1038/srep06406
145. Gao Y, Zhao MH. Review article: drug-induced anti-neutrophil cytoplasmic antibody-associated vasculitis. *Nephrology.* (2009) 14:33–41. doi: 10.1111/j.1440-1797.2009.01100.x
146. Wada N, Mukai M, Kohno M, Notoya A, Ito T, Yoshioka N. Prevalence of serum anti-myeloperoxidase antineutrophil cytoplasmic antibodies (MPO-ANCA) in patients with Graves' disease treated with propylthiouracil and thiamazole. *Endocr J.* (2002) 49:329–34. doi: 10.1507/endocrj.49.329
147. Kusunoki Y, Nakazawa D, Shida H, Hattanda F, Miyoshi A, Masuda S, et al. Peptidylarginine deiminase inhibitor suppresses neutrophil extracellular trap formation and MPO-ANCA production. *Front Immunol.* (2016) 7:227. doi: 10.3389/fimmu.2016.00227
148. Krebs CF, Paust HJ, Krohn S, Koyro T, Brix SR, Riedel JH, et al. Autoimmune renal disease is exacerbated by S1P-receptor-1-dependent intestinal Th17 cell migration to the kidney. *Immunity.* (2016) 45:1078–92. doi: 10.1016/j.immuni.2016.10.020

149. Unanue ER, Lee S, Dixon FJ, Feldman JD. Experimental glomerulonephritis. *VII.* The absence of an autoimmune antikidney response in nephrotoxic serum nephritis. *J Exp Med.* (1965) 122:565–78. doi: 10.1084/jem.122.3.565
150. Odobasic D, Kitching AR, Semple TJ, Holdsworth SR. Endogenous myeloperoxidase promotes neutrophil-mediated renal injury, but attenuates T cell immunity inducing crescentic glomerulonephritis. *J Am Soc Nephrol.* (2007) 18:760–70. doi: 10.1681/ASN.2006040375
151. Kuligowski MP, Kitching AR, Hickey MJ. Leukocyte recruitment to the inflamed glomerulus: a critical role for platelet-derived P-selectin in the absence of rolling. *J Immunol.* (2006) 176:6991–9. doi: 10.4049/jimmunol.176.11.6991
152. Huang XR, Tipping PG, Shuo L, Holdsworth SR. Th1 responsiveness to nephritogenic antigens determines susceptibility to crescentic glomerulonephritis in mice. *Kidney Int.* (1997) 51:94–103. doi: 10.1038/ki.1997.12
153. Tarzi RM, Davies KA, Robson MG, Fossati-Jimack L, Saito T, Walport MJ, et al. Nephrotoxic nephritis is mediated by Fcgamma receptors on circulating leukocytes and not intrinsic renal cells. *Kidney Int.* (2002) 62:2087–96. doi: 10.1046/j.1523-1755.2002.00687.x
154. Xie G, Roshandel D, Sherva R, Monach PA, Lu EY, Kung T, et al. Association of granulomatosis with polyangiitis (Wegener's) with HLA-DPB1*04 and SEMA6A gene variants: evidence from genome-wide analysis. *Arthritis Rheum.* (2013) 65:2457–68. doi: 10.1002/art.38036
155. Cao Y, Schmitz JL, Yang J, Hogan SL, Bunch D, Hu Y, et al. DRB1*15 allele is a risk factor for PR3-ANCA disease in African Americans. *J Am Soc Nephrol.* (2011) 22:1161–7. doi: 10.1681/ASN.2010101058
156. Pendergraft WF III, Preston GA, Shah RR, Tropsha A, Carter CW Jr., Falk RJ, et al. Autoimmunity is triggered by cPR-3(105-201), a protein complementary to human autoantigen proteinase-3. *Nat Med.* (2004) 10:72–9. doi: 10.1038/nm968
157. Ewert BH, Jennette JC, Falk RJ. Anti-myeloperoxidase antibodies stimulate neutrophils to damage human endothelial cells. *Kidney Int.* (1992) 41:375–83. doi: 10.1038/ki.1992.52
158. Salmela A, Rasmussen N, Tervaert JWC, Jayne DRW, Ekstrand A, European Vasculitis Study Group. Chronic nasal *Staphylococcus aureus* carriage identifies a subset of newly diagnosed granulomatosis with polyangiitis patients with high relapse rate. *Rheumatology.* (2017) 56:965–72. doi: 10.1093/rheumatology/kex001
159. Yang JJ, Tuttle RH, Hogan SL, Taylor JG, Phillips BD, Falk RJ, et al. Target antigens for anti-neutrophil cytoplasmic autoantibodies (ANCA) are on the surface of primed and apoptotic but not unstimulated neutrophils. *Clin Exp Immunol.* (2000) 121:165–72. doi: 10.1046/j.1365-2249.2000.01228.x
160. Gou SJ, Yuan J, Chen M, Yu F, Zhao MH. Circulating complement activation in patients with anti-neutrophil cytoplasmic antibody-associated vasculitis. *Kidney Int.* (2013) 83:129–37. doi: 10.1038/ki.2012.313
161. Soderberg D, Segelmark M. Neutrophil extracellular traps in ANCA-associated vasculitis. *Front Immunol.* (2016) 7:256. doi: 10.3389/fimmu.2016.00256
162. Quintana LF, Kronbichler A, Blasco M, Zhao MH, Jayne D. ANCA associated vasculitis: the journey to complement-targeted therapies. *Mol Immunol.* (2019) 112:394–8. doi: 10.1016/j.molimm.2019.06.018
163. Lyons PA, Rayner TF, Trivedi S, Holle JU, Watts RA, Jayne DR, et al. Genetically distinct subsets within ANCA-associated vasculitis. *N Engl J Med.* (2012) 367:214–23. doi: 10.1056/NEJMoa1108735
164. Hilhorst M, Arndt F, Joseph Kemna M, Wieczorek S, Donner Y, Wilde B, et al. HLA-DPB1 as a risk factor for relapse in antineutrophil cytoplasmic antibody-associated vasculitis: a cohort study. *Arthritis Rheumatol.* (2016) 68:1721–30. doi: 10.1002/art.39620
165. Lyons PA, Peters JE, Alberici F, Liley J, Coulson RMR, Astle W, et al. Genome-wide association study of eosinophilic granulomatosis with polyangiitis reveals genomic loci stratified by ANCA status. *Nat Commun.* (2019) 10:5120. doi: 10.1038/s41467-019-12515-9
166. Harper L, Savage CO. ANCA-associated renal vasculitis at the end of the twentieth century–a disease of older patients. *Rheumatology.* (2005) 44:495–501. doi: 10.1093/rheumatology/keh522
167. Nusser A, Nuber N, Wirz OF, Rolink H, Andersson J, Rolink A. The development of autoimmune features in aging mice is closely associated with alterations of the peripheral CD4(+) T-cell compartment. *Eur J Immunol.* (2014) 44:2893–902. doi: 10.1002/eji.201344408
168. Merkel PA, Xie G, Monach PA, Ji X, Ciavatta DJ, Byun J, et al. Identification of functional and expression polymorphisms associated with risk for antineutrophil cytoplasmic autoantibody-associated vasculitis. *Arthritis Rheumatol.* (2017) 69:1054–66. doi: 10.1002/art.40034
169. Morgan MD, Pachnio A, Begum J, Roberts D, Rasmussen N, Neil DA, et al. CD4+CD28- T cell expansion in granulomatosis with polyangiitis (Wegener's) is driven by latent cytomegalovirus infection and is associated with an increased risk of infection and mortality. *Arthritis Rheum.* (2011) 63:2127–37. doi: 10.1002/art.30366
170. Tao L, Reese TA. Making mouse models that reflect human immune responses. *Trends Immunol.* (2017) 38:181–93. doi: 10.1016/j.it.2016.12.007

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.

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: info@frontiersin.org | +41 21 510 17 00

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