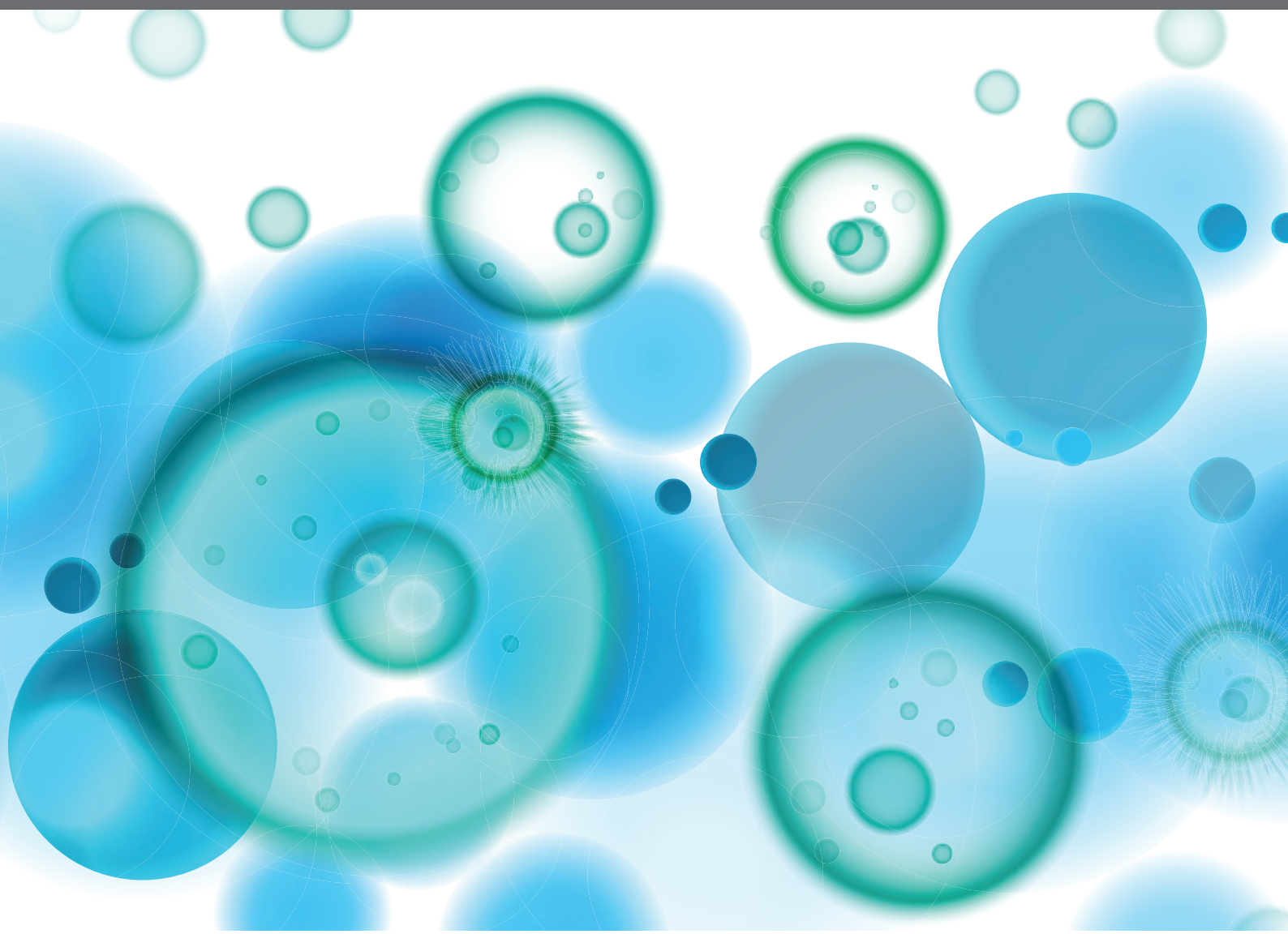
ADVANCES IN LIVER INFLAMMATION AND FIBROSIS DUE TO INFECTIOUS DISEASES

EDITED BY: M. Victoria Delpino, Guillermo Hernán Giambartolomei, Jorge Quarleri, Sergio C. Oliveira and Gary Splitter

PUBLISHED IN: Frontiers in Immunology and Frontiers in Cellular and Infection Microbiology



frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88966-166-4
DOI 10.3389/978-2-88966-166-4

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

ADVANCES IN LIVER INFLAMMATION AND FIBROSIS DUE TO INFECTIOUS DISEASES

Topic Editors:
M. Victoria Delpino, CONICET Institute of Immunology, Genetics and Metabolism (INIGEM), Argentina
Guillermo Hernán Giambartolomei, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina
Jorge Quarleri, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina
Sergio C. Oliveira, Federal University of Minas Gerais, Brazil
Gary Splitter, University of Wisconsin-Madison, United States

Citation: Delpino, M. V., Giambartolomei, G. H., Quarleri, J., Oliveira, S. C., Splitter, G., eds. (2020). Advances in Liver Inflammation and Fibrosis due to Infectious Diseases. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88966-166-4

Table of Contents

frontiers in Immunology

EDITORIAL
published: 05 August 2020
doi: 10.3389/fimmu.2020.01760

Editorial: Advances in Liver Inflammation and Fibrosis Due to Infectious Diseases

Sergio C. Oliveira[1*†], *M. Victoria Delpino*[2,3*†], *Guillermo Hernán Giambartolomei*[2,3*†], *Jorge Quarleri*[3,4*†] *and Gary Splitter*[5*†]

[1] Department of Biochemistry and Immunology, Federal University of Minas Gerais, Belo Horizonte, Brazil, [2] Facultad de Farmacia y Bioquímica, Instituto de Inmunología, Genética y Metabolismo (INIGEM), Universidad de Buenos Aires, Buenos Aires, Argentina, [3] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina, [4] Facultad de Medicina, Instituto de Investigaciones Biomédicas en Retrovirus y Sida (INBIRS), Universidad de Buenos Aires (UBA), Buenos Aires, Argentina, [5] Department of Pathobiological Sciences, University of Wisconsin-Madison, Madison, WI, United States

Keywords: fibrosis, HSC, liver, inflammasome, kupffer

OPEN ACCESS

Edited and reviewed by:
Ulrich Emil Schaible, Research Center Borstel (LG), Germany

***Correspondence:**
Sergio C. Oliveira
scozeus1@gmail.com
M. Victoria Delpino
mdelpino@ffyb.uba.ar
Guillermo Hernán Giambartolomei
ggiambart@ffyb.uba.ar
Jorge Quarleri
quarleri@fmed.uba.ar
Gary Splitter
gary.splitter@wisc.edu

†These authors have contributed equally to this work

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

Received: *14 May 2020*
Accepted: *30 June 2020*
Published: *05 August 2020*

Citation:
Oliveira SC, Delpino MV, Giambartolomei GH, Quarleri J and Splitter G (2020) Editorial: Advances in Liver Inflammation and Fibrosis Due to Infectious Diseases. Front. Immunol. 11:1760. doi: 10.3389/fimmu.2020.01760

Editorial on the Research Topic

Advances in Liver Inflammation and Fibrosis Due to Infectious Diseases

Liver inflammation is a common trigger of hepatic disease, and it is considered the main driver of tissue damage (1). Although the liver is able to regenerate, chronic liver inflammation leads to tissue damage with concomitant fibrosis, frequently leading to cirrhosis, and carcinogenesis (2).

The etiology of chronic liver inflammation may be infectious or not. Several microorganisms including bacteria, parasites, fungi, and virus could be involved. Although, other metabolic and immune disorders may also participate. Several proinflammatory cytokines including IL-1α, IL-1β, TNF-α, and IL-6 has been involved in liver disease (3). They are produced by resident macrophages (Kupffer cells) and also by recruited macrophages and neutrophils (4). The common mediators of fibrosis are IL-10 family cytokines, VEGF, EGF, and TGF-β that constitute the main profibrotic cytokines (5). TGF-β stimulate hepatic stellate cell trans-differentiation from a quiescent, vitamin A storing cell to a proliferative myofibroblast that is the central driver of fibrosis (6).

In this Research Topic, a series of articles provide new insights about the current view of liver pathology, inflammation and fibrosis, with original articles about the role of myeloid suppressor cells in fulminant hepatitis, inflammasome activation in brucellosis, new insights in preventing NALDF progression, and the role of NLRP6 in granuloma formation and liver disease associated with schistosomiasis.

This Research Topic also features three review articles regarding the role of glucocorticoids in therapy of liver failure, the interaction of liver and immune cells in brucellosis, the role of T cells in hepatic schistosomiasis, as well as the role of beneficial bacterial in liver disease progression. Also included is a perspective article with proposed mechanisms of interaction between human immunodeficiency virus (HIV) and Kupffer cells.

The original research articles included in this Research Topic were performed involving both *in vitro*, and *in vivo* experiments in the murine model. Such model has acquired particular relevance during the study of non-invasive biomarkers able to assess fibrosis in patients with chronic liver disease.

The liver is the most commonly affected organ in patients with active brucellosis. Accordingly, clinical and biochemical records of liver involvement have been observed in up to 50% of patients with active disease (7). However, the molecular mechanisms involved in liver damage has been

recently started to be elucidated. Giambartolomei and Delpino report current understanding of the interaction between liver structural cells including hepatocytes and hepatic stellate cells and immune system cells during *Brucella* infection. They highlight the role of the type IV secretion system (T4SS) and the effector protein BPE005 in the activation of hepatic stellate cells to induce fibrosis. It is likely that BPE005 could participate in granuloma formation that might act as a reservoir of bacteria contributing to the disease chronicity. Prior to the development of liver fibrosis may occur events of inflammation and cell death. The contribution of the inflammasomes activation due to *B. abortus* infection on liver fibrosis was explored in an original article. Arriola Benitez et al. demonstrate in a series of experiments performed using the hepatic stellate cell line LX-2, that inflammasomes NLRP3 and AIM2 are involved in the induction of a fibrotic phenotype in hepatic stellate cells during *B. abortus* infection in a mechanism that involved IL-1β. These experiments were further corroborated using knock out (KO) mice, and strongly suggest the main contribution of this inflammasomes in the liver fibrosis during *B. abortus* infection.

In the liver fibrosis induced by *Schistosoma mansoni* infection, inflammasomes has also involved. Sanches et al. have unraveled using the murine model, the crucial role of the NLRP6 inflammasome in the schistosomiasis-associated hepatic granulomas. Hence, the absence of NLRP6 correlates with a significant reduction in inflammation and collagen deposition, as well as α-SMA and IL-13 levels as fibrotic markers. Both articles stand out the role of inflammasomes as central contributors to liver fibrosis triggered by infectious hepatic diseases. Moreover, parasite worms such as *Schistosoma mansoni* and *S. japonicum* induce a dramatic granulomatous response in liver and intestines. Subsequently, infection may further develop into significant fibrosis and portal hypertension. Zheng et al. extensively review previous reports to elucidate the contribution of T lymphocytes and their secreted cytokines in the immunopathology of schistosomiasis. They clearly indicate the different roles developed by the T-cell subsets for regulating the pathological progression of schistosomiasis in the local microenvironment. Moreover, they highlight recent findings about Tfh and Th9 cells as promoters of liver granulomas and fibrogenesis.

The tumor progression locus 2 (TPL2) is a serine/threonine kinase acting as a key mediator in liver and systemic metabolic disorders with an inflammatory component (8). However, the function of TPL2 in regulating hepatocyte function and liver inflammation during fulminant hepatitis is poorly understood, Xu et al. shed light on the TPL2 role associated with the myeloid derived suppressor cells (MDSC)-mediated protection against fulminant hepatitis. Using the murine model, they demonstrate that TPL2 deficiency suppresses IL-25-induced chemokine CXCL1/2 expression in hepatocytes, thus impairing MDSC recruitment into the liver, and increasing the infiltrate $CD4^+$ lymphocyte proliferation that enhances fulminant hepatitis development. These findings strongly suggest that TPL2 is a potential target for the fulminant hepatitis treatment since it plays a critical role in MDSC recruitment.

The interaction between HIV and liver cell population has been previously explored. However, the relevance is still unclear in well-controlled patients on ART. In this context, the perspective article, Zhang and Bansal propose that during HIV infection, changes in the biology of Kupffer cells would create a microenvironment that drives hepatic pathology during microbial translocation. Targeting this pathway could help to improve liver-related consequences in HIV patients.

Liver damage associated to sterile inflammation was evaluated in a model of Non-alcoholic fatty liver disease (NAFLD). This pathology is associated to an excessive storage of fatty acids in the form of triglycerides in hepatocytes and it is considered the main causes of cirrhosis and major risk factors for hepatocellular carcinoma and liver-related death. Wang et al. reveal in a mice model that perforin regulates the abundance of hepatic IFN-γ-producing $CD4^+$ T cells with concomitant decrease of macrophage accumulation in liver. This indicate that perforin can act as an immune regulator to prevent NAFLD, suggesting its potential use to prevent hepatic steatosis and other related liver metabolic disorders.

Finally, the last two review article focus on the potentials therapy against liver pathology.

Excessive systemic inflammation is considered as the trigger of liver failure. Glucocorticoids (GCs) can rapidly suppress excessive inflammatory reactions and immune response. In a review article, Xue and Meng describe the current knowledge regarding glucocorticoid therapy in liver failure including emerging information. Although, the topic was addressed from basic research and clinical trials, the current understanding remains inconclusive for the application of GS treatment during liver failure.

The beneficial effect of probiotics has been extended to liver function in cirrhosis, nonalcoholic fatty liver disease, and alcoholic liver disease. Parker et al. review the current knowledge of bacteria of the genus *Alistipes* on its protective role during liver fibrosis among other diseases in animal models.

In summary, this Research Topic highlights the immunopathology of liver damage due to different pathologies. Knowledge acquired from articles contained in this special issue are relevant to the discovery of new targets for controlling liver inflammation and fibrosis.

We wish to express our appreciation to all the authors and reviewers who have participated in this Research Topic.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

REFERENCES

1. Rockey DC, Bell PD, Hill JA. Fibrosis–a common pathway to organ injury and failure. *N Engl J Med.* (2015) 372:1138–49. doi: 10.1056/NEJMra1300575
2. Tsochatzis EA, Bosch J, Burroughs AK. Liver cirrhosis. *Lancet.* (2014) 383:1749–61. doi: 10.1016/S0140-6736(14)60121-5
3. Zhangdi HJ, Su SB, Wang F, Liang ZY, Yan YD, Qin SY, et al. Crosstalk network among multiple inflammatory mediators in liver fibrosis. *World J Gastroenterol.* (2019) 25:4835–49. doi: 10.3748/wjg.v25.i33.4835
4. Tacke F, Zimmermann HW. Macrophage heterogeneity in liver injury and fibrosis. *J Hepatol.* (2014) 60:1090–6. doi: 10.1016/j.jhep.2013.12.025
5. Bataller R, Brenner DA. Liver fibrosis. *J Clin Invest.* (2005) 115:209–18. doi: 10.1172/JCI24282
6. Puche JE, Saiman Y, Friedman SL. Hepatic stellate cells and liver fibrosis. *Compr Physiol.* (2013) 3:1473–92. doi: 10.1002/cphy.c120035
7. Colmenero JD, Reguera JM, Martos F, Sanchez-De-Mora D, Delgado M, Causse M, et al. Complications associated with Brucella melitensis infection: a study of 530 cases. *Medicine (Baltimore).* (1996) 75:195–211. doi: 10.1097/00005792-199607000-00003
8. Gong J, Fang C, Zhang P, Wang PX, Qiu Y, Shen LJ, et al. Tumor progression locus 2 in hepatocytes potentiates both liver and systemic metabolic disorders in mice. *Hepatology.* (2019) 69:524–44. doi: 10.1002/hep.29820

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: 20 August 2019
doi: 10.3389/fimmu.2019.01980

Tpl2 Protects Against Fulminant Hepatitis Through Mobilization of Myeloid-Derived Suppressor Cells

Jing Xu[1,2], *Siyu Pei*[2], *Yan Wang*[2], *Junli Liu*[2], *Youcun Qian*[2], *Mingzhu Huang*[3]*, *Yanyun Zhang*[1,2]* and *Yichuan Xiao*[2]*

[1] The First Affiliated Hospital of Soochow University, Institutes for Translational Medicine, State Key Laboratory of Radiation Medicine and Protection, Key Laboratory of Stem Cells and Medical Biomaterials of Jiangsu Province, Medical College of Soochow University, Soochow University, Suzhou, China, [2] CAS Key Laboratory of Tissue Microenvironment and Tumor, Shanghai Institute of Nutrition and Health, Shanghai Institutes for Biological Sciences, University of Chinese Academy of Sciences, Chinese Academy of Sciences, Shanghai, China, [3] Department of Medical Oncology, Fudan University Shanghai Cancer Center, Shanghai, China

Myeloid derived suppressor cells (MDSC) in the liver microenvironment protects against the inflammation-induced liver injury in fulminant hepatitis (FH). However, the molecular mechanism through which MDSC is recruited into the inflamed liver remain elusive. Here we identified a protein kinase Tpl2 as a critical mediator of MDSC recruitment into liver during the pathogenesis of *Propionibacterium acnes*/LPS-induced FH. Loss of Tpl2 dramatically suppressed MDSC mobilization into liver, leading to exaggerated local inflammation and increased FH-induced mortality. Mechanistically, although the protective effect of Tpl2 for FH-induced mortality was dependent on the presence of MDSC, Tpl2 neither directly targeted myeloid cells nor T cells to regulate FH pathogenesis, but functioned in hepatocytes to mediate the induction of MDSC-attracting chemokine CXCL1 and CXCL2 through modulating IL-25 (also known as IL-17E) signaling. As a consequence, increased MDSC in the inflamed liver specifically restrained the local proliferation of infiltrated pathogenic $CD4^+$ T cells, and thus protected against the inflammation-induced acute liver failure. Together, our findings established Tpl2 as a critical mediator of MDSC recruitment and highlighted the therapeutic potential of Tpl2 for the treatment of FH.

Keywords: hepatitis, myeloid-derive suppressor cells (MDSCs), TPL2, IL-25, chemokine

OPEN ACCESS

Edited by:
Sergio C. Oliveira, Federal University of Minas Gerais, Brazil

Reviewed by:
Nagarkatti Prakash, University of South Carolina, United States
Patricia Lalor, University of Birmingham, United Kingdom

***Correspondence:**
Mingzhu Huang
mingzhuhuang0718@163.com
Yanyun Zhang
yyzhang@sibs.ac.cn
Yichuan Xiao
ycxiao@sibs.ac.cn

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

Received: *27 March 2019*
Accepted: *05 August 2019*
Published: *20 August 2019*

Citation:
Xu J, Pei S, Wang Y, Liu J, Qian Y, Huang M, Zhang Y and Xiao Y (2019) Tpl2 Protects Against Fulminant Hepatitis Through Mobilization of Myeloid-Derived Suppressor Cells. Front. Immunol. 10:1980. doi: 10.3389/fimmu.2019.01980

INTRODUCTION

Fulminant hepatitis (FH) is a dreaded disease characterized by rapid development of hepatocellular dysfunction, leading to the failure of hepatic regeneration (1). Published studies have shown that the pathogenesis of FH is associated with huge liver infiltration of immune cells, which secrete a large number of pro-inflammatory cytokines and thus induce acute inflammatory necrosis of hepatocytes (2, 3). The bacterial infection has been considered as a key factor that contribute to the development of FH pathology (4). Indeed, mice injected with heat-killed *Propionibacterium acnes* followed by lipopolysaccharide (LPS), one of the most commonly used FH animal models, phenocopy the inflammatory infiltration in hepatic parenchyma and finally lead to the acute liver failure (5–8). Although the pathogenesis of FH has been extensively investigated, there is no proper therapeutic strategies for this disease, leading to high mortality if there is no supportive management and/or liver transplantation (9).

Myeloid derived suppressor cells (MDSC) are a heterogeneous group of immune cells derived from bone marrow and have been implicated to play important immunosuppressive and protective roles in human hepatitis, hepatocellular carcinoma or various mouse hepatitis models through different mechanism. For example, MDSC inhibited T cell proliferation and IFN-γ production in chronic HCV patients (10), and suppressed NK cell function during the pathogenesis of human hepatocellular carcinoma (11). In hepatitis mouse models, MDSC also exhibited immunosuppressive function through inhibiting the T cells proliferation, activation and secretion of pro-inflammatory cytokines, and thus protected against hepatic inflammation and fibrosis through different mechanisms (12–14). Therefore, increasing the number of MDSC in the liver may help to inhibit the occurrence of local inflammation of the liver and protect against FH. Indeed, administration of IL-25 dramatically prevented and reverses acute liver damage through promoting the recruitment of the MDSC into liver in FH mouse (15).

IL-25, also known as IL-17E, belongs to IL-17 cytokine family, and was initially found to be highly expressed in T helper (Th) 2 cells and promote the proliferation of Th2 cells and eosinophils (16–18). In addition, it has been reported that IL-25 exhibited inhibitory effect of the proliferation of Th1 and Th17 cells and further suppressed the occurrence of autoimmune diseases in mice (19, 20). However, it is not clear how IL-25 initiates the signal pathway to mediate MDSC recruitment into liver during FH pathogenesis. Published study has identified that IL-25 can bind to the heterodimer receptor composed of IL-17RA and IL-17RB, which then recruit Act1 to activate downstream NF-κB and MAPK (21–23), suggesting a similarity with IL-17A-induced signaling pathway. Our previous study has demonstrated that the serine/threonine protein kinase Tpl2 is a key component in regulating the IL-17A signaling pathway, in which the activated Tpl2 directly bound to and phosphorylated TAK1 and further induce the activation of downstream NF-κB and MAPK (24, 25). Based on the similarity of IL-17A- and IL-25-induced signaling and the critical protective role of IL-25 in FH, we speculated that Tpl2 may also regulated the FH pathogenesis through modulation of IL-25 signaling.

In the present study, we found that Tpl2 protected against FH-induced acute liver injury and mouse mortality. Loss of Tpl2 in hepatocytes suppressed IL-25-induced chemokine CXCL1/2 expression, which impaired the recruitment of MDSC into the liver, leading to promoted proliferation of liver-infiltrating $CD4^+$ T cells and enhanced FH pathology.

RESULTS

Tpl2 Protected Against *P. acne*/LPS-Induced FH

To investigate the *in vivo* role of Tpl2 during FH pathogenesis, we induced a FH model by intravenously injecting the mice with heat-killed *P. acnes* and followed by LPS. In this model, only *P. acnes* priming is not lethal for the mice, and *P. acnes* priming plus LPS injection 7 days later will strongly induce acute liver damage, leading to FH-related mortality. However, *P. acnes* priming-induced liver inflammation is necessary and the reason for the mortality after LPS injection in this FH model (6, 7). As shown in **Figure 1A**, low dose of *P. acnes*/LPS priming provoked a non-lethal moderate hepatitis in wild-type (hereafter termed WT) mice. In contrast, *Tpl2*-deficient mice that induced with FH by using the same dose of *P. acnes*/LPS developed a much severer disease, leading to 86% lethality within 8 h (**Figure 1A**). Consistently, we observed the increased production of serum aspartate aminotransferase (AST) and higher ratio of AST/aminotransferase (ALT) levels, which is a hallmark of hepatitis-induced liver failure, in *Tpl2*-deficient mice accordingly (**Figure 1B**). In addition, histological analysis showed that there was more inflammatory infiltration observed in the *Tpl2*-deficient liver tissues on day 7 after *P. acnes* priming (**Figures 1C,D**). These results collectively suggested an important beneficial role of Tpl2 in protecting *P. acnes*/LPS-driven acute liver damage.

Tpl2 Deficiency Increased the Liver Infiltration of Pathogenic $CD4^+$ T Cells

The exaggerated FH in *Tpl2*-deficient mice promoted us to examine the cellular mechanism by which Tpl2 protect against liver failure during FH pathogenesis. We firstly examined the peripheral immune activation after *P. acnes* priming, and the results revealed that *Tpl2*-deficient and WT control mice had similar frequencies and absolute numbers of $CD4^+$ T cells, $CD8^+$ T cells, $CD11c^+$ dendritic cells, $B220^+$ B cells and $CD4^+Foxp3^+$ regulatory T cells (Treg) in the spleens 7 days after challenged with *P. acnes* (**Figures 2A–D**). In addition, the frequencies and absolute numbers of IFN-γ- and TNF-α-producing T helper (Th)1 $CD4^+$ T cells in the spleens were also comparable between the WT and *Tpl2*-deficient mice (**Figures 2E,F**). These data suggested Tpl2 does not affect peripheral immune activation during FH pathogenesis.

It is known that *P. acnes* priming promoted the liver infiltration of $CD4^+$ T cells, which contributed to the inflammation-induced liver injury (8). Although the frequencies and absolute cell numbers of $CD4^+$ T cells in the spleens or peripheral blood were comparable in WT and *Tpl2*-deficient mice during *P. acnes*-primed process (**Figures 2G–J**), loss of Tpl2 dramatically increased the frequencies and absolute numbers of $CD4^+$ T cells in the livers as compared with that in WT liver, whereas didn't affect the liver infiltration of $CD8^+$ T cells, dendritic cells, B cells and Treg cells at day 7 after *P. acnes* priming (**Figures 3A–E**). Since the Th1 cells are the major pathogenic contributor of *P. acnes*-induced liver injury (26), we next examined the TNF-α and IFN-γ production among the infiltrated $CD4^+$ T cells. Interestingly, *Tpl2* deficiency didn't affect the ability of liver-infiltrating $CD4^+$ T cells to produce TNF-α and IFN-γ, as reflected by comparable frequencies of IFN-γ- and TNF-α-producing Th1 cells in the inflamed livers of WT and *Tpl2*-deficient mice (**Figures 3F,G**). However, the absolute cell numbers of the pathogenic Th1 cells were dramatically increased in the inflamed livers of *Tpl2*-deficient mice (**Figures 3F,G**). Moreover, *Tpl2* deficiency gradually increased the liver infiltration of $CD4^+$ T cells, notably

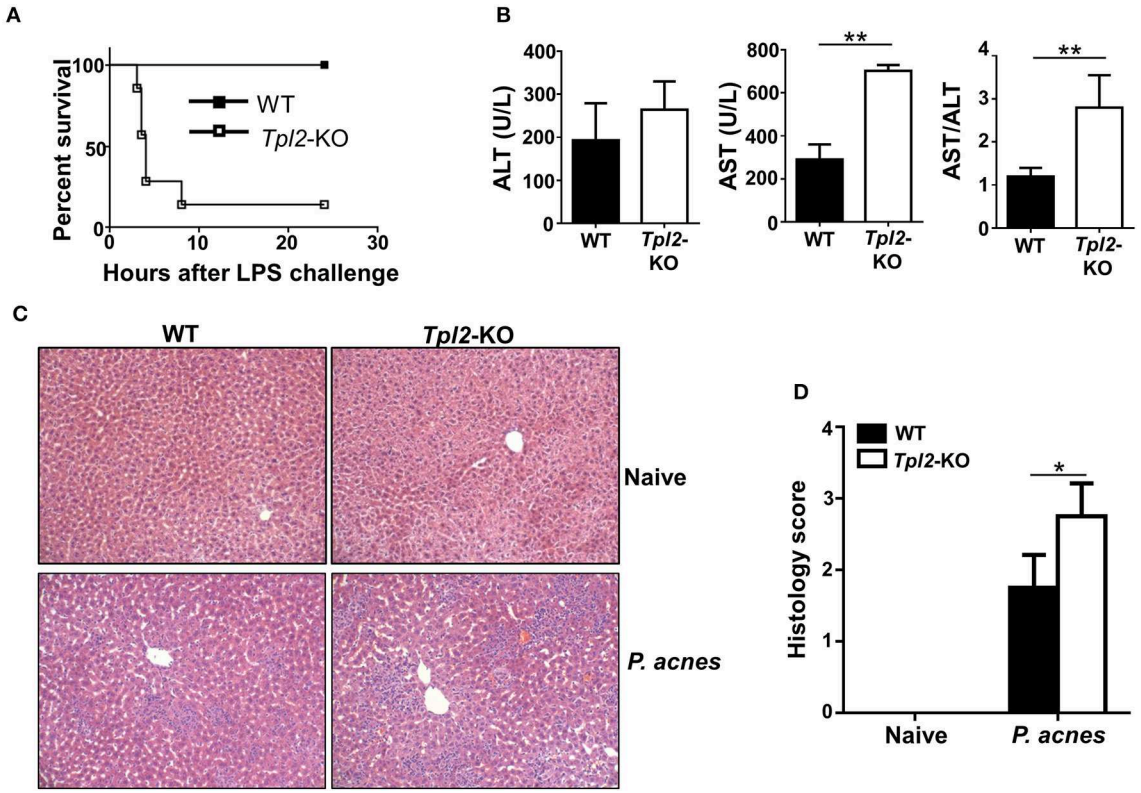


FIGURE 1 | *Tpl2* deficiency exaggerated *P. acnes*/LPS-induced FH. WT and *Tpl2*-KO mice were injected with 0.5 mg *P. acnes* suspended in 200 μl of phosphate-buffered saline (PBS), and then 1 μg of LPS in 200 μl of PBS was injected on day 7 to induce fulminant hepatitis (FH). **(A)** Cumulative survival rates of WT and *Tpl2*-KO mice were analyzed ($n = 7$ mice/group) after LPS injection. **(B)** Serum levels of aminotransferase (ALT), aspartate aminotransferase (AST) and the AST/ALT ratios ($n = 5$ mice per group) were measured on day 7 after *P. acnes* priming. **(C)** H&E staining showing the representative inflammatory infiltration in the livers of WT and *Tpl2*-KO mice that injected with *P. acnes* at day 7. The liver sections from WT and *Tpl2*-KO naive mice were stained as negative controls (magnification, ×200). **(D)** Semiquantitative analysis of inflammatory conditions in the livers from WT and *Tpl2*-KO naive and *P. acnes*-primed mice. Results are mean ± SD from three independent experiments. Two-tailed Student's *t*-tests were performed. $^*P < 0.05$; $^{**}P < 0.01$.

at day 5 and 7 after *P. acnes* priming (**Figures 3H,I**). These data collectively suggested Tpl2 may inhibited the liver infiltration of $CD4^+$ T cells during *P. acnes*-induced liver injury.

Tpl2 Specifically Restricted the Proliferation of Liver-Infiltrating $CD4^+$ T Cells

To confirm the pathogenic role of liver-infiltrating $CD4^+$ T cells after *P. acnes* priming in the FH model, we injected different dose of *P. acnes* and examined the survival rate and liver infiltration of $CD4^+$ T cells. The results revealed that after challenged with a single shot of same dose of LPS, higher dose of *P. acnes* priming dramatically increased the mouse mortality rate as compared with that primed with lower dose of *P. acnes* (**Figure 4A**). As expected, higher dose of *P. acnes* priming significantly increased the infiltration of $CD4^+$ T cells in the livers, along with decreased frequencies of liver infiltration of MDSC (**Figures 4B,C**), which is known to restrain the local inflammation in inflamed liver microenvironment through inhibiting T cell proliferation (15, 27). Therefore, we speculated that Tpl2 may regulate the proliferation of liver-infiltrating $CD4^+$ T cells, and then injected bromodeoxyuridine (BrdU), a synthetic nucleoside that could be incorporated into newly synthesized DNA to monitor the cell proliferation, into WT or *Tpl2*-deficient mice before *P. acnes* priming. The flow cytometric analysis revealed that *Tpl2* deficiency specifically increased the frequencies of $BrdU^+$ $CD4^+$ T cells that isolated from the inflamed livers, whereas the frequencies of $BrdU^+$ $CD4^+$ T cells were comparable in the spleens between WT and *Tpl2*-deficient mice after *P. acnes* priming (**Figures 4D,E**), suggesting Tpl2 may indirectly regulate the proliferation of $CD4^+$ T cells in the inflamed liver microenvironment. Indeed, Tpl2 is dispensable for the *in vitro* $CD4^+$ T cell proliferation, as characterized by comparable proliferation rate of naïve WT and *Tpl2*-deficient $CD4^+$ T cells upon the stimulation of anti-CD3 plus anti-CD28 antibodies (**Figure 4F**).

To further exclude the possibility that Tpl2 may directly function in $CD4^+$ T cell to regulate FH pathogenesis, we adoptively transferred WT or *Tpl2*-deficient $CD4^+$ T cells into T cell-deficient *Rag1*-KO mice, which were then induced the FH model by injecting *P. acnes*/LPS. Expectedly, the FH-induced mortality rates are comparable in recipient *Rag1*-KO

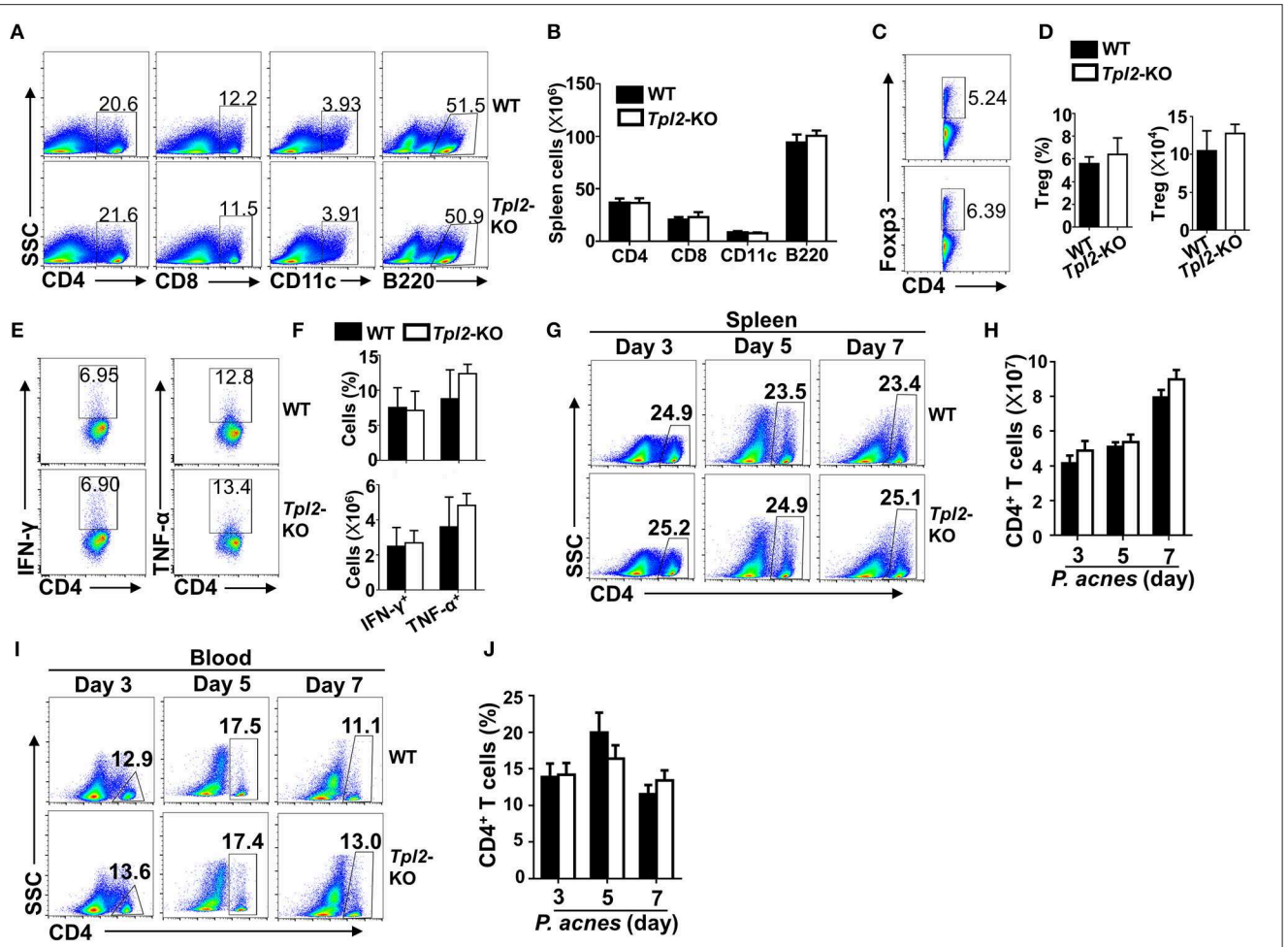


FIGURE 2 | Tpl2 didn't affect peripheral immune activation during FH pathogenesis. The splenic cells or peripheral blood immune cells were isolated from *P. acnes*-primed WT and *Tpl2*-KO mice at day 3, 5, and 7 as described in Materials and methods ($n = 4$ mice/group), and subjected for flow cytometry analysis. **(A–F)** Flow cytometry analysis of the frequencies and absolute numbers of $CD4^+$ T cells, $CD8^+$ T cells, $B220^+$ B cells, $CD11c^+$ dendritic cells **(A,B)**, $CD4^+Foxp3^+$ Treg cells **(C,D)**, and IFN-γ- and TNF-α-producing pathogenic Th1 cells **(E,F)** in the spleens of WT and *Tpl2*-KO mice at day 7 after *P. acnes* priming. Data are presented as representative plots of the frequencies of immune cell subpopulations **(A,C,E)** and a summary graph of the cell frequencies or absolute cell numbers **(B,D,F)**. **(G–J)** Flow cytometry analysis of the frequencies and absolute numbers of $CD4^+$ T cells in the spleens **(G,H)** or peripheral blood **(I,J)** of WT and *Tpl2*-KO mice at day 3, 5, and 7 after *P. acnes* priming. Data are presented as representative plots of the frequencies of immune cell subpopulations **(G,I)** and a summary graph of the cell frequencies or absolute cell numbers **(H,J)**. Results are mean ± SD from three independent experiments.

mice that either transferred with WT or *Tpl2*-deficient $CD4^+$ T cells (**Figure 4G**). Collectively, these results suggested that Tpl2 specifically restricted the proliferation of liver-infiltrating $CD4^+$ T cells through an indirect mechanism during FH pathogenesis.

Tpl2 Mediated the Recruitment of MDSC Into Liver

Considering the indirect function of Tpl2 in regulating $CD4^+$ T cell proliferation, we examined the infiltration status of MDSC in inflamed liver. In contrast to the increased infiltrating rate of $CD4^+$ T cells in inflamed liver (**Figures 3H,I**), *Tpl2* deficiency gradually decreased the frequencies and absolute numbers of liver-infiltrating $CD11b^+Gr\text{-}1^+$ MDSC, notably at day 5 and 7 after *P. acnes* priming, as compared with that of WT mice (**Figures 5A,B**). The immunofluorescence analysis also confirmed the decreased infiltration of MDSC in the hepatic parenchyma of *Tpl2*-KO mice at day 7 after *P. acnes* priming (**Figure 5C**). However, loss of Tpl2 neither altered the frequencies and absolute numbers of MDSC in the spleens nor affected the peripheral distribution of MDSC in the circulation system during *P. acnes*-primed process (**Figures 5D–G**). In addition, *in vitro* proliferation assay revealed that *Tpl2*-deficient MDSC exhibited similar ability as WT MDSC to suppress either WT or *Tpl2*-deficient T cell proliferation after cocultured with $CD4^+$ T cell that stimulated with anti-CD3 plus anti-CD28 antibodies (**Figures 5H,I**). These data suggested that *Tpl2* deficiency suppressed the liver recruitment of MDSC without affecting their immunosuppressive function.

We next examined whether the impaired liver recruitment of MDSC contributed to the enhanced mortality in *Tpl2*-deficient FH mice. To this end, we specifically deleted the MDSC by injection of anti-Ly-6G neutralizing antibody

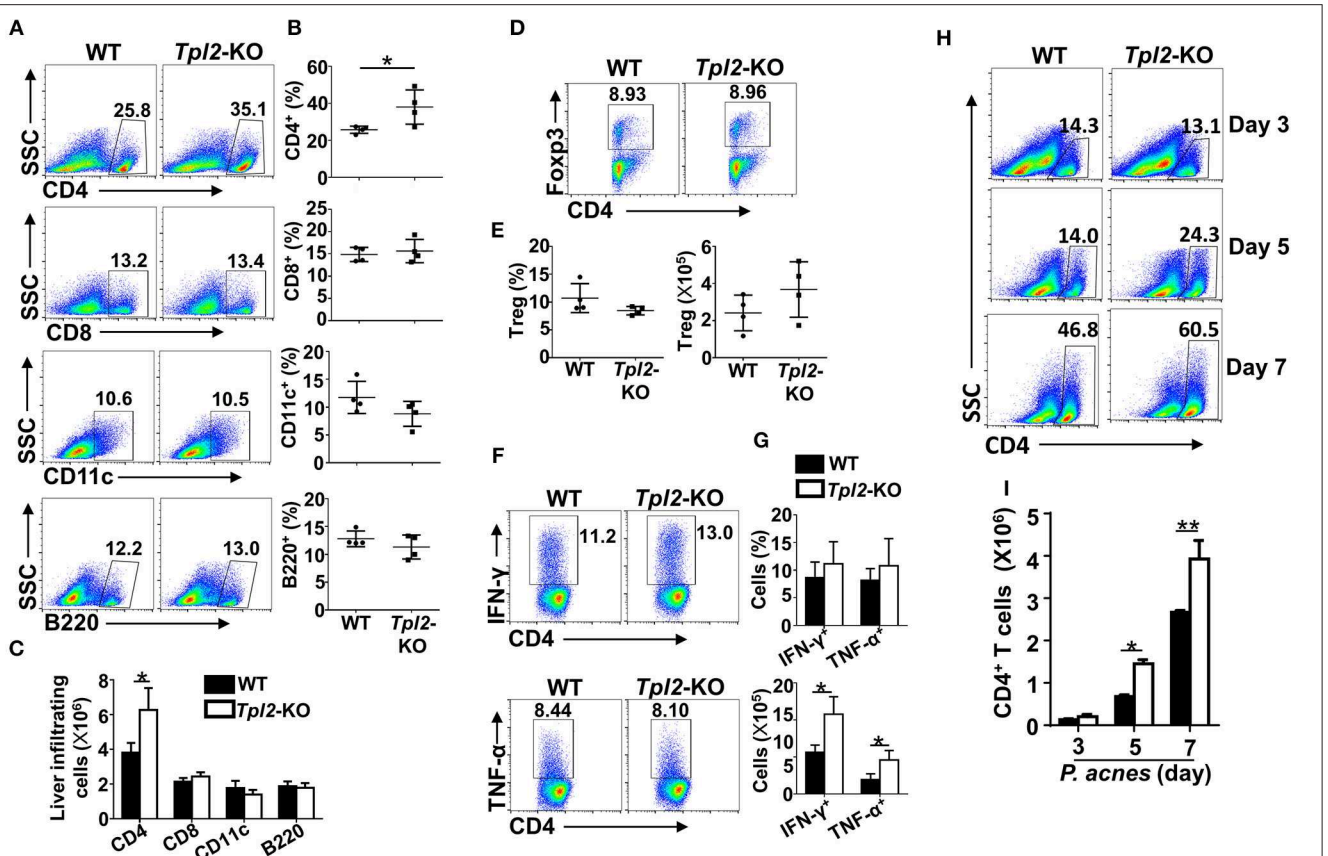


FIGURE 3 | Tpl2 reduced liver infiltration of pathogenic CD4$^+$ T cells. Liver-infiltrating immune cells were isolated from *P. acnes*-primed WT and *Tpl2*-KO mice at day 3, 5, and 7 as described in Materials and methods ($n = 4$ mice/group). **(A–G)** Flow cytometry analysis of the frequencies and absolute numbers of CD4$^+$ and CD8$^+$ T cells, B220$^+$ B cell, CD11c$^+$ dendritic cells **(A–C)**, CD4$^+$Foxp3$^+$ Treg cells **(D,E)**, and IFN-γ- and TNF-α-producing pathogenic Th1 cells **(F,G)** in the livers of WT and *Tpl2*-KO mice at day 7 after *P. acnes* priming. Data are presented as representative plots of the frequencies of immune cell subpopulations **(A,D,F)** and a summary graph of the frequencies and absolute cell numbers **(B,C,E,G)**. **(H,I)** Flow cytometry analysis of the frequencies and absolute numbers of CD4$^+$ T cells in the livers of WT and *Tpl2*-KO mice at day 3, 5, and 7 after *P. acnes* priming. Data are presented as representative plots of the frequencies of immune cell subpopulations **(H)** and a summary graph of the absolute cell numbers **(I)**. Results are mean ± SD from three independent experiments. Two-tailed Student's *t*-tests were performed. $^*P < 0.05$; $^{**}P < 0.01$.

(**Supplementary Figures 1A,B**), and challenged mice with *P. acnes*/LPS to induce FH. As expected, *in vivo* MDSC depletion dramatically increased the mortality rate of WT FH mice, and largely abolished the difference of the survival rate between WT and *Tpl2*-deficient mice that injected with *P. acnes*/LPS (**Figure 5J**). These results collectively established Tpl2 as a critical mediator of MDSC mobilization into liver to protect inflammation-induced liver injury during FH pathogenesis.

Tpl2 Functioned in Liver-Resident Cells to Protect Against FH

To figure out which type of cells *in vivo* was directly targeted by Tpl2 to protect against FH-induced liver failure, we generated the mixed bone marrow (BM) chimeric mice by reconstituting the lethally irradiated WT mice with WT or *Tpl2*-deficient BM, which were then challenged with *P. acnes*/LPS to induce FH. Unexpectedly, *Tpl2*-deficient BM reconstituted chimeric mice were totally resistant to FH-induced death (**Figure 6A**), suggesting that *Tpl2* deficiency in myeloid cells (including macrophages and MDSC) does not contribute to the aggregation of FH-induced mortality. However, when reconstituting the WT BM into lethally irradiated WT or *Tpl2*-deficient mice, we found that WT BM failed to induce FH-mediated death in WT recipient mice, whereas dramatically promoted the mortality rate of *Tpl2*-deficient recipient mice (**Figure 6B**). These data collectively suggested that Tpl2 didn't target myeloid cells, but functioned in liver-resident cells to protect against FH pathology.

Next, we examined the proinflammatory cytokine and chemokine induction in the livers at the early priming phase of FH model. After 3 days of *P. acnes* challenge, the expression of Th1 cytokine genes *Ifng* and *Tnf* in the *Tpl2*-deficient inflamed livers were much higher than that in WT livers (**Figure 6C**), which suggested that the increased liver infiltration of pathogenic Th1 cells as shown in **Figure 3G** may contribute to the enhanced expression of these pro-inflammatory genes. Accordingly, the expression of MDSC-attracting chemokine genes *Cxcl1* and *Cxcl2* were dramatically suppressed in the *Tpl2*-deficient livers as compared with that in WT livers (**Figure 6C**). In addition, the

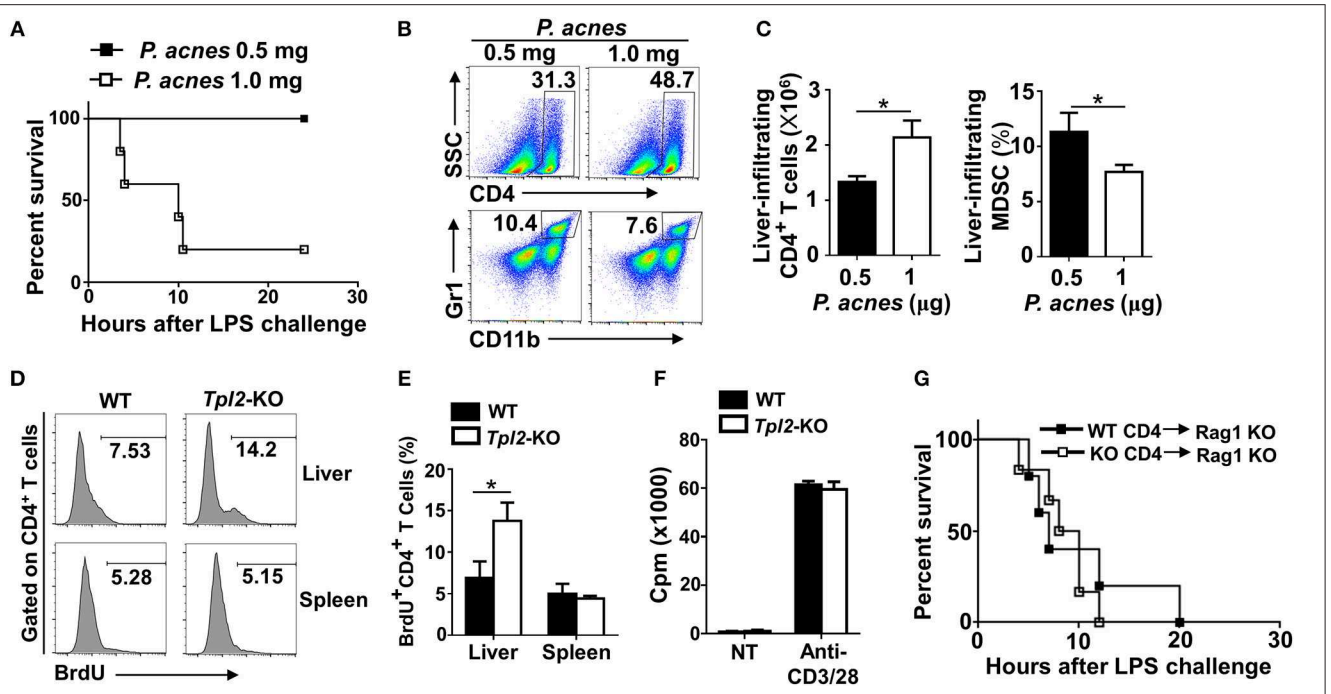


FIGURE 4 | *Tpl2* deficiency specifically inhibited the proliferation of liver-infiltrated CD4$^+$ T cells. **(A)** Cumulative survival analysis of WT C57BL/6 mice ($n = 5$ mice/group) that were injected with 0.5 or 1.0 mg *P. acnes* suspended in 200 μl of phosphate-buffered saline (PBS), and then with 1 μg of LPS in 200 μl of PBS at day 7 to induce fulminant hepatitis (FH). **(B,C)** Flow cytometry analysis of the frequencies and absolute numbers of CD4$^+$ and Gr-1$^+$CD11b$^+$ MDSC in the livers of WT C57BL/6 mice at day 7 after different dose of *P. acnes* priming. Data are presented as representative plots of the frequencies of immune cell subpopulations **(B)** and a summary graph of the frequencies or absolute cell numbers **(C)**. **(D,E)** Flow cytometry analysis of CD4$^+$ T cell proliferation in the spleens and livers of WT and *Tpl2*-KO mice at day 7 after *P. acnes* priming ($n = 4$ mice/group). Data are presented as a representative histogram **(D)** and a summary bar graph **(E)** showing the frequencies of proliferating BrdU$^+$ CD4$^+$ T cells. **(F)** Proliferation of WT and *Tpl2*-KO CD4$^+$ T cells in the absence (NT) or presence of anti-CD3/CD28 antibodies, then assessed by [^{3}H] thymidine incorporation. **(G)** Cumulative survival rates of age- and sex-matched *Rag1*-KO mice that adoptively transferred with WT and *Tpl2*-KO CD4$^+$ T cells and then subjected to *P. acnes*/LPS-mediated FH induction ($n = 5$ mice/group). Results are mean ± SD from three independent experiments. Two-tailed Student's *t*-tests were performed. *$P < 0.05$.

expression of the genes that encoding DC-recruiting chemokine MIP-1α (6) and other two MDSC-attracting chemokine CCL17 and CCL19 (15) were not affected in the livers of *P. acnes*-primed *Tpl2*-deficient mice (**Supplementary Figures 1C,D**). Moreover, the enzyme-linked immunosorbent assay confirmed that *Tpl2* ablation inhibited *P. acnes*-induced CXCL1 chemokine protein production in the liver parenchyma as compared with that in WT livers (**Figure 6D**). Together, these results suggested that Tpl2 directly functioned in liver-resident cells, but not in peripheral immune cells, to mediate MDSC recruitment, and thus protect against FH pathology.

Tpl2 Regulated IL-25 Signaling in Hepatocytes

Published study has suggested that IL-25 is highly produced in both human and mouse livers, and it is critical for the liver recruitment of MDSC in D-Gal/LPS-induced FH mouse model (15). In addition, we have previously demonstrated that Tpl2 mediates the activation of signaling pathway induced by IL-17A, which belongs to the same IL-17 family as IL-25 (24, 25). Therefore, we speculated that Tpl2 may potentially modulates IL-25 signaling in the liver-resident cells to regulate FH pathogenesis. To test this hypothesis, we firstly examined the IL-25 production in the livers, and found that there is no significant difference of IL-25 levels in the liver homogenates between WT and *Tpl2*-deficient mice that were either under physiological condition or challenged with *P. acnes* (**Figure 7A**), suggesting Tpl2 is dispensable for the IL-25 secretion in the liver tissue. In addition, we observed that IL-25 production in the livers of *P. acnes*-primed mice were comparable with that of naïve mice (**Figure 7A**), which is different from the D-Gal/LPS-induced FH model that IL-25 levels are decreased in the livers of FH mice (15). Next, we generated the *Tpl2*/*Il25* double knockout mice and examined the potential *in vivo* link between Tpl2 and IL-25. Expectedly, IL-25 deletion under WT or *Tpl2*-KO background both dramatically increased the mortality rate of FH mice, and suppressed the expression of MDSC-recruiting chemokine genes *Cxcl1* and *Cxcl2* in *P. acnes*-primed livers (**Figures 7B,C**), implying IL-25 is also critical for the liver recruitment of MDSC and thus protect against *P. acnes*/LPS-induced FH. Interestingly, *Tpl2*/*Il25* double knockout mice didn't further exaggerated the disease severity of *P. acnes*/LPS-induced FH when compared with *Tpl2*-deficient mice, as reflected by comparable mortality rate of these two strains of FH mice (**Figure 7B**), suggesting Tpl2-mediated prevention of FH is indeed through IL-25 signaling.

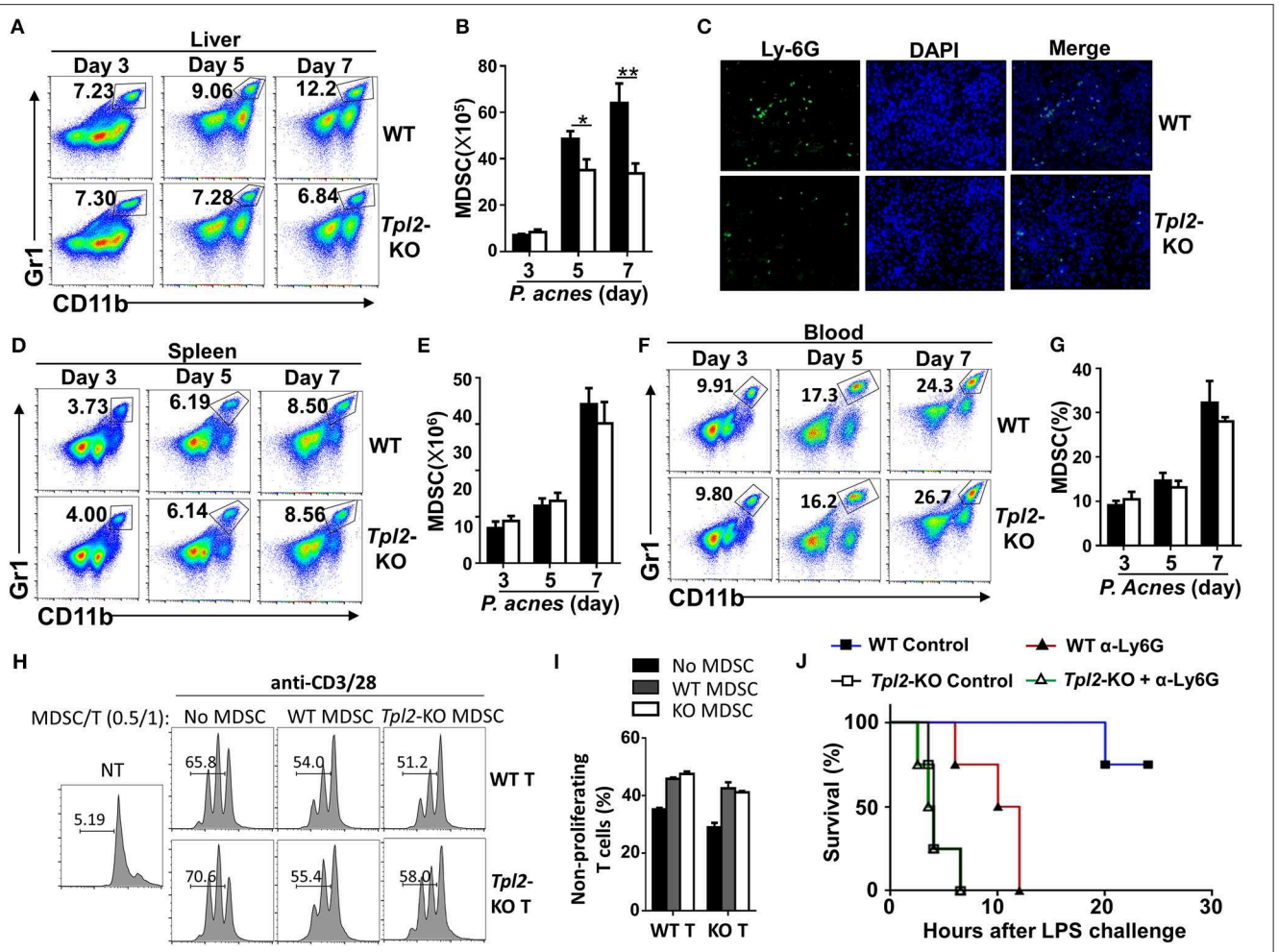


FIGURE 5 | Tpl2 mediated the liver recruitment of MDSC during FH pathogenesis. **(A,B,D–G)** Flow cytometric analysis of Gr-1$^+$CD11b$^+$ MDSC in the livers **(A,B)** or spleens **(D,E)** or peripheral blood **(F,G)** of WT and *Tpl2*-KO mice at day 3, 5, and 7 after *P. acnes* priming ($n = 4$ mice/group). Data are presented as representative plots **(A,D,F)** and summary bar graph **(B,E,G)** showing the absolute numbers or frequencies of MDSC. **(C)** Immunofluorescence images showing the infiltrated MDSC by using the anti-Ly6G antibody in the liver sections obtained from WT and *Tpl2*-KO mice at day 7 after *P. acnes* priming (magnification, ×200). **(H,I)** Flow cytometry analysis of the proliferation of WT CD4$^+$ T cells that labeled with CFSE, and then cocultured with WT or *Tpl2*-KO MDSC at the indicated ratio in the absence (NT) or presence of anti-CD3/28 antibodies for 72 h. Data are presented representative histograms **(H)** and bar graph **(I)**. **(J)** Cumulative survival rates of WT and *Tpl2*-KO mice that injected with anti-Ly6G antibody (200 μg/mouse, three times) to deplete *in vivo* MDSC or control antibody, and then subjected to *P. acnes*/LPS-mediated FH induction ($n = 4$ mice/group). Results are mean ± SD from three independent experiments. Two-tailed Student's *t*-tests were performed. $^*P < 0.05$; $^{**}P < 0.01$.

Next, we examined the cellular source of CXCL1 and CXCL2 in the *P. acnes*-primed livers. The results revealed that *Tpl2* deficiency didn't affect the expression of *Cxcl1* and *Cxcl2* in *P. acnes*-primed liver CD11b$^+$F4/80$^+$GR-1$^-$ kuffer cells and macrophages (**Supplementary Figure 1E**). However, loss of Tpl2 or IL-25 both significantly suppressed these two genes' expression in the hepatocytes that isolated from *P. acnes*-primed livers (**Figure 7D**). In addition, the RNA sequencing analysis showed that the expression of *Cxcl1*, *Cxcl2,* and other IL-25-response genes are dramatically decreased in IL-25-stimulated *Tpl2*-deficient primary mouse hepatocytes as compared with that of WT cells (**Supplementary Figures 2A–D**, **Figure 7E**). Moreover, the quantitative PCR resulted also confirmed that Tpl2 is indispensable for the constitutively and IL-25-induced expression of *Cxcl1* and *Cxcl2* in primary mouse hepatocytes (**Figure 7F**). Collectively, these results suggested that Tpl2 mediated IL-25 signaling in hepatocyte to protect against FH.

DISCUSSION

FH is a life-threatening disease and liver transplantation is the only definitive treatment for the acute liver injury. However, the obvious side-effects of transplantation, such as donor shortage, immune rejection, detrimental effect of immunosuppressive drugs, etc., suggested an urgent to develop novel therapeutic strategies (1, 9, 28). Recently, accumulating evidences suggested that MDSC is critical to maintain the immunosuppressive niche in inflamed liver during the pathogenesis of various kinds of human hepatitis and related mouse models, and

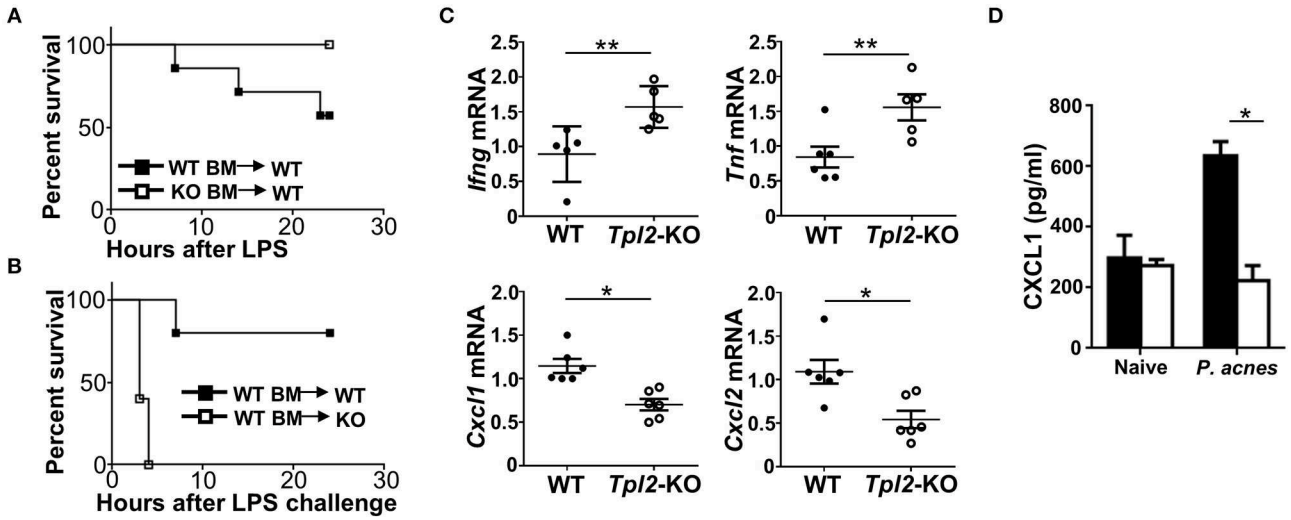


FIGURE 6 | Tpl2 functioned in liver-resident cells to protect against FH. **(A,B)** Cumulative survival rates of the lethally irradiated bone marrow (BM) chimeric WT mice that reconstituted with WT or *Tpl2*-KO BM **(A)** or the BM chimeric WT or *Tpl2*-KO mice that reconstituted with WT BM **(B)**, and then subjected to *P. acnes*/LPS-mediated FH induction ($n = 7$ or 5 mice/group). **(C)** QPCR analysis to determine the relative mRNA expression level of proinflammatory genes in livers of WT and *Tpl2*-KO mice at day 3 after *P. acnes* priming ($n = 6$ mice/group). Data were normalized to a reference gene, *Actb*. **(D)** Enzyme-linked immunosorbent assay of CXCL1 cytokine secretion in the supernatants of liver homogenates from WT and *Tpl2*-KO naïve or *P. acnes*-primed mice at day 3 ($n = 4$). Results are mean ± SD from three independent experiments. Two-tailed Student's *t*-tests were performed. $^*P < 0.05$; $^{**}P < 0.01$.

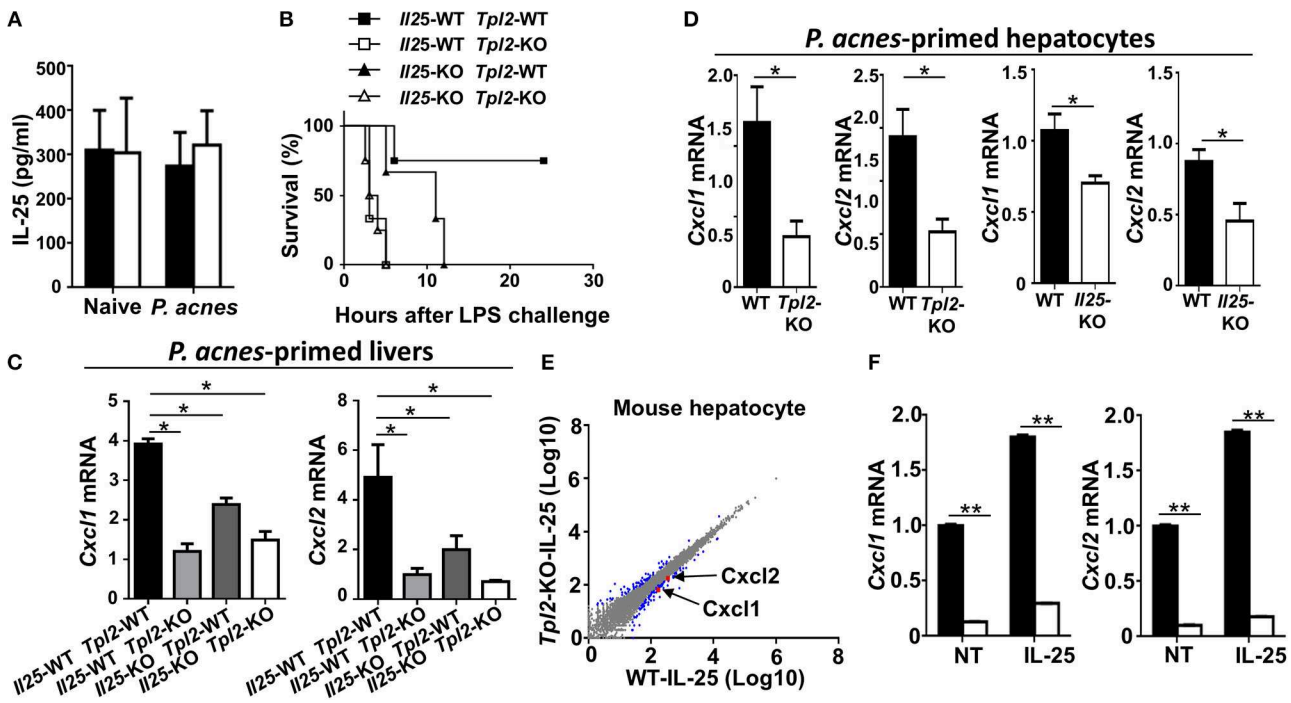


FIGURE 7 | Tpl2 regulated IL-25 signaling in hepatocytes. **(A)** Enzyme-linked immunosorbent assay showing IL-25 cytokine secretion in the supernatants of liver homogenates from WT and *Tpl2*-KO naïve or *P. acnes*-primed mice at day 3 ($n = 3$ or 4). **(B)** Cumulative survival rates of WT, *Tpl2*-KO, *Il25*-KO, *Tpl2*/*Il25* double knockout mice that subjected to *P. acnes*/LPS-mediated FH induction ($n = 3$ or 4 mice/group). **(C,D)** QPCR analysis of *Cxcl1* and *Cxcl2* genes' expression of livers **(C)** or hepatocytes **(D)** in WT, *Tpl2*-KO, *Il25*-KO, *Tpl2*/*Il25* double knockout mice at day 3 after *P. acnes* priming ($n = 6$ mice/group). **(E)** Scatter diagram showing the RNA-sequencing analysis of the gene expression pattern in WT and *Tpl2*-KO primary hepatocytes that stimulated by IL-25 for 8 h. The blue dots indicated the IL-25-induced up- or down-regulated genes, and the red dots indicated the down-regulation of *Cxcl1* and *Cxcl2* in *Tpl2*-KO hepatocytes. **(F)** QPCR determining the relative mRNA expression level of *Cxcl1* and *Cxcl2* in WT and *Tpl2*-KO hepatocytes that left untreated (NT) or stimulated with IL-25 for 8 h. Data were normalized to a reference gene, *Actb*. Results are mean ± SD from three independent experiments. Two-tailed Student's *t*-tests were performed. $^*P < 0.05$; $^{**}P < 0.01$.

increased infiltration of MDSC effectively attenuated the liver inflammation and protected FH-induced acute liver failure (10–15, 27). However, the molecular mechanism through which driving MDSC mobilization into inflamed liver remain elusive. Here we identified the protein kinase Tpl2 as an essential mediator to mobilize MDSC into liver during FH pathogenesis, and thus Tpl2 effectively protected the mice against FH-induced acute liver failure and mortality. Therefore, Tpl2 may have therapeutic potential for the treatment of FH.

Tpl2 is a protein kinase that was initially identified as protooncogene due to the tumor promoting function of its C-terminal truncation (29, 30). The expression of Tpl2 is universal and it is found to expressed in both innate and adaptive immune cells and in diverse tissues, including the liver, lung, and intestines (30–33). The immune-regulatory function of Tpl2 is largely attributed to its activation of the MEK/ERK pathway in toll-like receptor (TLR), interleukin-1 receptor (IL-1R), or tumor necrosis factor receptor (TNFR) signaling (34, 35). In addition, Tpl2 also modulate the activation of p38, JNK, protein kinase B, and mammalian target of rapamycin in a context-dependent manner (25, 36). We previously found that Tpl2 functions in astrocytes to mediate IL-17A-induced chemokine (*Cxcl1/2*) expression through promoting TAK1 phosphorylation and its downstream NF-κB, p38, and JNK activation, whereas ERK activation is not affected (24). Therefore, it is not surprising we found in the present study that Tpl2 functioned in hepatocyte to modulate *Cxcl1/2* expression, which then modulated the recruitment of MDSC into liver during FH pathogenesis. A recent study has suggested that Tpl2 exhibited neutrophil intrinsic function to mediate the trafficking of this type of immune cells (37), so it is also possible that Tpl2 may functioned directly in MDSC to promote its liver mobilization in FH mice. However, the increased mortality was only observed in Tpl2 germline knockout FH mice or *Tpl2*-deficient recipient chimeric FH mice that adoptively transferred with WT BM, but not in the WT recipient chimeric FH mice that adoptively transferred with either WT or *Tpl2*-deficient BM, suggesting the liver MDSC mobilization during FH pathogenesis is not attributed to the direct intrinsic function of Tpl2 in MDSC, but in hepatocytes.

Although IL-25 is one of the IL-17 family protein, there is no functional similarity of IL-25 as compared with the pro-inflammatory IL-17A (16, 17). For example, IL-25 augments type 2 immune responses and promote the airway inflammation of patients with asthma (38). A recent study suggested that IL-25 is highly expressed in both human and mouse liver, and plays a critical function in maintaining the homeostasis and limiting local inflammation through recruiting the immunosuppressive MDSC (15). Nevertheless, the molecular mechanism through which IL-25 recruit MDSC into liver is not clear. Our present study provided a Tpl2 link between IL-25 and MDSC mobilization, and established Tpl2 as a key mediator of IL-25-induced signaling that contribute to the MDSC recruitment. In addition, during *P. acnes*/LPS-induced FH pathogenesis, Tpl2 seemed specifically mediate IL-25-induced expression of CXCL1/2, but not affected the induction of CCL17, a previously reported MDSC-attracting chemokine that could be induced by IL-25 administration in D-Gal/LPS-induced FH mice (15), suggesting Tpl2 modulated IL-25-induced chemokine expression in a context-dependent manner.

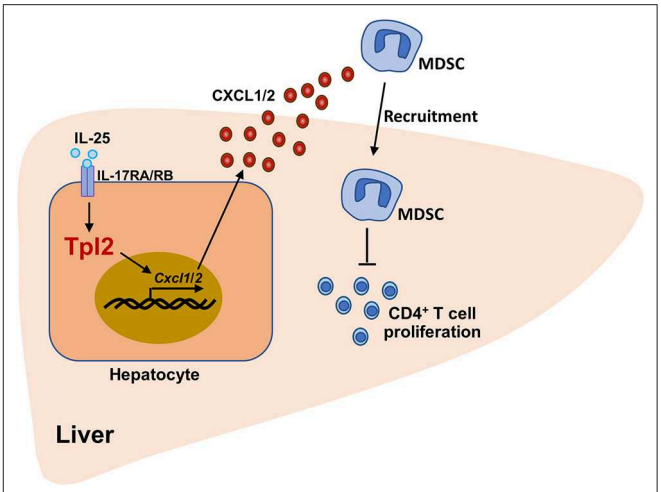


FIGURE 8 | The working model of Tpl2 in protecting against fulminant hepatitis. During the pathogenesis of *P. acnes*/LPS-induced FH, high levels of IL-25 in the liver microenvironment activated the signaling pathway mediated by IL-17RA/IL-17RB heterodimer receptor in hepatocytes, which were induced the expression of *Cxcl1/2* on a Tpl2-dependent manner. Increased CXCL1/2 production promoted the liver recruitment of the immunosuppressive MDSC, which further impaired the proliferation of liver-infiltrated pathogenic $CD4^+$ T cells, and finally suppressed the inflammation-induced acute liver injury.

Our previously study has suggested that Tpl2 critically regulate IL-17A-induced signaling in astrocytes to mediate autoimmune inflammation, here we also demonstrated that Tpl2 is a key modulator in IL-25-induced signaling in hepatocyte to restrain hepatitis. This functional controversy may be due to Tpl2 regulates the function of different cells upon different stimulus, and suggest Tpl2 have a dual role in promoting or restraining inflammatory processes in a context-dependent manner.

In conclusion, our findings demonstrated that Tpl2 effectively attenuated the severity of acute liver injury and increased the survival rate of FH mice. Mechanistically, Tpl2 functioned in hepatocytes to mediate IL-25-induced CXCL1/2 chemokines, which promoted the recruitment of MDSC to suppress Th1-mediated local inflammation, resulting in the amelioration of FH (**Figure 8**). Our data not only highlighted a novel function of Tpl2 in mediating IL-25 signaling, but also raised the possibility to develop Tpl2-based therapeutic strategies against this dreaded disease.

MATERIALS AND METHODS

Mice

Tpl2-deficient mice (C57BL/6 background) were described as previously (24). The $Tpl2^{+/-}$ mice were bred to generate age-matched $Tpl2^{-/-}$ (*Tpl2*-KO) and $Tpl2^{+/+}$ (WT) mice. The *Il25*-defcient mice (C57BL/6 background) were provided by Dr. Y. Qian (Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences). In some experiments, $Tpl2^{-/-}$ mice

were crossed with $Il25^{-/-}$ mice to generate $Tpl2^{+/-}Il25^{+/-}$ mouse, which were then bred to generate age-matched $Tpl2^{+/+}Il25^{+/+}$, $Tpl2^{-/-}Il25^{+/+}$, $Tpl2^{+/+}Il25^{-/-}$, and $Tpl2^{-/-}Il25^{-/-}$ mice. $Rag1^{-/-}$ mice (NM-KO-00069) were purchased from Shanghai Model Organisms Center. Mice were maintained in a specific pathogen-free facility, and all animal experiments were in accordance with protocols approved by the institutional Biomedical Research Ethics Committee, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences.

Induction of FH Mouse Model

For the induction of FH model, the age- and sex-matched mice were intravenously injected with 0.5 mg of heat-killed *P. acnes* suspended in 200 μl of phosphate-buffered saline (PBS) and after 7 days mice were injected intravenously with 1 μg of LPS and were monitored the survival rate. In some experiments, the WT and *Tpl2*-deficient mice were intravenously injected with 200 μg anti-Ly6G antibody for 3 times to deplete MDSC *in vivo*, or the *Rag1*-KO mice were adoptively transferred with WT or *Tpl2*-deficient $CD4^+$ T cells, or the lethally irradiated mice that were reconstituted with WT or *Tpl2*-deficient bone marrows, and then these mice were injected with *P. acnes*/LPS to induce FH and were monitored the survival rate.

Antibodies and Reagents

APC conjugated anti-mouse CD4 (17-0041-83), PB conjugated anti-mouse CD4 (48-0042-82), PE-cy7 conjugated anti-mouse CD8 (25-0081-82), APC-cy7 conjugated anti-mouse CD11b (47-0112-82), PB conjugated anti-mouse CD11c (48-0114-82), PerCP conjugated anti-mouse Ly6G (46-9668-82), FITC conjugated anti-mouse B220 (11-0452-85), PE conjugated anti-mouse CD45 (12-0451-83), APC conjugated anti-mouse IFNγ (17-7311-82), PE conjugated anti-mouse TNFα (12-7321-82), PE-cy7 conjugated anti-mouse CD25 (25-0251-82), APC conjugated anti-mouse Foxp3 (17-5773-82), PE conjugated anti-mouse CD44 (12-0441-83), FITC conjugated anti-mouse CD62L (11-0621-86), anti-mouse CD3 (16-0031-86), and anti-mouse CD28 (16-0281-86) antibodies were purchased from eBioscience. BrdU Flow Kits (559619) were purchased from BD Biosciences. Anti-mouse Ly6G (BE0075) antibody was purchased from Bioxcell. Alexa Fluor 488-conjugated anti-Rat IgG secondary antibody (A-21210) was from Thermo Fisher. Mouse anti-CD4 (L3T4, 130-049-201) and mouse anti-Ly-6G (130-092-332) Micro Beads were purchased from Miltenyi Biotec. Murine IL-25 (1399) were purchased from R&D. Lipopolysaccharides (LPS, L3129) were purchased from Sigma. *P. acnes* were prepared as previously described (7).

Flow Cytometry

The infiltrated immune cells from WT and *Tpl2*-deficient inflamed livers were prepared through 33% Percoll gradient as previously described (6). The collected liver-infiltrated immune cells or splenic cell suspensions were stained with the indicated antibodies and were subjected to flow cytometry analyses as previously described by using a Beckman Gallios flow cytometer (39). For the intracellular staining of TNF-α, IFN-γ, and Foxp3, the cells were fixed and permeabilized by fixation/permeabilization buffer (Thermo Fisher) before staining these antibodies, and then detected by flow cytometer. The absolute numbers of splenic and liver-infiltrating immune cells subpopulations were calculated based on their frequencies and the total number of isolated splenic and liver immune cells, and the data were presented as the average numbers of immune cell subpopulations per one spleen or liver of one mouse.

Histology and Immunofluorescence Analysis

Liver specimens were fixed in 4% paraformaldehyde and paraffin-embedded. Deparaffinized sections (8 μm) were stained with hematoxylin and eosin. Semiquantitative analysis of the status of liver inflammation was performed in a blinded manner as previously described (40). Briefly, the H&E stained liver slides were scored by a pathologist in a "blinded fashion" to determine the degree of inflammatory condition as follows: 0 = no infiltration, 1 = minimal/slight infiltration, 2 = moderate infiltration, 3 = severe infiltration. For immunofluorescence staining, the frozen sections (10 μm) from liver specimens were incubated with rat anti-mouse Ly6G (BE0075, Bioxcell) and were then labeled with Alexa Fluor 488-conjugated rabbit anti-rat IgG (A21210, Invitrogen), and the nuclei were stained by using DAPI (28718-90-3, Sangon Biotech).

Bone Marrow Chimeras

The bone marrow cells were prepared from WT or *Tpl2*-deficient mice and adoptively transferred into lethally irradiated (^{137}Cs, γ-ray, 950 rad) WT or *Tpl2*-deficient mice (around 7-week-old; 10^7 cells/mouse) as previously described (41). The lethal-dose irradiation would eliminate the bone marrow and peripheral immune cells without affecting the radioresistant liver-resident cells, and the bone marrow chimeric mice would thus have their peripheral immune system reconstituted. After 8 weeks, the chimeric mice were applied for the indicated experiments.

Mouse Hepatocyte Isolation

The mouse primary hepatocytes were prepared as previously described (42). In brief, the livers were sequentially perfused with Earle's balanced salt solutions (EBSS) without Ca^+ and Mg^+ containing EGTA, EBSS with Ca^+ and Mg^+ containing Hepes, EBSS with Ca^+ and Mg^+ containing Hepes and Collagenase IV, and the liver cells were squeezed out to obtain cell suspension in DMEM medium, which were then applied for centrifugation over a mixture of 9 ml Percoll, 1 ml 10 × EBSS and 10 ml DMEM. The precipitated hepatocytes were suspended and cultured with DMEM complete medium in a collagen-coated culture dish. Cell viability was determined by using Trypan blue exclusion assay.

In vivo BrdU Incorporation

Seven days after *P. acnes* priming, 2 mg BrdU (559619, BD) in 200 μl PBS was intraperitoneally injected into WT or *Tpl2*-deficient mice. The mice were sacrificed 2 h after the BrdU administration, and the immune cell suspensions from livers or spleens were prepared for flow cytometric analysis.

T Cell Proliferation Assay

The WT $CD4^+$ T cells were purified by MACS sorting and were labeled with 5 μM CFSE. The labeled cells were then seeded in the anti-CD3/CD28 antibodies-pre-coated plates and cocultured with MDSCs that isolated from WT or *Tpl2*-deficient bone marrow at the indicated ratio for 72 h. The cell proliferation was then determined by flow cytometry. In some experiment, WT or *Tpl2*-deficient $CD4^+$ T cells were seeded in anti-CD3/CD28 antibodies-pre-coated plates with 3 replicates and cultured for a total 72 h. The cell proliferation was recorded based on the $[^3H]$ thymidine labeling 8 h before examination.

RNA-Seq Analysis

Total RNA isolated from WT and *Tpl2*-KO hepatocytes stimulated with IL-25 were subjected to RNA-sequencing analysis. RNA sequencing was performed by BGI Tech Solutions. Transcriptomic reads from the RNA-Seq experiments were mapped to a reference genome (build mm 10) by using Bowtie. Gene expression levels were quantified by using the RSEM software package. Significant genes were defined by the *p*-value and false discovery rate of cutoff of 0.05 and fold changes $\geq$1.5. Differentially expressed genes were analyzed by the IPA and DAVID bioinformatics platform.

Quantitative RT-PCR

Liver tissues or cell samples were homogenized in Trizol reagent (Invitrogen). The cDNA was synthesized from 500 ng of extracted total RNA using M-MLV Reverse Transcriptase kit (Takara) according to the manufacturer's instructions. Quantitative PCR was performed with SYBR-Green premix ExTaq (Roche) and detected by a Real-time PCR System by using gene-specific primers. Gene expression was assessed in triplicate and normalized to a reference gene, β-actin. The gene-specific PCR primers are listed in **Supplementary Table 1**.

Quantification and Statistical Analysis

Statistical analyses were measured by GraphPad Software. Except where otherwise indicated, all the presented data are representative results of at least three independent repeats. Data are presented as mean ± SD, and the *P*-values were determined by two-tailed Student's *t*-tests. The *P*-values <0.05 were considered statistically significant.

DATA AVAILABILITY

The RNA-Sequencing data have been deposited into the Gene Expression Omnibus (accession code GSE125764). All other data supporting the findings of this study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of animal protocols that approved by Biomedical Research Ethics Committee, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. The protocol was approved by the Biomedical Research Ethics Committee, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences.

AUTHOR CONTRIBUTIONS

JX designed and performed the experiments, prepared the figures, and wrote part of the manuscript. SP, YW, and JL contributed to part of the experiments. YQ provided the *Il25*-deficient mice. MH and YZ supervised the work and contributed to data analysis. YX designed and supervised the work, prepared the figures, and wrote the manuscript.

FUNDING

We thank Dr. Shao-Cong Sun (Department of Immunology, MD Anderson Cancer Center, The University of Texas) for providing the *Tpl2*-deficient mice. This research was supported by the grants from the National Natural Science Foundation of China (81770567 and 81571545), the National Key R&D Program of China (2018YFA0107201, 2018YFA0902700), the Strategic Priority Research Program of the Chinese Academy of Sciences (XDPB10), the Key Arrangements of the Chinese Academy of Sciences (KFZD-SW-216), the Thousand Young Talents Plan of China, CAS Key Laboratory of Tissue Microenvironment and Tumor.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01980/full#supplementary-material

REFERENCES

1. Bernal W, Auzinger G, Dhawan A, Wendon J. Acute liver failure. *Lancet*. (2010) 376:190–201. doi: 10.1016/S0140-6736(10)60274-7
2. Antoniades CG, Berry PA, Wendon JA, Vergani D. The importance of immune dysfunction in determining outcome in acute liver failure. *J Hepatol*. (2008) 49:845–61. doi: 10.1016/j.jhep.2008.08.009
3. Crispe IN. The liver as a lymphoid organ. *Annu Rev Immunol*. (2009) 27:147–63. doi: 10.1146/annurev.immunol.021908.132629
4. Rolando N, Harvey F, Brahm J, Philpott-Howard J, Alexander G, Gimson A, et al. Prospective study of bacterial infection in acute liver failure: an analysis of fifty patients. *Hepatology*. (1990) 11:49–53. doi: 10.1002/hep.1840110110
5. Nakayama Y, Shimizu Y, Hirano K, Ebata K, Minemura M, Watanabe A, et al. CTLA-4Ig suppresses liver injury by inhibiting acquired immune responses in a mouse model of fulminant hepatitis. *Hepatology*. (2005) 42:915–24. doi: 10.1002/hep.20872
6. Xiao Y, Xu J, Mao C, Jin M, Wu Q, Zou J, et al. 18Beta-glycyrrhetinic acid ameliorates acute Propionibacterium acnes-induced liver injury through inhibition of macrophage inflammatory protein-1alpha. *J Biol Chem*. (2010) 285:1128–37. doi: 10.1074/jbc.M109.037705
7. Zhang Y, Cai W, Huang Q, Gu Y, Shi Y, Huang J, et al. Mesenchymal stem cells alleviate bacteria-induced liver injury in mice by inducing regulatory dendritic cells. *Hepatology*. (2014) 59:671–82. doi: 10.1002/hep.26670

8. Zhang Y, Yoneyama H, Wang Y, Ishikawa S, Hashimoto S, Gao JL, et al. Mobilization of dendritic cell precursors into the circulation by administration of MIP-1alpha in mice. *J Natl Cancer Inst.* (2004) 96:201–9. doi: 10.1093/jnci/djh024
9. Nemes B, Gelley F, Piros L, Zadori G, Gorog D, Fehervari I, et al. The impact of Milan criteria on liver transplantation for hepatocellular carcinoma: first 15 years' experience of the Hungarian Liver Transplant Program. *Transplant Proc*. (2011) 43:1272–4. doi: 10.1016/j.transproceed.2011.03.077
10. Cai W, Qin A, Guo P, Yan D, Hu F, Yang Q, et al. Clinical significance and functional studies of myeloid-derived suppressor cells in chronic hepatitis C patients. *J Clin Immunol*. (2013) 33:798–808. doi: 10.1007/s10875-012-9861-2
11. Hoechst B, Voigtlaender T, Ormandy L, Gamrekelashvili J, Zhao F, Wedemeyer H, et al. Myeloid derived suppressor cells inhibit natural killer cells in patients with hepatocellular carcinoma via the NKp30 receptor. *Hepatology*. (2009) 50:799–807. doi: 10.1002/hep.23054
12. Cripps JG, Wang J, Maria A, Blumenthal I, Gorham JD. Type 1 T helper cells induce the accumulation of myeloid-derived suppressor cells in the inflamed Tgfb1 knockout mouse liver. *Hepatology*. (2010) 52:1350–9. doi: 10.1002/hep.23841
13. Jenne CN, Wong CH, Zemp FJ, McDonald B, Rahman MM, Forsyth PA, et al. Neutrophils recruited to sites of infection protect from virus challenge by releasing neutrophil extracellular traps. *Cell Host Microbe*. (2013) 13:169–80. doi: 10.1016/j.chom.2013.01.005
14. Suh YG, Kim JK, Byun JS, Yi HS, Lee YS, Eun HS, et al. CD11b(+) Gr1(+) bone marrow cells ameliorate liver fibrosis by producing interleukin-10 in mice. *Hepatology*. (2012) 56:1902–12. doi: 10.1002/hep.25817
15. Sarra M, Cupi ML, Bernardini R, Ronchetti G, Monteleone I, Ranalli M, et al. IL-25 prevents and cures fulminant hepatitis in mice through a myeloid-derived suppressor cell-dependent mechanism. *Hepatology*. (2013) 58:1436–50. doi: 10.1002/hep.26446
16. Angkasekwinai P, Park H, Wang YH, Wang YH, Chang SH, Corry DB, et al. Interleukin 25 promotes the initiation of proallergic type 2 responses. *J Exp Med*. (2007) 204:1509–17. doi: 10.1084/jem.20061675
17. Fort MM, Cheung J, Yen D, Li J, Zurawski SM, Lo S, et al. IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies *in vivo*. *Immunity*. (2001) 15:985–95. doi: 10.1016/S1074-7613(01)00243-6
18. Kim MR, Manoukian R, Yeh R, Silbiger SM, Danilenko DM, Scully S, et al. Transgenic overexpression of human IL-17E results in eosinophilia, B-lymphocyte hyperplasia, and altered antibody production. *Blood*. (2002) 100:2330–40. doi: 10.1182/blood-2002-01-0012
19. Caruso R, Sarra M, Stolfi C, Rizzo A, Fina D, Fantini MC, et al. Interleukin-25 inhibits interleukin-12 production and Th1 cell-driven inflammation in the gut. *Gastroenterology*. (2009) 136:2270–9. doi: 10.1053/j.gastro.2009.02.049
20. Liu D, Cao T, Wang N, Liu C, Ma N, Tu R, et al. IL-25 attenuates rheumatoid arthritis through suppression of Th17 immune responses in an IL-13-dependent manner. *Sci Rep*. (2016) 6:36002. doi: 10.1038/srep36002
21. Gu C, Wu L, Li X. IL-17 family: cytokines, receptors and signaling. *Cytokine*. (2013) 64:477–85. doi: 10.1016/j.cyto.2013.07.022
22. Kang Z, Swaidani S, Yin W, Wang C, Barlow JL, Gulen MF, et al. Epithelial cell-specific Act1 adaptor mediates interleukin-25-dependent helminth expulsion through expansion of Lin(-)c-Kit(+) innate cell population. *Immunity*. (2012) 36:821–33. doi: 10.1016/j.immuni.2012.03.021
23. Swaidani S, Bulek K, Kang Z, Liu C, Lu Y, Yin W, et al. The critical role of epithelial-derived Act1 in IL-17- and IL-25-mediated pulmonary inflammation. *J Immunol*. (2009) 182:1631–40. doi: 10.4049/jimmunol.182.3.1631
24. Xiao Y, Jin J, Chang M, Nakaya M, Hu H, Zou Q, et al. TPL2 mediates autoimmune inflammation through activation of the TAK1 axis of IL-17 signaling. *J Exp Med*. (2014) 211:1689–702. doi: 10.1084/jem.20132640
25. Xiao Y, Sun SC. TPL2 mediates IL-17R signaling in neuroinflammation. *Oncotarget*. (2015) 6:21789–90. doi: 10.18632/oncotarget.4888
26. Yoneyama H, Harada A, Imai T, Baba M, Yoshie O, Zhang Y, et al. Pivotal role of TARC, a CC chemokine, in bacteria-induced fulminant hepatic failure in mice. *J Clin Invest*. (1998) 102:1933–41. doi: 10.1172/JCI4619
27. Pallett LJ, Gill US, Quaglia A, Sinclair LV, Jover-Cobos M, Schurich A, et al. Metabolic regulation of hepatitis B immunopathology by myeloid-derived suppressor cells. *Nat Med*. (2015) 21:591–600. doi: 10.1038/nm.3856
28. Stravitz RT, Kramer DJ. Management of acute liver failure. *Nat Rev Gastroenterol Hepatol*. (2009) 6:542–53. doi: 10.1038/nrgastro.2009.127
29. Ceci JD, Patriotis CP, Tsatsanis C, Makris AM, Kovatch R, Swing DA, et al. Tpl-2 is an oncogenic kinase that is activated by carboxy-terminal truncation. *Genes Dev*. (1997) 11:688–700. doi: 10.1101/gad.11.6.688
30. Patriotis C, Makris A, Bear SE, Tsichlis PN. Tumor progression locus 2 (Tpl-2) encodes a protein kinase involved in the progression of rodent T-cell lymphomas and in T-cell activation. *Proc Natl Acad Sci USA*. (1993) 90:2251–5. doi: 10.1073/pnas.90.6.2251
31. Hedl M, Abraham C. A TPL2 (MAP3K8) disease-risk polymorphism increases TPL2 expression thereby leading to increased pattern recognition receptor-initiated caspase-1 and caspase-8 activation, signalling and cytokine secretion. *Gut*. (2016) 65:1799–811. doi: 10.1136/gutjnl-2014-308922
32. Chowdhury FZ, Estrada LD, Murray S, Forman J, Farrar JD. Pharmacological inhibition of TPL2/MAP3K8 blocks human cytotoxic T lymphocyte effector functions. *PLoS ONE*. (2014) 9:e92187. doi: 10.1371/journal.pone.0092187
33. Watford WT, Wang CC, Tsatsanis C, Mielke LA, Eliopoulos AG, Daskalakis C, et al. Ablation of tumor progression locus 2 promotes a type 2 Th cell response in Ovalbumin-immunized mice. *J Immunol*. (2010) 184:105–13. doi: 10.4049/jimmunol.0803730
34. Vougioukalaki M, Kanellis DC, Gkouskou K, Eliopoulos AG. Tpl2 kinase signal transduction in inflammation and cancer. *Cancer Lett*. (2011) 304:80–9. doi: 10.1016/j.canlet.2011.02.004
35. Perugorria MJ, Murphy LB, Fullard N, Chakraborty JB, Vyrla D, Wilson CL, et al. Tumor progression locus 2/Cot is required for activation of extracellular regulated kinase in liver injury and toll-like receptor-induced TIMP-1 gene transcription in hepatic stellate cells in mice. *Hepatology*. (2013) 57:1238–49. doi: 10.1002/hep.26108
36. Li X, Acuff NV, Peeks AR, Kirkland R, Wyatt KD, Nagy T, et al. Tumor Progression Locus 2 (Tpl2) activates the Mammalian Target of Rapamycin (mTOR) pathway, inhibits Forkhead Box P3 (FoxP3) expression, and limits Regulatory T Cell (Treg) immunosuppressive functions. *J Biol Chem*. (2016) 291:16802–15. doi: 10.1074/jbc.M116.718783
37. Acuff NV, Li X, Elmore J, Rada B, Watford WT. Tpl2 promotes neutrophil trafficking, oxidative burst, and bacterial killing. *J Leukoc Biol*. (2017) 101:1325–33. doi: 10.1189/jlb.3A0316-146R
38. Wang YH, Angkasekwinai P, Lu N, Voo KS, Arima K, Hanabuchi S, et al. IL-25 augments type 2 immune responses by enhancing the expansion and functions of TSLP-DC-activated Th2 memory cells. *J Exp Med*. (2007) 204:1837–47. doi: 10.1084/jem.20070406
39. Liu J, Huang X, Hao S, Wang Y, Liu M, Xu J, et al. Peli1 negatively regulates noncanonical NF-kappaB signaling to restrain systemic lupus erythematosus. *Nat Commun*. (2018) 9:1136. doi: 10.1038/s41467-018-03530-3
40. Xiao Y, Xu J, Wang S, Mao C, Jin M, Ning G, et al. Genetic ablation of steroid receptor coactivator-3 promotes PPAR-beta-mediated alternative activation of microglia in experimental autoimmune encephalomyelitis. *Glia*. (2010) 58:932–42. doi: 10.1002/glia.20975
41. Zhang X, Wang Y, Yuan J, Li N, Pei S, Xu J, et al. Macrophage/microglial Ezh2 facilitates autoimmune inflammation through inhibition of Socs3. *J Exp Med*. (2018) 215:1365–82. doi: 10.1084/jem.20171417
42. Li D, Cen J, Chen X, Conway EM, Ji Y, Hui L. Hepatic loss of survivin impairs postnatal liver development and promotes expansion of hepatic progenitor cells in mice. *Hepatology*. (2013) 58:2109–21. doi: 10.1002/hep.26601

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

MINI REVIEW
published: 23 October 2019
doi: 10.3389/fimmu.2019.02490

The Management of Glucocorticoid Therapy in Liver Failure

Ran Xue[1,2] *and Qinghua Meng*[1]*

[1] Department of Critical Care Medicine of Liver Disease, Beijing You-An Hospital, Capital Medical University, Beijing, China, [2] Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education), Department of Gastrointestinal Oncology, Peking University Cancer Hospital & Institute, Beijing, China

Liver failure is characterized by rapid progression and high mortality. Excessive systemic inflammation is considered as the trigger of liver failure. Glucocorticoids (GCs) can rapidly suppress excessive inflammatory reactions and immune response. GCs have been applied in the treatment of liver failure since the 1970s. However, until now, the use of GCs in the treatment of liver failure has been somewhat unclear and controversial. New research regarding the molecular mechanisms of GCs may explain the controversial actions of GCs in liver failure. More results should be confirmed in a larger randomized clinical trial; this can aid the discovery of better definitions in terms of treatment schedules according to different clinical settings. Meanwhile, the timing and dosing of GCs in the treatment of liver failure should also be explored.

Keywords: glucocorticoids, liver failure, timing, dosing, mechanism

OPEN ACCESS

Edited by:
M. Victoria Delpino, CONICET Institute of Immunology, Genetics and Metabolism (INIGEM), Argentina

Reviewed by:
Oscar Bottasso, National University of Rosario, Argentina
Carolina Demarchi Munhoz, University of São Paulo, Brazil

***Correspondence:**
Qinghua Meng
meng_qh@126.com

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

Received: *16 April 2019*
Accepted: *04 October 2019*
Published: *23 October 2019*

Citation:
Xue R and Meng Q (2019) The Management of Glucocorticoid Therapy in Liver Failure. Front. Immunol. 10:2490. doi: 10.3389/fimmu.2019.02490

BACKGROUND

Liver failure is a life-threatening clinical syndrome with heterogeneous etiology that can cause serious disorders, such as coagulation disorders, icteria, hepatic encephalopathy (HE), and ascites (1, 2). Despite significant advances in artificial liver support system (ALSS) and liver transplantation (LT), these techniques are still difficult to apply more widely due to many restrictions, such as the amount of plasma, the limitation of liver donors, and the patient's economic situation, and so the mortality of liver failure is still high (3–5). It is therefore essential to develop more effective therapies for liver failure.

Glucocorticoids (GCs) have been applied to the clinical treatment of liver failure for many years. The first paper on GCs therapy for liver failure was published in the 1960s. Nowadays, many basic and clinical studies have explored the feasibility of GCs treatment in liver failure (6–12), but they remain inconclusive for the application of GCs treatment in liver failure.

THE APPLIED STATUS OF GCS THERAPY IN LIVER FAILURE

Among the different liver diseases, the most authoritative clinical indication of GCs therapy is autoimmune hepatitis (AIH) (7). However, in patients with suspected drug-induced AIH who are undergoing GCs therapy, withdrawal of treatment once the liver injury has resolved should be accompanied by close monitoring (13). A recent report from APASL ACLF Research Consortium Working Party defined the histopathological, clinical spectrum, and role of GCs therapy in patients with AIH-ACLF. It was shown that early stratification to LT or GCs therapy (hepatic encephalopathy in $\geq$F3, MELD>27) would improve outcomes and reduce ICU stay in patients with AIH-ACLF (14).

GCs therapy is also recommended as a first-line treatment strategy in patients with severe alcoholic hepatitis, hepatic encephalopathy, or maddrey discriminant function $\geq$32 (6).

Meanwhile, GCs would not increase occurrence of or mortality from bacterial infections in patients with severe alcoholic hepatitis (15). However, a recent meta-analysis showed that it could not determine whether GCs had a positive or negative effect on people with alcoholic liver disease because available data were still insufficient to produce robust results, trials were small, and the included participants differed in severity of disease (16).

Drug-induced liver failure requires evidence of immunopathogenicity to reverse the condition through GCs blocking immune responses. A recent study showed that short-term use of GCs was strongly recommended for severe DILI patients with hyperbilirubinemia (TBil >243 μmol/L) (17). However, Wan et al. found that prednisone was not beneficial for the treatment of severe drug-induced liver injury (18). The newest EASL clinical practice guidelines for drug-induced liver injury consider how GCs are often given when all else fails to procedure results (19). Early trials of GCs therapies, for all forms of ALF, demonstrated limited benefits (10, 20). GCs are also applied to treat drug-induced cholestatic hepatitis, especially in patients with allergic manifestations such as fever, eosinophilia, and rash. Liver injury caused by antiepileptic drugs are commonly related to features of hypersensitivity and may respond to GCs (21).

There exist significant differences in the etiology of liver diseases between the East and West. HBV is the leading cause of chronic liver disease in the Asia-Pacific region, including China and India (2). HBV-activated immune response and immune pathology caused by liver cell inflammation and necrosis are the initiated factors of liver failure. Although a large number of studies reported that GC therapy is effective in liver failure (22, 23), GC therapy is only recommended for the treatment of early stages of liver failure, and there is little evidence to support its effectiveness.

However, with the arrival of nucleoside analogs (NAs), more and more guidelines have recommended NAs to be used in patients with acute exacerbation of chronic HBV infection. The early combined use of NAs and GCs could be a good option to reverse the potential deterioration in patients with HBV-related liver failure. A recent study reported that early combination therapy with corticosteroid and NAs induces rapid resolution of inflammation in ALF due to transient HBV infection (24). It has been shown that with sufficient doses of NAs, GCs cannot affect the replication of HBV (12). However, Huang et al. (12) investigated retrospectively the efficacy of GCs in patients with hepatitis B virus-related acute-on-chronic liver failure (HBV-ACLF). It was indicated that GC treatment did not improve transplant-free survival in patients with HBV-ACLF.

It is not rare for GCs to be abused in the treatment of liver failure as "reduced bilirubin drugs." Therefore, its use in terms of liver failure therapy should not be exaggerated, although some patients with liver failure can indeed benefit from GCs therapy. As a "double-edged sword," the timing, dosage, and clinical indication of GC therapy are the key points to better definitions in terms of treatment schedules according to different clinical settings in the future.

THE TIMING OF GC THERAPY IN LIVER FAILURE

In the Asia-Pacific region, the most common type of liver failure is HBV-ACLF. The clinical stage of HBV-ACLF can be divided into four stages: early stage of ascending period, late stage of ascending period, platform period, and recovery period. Immune injury is the major event in the early stage of ascending period. The pathogenesis in the late stage of ascending period is involved in ischemia, immune injury, and hypoxia injury (25). During the platform period, body conditions achieve an immunosuppression state.

Endotoxemia is an important factor during the initiation of liver injury. Recent studies have shown that there was an inflammatory cascade in the early period of HBV-ACLF (26, 27). The sooner systemic inflammatory response syndromes (SIRS) occurred, the higher the mortality rate would be. GCs can inhibit inflammation, stabilize the liver cell membrane, and prevent further necrosis of liver cells (28). Therefore, early application of GC therapy can inhibit immune responses. The inhibition of systemic inflammation delays rapid progression and improves the survival rate of patients with ACLF.

Zhao et al. (11) found that patients responding best to GCs were those with less severe liver failure and a higher risk of rapid disease progression, with lower HE grades and MELD scores but extremely high ALT levels. The optimal time of intervention with GCs was within 14 days of the onset of symptoms.

We consider that the efficacy of GC treatment is primarily associated with the timing of GC administration. Meanwhile, the first-time physician, age, basic condition, and complications should also be considered for GC administration. Patients with some specified indicators can benefit more from GC therapy; these could be indicators such as ALT>1,000 U/L, TBIL in the 10∼20 × ULN, PTA≥30%, MELD score<28, no obvious signs of infection, hepatic encephalopathy Stage<II, no liver and kidney syndrome trends, as well as overactive immunological responses. However, until now, there has been a lack of accurate quantitative indicators for GC therapy. Therefore, it is particularly important for doctors to accumulate more and more clinical experience.

THE DOSE SELECTION FOR GC THERAPY IN LIVER FAILURE

Today, the ideal choices regarding GC type and does remains inconclusive. Based on current clinical reports, GC dose is generally controlled in 1∼2 g/kg/d (methylprednisolone). Kotoh et al. (23) explored the feasibility of large doses of GC treatment for the treatment of liver failure. They divided 34 patients with ALF into two groups; 17 patients were given methylprednisolone

Abbreviations: ALF, acute liver failure; GCs, glucocorticoids; HBV, hepatitis B virus; HBV-ACLF, hepatitis B virus-related acute-on-chronic liver failure; HE, hepatic encephalopathy; INF-α, interferon-α; LPS, lipopolysaccharide; SALF, subacute liver failure; SIRS, systemic inflammatory response syndromes; TNF-α, tumor necrosis factor-α.

1,000 mg/d via hepatic artery continuously for 3 days. As a result, 13 patients were cured, 2 patients died, and 2 patients underwent LT without serious complications. Fujiwara et al. (29) discussed the value of high-dose GCs in the treatment of HBV-related liver failure. It was found that the survival rate and liver regeneration in the GC-treated group showed a slim advantage, but there was no statistical difference, while patients with HBV infection and a poor basic condition had an unfavorable prognosis.

When the efficacy of GCs therapy cannot be determined in clinic, it is required that possible side effects of GCs are kept within a controllable range based on the principle of safety. GCs can significantly inhibit the presence of phagocytic cells to the antigen, promote the destruction and disintegration of lymphocytes, and develop the removal of lymphocytes from blood vessels so as to reduce the number of lymphocytes in circulation (30). Small doses of GCs mainly inhibit cellular immunity, while high doses of GCs can suppress humoral immune function by inhibiting B cells and antibody production (31).

The number of liver surface glucocorticoid receptors (GRs) may be reduced in liver failure (27). If greater doses of GCs are given, the GCs cannot play a role during the presence of receptor saturation, but may increase the incidence of side effects of GCs. Therefore, patients with liver failure, especially those with cirrhosis, are not recommended to use high-dose GCs. Although GCs can increase the incidence of infection and upper gastrointestinal bleeding, as well as other complications, the side effects of GCs are controllable. Therefore, it is essential to screen and monitor the side effects during GCs therapy in patients with liver failure.

THE MECHANISM OF THE POTENTIAL BENEFIT OF GC THERAPY IN LIVER FAILURE

The Core Pathogenesis of Liver Failure

Currently, it is widely accepted that "endotoxin-macrophage-cytokine storm" is the core pathogenesis of liver failure, combined with the immune injury as the initial factor in the development of liver failure, especially in the early stage of liver failure (27, 32).

The chemical essence of endotoxin is lipopolysaccharide [LPS, recognized by the pattern-recognition receptor toll-like receptor 4 (TLR4)] (33). With the interaction of LPS-binding proteins, it binds to a variety of cell membranes with receptor CD14, transmitting signals from the outside of the cell to nucleus and stimulating the synthesis and release of cytokines, which involves tumor necrosis factor-α (TNF-α), interferon-α (INF-α), IL-1, and IL-6 and simultaneously induces macrophages to secrete nitric oxide and large amounts of oxygen-free radicals (34–37). The liver cells are injured by delayed type hypersensitivity, oxidative stress, and apoptosis. If the immune response cannot be suspended in time, it would lead to a vicious cycle, resulting in significant liver cell necrosis, apoptosis, and liver failure (38–40). Peripheral blood mononuclear cells (PBMCs) and monocytes from patients with cirrhosis respond stronger to LPS stimulation (41). Heat shock proteins (HSPs) are well-known as protective proteins that make cells resistant to stress-induced cell damage. However, simultaneous activation of TLR4 by HSPs causes enhanced tissue injury (42).

Immune injury is considered as the first blow in the "triple hit theory" of liver failure, and timely suspension of its excessive immune response may reduce or even reverse its condition (43, 44). As the most commonly used anti-inflammatory and immunosuppressive agents, GCs can inhibit macrophage phagocytosis and antigen treatment and suppress the production of inflammatory cytokines. Therefore, GCs have the theoretical basis for the treatment of liver failure.

The Anti-inflammatory Mechanisms of GCs

Aside from rapid non-genomic effects, GCs exert genomic effects by binding to the glucocorticoid receptor (GR), a member of the nuclear receptor family of transcription factors (45). Upon ligand binding, the GR translocates to the nucleus, where it acts either as a monomeric protein that affects transcription with other transcription factors or as a homodimeric transcription factor, which binds glucocorticoid response elements (GREs) in promoter regions of GC-inducible genes (46). Some reports have clearly showed that GR dimer-dependent transactivation is essential in the anti-inflammatory activities of GR (47–49). $GR^{dim/dim}$ mutant mice were used to show reduced GR dimerization, and hence GC cannot control inflammation (50, 51).

THE POTENTIAL MECHANISM FOR CONTROVERSIAL ACTIONS OF GC THERAPY IN LIVER FAILURE

The Pro-inflammatory Mechanisms of GC

Emerging studies have shown that GCs have a two-way regulation for inflammatory and immune responses (52). The basal state of the immune system and the type of exposure to GCs are significant factors influencing the effects of GCs (53). For instance, while chronic exposure to GCs seems to be immunosuppressive, acute exposure increases the peripheral immune response (54).

It was found that GCs can induce the expression of several innate immune-related genes, including several members of the Toll-like receptor (TLR) family, such as TLR2 and TLR4 (55–57). The activation of TLRs via the repression of NF-κB and AP-1 or via the induction of GC-induced leucine zipper (GILZ) or MKP-1 is a hallmark feature of inflammation (55).

The GR signaling interplays with the TLRs signaling pathway via several mechanisms (58). Hermoso et al. (59) found that dexamethasone increased TNF-α induction of TLR2 through the activation of GR, supporting the existence of positive feedback between the activation of the TLR signaling pathway and GC secretion. Meanwhile, GCs may exert pro-inflammatory actions through interactions with inflammatory cytokines such as TNF-α and acute phase protein serpinA3 (60). Besides, some studies indicated that GCs can work synergistically with pro-inflammatory mediators to enhance the defense mechanisms to

ensure removal and clearance of pathogens in the hepatic acute-phase response (58, 61). GC-mediated activation of NLRP3, TLR2, and P2Y2R and the potentiation of LIF and TNF-α regulated pro-inflammatory genes (58, 62). All these results provide a potential explanation for the controversial actions of GC therapy in liver failure. More studies are required to characterize the liver-specific effects of the anti- and pro-inflammatory roles of GR signaling.

GC Resistance (GCR)

There are two types of GCR, inherited or familial GCR and acquired GCR (63). It is accepted that a pro-inflammatory environment can negatively affect GR sensitivity (64, 65). The mechanisms contributing to reduced GC responsiveness are heterogeneous as they involve various cytokines and cell types. The mechanisms of GCR are still unclear. As GCR occurs in many inflammatory diseases, it is widely considered that GCR is a heterogeneous phenomenon with multiple underlying mechanisms (66). Some of these involve problems with the GR protein itself, but many others are independent from GR and involved in mutations in GR-induced genes and problems with chaperones or cofactors (63).

Meanwhile, the down-regulation of the GR protein is associated with GRC, involving many different mechanisms such as reduced transcription and homologous down-regulation (67), GR protein degradation (68), and decreased stability of GR mRNA (the involvement of AUUUA motifs in the 3′UTR of GR mRNA) (69). Moreover, post-translational modifications of GR also contribute to a reduced GC response, such as ubiquitination of the GR (Lys-426 within a PEST element) and phosphorylation of the GR (68, 70). Besides, some research also showed miRNAs have a prominent role in the regulation of GR mRNA turnover and the occurrence of GCR (71, 72).

In addition to the non-genomic and genomic actions of GCs, GR signaling also relies on the existence of post-translational modifications (PTMs) and multiple receptor isoforms. GR is transcribed from a single gene, NR3C1; however, alternative splicing of this gene generates GRα and GRβ isoforms (73). The GRβ isoform also participates in the GCR. It was found that up-regulation of the dominant negative GRβ isoform was correlated with GR insensitivity via inhibiting GR-induced transactivation and GR nuclear translocation (74).

The Possible Factors of GC Refractoriness in Liver Failure: Sepsis

Sepsis is a common complication of ACLF, which is an acute systemic inflammatory disease (75). However, GCs are hardly useful in sepsis (63). Thus, sepsis is considered a GCR disease. GCR is an essential problem in sepsis and leads to: lack of transport and removal of bile acids in the liver, resulting in cholestasis; increased production and reduced removal of L-lactate, resulting in lactic acidosis; GCs having no anti-inflammatory effects.

Our previous study proposed that the diagnostic criteria of sepsis are not suitable for patients in HBV-ACLF with sepsis, because patients with underlying chronic liver disease and cirrhosis may have deranged clinical parameters (76). Therefore, it is essential to establish compatible diagnostic criteria for sepsis in patients with ACLF. When sepsis occurred, the serum TBiL level and WBC count elevated significantly while PLT count decreased significantly. We argue that when sepsis occurs during the process of liver failure GCs are not recommended for patients.

CONCLUSIONS AND FUTURE PERSPECTIVES

The idea of using GCs during acute liver failure has circulated for so many years, but, so far, no meaningful work has provided conclusive evidence of its therapeutic efficacy, except in the field of autoimmune etiology. Beyond the east/west demarcation, current data availing GC's use in liver failure revealed benefits that appeared marginal and were no longer present upon adjustment (10), came from evidence recorded in non-randomized studies (22), or were other ones carried out in small groups of patients (23, 24). More results should be confirmed using a larger randomized clinical trial to in order to arrive at better definitions in terms of treatment schedules according to different clinical settings.

Meanwhile, due to the complicated pathophysiology of liver failure, the exploration of immunological manifestations with different etiology and different clinical staging of patients with liver failure is needed urgently. This is a prerequisite for the feasibility and safety of GC applications. With an in-depth study, we can find the accurate timing, dosage, and clinical indicators of GC therapy for the clinical management of liver failure, so that clinicians can make timely treatment options so as to obtain the greatest benefits for patients.

AUTHOR CONTRIBUTIONS

RX wrote this manuscript. QM designed this manuscript.

FUNDING

This study was funded by the National Natural Science Foundation of China (No. 81470877).

REFERENCES

1. Ran X, Zhonghui D, Haixia L, Li C, Hongwei Y, Meixin R, et al. A novel dynamic model for predicting outcome in patients with hepatitis B virus related acute-on-chronic liver failure. *Oncotarget.* (2017) 8:108970–80. doi: 10.18632/oncotarget.22447
2. Bernal W, Jalan R, Quaglia A, Simpson K, Wendon J, Burroughs A. Acute-on-chronic liver failure. *Lancet.* (2015) 386:1576–87. doi: 10.1016/S0140-6736(15)00309-8

3. Asrani SK, Simonetto DA, Kamath PS. Acute-on-chronic liver failure. *Clin Gastroenterol Hepatol.* (2015) 13:2128–39. doi: 10.1016/j.cgh.2015.07.008
4. Blasco-Algora S, Masegosa-Ataz J, Gutiérrez-García ML, Alonso-López S, Fernández-Rodríguez CM. Acute-on-chronic liver failure: pathogenesis, prognostic factors and management. *World J Gastroenterol.* (2015) 21:12125–40. doi: 10.3748/wjg.v21.i42.12125
5. Singanayagam A, Bernal W. Update on acute liver failure. *Curr Opin Crit Care.* (2015) 21:134–41. doi: 10.1097/MCC.0000000000000187
6. Wang F, Wang BY. Corticosteroids or non-corticosteroids:a fresh perspective on alcoholic hepatitis treatment. *Hepatobiliary Pancreat Dis Int.* (2011) 10:458–64. doi: 10.1016/S1499-3872(11)60079-9
7. Yeoman AD, Westbrook RH, Zen Y, Bernal W, Al-Chalabi T, Wendon JA, et a1. Prognosis of acute severe autoimmune hepatitis(AS-AIH): the role of eortieosteroids in modifying outcome. *J Hepatol.* (2014) 61:876–82. doi: 10.1016/j.jhep.2014.05.021
8. Yang CH, Wu TS, Chiu CT. Chronic hepatitis B reactivation: a word of caution regarding the use of systemic glueoeortieosteroid therapy. *Br J Dermatol.* (2007) 157:587–90. doi: 10.1111/j.1365-2133.2007.08058.x
9. Ramachandran J, Sajith KG, Pal S, Rasak JV, Prakash JA, Ramakrishna B. Clinicopathological profile and management of severe autoimmune hepatitis. *Trop Gastmenterol.* (2014) 35:25–31. doi: 10.7869/tg.160
10. Karkhanis J, Verna EC, Chang MS, Stravitz RT, Schilsky M, Lee WM, et al. acute liver failure study group. steroid use in acute liver failure. *Hepatology.* (2014) 59:612–21. doi: 10.1002/hep.26678
11. Zhao B, Zhang HY, Xie GJ, Liu HM, Chen Q, Li RF, et al. Evaluation of the efficacy of steroid therapy on acute liver failure. *Exp Ther Med.* (2016) 12:3121–9. doi: 10.3892/etm.2016.3720
12. Huang C, Yu KK, Zheng JM, Li N. Steroid treatment in patients with acute-on-chronic liver failure precipitated by hepatitis B: A 10-year cohort study in a university hospital in East China. *J Dig Dis.* (2019) 20:38–44. doi: 10.1111/1751-2980.12691
13. Björnsson ES, Bergmann O, Jonasson JG, Grondal G, Gudbjornsson B, Olafsson S. Drug-induced autoimmune hepatitis: response to corticosteroids and lack of relapse after cessation of steroids. *Clin Gastroenterol Hepatol.* (2017) 15:1635–6. doi: 10.1016/j.cgh.2017.05.027
14. Anand L, Choudhury A, Bihari C, Sharma BC, Kumar M, Maiwall R, et al. Flare of autoimmune hepatitis causing acute on chronic liver failure: diagnosis and response to corticosteroid therapy. *Hepatology.* (2018) 70:587–96. doi: 10.1002/hep.30205
15. Hmoud BS, Patel K, Bataller R, Singal AK. Corticosteroids and occurrence of and mortality from infections in severe alcoholic hepatitis: a meta-analysis of randomized trials. *Liver Int.* (2016) 36:721–8. doi: 10.1111/liv.12939
16. Pavlov CS, Varganova DL, Casazza G, Tsochatzis E, Nikolova D, Gluud C. Glucocorticosteroids for people with alcoholic hepatitis. *Cochrane Database Syst Rev.* (2019) 4:CD001511. doi: 10.1002/14651858.CD001511.pub4
17. Wang PQ, Chen H, Hu XF, Xie QP, Shi J, Lin L, et al. Beneficial effect of corticosteroids for patients with severe drug-induced liver injury. *J Dig Dis.* (2016) 17:618–27. doi: 10.1111/1751-2980.12383
18. Wan YM, Wu JF, Li YH, Wu HM, Wu XN, Xu Y. Prednisone is not beneficial for the treatment of severe drug-induced liver injury: an observational study (STROBE compliant). *Medicine.* (2019) 98:e15886. doi: 10.1097/MD.0000000000015886
19. European Association for the Study of the Liver. EASL clinical practice guidelines: drug-induced liver injury. *J Hepatol.* (2019) 70:1222–61. doi: 10.1016/j.jhep.2019.02.014
20. Tujios SR, Lee WM. Acute liver failure induced by idiosyncratic reaction to drugs: challenges in diagnosis and therapy. *Liver Int.* (2018) 38:6–14. doi: 10.1111/liv.13535
21. Björnsson E. Hepatotoxicity associated with antiepileptic drugs. *Acta Neurol Scand.* (2008) 118:281–90. doi: 10.1111/j.1600-0404.2008.01009.x
22. Zhang XQ, Jiang L, You JP, Liu YY, Peng J, Zhang HY, et al. Efficacy of short-term dexamethasone therapy in acute-on-chronic pre-liver failure. *Hepatol Res.* (2011) 41:46–53. doi: 10.1111/j.1872-034X.2010.00740.x
23. Kotoh K, Enjoji M, Nakamuta M, Yoshimoto T, Kohjima M, Morizono S, et al. Arterial steroid injection therapy can inhibit the progression of severe acute hepatic failure toward fulminant liver failure. *World J Gastroenterol.* (2006) 12:6678–82. doi: 10.3748/wjg.v12.i41.6678
24. Fujiwara K, Yasui S, Haga Y, Nakamura M, Yonemitsu Y, Arai M, et al. Early combination therapy with corticosteroid and nucleoside analogue induces rapid resolution of inflammation in acute liver failure due to transient hepatitis B virus infection. *Intern Med.* (2018) 57:1543–52. doi: 10.2169/internalmedicine.9670-17
25. Zheng YB, Huang ZL, Wu ZB, Zhang M, Gu YR, Su YJ, et al. Dynamic changes of clinical features that predict the prognosis of acute-on-chronic hepatitis B liver failure: a retrospective cohort study. *Int J Med Sci.* (2013) 10:1658–64. doi: 10.7150/ijms.6415
26. Xing T, Li L, Cao H, Huang J. Altered immune function of monocytes in different stages of patients with acute on chronic liver failure. *Clin Exp Immunol.* (2007) 147:184–8.
27. Chen P, Wang YY, Chen C, Guan J, Zhu HH, Chen Z. The immunological roles in acute-on-chronic liver failure: an update. *Hepatobiliary Pancreat Dis Int.* (2019) 18:403–11. doi: 10.1016/j.hbpd.2019.07.003
28. Ye Y. Three attacks in the development of HBV-related liver failure. *Infect Dis Inform.* (2009) 22:276–9.
29. Fujiwara K, Yasui S, Okitsu K, Yonemitsu Y, Oda S, Yokosuka O. The requirement for a sufficient period of corticosteroid treatment in combination with nucleoside analogue for severe acute exacerbation of chronic hepatitis B. *J Gastroenterol.* (2010) 45:1255–62. doi: 10.1007/s00535-010-0280-y
30. Hämäläinen M, Lilja R, Kankaanranta H, Moilanen E. Inhibition of iNOS expression and NO production by anti-inflammatory steroids. Reversal by histone deacetylase inhibitors. *Pulm Pharmacol Ther.* (2008) 21:331–9. doi: 10.1016/j.pupt.2007.08.003
31. Lim HY, Müller N, Herold MJ, van den Brandt J, Reichardt HM. Glucocorticoids exert opposing effects on macrophage function dependent on their concentration. *Immunology.* (2007) 122:47–53. doi: 10.1111/j.1365-2567.2007.02611.x
32. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. *Cell.* (2010) 140:805–20. doi: 10.1016/j.cell.2010.01.022
33. Jerala R. Structural biology of the LPS recognition. *Int J Med Microbiol.* (2007) 297:353–63. doi: 10.1016/j.ijmm.2007.04.001
34. Dajani R, Sanlioglu S, Zhang Y, Li Q, Monick MM, Lazartigues E, et al. Pleiotropic functions of TNF-alpha determine distinct IKKbeta-dependent hepatocellular fates in response to LPS. *Am J Physiol Gastrointest Liver Physiol.* (2007) 292:242–52. doi: 10.1152/ajpgi.00043.2006
35. Nardo B, Montalti R, Puviani L, Pacilè V, Beltempo P, Bertelli R, et al. Portal vein oxygen supply through a liver extracorporeal device to treat acute liver failure in swine induced by subtotal hepatectomy: preliminary data. *Transplant Proc.* (2006) 38:1190–2. doi: 10.1016/j.transproceed.2006.03.057
36. Radziewicz H, Hanson HL, Ahmed R, Grakoui A. Unraveling the role of PD-1/PD-L interactions in persistent hepatotropic infections: potential for therapeutic application? *Gastroenterology.* (2008) 134:2168–71. doi: 10.1053/j.gastro.2008.04.012
37. Zhang Z, Zhang JY, Wherry EJ, Jin B, Xu B, Zou ZS, et al. Dynamic programmed death 1 expression by virus-specific CD8 T cells correlates with the outcome of acute hepatitis B. *Gastroenterology.* (2008) 134:1938–49. doi: 10.1053/j.gastro.2008.03.037
38. Tavakoli S, Mederacke I, Herzog-Hauff S, Glebe D, Grün S, Strand D, et al. Peripheral blood dendritic cells are phenotypically and functionally intact in chronic hepatitis B virus (HBV) infection. *Clin Exp Immunol.* (2008) 151:61–70. doi: 10.1111/j.1365-2249.2007.03547.x
39. Op den Brouw ML, Binda RS, van Roosmalen MH, Protzer U, Janssen HL, van der Molen RG, et al. Hepatitis B virus surface antigen impairs myeloid dendritic cell function: a possible immune escape mechanism of hepatitis B virus. *Immunology.* (2009) 126:280-289. doi: 10.1111/j.1365-2567.2008.02896.x
40. Xie Q, Shen HC, Jia NN, Wang H, Lin LY, An BY, et al. Patients with chronic hepatitis B infection display deficiency of plasmacytoid dendritic cells with reduced expression of TLR9. *Microbes Infect.* (2009) 11:515–23. doi: 10.1016/j.micinf.2009.02.008
41. Gandoura S, Weiss E, Rautou PE, Fasseu M, Gustot T, Lemoine F, et al. Gene- and exon-expression profiling reveals an extensive LPS-induced response in immune cells in patients with cirrhosis. *J Hepatol.* (2013) 58:936–48. doi: 10.1016/j.jhep.2012.12.025
42. Rosenberger K, Derkow K, Dembny P, Krüger C, Schott E, Lehnardt S. The impact of single and pairwise Toll-like receptor activation on

neuroinflammation and neurodegeneration. *J Neuroinflammation*. (2014) 11:166. doi: 10.1186/s12974-014-0166-7
43. Zhang Z, Zou ZS, Fu JL, Cai L, Jin L, Liu YJ, et al. Severe dendritic cell perturbationisactively involvedin the pathogenesis of acute-on-chronic hepatitis B liver failure. *J Hepatol.* (2008) 49:396–406. doi: 10.1016/j.jhep.2008.05.017
44. Malhi H, Gores GJ. Cellular and molecular mechanisms of liver injury. *Gastroenterology*. (2008) 134:1641–54. doi: 10.1053/j.gastro.2008.03.002
45. Scheschowitsch K, Leite J, Assreuy J. New insights in glucocorticoid receptor signaling-more than just a ligand-binding receptor. *Front Endocrinol.* (2017) 8:16. doi: 10.3389/fendo.2017.00016
46. Galon J, Franchimont D, Hiroi N, Frey G, Boettner A, Ehrhart-Bornstein M, et al. Gene profiling reveals unknown enhancing and suppressive actions of glucocorticoids on immune cells. *FASEB J.* (2002) 16:61–71. doi: 10.1096/fj.01-0245com
47. Vandevyver S, Dejager L, Tuckermann J, Libert C. New insights into the anti-inflammatory mechanisms of glucocorticoids: an emerging role for glucocorticoid-receptor-mediated transactivation. *Endocrinology*. (2013) 154:993–1007. doi: 10.1210/en.2012-2045
48. Reichardt HM, Tuckermann JP, Göttlicher M, Vujic M, Weih F, Angel P, et al. Repression of inflammatory responses in the absence of DNA binding by the glucocorticoid receptor. *Embo J.* (2001) 20:7168–73. doi: 10.1093/emboj/20.24.7168
49. Smoak KA, Cidlowski JA. Mechanisms of glucocorticoid receptor signaling during inflammation. *Mech Ageing Dev*. (2004) 125:697–706. doi: 10.1016/j.mad.2004.06.010
50. Jewell CM, Scoltock AB, Hamel BL, Yudt MR, Cidlowski JA. Complexhumanglucocorticoid receptor dim mutations define glucocorticoid induced apoptotic resistance in bone cells. *Mol Endocrinol.* (2012) 26:244–56. doi: 10.1210/me.2011-1116
51. Kleiman A, Hubner S, Rodriguez Parkitna JM, Neumann A, Hofer S, Weigand MA, et al. Glucocorticoid receptor dimerization is required for survival in septic shock via suppression of interleukin-1 in macrophages. *Faseb J.* (2012) 26:722–9. doi: 10.1096/fj.11-192112
52. Cruz-Topete D, Cidlowski JA. One hormone, two actions: anti- and pro-inflammatory effects of glucocorticoids. *Neuroimmunomodulation*. (2015) 22:20–32. doi: 10.1159/000362724
53. Dhabhar FS. A hassle a day may keep the doctor away: stress and the augmentation of immune function. *Integr Comp Biol.* (2002) 42:556–64. doi: 10.1093/icb/42.3.556
54. Dhabhar FS. Stress-induced augmentation of immune function–the role of stress hormones, leukocyte trafficking, and cytokines. *Brain Behav Immun.* (2002) 16:785–98. doi: 10.1016/S0889-1591(02)00036-3
55. Chinenov Y, Rogatsky I. Glucocorticoids and the innate immune system: crosstalk with the toll-like receptor signaling network. *Mol Cell Endocrinol.* (2007) 275:30–42. doi: 10.1016/j.mce.2007.04.014
56. Xie Y, Tolmeijer S, Oskam JM, Tonkens T, Meijer AH, Schaaf MJM. Glucocorticoids inhibit macrophage differentiation towards a pro-inflammatory phenotype upon wounding without affecting their migration. *Dis Model Mech*. (2019) 12:dmm037887. doi: 10.1242/dmm.037887
57. Desmet SJ, De Bosscher K. Glucocorticoid receptors: finding the middle ground. *J Clin Invest*. (2017) 127:1136–45. doi: 10.1172/JCI88886
58. Busillo JM, Cidlowski JA. The five Rs of glucocorticoid action during inflammation: ready, reinforce, repress, resolve, and restore. *Trends Endocrinol Metab.* (2013) 24:109–119. doi: 10.1016/j.tem.2012.11.005
59. Hermoso MA, Matsuguchi T, Smoak K, Cidlowski JA. Glucocorticoids and tumor necrosis factor alpha cooperatively regulate toll-like receptor 2 gene expression. *Mol Cell Biol.* (2004) 24:4743–56. doi: 10.1128/MCB.24.11.4743-4756.2004
60. Lannan EA, Galliher-Beckley AJ, Scoltock AB, Cidlowski JA. Proinflammatory actions of glucocorticoids: glucocorticoids and TNFalpha coregulate gene expression *in vitro* and *in vivo*. *Endocrinology*. (2012) 153:3701–12. doi: 10.1210/en.2012-1020
61. Langlais D, Couture C, Balsalobre A, Drouin J. Regulatory network analyses reveal genome-wide potentiation of LIF signaling by glucocorticoids and define an innate cell defense response. *PLoS Genet*. (2008) 4:e1000224. doi: 10.1371/journal.pgen.1000224
62. Busillo JM, Azzam KM, Cidlowski JA. Glucocorticoids sensitize the innate immune system through regulation of the NLRP3 inflammasome. *J Biol Chem.* (2011) 286:38703–13. doi: 10.1074/jbc.M111.275370
63. Dendoncker K, Libert C. Glucocorticoid resistance as a major drive in sepsis pathology. *Cytokine Growth Factor Rev.* (2017) 35:85–96. doi: 10.1016/j.cytogfr.2017.04.002
64. Dejager L, Vandevyver S, Petta I, Libert C. Dominance of the strongest: inflammatory cytokines versus glucocorticoids. *Cytokine Growth Factor Rev*. (2014) 25:21–33. doi: 10.1016/j.cytogfr.2013.12.006
65. Van Bogaert T, De Bosscher K, Libert C. Crosstalk between TNF and glucocorticoid receptor signaling pathways. *Cytokine Growth Factor Rev*. (2010) 21:275–86. doi: 10.1016/j.cytogfr.2010.04.003
66. Matsumura Y. Heterogeneity of glucocorticoid resistance in patients with bronchial asthma. *Int J Biomed Sci*. (2010) 6:158–66.
67. Drouin J, Trifiro MA, Plante RK, Nemer M, Eriksson P, Wrange O. Glucocorticoid receptor binding to a specific DNA sequence is required for hormone-dependent repression of proopiomelanocortin gene transcription. *Mol Cell Biol.* (1989) 9:5305–14. doi: 10.1128/MCB.9.12.5305
68. Wallace AD, Cidlowski JA. Proteasome-mediated glucocorticoid receptor degradation restricts transcriptional signaling by glucocorticoids. *J Biol Chem*. (2001) 276:42714–21. doi: 10.1074/jbc.M106033200
69. Green TL, Leventhal SM, Lim D, Cho K, Greenhalgh DG. A Tri-nucleotide pattern in a 3' UTR segment affects the activity of a human glucocorticoid receptor isoform. *Shock.* (2017) 47:148–57. doi: 10.1097/SHK.0000000000000750
70. Pazdrak K, Straub C, Maroto R, Stafford S, White WI, Calhoun WJ, et al. Cytokine-induced glucocorticoid resistance from eosinophil activation: protein phosphatase 5 modulation of glucocorticoid receptor phosphorylation and signaling. *J Immunol.* (2016) 197:3782–91. doi: 10.4049/jimmunol.1601029
71. Li JJ, Tay HL, Maltby S, Xiang Y, Eyers F, Hatchwell L, et al. MicroRNA-9 regulates steroid-resistant airway hyperresponsiveness by reducing protein phosphatase 2A activity, *J Allergy Clin Immunol.* (2015) 136:462–73. doi: 10.1016/j.jaci.2014.11.044
72. Ledderose C, Möhnle P, Limbeck E, Schütz S, Weis F, Rink J, et al. Corticosteroid resistance in sepsis is influenced by microRNA-124–induced downregulation of glucocorticoid receptor-α. *Crit Care Med*. (2012) 40:2745–53. doi: 10.1097/CCM.0b013e31825b8ebc
73. Yudt MR, Cidlowski JA. Molecular identification and characterization of a and b forms of the glucocorticoid receptor. *Mol Endocrinol.* (2001) 15:1093–103. doi: 10.1210/mend.15.7.0667
74. Bamberger CM, Bamberger AM, de Castro M, Chrousos GP. Glucocorticoid receptor beta, a potential endogenous inhibitor of glucocorticoid action in humans. *J Clin Investig.* (1995) 95:2435–41. doi: 10.1172/JCI117943
75. Hernaez R, Solà E, Moreau R, Ginès P. Acute-on-chronic liver failure: an update. *Gut*. (2017) 66:541–53. doi: 10.1136/gutjnl-2016-312670
76. Xue R, Zhu Y, Liu H, Meng Q. The clinical parameters for the diagnosis of hepatitis B virus related acute-on-chronic liver failure with sepsis. *Sci Rep*. (2019) 9:2558. doi: 10.1038/s41598-019-38866-3

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 Cellular and Infection Microbiology

REVIEW
published: 20 December 2019
doi: 10.3389/fcimb.2019.00423

Immunopathogenesis of Hepatic Brucellosis

*Guillermo Hernán Giambartolomei and María Victoria Delpino**

Instituto de Inmunología, Genética y Metabolismo (INIGEM), Universidad de Buenos Aires, Buenos Aires (UBA), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina

The hepatic immune system can induce rapid and controlled responses to pathogenic microorganisms and tumor cells. Accordingly, most of the microorganisms that reach the liver through the blood are eliminated. However, some of them, including *Brucella* spp., take advantage of the immunotolerant capacity of the liver to persist in the host. *Brucella* has a predilection for surviving in the reticuloendothelial system, with the liver being the largest organ of this system in the human body. Therefore, its involvement in brucellosis is practically invariable. In patients with active brucellosis, the liver is commonly affected, and the most frequent clinical manifestation is hepatosplenomegaly. The molecular mechanisms implicated in liver damage have been recently elucidated. It has been demonstrated how *Brucella* interacts with hepatocytes inducing its death by apoptosis. The inflammatory microenvironment and the direct effect of *Brucella* on hepatic stellate cells (HSC) induce their activation and turn these cells from its quiescent form to their fibrogenic phenotype. This HSC activation induced by *Brucella* infection relies on the presence of a functional type IV secretion system and the effector protein BPE005 through a mechanism involved in the activation of the autophagic pathway. Finally, the molecular mechanisms of liver brucellosis observed so far are shedding light on how the interaction of *Brucella* with liver cells may play an important role in the discovery of new targets to control the infection. In this review, we report the current understanding of the interaction between liver structural cells and immune system cells during *Brucella* infection.

Keywords: liver, fibrosis, inflamación, hepatocite, stellate cells (Ito cells)

OPEN ACCESS

Edited by:
Nesrin Ozoren, Bogaziçi University, Turkey

Reviewed by:
Kai Yang, Indiana University School of Medicine—Lafayette, United States
Thorsten Maretzky, The University of Iowa, United States

****Correspondence:***
María Victoria Delpino
mdelpino@ffyb.uba.ar

Specialty section:
This article was submitted to Microbes and Innate Immunity, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *27 August 2019*
Accepted: *28 November 2019*
Published: *20 December 2019*

Citation:
Giambartolomei GH and Delpino MV (2019) Immunopathogenesis of Hepatic Brucellosis. Front. Cell. Infect. Microbiol. 9:423. doi: 10.3389/fcimb.2019.00423

BRUCELLOSIS

Brucellosis is a common zoonotic disease distributed around the world. The disease is rare in industrialized countries because of the routine screening of domestic livestock and animal vaccination program (Pappas et al., 2006; Mancini et al., 2014). Recently, case studies and review of published cases have been carried out to determine the prevalence of brucellosis in endemic regions. However, it should be borne in mind that because many areas with endemic brucellosis have poor infrastructure, it is likely that the incidence of the disease is underestimated. There are a reported 500,000 incident cases of human brucellosis per year. However, true incidence is estimated to be 5,000,000 to 12,500,000 cases annually (Godfroid et al., 2013; Hull and Schumaker, 2018). The clinical disease is common in the Middle East, Asia, Africa, South and Central America, the Mediterranean Basin, and the Caribbean (Pappas et al., 2006; Mancini et al., 2014; Cross et al., 2019).

At present, 12 species of *Brucella* genus have been described, each of which has its natural host, including domestic and farm animals, as well as wild animals, such as camels, bison, foxes, cetaceans, among others. *Brucella* is an expanding genus, and the most recent species were isolated from amphibians (Eisenberg et al., 2012; Fischer et al., 2012). The bacteria are transmitted from animals to human by ingestion of infected food products (meat or raw milk), direct contact with infected animals or their tissues, or inhalation of aerosols. Humans are accidental hosts, but brucellosis continues to be a major public health concern worldwide and is the most widespread zoonotic infection.

Brucella genus does not exhibit classic virulence factors, such as exotoxins, exoproteases, cytolysins, or other exoenzymes (Moreno and Moriyon, 2002). Observed tissue harm is a result of inflammatory immune responses through the activation of host immune responses after recognition of brucellar antigens by immune receptors, such as Toll-like receptors (TLR) and inflammasomes (Campos et al., 2004, 2017; Giambartolomei et al., 2004; Zwerdling et al., 2008, 2009; García Samartino et al., 2010; de Almeida et al., 2011, 2013; Delpino et al., 2012; Gomes et al., 2013). Notwithstanding, with its intracellular lifestyle, *Brucella* limits exposure to innate and adaptive immune responses and leads the clinical manifestations of the disease and pathology. *Brucella* takes advantage of intracellular destruction by restringing fusion of *Brucella*-containing vacuoles with lysosomal compartments in a mechanism mediated by the type IV secretion system (T4SS) (Comerci et al., 2001). Also, *Brucella* inhibits the apoptosis of infected macrophages and prevents the development of adequate adaptive immune response by the inhibition of antigen presentation (de Figueiredo et al., 2015; Barrionuevo and Giambartolomei, 2019).

CLINICAL FEATURES OF *BRUCELLA* LIVER INFECTION

The liver is the most commonly affected organ in patients with active brucellosis. Accordingly, clinical and biochemical records of liver involvement have been observed in up to 50% of patients with active disease (Colmenero et al., 1996). Histopathological analyses of liver biopsies from a large number of patients with brucellosis have revealed liver parenchyma lesions due to inflammation, including focal areas of cellular inflammation with minimal necrosis of liver cells or the presence of granulomas with different localizations in parenchymal tissue and portal space (Akritidis et al., 2007). The pathology report on liver granulomas in *Brucella* infection usually shows necrotizing granulomas with a peripheral halo of epithelioid cells, lymphocytes, and plasma cells, as well as polymorphonuclear infiltrate in the necrotic area (Colmenero Jde et al., 2002; Villar et al., 2002). There are differences in the histological evaluation of liver manifestations in human brucellosis due to several causes. One of them is that most reports are retrospective and lack bacteriological confirmation. Also, previous reports with bacteriological confirmation did not always specify the species of *Brucella* involved. Moreover, not all of them used the same criteria to define granuloma (for example, presence of epithelioid cells or presence of giant cells) (Adams, 1976).

Liver biopsies also presented evidence of hepatitis (Akritidis et al., 2007). The liver conducts the metabolism of carbohydrates, proteins, and fats. Some of the enzymes and the end products of these metabolic pathways may be utilized as biochemical markers of liver dysfunction because they are very sensitive to any abnormality that takes place. Despite the abovementioned, the liver function is frequently normal in *Brucella*-infected individuals. The total serum bilirubin may be slightly increased, and the total serum protein, albumin, and globulin are usually normal. The most frequent abnormalities are shown by the increase in transaminases and alkaline phosphatase, although they are non-specific (Young, 1995; Madkour, 2001). In these patients, the presence of inflammatory infiltrates is also common, and most of them present parenchymal necrosis (Madkour, 2001; Akritidis et al., 2007).

THE LIVER AS AN IMMUNE ORGAN

The liver is usually regarded only as a non-immunological organ involved mainly in metabolic, nutrient storage, and detoxification activities. However, it is a member of the immune system responsible for the production of acute-phase proteins, chemokines, cytokines, complement proteins, and it also carries diverse populations of resident immune cells (O'Farrelly and Crispe, 1999; Crispe, 2009; Nemeth et al., 2009). Notwithstanding, the spleen is the main critical mediator in the clearance of blood pathogens, which overlaps with the function of the liver (Robinson et al., 2016).

Lymphocyte populations are present in the parenchyma and the portal tracts of the liver. These populations consist of conventional and unconventional lymphocytes of innate immunity, NKT and NK cells, as well as cells of the adaptive immune system, T and B cells (Freitas-Lopes et al., 2017). Resident antigen-presenting cells are also abundant in the liver. These cells are able to capture antigens that enter through the liver or are released by dead or infected hepatocytes. The group of resident antigen-presenting cells comprises Kupffer cells (members of the reticuloendothelial system) (Gale et al., 1978), liver sinusoidal endothelial cells (LSEC) (a particular type of vascular endothelial cells) (Steffan et al., 1986), and dendritic cells (DC) (Prickett et al., 1988; Lau and Thomson, 2003). The presence of these cells is essential for the maintenance of liver tolerance in physiological conditions (Robinson et al., 2016).

Kupffer cells are the liver resident macrophages that adhere to sinusoidal endothelial cells inside the sinusoids (Smith, 2013). This localization is adequate to perform its function as a scavenger removing protein complexes, small particles, senescent red blood cells, and cell debris from the portal blood via pattern recognition receptors (PRRs) (Petrasek et al., 2012).

LSEC are specialized endothelial cells that by the absence of diaphragm and lack of basement membrane are the most permeable endothelial cells in the body. In physiological conditions, these cells maintain hepatic stellate cell (HSC)

quiescence inhibiting fibrosis development. These cells have a high phagocytic capacity and the molecules that promote antigen processing and presentation with efficacy similar to that of DC (Steffan et al., 1986; Lohse et al., 1996; Knolle et al., 1999).

Resident hepatic DC are located around the central veins and portal tracts. The presence of interleukin (IL)-10 and transforming growth factor (TGF)-β secreted by Kupffer and LSEC cells in the absence of infection provides a microenvironment capable of generating tolerant resident DC. The activation of these cells increases their capacity to migrate via the space of Disse to the lymphatic vessels in the portal tracts and then to the extrahepatic lymph nodes (Matsuno et al., 1996; Kudo et al., 1997).

Additionally, HSC and hepatocytes contribute to the immune homeostasis of the liver. Quiescent HSC exert immunoregulatory roles secreting chemokines, chemokine receptors, macrophage inflammatory proteins (MIPs), and TLR and also function as antigen-presenting cells (Friedman, 2008; Hernandez-Gea and Friedman, 2011). Hepatocytes can also participate as antigen-presenting cells, but they do not express the costimulatory molecules CD80 and CD86 (Bertolino et al., 1998). Therefore, hepatocytes induce T-cell functional activation but fail to promote survival (Bertolino et al., 1998). In addition, hepatocytes can also express PD-L1 in response to interferon (IFN) of type I and II with the consequent induction of T-cell apoptosis contributing to liver tolerance (Mühlbauer et al., 2006). This immunotolerant capacity of the liver is due to its structure, with resident immune cells in constant stimulation and the hepatic blood supply that creates a unique cytokine and growth factor milieu. This microenvironment determines the balance between tolerance and inflammation in the healthy liver. The complex of cytokine milieu in adult liver, in the absence of pathological inflammation, includes basal expression of proinflammatory cytokines IL-15, IL-7, IL-2, IL-12, IFN-γ, and the anti-inflammatory cytokines IL-10, IL-13, and TFG-β (Golden-Mason et al., 2004; Kelly et al., 2006). The tolerogenic environment is maintained by regulatory myeloid populations, such as myeloid-derived suppressor cells (MDSC), that mediate their suppressive activity through the production of IL-10 and TGF-β (Gabrilovich and Nagaraj, 2009). The transmigration of monocytes leads to MDSC differentiation and activation, contributing to the immunoregulatory capacity of the liver (Sander et al., 2010; Zimmermann et al., 2015). In particular, the impact of IL-10 in *Brucella* persistence and establishment of chronic infection through the modulation of macrophages has been demonstrated previously by using IL-10-deficient mice (Xavier et al., 2013).

Nevertheless, this immune tolerance, the hepatic immune system is able to induce rapid and controlled responses to pathogenic microorganisms and tumor cells (Robinson et al., 2016). Accordingly, most of the microorganisms that reach the liver through the blood are eliminated. Although the liver has several mechanisms to resist and to eliminate infectious agents, some of them, such as *Brucella* spp, take advantage of the immunotolerant capacity of the liver to escape from the immune response and persist in the host. Besides, because the liver is the organ with the largest number of resident macrophages (Heymann and Tacke, 2016), we can speculate that the liver constitutes a place for *Brucella* persistence.

BRUCELLA AND HSC IN FIBROSIS

HSC reside in the liver between the hepatocytes and the small blood vessels. These cells are characterized as containing intracellular lipid droplets and protrusions that extend around the blood vessels. During liver damage, these cells are activated and secrete collagen with the formation of scar tissue, leading to chronic fibrosis or cirrhosis (Xu et al., 2012).

Brucella is an infectious stimulus that causes HSC activation that involves the conversion of quiescent cells into myofibroblasts, as revealed by the increase in α-smooth muscle actin (SMA) expression, the increase in collagen secretion, the inhibition of matrix metalloproteinase (MMP)-9 secretion, the induction of the tissue inhibitor of metalloproteinases (TIMP)-1, and the secretion of the master regulator of fibrosis TGF-β (Arriola Benitez et al., 2013). TGF-β was identified as a main driver of HSC activation, and several approaches targeting the TGF-β signaling pathway were successfully used to engage fibrosis in animal models of chronic liver diseases (Dooley and ten Dijke, 2012; Puche et al., 2013).

HSC secrete the chemokines IL-8 and the monocyte chemotactic protein (MCP)-1 in response to *Brucella abortus* infection that could attract to the site of infection, neutrophils and monocytes, respectively (Arriola Benitez et al., 2013). It has been demonstrated that *B. abortus*-infected monocytes could not reverse the fibrotic phenotype in *B. abortus*-infected HSC. However, *B. abortus*-infected monocytes inhibit collagen deposition and induce MMP-9 secretion in uninfected HSC (Arriola Benitez et al., 2013). This may explain, at least in part, why many patients with brucellosis have an inflammatory infiltrate, but only some of them develop cirrhosis. Moreover, in patients with brucellosis and cirrhosis, other causes such as viral hepatitis or alcoholic cirrhosis, have not been ruled out (Madkour, 2001).

Until now, it is unclear that *Brucella* infection can directly or indirectly modulate HSC activity and, consequently, the deposition of extracellular matrix. As in other liver diseases, HSC activation during *B. abortus* infection could contribute to granuloma formation by laying down a ring of collagen to encapsulate the granuloma (Chuah et al., 2014) (**Figure 1**).

BRUCELLA TYPE IV SECRETION SYSTEM IN FIBROSIS

T4SSs are a multiprotein complex involved in the translocation of nucleoproteins and/or protein substrates across the bacterial cell envelope to the host cell (Zechner et al., 2012). In *Brucella*, T4SS is encoded by the *virB* operon, which consists of 12 genes (virB1-12) located on chromosome II. The transcription of the *virB* operon is controlled by the promoter upstream of *virB*1 (O'Callaghan et al., 1999; Sieira et al., 2000). T4SS protein substrates have been shown to modulate several cellular processes in the host cell, such as apoptosis, vesicle trafficking, ubiquitination, and

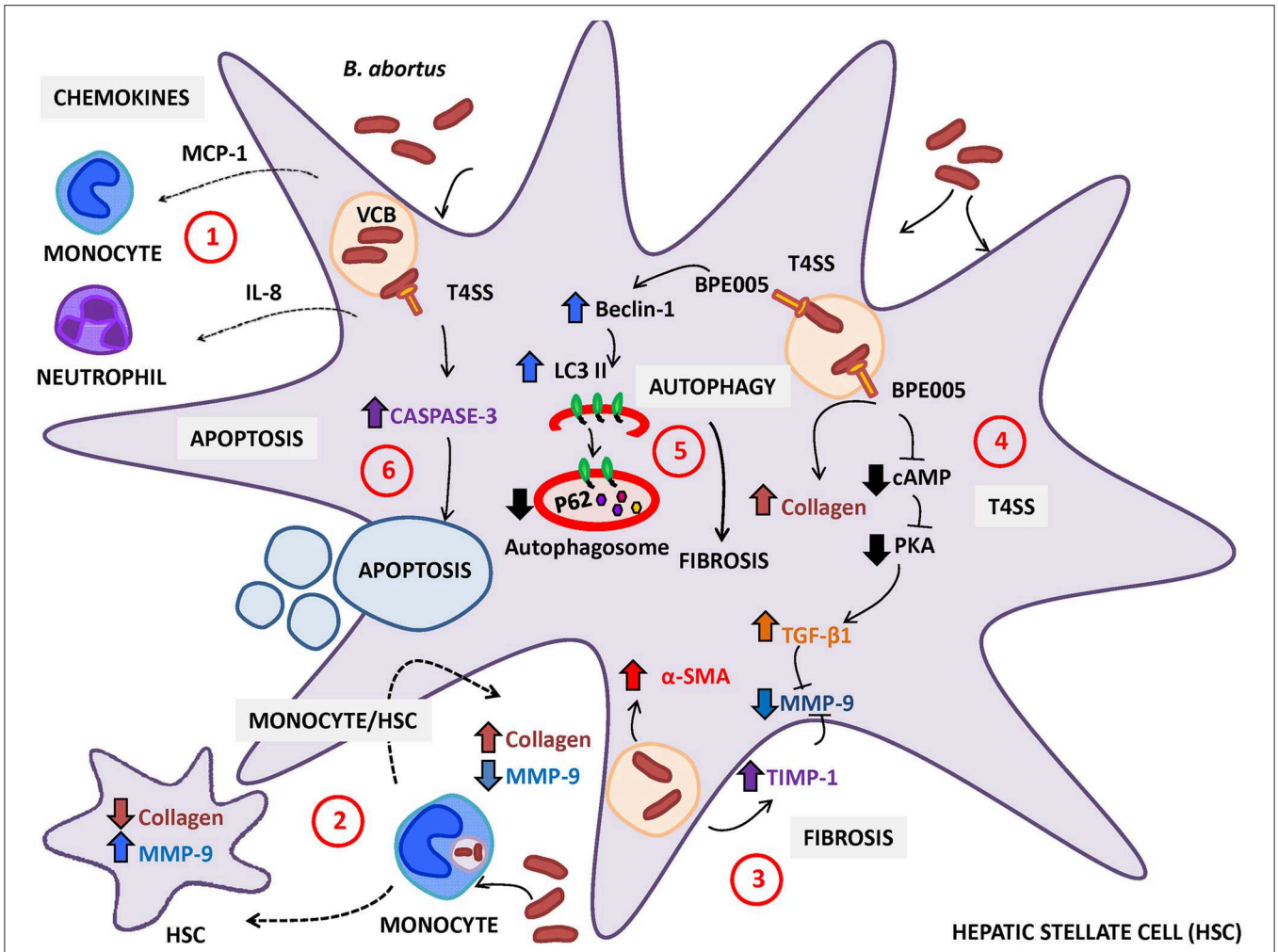


FIGURE 1 | *Brucella abortus* infection modulates the activation of hepatic stellate cells (HSC). (1) HSC secrete monocyte chemotactic protein (MCP)-1 and interleukin (IL)-8 (chemoattractant of monocytes and neutrophils, respectively) in response to *B. abortus* infection. (2) Resident macrophages or monocytes attracted to the site of infection could be infected by *B. abortus* and then modulate the expression of collagen and MMP-9 depending on the status of HSC (infected or not). In non-infected HSC, *B. abortus*-infected monocytes induce an inflammatory phenotype characterized by the increase in MMP-9 expression and the inhibition of collagen deposition. In contrast, *B. abortus*-infected monocytes induce the inhibition of matrix metalloproteinase (MMP)-9 secretion and the increase in collagen deposition in infected HSC. (3) *B. abortus* infection of HSC induces HSC activation by inducing a fibrotic phenotype characterized by the expression of α-smooth muscle actin (α-SMA), collagen deposition, and inhibition of MMP-9 through a mechanism that is dependent on the increase in transforming growth factor (TGF)-β and tissue inhibitor of metalloproteinase (TIMP)-1. (4) Fibrosis induction by *B. abortus* infection was dependent on the Type IV secretion system (T4SS) and its secreted effector BPE005 in a mechanism that depends on the cyclic AMP (cAMP)/protein kinase A (PKA) signaling pathway. (5) Fibrosis induction is also dependent on autophagy pathway induction characterized by the increase in the expression of LC3II and Beclin-1 and the inhibition of p62 expression. (6) Then, *B. abortus* infection induces cell death by apoptosis of activated HSC in a mechanism that is dependent on a functional T4SS and caspase 3 cleavage.

so on (Ninio and Roy, 2007; Franco et al., 2009). In *Brucella*, T4SS has been shown to be involved in the modulation of the immune system during infection (Roux et al., 2007; Rolán and Tsolis, 2008; Gomes et al., 2013); *Brucella* protein effector (BPE)005 is particularly involved in liver fibrosis (Arriola Benitez et al., 2016). The predicted structure BPE005 suggests that it might have an effect on cyclic AMP (cAMP) signaling pathways (Marchesini et al., 2011). In the HSC, BPE005 may mediate its effect by blocking the binding between cAMP and protein kinase A (PKA) with the concomitant fibrosis induction, as demonstrated in studies performed *in vitro* (Arriola Benitez et al., 2016). Interestingly, the role of BPE005 in the modulation of the fibrotic phenotype during *B. abortus* infection was confirmed in an *in vivo* model in mice infected with a *B. abortus* BPE005 mutant. The histological analysis by Masson's trichrome staining revealed that the level of fibrotic patches is lower in mice infected with BPE005 mutants than in those infected with the wild-type counterpart. Accordingly, levels of collagen and TGF-β were lower in mice infected with the *B. abortus* BPE005 mutant (Arriola Benitez et al., 2016).

The role of the cAMP/PKA signaling pathway in the liver has been reported previously. The involvement of this

pathway was demonstrated in various metabolic functions with the main effect in the facilitation of carbohydrate and lipid metabolism. cAMP plays a major role in the modulation of the HSC function by inhibiting profibrogenic pathways in HSC (Lopez-Sanchez et al., 2014).

Autophagy is involved in the fibrotic response during chronic hepatic lesion caused by hepatitis virus infection, alcohol abuse, and nonalcoholic steatohepatitis (Song et al., 2014).

Autophagy is a catabolic intracellular pathway that targets defective or excessive organelles to the lysosomes for degradation into amino acids, free fatty acids, or other small molecules used for material recycling or energy harvesting (Mao and Fan, 2015). Autophagy, usually stimulated by energy restriction, stress, or inflammation, is regarded as a survival mechanism that plays a critical role in maintaining cellular homeostasis, which is involved in many human disorders, such as fibrotic disease (Yin et al., 2008). In fibrosis, autophagy is mostly a cell survival mechanism that attenuates hepatic inflammatory injury and ultimately induces liver fibrosis (Mao and Fan, 2015). In addition, and supporting the role of BPE005 in the induction of HSC activation to a fibrotic phenotype, it has been demonstrated that autophagy is involved in the fibrotic response due to *B. abortus*, which depends on a functional T4SS and its effector BPE005 (Arriola Benitez et al., 2018). Autophagy was revealed by the upregulation of the LC3II/LC3I ratio and Beclin-1 expression and by the inhibition of p62 expression in infected cells (Arriola Benitez et al., 2018). However, further studies are necessary to determine if BPE005 could be a therapeutic target because this factor induces a fibrogenic phenotype that could help not only the host in the response to the inflammatory damage but also in the formation of granulomas likely favoring the persistence of bacterium.

Of note, the presence of liver cirrhosis during hepatic brucellosis is a debatable issue (Madkour, 2001). In addition, in murine models, cirrhosis was not observed upon infection with *B. abortus* (Arriola Benitez et al., 2013). In line with previous observations, *B. abortus* infection induces the clearance of activated HSC by apoptosis through a mechanism that is dependent on a functional T4SS and involved caspase 3 cleavage, triggering the recovery of liver fibrosis (Arriola Benitez et al., 2018) (**Figure 1**).

BRUCELLA AND HEPATOCYTES

Hepatocytes are the main cells of the liver parenchyma tissue and occupy around 70–85% of the liver volume (Kmieć, 2001). Hepatocytes are known to be involved in the synthesis of proteins, glycoproteins, cholesterol, bile salts, and phospholipids. On the other hand, they contribute to the detoxification and excretion of substances. However, these cells also participate in the immune response against pathogens. Hepatocytes respond to viral, bacterial, and parasitic infections by secreting pro-inflammatory cytokines and chemokines as mentioned elsewhere (Santos et al., 2005; McCord et al., 2006; Costa et al., 2008; Heydtmann and Adams, 2009), and *B. abortus* is not the exception. *B. abortus* infects hepatocytes and induces the secretion of IL-8, the main chemoattractant for neutrophil (Delpino et al., 2010), which correlates with the neutrophil infiltration observed in the liver of *Brucella*-infected patients (Hunt and Bothwell, 1967; Colmenero Jde et al., 2002). *B. abortus* infection also induces intercellular adhesion molecule (ICAM-1) and MMP-9 secretion by hepatocytes (Delpino et al., 2010). These molecules ensure the influx of neutrophils to the tissues. ICAM-1 could facilitate the interaction of hepatocytes with neutrophils, and MMP-9 promotes neutrophil transmigration through degradation of extracellular matrix. These neutrophils could be also infected by *B. abortus*, and in response to this infection, they can contribute to the inflammatory reaction through the secretion of IL-8 and MMP-9 by inducing the expression of ICAM-1 by hepatocytes (Delpino et al., 2010).

Presence of liver parenchymal necrosis has been demonstrated in patients with hepatic manifestations of brucellosis (Akritidis et al., 2007), and hepatocytes occupy most of the parenchymal tissue. The analysis of the effects of *B. abortus* infection on hepatocyte viability revealed the induction of cell death by apoptosis and cytotoxic release of lactate dehydrogenase (LDH) in a T4SS-dependent manner (Delpino et al., 2010). Neutrophils also contribute to the induction of apoptosis and LDH release by hepatocytes as revealed by the stimulation with culture supernatants from *B. abortus*-infected neutrophils (Delpino et al., 2010).

B. abortus-infected hepatocytes can also modulate HSC function. Supernatants from *B. abortus*-infected hepatocytes induce MMP-9 secretion and inhibit collagen deposition in LX-2 cells (Arriola Benitez et al., 2013). However, when stimulation was performed on *B. abortus*-infected HSC, supernatants from *B. abortus*-infected hepatocytes were unable to reverse the inhibitory effect of *B. abortus* infection in the inhibition of MMP-9 secretion and the induction of collagen deposition on HSC (Arriola Benitez et al., 2013). The status of HSC (infected or not) defines if *B. abortus*-infected hepatocytes induce a fibrotic or an inflammatory phenotype in these cells (**Figure 2**).

IN VIVO STUDIES FOR LIVER BRUCELLOSIS

The absence of a suitable animal model that can reproduce, after experimental infection, the variety of disease manifestations of human brucellosis has determined the slow progress in most of the pathobiology of focal forms of the disease. However, while laboratory rodents do not mimic all of the spectrum of clinical signs and symptoms of human disease, there are focal manifestations of liver brucellosis (Olsen and Palmer, 2014). This allows characterizing at least in part, the pathophysiological and immune manifestations of hepatic brucellosis.

Studies performed in mice infected with *Brucella melitensis* stated that most of the infected cells in the liver expressed F4/80 myeloid marker. In the peak of infection, granuloma formation occurs. Granulomas are mainly composed by CD11b+ F4/80+ MHC-II+ cells expressing iNOS/NOS2 enzyme (Copin et al., 2012). A population of these cells also expressed

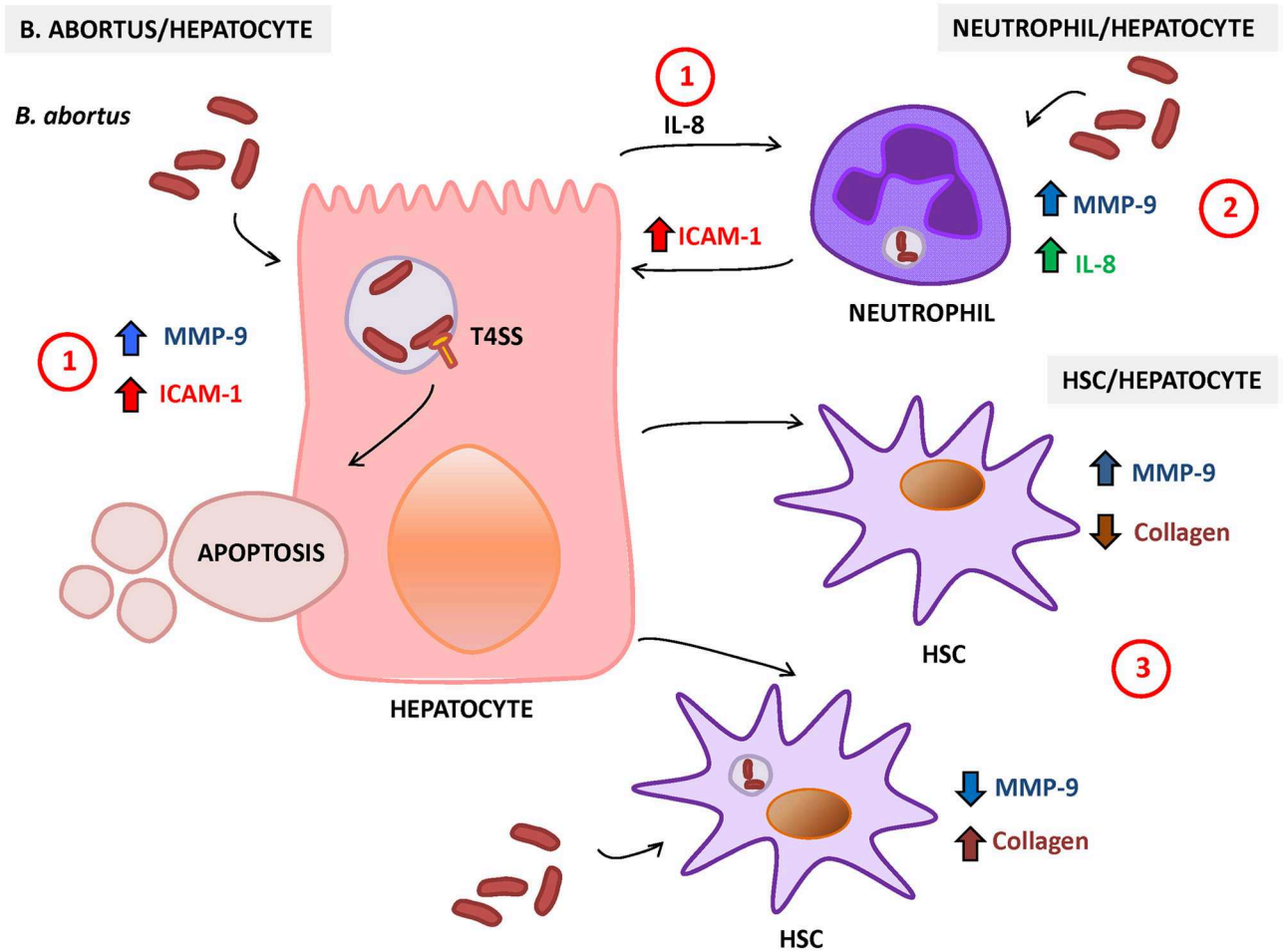


FIGURE 2 | Response of hepatocytes to *Brucella abortus* infection. (1) *B. abortus* infection of hepatocyte induces the secretion of matrix metalloproteinase (MMP)-9 and interleukin (IL)-8 (chemoattractant of neutrophils) and increases the surface expression of intercellular adhesion molecule (ICAM)-1 and cell death by apoptosis. (2) *B. abortus*-infected neutrophils secrete IL-8 and MMP-9 and induce the expression of ICAM-1 on hepatocytes. (3) HSC also interact with hepatocytes, and its response is dependent on the status of HSC (infected or not). In non-infected HSC, *B. abortus*-infected hepatocytes induce an inflammatory phenotype characterized by the increase in MMP-9 expression and the inhibition of collagen deposition. In contrast, *B. abortus*-infected hepatocytes induce the inhibition of MMP-9 secretion and the increase in collagen deposition in infected HSC.

CD11c marker, indicating the presence of inflammatory DC. However, further analyses are necessary to determine if *Brucella* will remain in these cells or will migrate to others that protect them from the immune response (Copin et al., 2012). The delay in conducting these studies is due to the fact that the murine model does not represent all of the clinical manifestations of the disease, and that the liver biopsy is not a medical practice for the diagnosis of brucellosis.

In vivo studies also revealed that IL-10 production by $CD25^{+}CD4^{+}$ T cells that modulate macrophage function enhances bacterial survival and persistence in liver through the modulation of the balance between pro-inflammatory and anti-inflammatory cytokines (Xavier et al., 2013). In addition, liver histopatology from infected IL-$10^{-}/^{-}$ mice at 3 weeks revealed multifocal granulomas and liver necrosis, as occurs in wild-type mice. However, at 6 weeks postinfection, the number of granulomas was reduced in IL-$10^{-}/^{-}$ mice with respect to wild-type controls. This reduction in liver pathology was accompanied by the increase of $CD4^{+}CD25^{+}$ $foxp3^{+}$ T cells with the expression of TGF-β (Corsetti et al., 2013). We can speculate that this reduction in liver pathology despite the increase in TGF-β can be attributed to the reduction of bacterial number and the consequent reduction in liver damage. However, further studies would be necessary to determine if the TGF-β levels produced are capable of causing liver fibrosis in these mice.

In addition, intraperitoneal infection of mice with *B. abortus* allows corroborating *in vitro* findings, among them the role of BPE005 in the induction of liver fibrosis revealed by the increase in collagen deposition in liver and the expression of TGF-β in HSC, as well as the presence of lymphocytic inflammatory infiltrate (Arriola Benitez et al., 2013, 2016).

Despite the limitations of the rodent model, it allowed to corroborate the most relevant *in vitro* findings related to human clinical outcomes.

CONCLUDING REMARKS

Liver involvement in active brucellosis has ranged from 5 to 52% or more (Madkour, 2001). While the clinical and imaging aspect of hepatic brucellosis has been widely described, the molecular and cellular mechanisms involved in the damage and the immune response in the liver have only been partially clarified over the past 10 years. The findings summarized in this review attempt to answer the key questions about the soluble mediators, local and infiltrating cells involved in inflammatory damage during liver brucellosis.

The infection through the T4SS effector, BPE005, could modulate HSC activation to control the fibrotic process. It is likely that BPE005 could participate in granuloma formation that might act as a reservoir of bacteria contributing to the disease chronicity. Additionally, and in line with the presence of liver parenchymal necrosis observed in patients (Akritidis et al., 2007), *B. abortus* induces cell death of hepatocytes in a mechanism that also depends on a functional T4SS (Arriola Benitez et al., 2018). Altogether, this knowledge could frame the first approach toward the discovery of a new therapeutic target leading to new treatments that could be coadministrated with antibiotics aimed at reducing liver damage.

AUTHOR CONTRIBUTIONS

GG helped draft and correct the manuscript. MD drafted the manuscript.

FUNDING

This work was supported by grants PICT 2014-1111 and PICT 2015-0316 from Agencia Nacional of Promoción Científica y Tecnológica (ANPCYT, Argentina), by grant PIP 2015-2017-0200 from Consejo Nacional de Investigaciones científicas y técnicas (CONICET).

REFERENCES

Adams, D. O. (1976). The granulomatous inflammatory response. A review. *Am. J. Pathol.* 84, 164–192.

Akritidis, N., Tzivras, M., Delladetsima, I., Stefanaki, S., Moutsopoulos, H. M., and Pappas, G. (2007). The liver in brucellosis. *Clin. Gastroenterol. Hepatol.* 5, 1109–1112. doi: 10.1016/j.cgh.2006.08.010

Arriola Benitez, P. C., Pesce Viglietti, A. I., Herrmann, C. K., Dennis, V. A., Comerci, D. J., Giambartolomei, G. H., et al. (2018). *Brucella abortus* promotes a fibrotic phenotype in hepatic stellate cells, with concomitant activation of the autophagy pathway. *Infect. Immun.* 86, e00522–17. doi: 10.1128/IAI.00522-17

Arriola Benitez, P. C., Rey Serantes, D., Herrmann, C. K., Pesce Viglietti, A. I., Vanzulli, S., Giambartolomei, G. H., et al. (2016). The effector protein BPE005 from *Brucella abortus* induces collagen deposition and matrix metalloproteinase 9 downmodulation via transforming growth factor beta1 in hepatic stellate cells. *Infect. Immun.* 84, 598–606. doi: 10.1128/IAI.01227-15

Arriola Benitez, P. C., Scian, R., Comerci, D. J., Serantes, D. R., Vanzulli, S., Fossati, C. A., et al. (2013). *Brucella abortus* induces collagen deposition and MMP-9 down-modulation in hepatic stellate cells via TGF-beta1 production. *Am. J. Pathol.* 183, 1918–1927. doi: 10.1016/j.ajpath.2013.08.006

Barrionuevo, P., and Giambartolomei, G. H. (2019). Inhibition of antigen presentation by *Brucella*: many more than many ways. *Microbes Infect.* 21, 136–142. doi: 10.1016/j.micinf.2018.12.004

Bertolino, P., Trescol-Biémont, M. C., and Rabourdin-Combe, C. (1998). Hepatocytes induce functional activation of naive CD8+ T lymphocytes but fail to promote survival. *Eur. J. Immunol.* 28, 221–36.

Campos, M. A., Rosinha, G. M., Almeida, I. C., Salgueiro, X. S., Jarvis, B. W., Splitter, G. A., et al. (2004). Role of Toll-like receptor 4 in induction of cell-mediated immunity and resistance to *Brucella abortus* infection in mice. *Infect. Immun.* 72, 176–186. doi: 10.1128/IAI.72.1.176-186.2004

Campos, P. C., Gomes, M. T., Guimarães, E. S., Guimarães, G., and Oliveira, S. C. (2017). TLR7 and TLR3 sense *Brucella abortus* RNA to induce proinflammatory cytokine production but they are dispensable for host control of infection. *Front. Immunol.* 8:28. doi: 10.3389/fimmu.2017.00028

Chuah, C., Jones, M. K., Burke, M. L., McManus, D. P., and Gobert, G. N. (2014). Cellular and chemokine-mediated regulation in schistosome-induced hepatic pathology. *Trends Parasitol.* 30, 141–150. doi: 10.1016/j.pt.2013.12.009

Colmenero Jde, D., Queipo-Ortuño, M. I., Maria Reguera, J., Angel Suarez-Muñoz, M., Martín-Carballino, S., and Morata, P. (2002). Chronic hepatosplenic abscesses in Brucellosis. Clinico-therapeutic features and molecular diagnostic approach. *Diagn. Microbiol. Infect. Dis.* 42, 159–167. doi: 10.1016/S0732-8893(01)00344-3

Colmenero, J. D., Reguera, J. M., Martos, F., Sánchez-De-Mora, D., Delgado, M., Causse, M., et al. (1996). Complications associated with *Brucella melitensis* infection: a study of 530 cases. *Medicine* 75, 195–211. doi: 10.1097/00005792-199607000-00003

Comerci, D. J., Martínez-Lorenzo, M. J., Sieira, R., Gorvel, J. P., and Ugalde, R. A. (2001). Essential role of the VirB machinery in the maturation of the *Brucella abortus*-containing vacuole. *Cell. Microbiol.* 3, 159–168. doi: 10.1046/j.1462-5822.2001.00102.x

Copin, R., Vitry, M. A., Hanot Mambres, D., Machelart, A., De Trez, C., Vanderwinden, J. M., et al. (2012). *In situ* microscopy analysis reveals local innate immune response developed around *Brucella* infected cells in resistant and susceptible mice. *PLoS Pathog.* 8:e1002575. doi: 10.1371/journal.ppat.1002575

Corsetti, P. P., de Almeida, L. A., Carvalho, N. B., Azevedo, V., Silva, T. M., Teixeira, H. C., et al. (2013). Lack of endogenous IL-10 enhances production of proinflammatory cytokines and leads to *Brucella abortus* clearance in mice. *PLoS ONE* 8:e74729. doi: 10.1371/journal.pone.0074729

Costa, J. D., Nogueira de Melo, A. C., Vermelho, A. B., Meirelles Mde, N., and Porrozzi, R. (2008). *In vitro* evidence for metallopeptidase participation in hepatocyte damage induced by Leishmania chagasi-infected macrophages. *Acta Trop.* 106, 175–183. doi: 10.1016/j.actatropica.2008.03.006

Crispe, I. N. (2009). The liver as a lymphoid organ. *Annu. Rev. Immunol.* 27, 147–163. doi: 10.1146/annurev.immunol.021908.132629

Cross, A. R., Baldwin, V. M., Roy, S., Essex-Lopresti, A. E., Prior, J. L., and Harmer, N. J. (2019). Zoonoses under our noses. *Microbes Infect.* 21, 10–19. doi: 10.1016/j.micinf.2018.06.001

de Almeida, L. A., Carvalho, N. B., Oliveira, F. S., Lacerda, T. L., Vasconcelos, A. C., Nogueira, L., et al. (2011). MyD88 and STING signaling pathways are required for IRF3-mediated IFN-beta induction in response to *Brucella abortus* infection. *PLoS ONE* 6:e23135. doi: 10.1371/journal.pone.0023135

de Almeida, L. A., Macedo, G. C., Marinho, F. A., Gomes, M. T., Corsetti, P. P., Silva, A. M., et al. (2013). Toll-like receptor 6 plays an important role in host innate resistance to *Brucella abortus* infection in mice. *Infect. Immun.* 81, 1654–1662. doi: 10.1128/IAI.01356-12

de Figueiredo, P., Ficht, T. A., Rice-Ficht, A., Rossetti, C. A., and Adams, L. G. (2015). Pathogenesis and immunobiology of brucellosis: review of Brucella-host interactions. *Am. J. Pathol.* 185, 1505–1517. doi: 10.1016/j.ajpath.2015.03.003

Delpino, M. V., Barrionuevo, P., Macedo, G. C., Oliveira, S. C., Genaro, S. D., Scian, R., et al. (2012). Macrophage-elicited osteoclastogenesis in response to *Brucella abortus* infection requires TLR2/MyD88-dependent TNF-alpha production. *J. Leukoc. Biol.* 91, 285–298. doi: 10.1189/jlb.04111185

Delpino, M. V., Barrionuevo, P., Scian, R., Fossati, C. A., and Baldi, P. C. (2010). *Brucella*-infected hepatocytes mediate potentially tissue-damaging immune responses. *J. Hepatol.* 53, 145–154. doi: 10.1016/j.jhep.2010.02.028

Dooley, S., and ten Dijke, P. (2012). TGF-beta in progression of liver disease. *Cell Tissue Res.* 347, 245–256. doi: 10.1007/s00441-011-1246-y

Eisenberg, T., Hamann, H. P., Kaim, U., Schlez, K., Seeger, H., Schauerte, N., et al. (2012). Isolation of potentially novel *Brucella* spp. from frogs. *Appl. Environ. Microbiol.* 78, 3753–3755. doi: 10.1128/AEM.07509-11

Fischer, D., Lorenz, N., Heuser, W., Kämpfer, P., Scholz, H. C., and Lierz, M. (2012). Abscesses associated with a *Brucella* inopinata-like bacterium in a big-eyed tree frog (*Leptopelis vermiculatus*). *J. Zoo Wildl. Med.* 43, 625–628. doi: 10.1638/2011-0005R2.1

Franco, I. S., Shuman, H. A., and Charpentier, X. (2009). The perplexing functions and surprising origins of Legionella pneumophila type IV secretion effectors. *Cell. Microbiol.* 11, 1435–1443. doi: 10.1111/j.1462-5822.2009.01351.x

Freitas-Lopes, M. A., Mafra, K., David, B. A., Carvalho-Gontijo, R., and Menezes, G. B. (2017). Differential location and distribution of hepatic immune cells. *Cells* 6:E48. doi: 10.3390/cells6040048

Friedman, S. L. (2008). Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver. *Physiol. Rev.* 88, 125–172. doi: 10.1152/physrev.00013.2007

Gabrilovich, D. I., and Nagaraj, S. (2009). Myeloid-derived suppressor cells as regulators of the immune system. *Nat. Rev. Immunol.* 9, 162–174. doi: 10.1038/nri2506

Gale, R. P., Sparkes, R. S., and Golde, D. W. (1978). Bone marrow origin of hepatic macrophages (Kupffer cells) in humans. *Science* 201, 937–938. doi: 10.1126/science.356266

García Samartino, C., Delpino, M. V., Pott Godoy, C., Di Genaro, M. S., Pasquevich, K. A., Zwerdling, A., et al. (2010). *Brucella abortus* induces the secretion of proinflammatory mediators from glial cells leading to astrocyte apoptosis. *Am. J. Pathol.* 176, 1323–1338. doi: 10.2353/ajpath.2010.090503

Giambartolomei, G. H., Zwerdling, A., Cassataro, J., Bruno, L., Fossati, C. A., and Philipp, M. T. (2004). Lipoproteins, not lipopolysaccharide, are the key mediators of the proinflammatory response elicited by heat-killed *Brucella abortus*. *J. Immunol.* 173, 4635–4642. doi: 10.4049/jimmunol.173.7.4635

Godfroid, J., Al Dahouk, S., Pappas, G., Roth, F., Matope, G., Muma, J., et al. (2013). A "One Health" surveillance and control of brucellosis in developing countries: moving away from improvisation. *Comp. Immunol. Microbiol. Infect. Dis.* 36, 241–248. doi: 10.1016/j.cimid.2012.09.001

Golden-Mason, L., Kelly, A. M., Doherty, D. G., Traynor, O., McEntee, G., Kelly, J., et al. (2004). Hepatic interleuklin 15 (IL-15) expression: implications for local NK/NKT cell homeostasis and development. *Clin. Exp. Immunol.* 138, 94–101. doi: 10.1111/j.1365-2249.2004.02586.x

Gomes, M. T., Campos, P. C., Oliveira, F. S., Corsetti, P. P., Bortoluci, K. R., Cunha, L. D., et al. (2013). Critical role of ASC inflammasomes and bacterial type IV secretion system in caspase-1 activation and host innate resistance to *Brucella abortus* infection. *J. Immunol.* 190, 3629–3638. doi: 10.4049/jimmunol.1202817

Hernandez-Gea, V., and Friedman, S. L. (2011). Pathogenesis of liver fibrosis. *Annu. Rev. Pathol.* 6, 425–456. doi: 10.1146/annurev-pathol-011110-130246

Heydtmann, M., and Adams, D. H. (2009). Chemokines in the immunopathogenesis of hepatitis C infection. *Hepatology* 49, 676–688. doi: 10.1002/hep.22763

Heymann, F., and Tacke, F. (2016). Immunology in the liver–from homeostasis to disease. *Nat. Rev. Gastroenterol. Hepatol.* 13, 88–110. doi: 10.1038/nrgastro.2015.200

Hull, N. C., and Schumaker, B. A. (2018). Comparisons of brucellosis between human and veterinary medicine. *Infect. Ecol. Epidemiol.* 8:1500846. doi: 10.1080/20008686.2018.1500846

Hunt, A. C., and Bothwell, P. W. (1967). Histological findings in human brucellosis. *J. Clin. Pathol.* 20, 267–272. doi: 10.1136/jcp.20.3.267

Kelly, A. M., Golden-Mason, L., Traynor, O., Geoghegan, J., McEntee, G., Hegarty, J. E., et al. (2006). Changes in hepatic immunoregulatory cytokines in patients with metastatic colorectal carcinoma: implications for hepatic anti-tumour immunity. *Cytokine* 35, 171–179. doi: 10.1016/j.cyto.2006.07.019

Kmieć, Z. (2001). Cooperation of liver cells in health and disease. *Adv. Anat. Embryol. Cell Biol*/ 161:III-XIII, 1-151. doi: 10.1007/978-3-642-56553-3

Knolle, P. A., Schmitt, E., Jin, S., Germann, T., Duchmann, R., Hegenbarth, S., et al. (1999). Induction of cytokine production in naive CD4(+) T cells by antigen-presenting murine liver sinusoidal endothelial cells but failure to induce differentiation toward Th1 cells. *Gastroenterology* 116, 1428–1440. doi: 10.1016/S0016-5085(99)70508-1

Kudo, S., Matsuno, K., Ezaki, T., and Ogawa, M. (1997). A novel migration pathway for rat dendritic cells from the blood: hepatic sinusoids-lymph translocation. *J. Exp. Med.* 185, 777–784. doi: 10.1084/jem.185.4.777

Lau, A. H., and Thomson, A. W. (2003). Dendritic cells and immune regulation in the liver. *Gut* 52, 307–314. doi: 10.1136/gut.52.2.307

Lohse, A. W., Knolle, P. A., Bilo, K., Uhrig, A., Waldmann, C., Ibe, M., et al. (1996). Antigen-presenting function and B7 expression of murine sinusoidal endothelial cells and Kupffer cells. *Gastroenterology* 110, 1175–1181. doi: 10.1053/gast.1996.v110.pm8613007

Lopez-Sanchez, I., Dunkel, Y., Roh, Y. S., Mittal, Y., De Minicis, S., Muranyi, A., et al. (2014). GIV/Girdin is a central hub for profibrogenic signalling networks during liver fibrosis. *Nat. Commun.* 5:4451. doi: 10.1038/ncomms5451

Madkour, M. M. (2001). "Gastrointestinal brucellosis," in *Madkour's Brucellosis*, *2nd ed,* eds M. M. Madkour (Berlin: Springer-Verlag), 150–158. doi: 10.1007/978-3-642-59533-2_13

Mancini, F. R., Bella, A., Graziani, C., Marianelli, C., Mughini-Gras, L., Pasquali, P., et al. (2014). Trends of human brucellosis in Italy, 1998-2010. *Epidemiol. Infect.* 142, 1188–1195. doi: 10.1017/S0950268813002227

Mao, Y. Q., and Fan, X. M. (2015). Autophagy: a new therapeutic target for liver fibrosis. *World J. Hepatol.* 7, 1982–1986. doi: 10.4254/wjh.v7.i16.1982

Marchesini, M. I., Herrmann, C. K., Salcedo, S. P., Gorvel, J. P., and Comerci, D. J. (2011). In search of *Brucella abortus* type IV secretion substrates: screening and identification of four proteins translocated into host cells through VirB system. *Cell. Microbiol.* 13, 1261–1274. doi: 10.1111/j.1462-5822.2011.01618.x

Matsuno, K., Ezaki, T., Kudo, S., and Uehara, Y. (1996). A life stage of particle-laden rat dendritic cells *in vivo*: their terminal division, active phagocytosis, and translocation from the liver to the draining lymph. *J. Exp. Med.* 183, 1865–1878. doi: 10.1084/jem.183.4.1865

McCord, A. M., Resto-Ruiz, S. I., and Anderson, B. E. (2006). Autocrine role for interleukin-8 in Bartonella henselae-induced angiogenesis. *Infect. Immun.* 74, 5185–5190. doi: 10.1128/IAI.00622-06

Moreno, E., and Moriyon, I. (2002). *Brucella melitensis*: a nasty bug with hidden credentials for virulence. *Proc. Natl. Acad. Sci. U.S.A.* 99, 1–3. doi: 10.1073/pnas.022622699

Mühlbauer, M., Fleck, M., Schütz, C., Weiss, T., Froh, M., Blank, C., et al. (2006). PD-L1 is induced in hepatocytes by viral infection and by interferon-alpha and -gamma and mediates T cell apoptosis. *J. Hepatol.* 45, 520–528. doi: 10.1016/j.jhep.2006.05.007

Nemeth, E., Baird, A. W., and O'Farrelly, C. (2009). Microanatomy of the liver immune system. *Semin. Immunopathol.* 31, 333–343. doi: 10.1007/s00281-009-0173-4

Ninio, S., and Roy, C. R. (2007). Effector proteins translocated by Legionella pneumophila: strength in numbers. *Trends Microbiol.* 15, 372–380. doi: 10.1016/j.tim.2007.06.006

O'Callaghan, D., Cazevieille, C., Allardet-Servent, A., Boschiroli, M. L., Bourg, G., Foulongne, V., et al. (1999). A homologue of the Agrobacterium tumefaciens VirB and Bordetella pertussis Ptl type IV secretion systems is essential for intracellular survival of Brucella suis. *Mol. Microbiol.* 33, 1210–1220. doi: 10.1046/j.1365-2958.1999.01569.x

O'Farrelly, C., and Crispe, I. N. (1999). Prometheus through the looking glass: reflections on the hepatic immune system. *Immunol. Today* 20, 394–398. doi: 10.1016/S0167-5699(99)01518-2

Olsen, S. C., and Palmer, M. V. (2014). Advancement of knowledge of Brucella over the past 50 years. *Vet. Pathol.* 51, 1076–1089. doi: 10.1177/0300985814540545

Pappas, G., Papadimitriou, P., Akritidis, N., Christou, L., and Tsianos, E. V. (2006). The new global map of human brucellosis. *Lancet Infect. Dis.* 6, 91–99. doi: 10.1016/S1473-3099(06)70382-6

Petrasek, J., Bala, S., Csak, T., Lippai, D., Kodys, K., Menashy, V., et al. (2012). IL-1 receptor antagonist ameliorates inflammasome-dependent alcoholic steatohepatitis in mice. *J. Clin. Invest.* 122, 3476–3489. doi: 10.1172/JCI60777

Prickett, T. C., McKenzie, J. L., and Hart, D. N. (1988). Characterization of interstitial dendritic cells in human liver. *Transplantation* 46, 754–761. doi: 10.1097/00007890-198811000-00024

Puche, J. E., Lee, Y. A., Jiao, J., Aloman, C., Fiel, M. I., Muñoz, U., et al. (2013). A novel murine model to deplete hepatic stellate cells uncovers their role in amplifying liver damage in mice. *Hepatology* 57, 339–350. doi: 10.1002/hep.26053

Robinson, M. W., Harmon, C., and O'Farrelly, C. (2016). Liver immunology and its role in inflammation and homeostasis. *Cell. Mol. Immunol.* 13, 267–276. doi: 10.1038/cmi.2016.3

Rolán, H. G., and Tsolis, R. M. (2008). Inactivation of the type IV secretion system reduces the Th1 polarization of the immune response to *Brucella abortus* infection. *Infect. Immun.* 76, 3207–3213. doi: 10.1128/IAI.00203-08

Roux, C. M., Rolán, H. G., Santos, R. L., Beremand, P. D., Thomas, T. L., Adams, L. G., et al. (2007). Brucella requires a functional Type IV secretion system to elicit innate immune responses in mice. *Cell. Microbiol.* 9, 1851–1869. doi: 10.1111/j.1462-5822.2007.00922.x

Sander, L. E., Sackett, S. D., Dierssen, U., Beraza, N., Linke, R. P., Müller, M., et al. (2010). Hepatic acute-phase proteins control innate immune responses during infection by promoting myeloid-derived suppressor cell function. *J. Exp. Med.* 207, 1453–1464. doi: 10.1084/jem.20091474

Santos, S. A., Andrade, D. R., and Andrade Júnior, D. R. (2005). Rat hepatocyte invasion by Listeria monocytogenes and analysis of TNF-alpha role in apoptosis. *Rev. Inst. Med. Trop. São Paulo* 47, 73–80. doi: 10.1590/S0036-46652005000200003

Sieira, R., Comerci, D. J., Sánchez, D. O., and Ugalde, R. A. (2000). A homologue of an operon required for DNA transfer in Agrobacterium is required in *Brucella abortus* for virulence and intracellular multiplication. *J. Bacteriol.* 182, 4849–4855. doi: 10.1128/JB.182.17.4849-4855.2000

Smith, K. (2013). Liver disease: kupffer cells regulate the progression of ALD and NAFLD. *Nat. Rev. Gastroenterol. Hepatol.* 10:503. doi: 10.1038/nrgastro.2013.140

Song, Y., Zhao, Y., Wang, F., Tao, L., Xiao, J., and Yang, C. (2014). Autophagy in hepatic fibrosis. *Biomed Res. Int.* 2014:436242. doi: 10.1155/2014/436242

Steffan, A. M., Gendrault, J. L., McCuskey, R. S., McCuskey, P. A., and Kirn, A. (1986). Phagocytosis, an unrecognized property of murine endothelial liver cells. *Hepatology* 6, 830–836. doi: 10.1002/hep.1840060505

Villar, J. M., Garrote, D., Villegas, M. T., Alvarez, M. J., Mansilla, A., and Ferrón, J. A. (2002). Hepatic brucelloma. *J. Am. Coll. Surg.* 194:86. doi: 10.1016/S1072-7515(01)01111-5

Xavier, M. N., Winter, M. G., Spees, A. M., Nguyen, K., Atluri, V. L., Silva, T. M., et al. (2013). CD4+ T cell-derived IL-10 promotes *Brucella abortus* persistence via modulation of macrophage function. *PLoS Pathog.* 9:e1003454. doi: 10.1371/journal.ppat.1003454

Xu, R., Zhang, Z., and Wang, F. S. (2012). Liver fibrosis: mechanisms of immune-mediated liver injury. *Cell. Mol. Immunol.* 9, 296–301. doi: 10.1038/cmi.2011.53

Yin, X. M., Ding, W. X., and Gao, W. (2008). Autophagy in the liver. *Hepatology* 47, 1773–1785. doi: 10.1002/hep.22146

Young, E. J. (1995). An overview of human brucellosis. *Clin. Infect. Dis.* 21, 283–9; quiz 90. doi: 10.1093/clinids/21.2.283

Zechner, E. L., Lang, S., and Schildbach, J. F. (2012). Assembly and mechanisms of bacterial type IV secretion machines. *Philos. Trans. R. Soc. Lond,. B,. Biol. Sci.* 367, 1073–1087. doi: 10.1098/rstb.2011.0207

Zimmermann, H. W., Bruns, T., Weston, C. J., Curbishley, S. M., Liaskou, E., Li, K. K., et al. (2015). Bidirectional transendothelial migration of monocytes across hepatic sinusoidal endothelium shapes monocyte differentiation and regulates the balance between immunity and tolerance in liver. *Hepatology* 63, 233–246. doi: 10.1002/hep.28285

Zwerdling, A., Delpino, M. V., Barrionuevo, P., Cassataro, J., Pasquevich, K. A., García Samartino, C., et al. (2008). Brucella lipoproteins mimic dendritic cell maturation induced by *Brucella abortus*. *Microbes Infect.* 10, 1346–1354. doi: 10.1016/j.micinf.2008.07.035

Zwerdling, A., Delpino, M. V., Pasquevich, K. A., Barrionuevo, P., Cassataro, J., García Samartino, C., et al. (2009). *Brucella abortus* activates human neutrophils. *Microbes Infect.* 11, 689–697. doi: 10.1016/j.micinf.2009.04.010

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: 21 January 2020
doi: 10.3389/fimmu.2019.03036

Brucella abortus Infection Elicited Hepatic Stellate Cell-Mediated Fibrosis Through Inflammasome-Dependent IL-1β Production

*Paula Constanza Arriola Benitez[1], Ayelén Ivana Pesce Viglietti[1], Marco Tulio R. Gomes[2], Sergio Costa Oliveira[2], Jorge Fabián Quarleri[3], Guillermo Hernán Giambartolomei[1] and María Victoria Delpino[1]**

[1] Instituto de Inmunología, Genética y Metabolismo (INIGEM), Universidad de Buenos Aires, CONICET, Buenos Aires, Argentina, [2] Department of Biochemistry and Immunology, Institute of Biological Sciences, Federal University of Minas Gerais, Belo Horizonte, Brazil, [3] Instituto de Investigaciones Biomédicas en Retrovirus y Sida (INBIRS), Universidad de Buenos Aires, CONICET, Buenos Aires, Argentina

OPEN ACCESS

Edited by:
Juarez Antonio Simões Quaresma, Instituto Evandro Chagas, Brazil

Reviewed by:
Leticia Cedillo-Barron, Center for Research and Advanced Studies (CINVESTAV), Mexico
Rebecca Leigh Schmidt, Upper Iowa University, United States

***Correspondence:**
María Victoria Delpino
mdelpino@ffyb.uba.ar

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

Received: 13 October 2019
Accepted: 11 December 2019
Published: 21 January 2020

Citation:
Arriola Benitez PC, Pesce Viglietti AI, Gomes MTR, Oliveira SC, Quarleri JF, Giambartolomei GH and Delpino MV (2020) Brucella abortus Infection Elicited Hepatic Stellate Cell-Mediated Fibrosis Through Inflammasome-Dependent IL-1β Production. Front. Immunol. 10:3036. doi: 10.3389/fimmu.2019.03036

In human brucellosis, the liver is frequently affected. *Brucella abortus* triggers a profibrotic response on hepatic stellate cells (HSCs) characterized by inhibition of MMP-9 with concomitant collagen deposition and TGF-β1 secretion through type 4 secretion system (T4SS). Taking into account that it has been reported that the inflammasome is necessary to induce a fibrotic phenotype in HSC, we hypothesized that *Brucella* infection might create a microenvironment that would promote inflammasome activation with concomitant profibrogenic phenotype in HSCs. *B. abortus* infection induces IL-1β secretion in HSCs in a T4SS-dependent manner. The expression of caspase-1 (Casp-1), absent in melanoma 2 (AIM2), Nod-like receptor (NLR) containing a pyrin domain 3 (NLRP3), and apoptosis-associated speck-like protein containing a CARD (ASC) was increased in *B. abortus*-infected HSC. When infection experiments were performed in the presence of glyburide, a compound that inhibits NLRP3 inflammasome, or A151, a specific AIM2 inhibitor, the secretion of IL-1β was significantly inhibited with respect to uninfected controls. The role of inflammasome activation in the induction of a fibrogenic phenotype in HSCs was determined by performing *B. abortus* infection experiments in the presence of the inhibitors Ac-YVAD-cmk and glyburide. Both inhibitors were able to reverse the effect of *B. abortus* infection on the fibrotic phenotype in HSCs. Finally, the role of inflammasome in fibrosis was corroborated *in vivo* by the reduction of fibrotic patches in liver from *B. abortus*-infected ASC, NLRP, AIM2, and cCasp-1/11 knock-out (KO) mice with respect to infected wild-type mice.

Keywords: *Brucella*, inflammasome, fibrosis, hepatic stellate cells, IL-1β

INTRODUCTION

Human brucellosis is a zoonosis that induces a chronic and debilitating disease caused by *Brucella* species that manifests itself with a broad clinical spectrum (1, 2). Liver involvement in human brucellosis is usually documented, given the well-characterized tropism of *Brucella* for the reticuloendothelial system (1, 2). The incidence of liver involvement in active brucellosis has ranged from 5 to 53% or more (2).

Inflammasome activation has been documented in several liver diseases. Accordingly, it has been postulated that the upregulation of IL-1β and IL-18 secretion leads to myofibroblast differentiation with concomitant increase of collagen and TGF-β expression (3). In addition, it was established that inflammasome components are present in hepatic stellate cells (HSCs) and could regulate their function (3). The consequences of activation of inflammasome pathway were also confirmed *in vivo*, demonstrating its key role in liver fibrosis (4).

The activation and release of IL-1β and IL-18 requires two distinct signals. TLR engagement by pathogen or endogenous signal induces the expression of the precursor forms of these cytokines (pro–IL-1β and pro–IL-18), after which NLR-dependent activation of caspase-1 regulates their proteolytic processing and release (5). Activation of inflammasome by *Brucella abortus* infection has been previously demonstrated in bone marrow-derived macrophages and dendritic cells (6, 7). In these cells, *B. abortus* induces the secretion of IL-1β, in a process in which NLRP3 is necessary for activation of ASC inflammasome and the concomitant activation of caspase-1 and maturation and secretion of IL-1β (6, 7). In addition, ASC inflammasomes are also essential for IL-1β secretion induced by *B. abortus* infection in astrocytes and microglia (8). The first signal can be triggered by various pathogen-associated molecular patterns (PAMPs) via TLR activation. In the case of *B. abortus* infection inflammasome activation, the second signal involved the presence of a functional type 4 secretion system (T4SS) and DNA-sensing inflammasome receptor AIM2, in bone marrow-derived macrophages, and Mal/TIRAP and TLR-2 are the main signaling involved in astrocytes and microglia (8).

Previously, we have demonstrated that upon infection of HSCs, *B. abortus* triggers a profibrotic response characterized by inhibition of MMP-9 secretion inducing concomitant collagen deposition and transforming growth factor (TGF)-β1 secretion in a way that involves a functional T4SS and its effectors protein BPE005 (9). Taking into account that inflammasome has been documented to be necessary to induce activation to a fibrotic phenotype of HSCs, we hypothesized that *Brucella* infection might create a microenvironment that would promote inflammasome activation and concomitant profibrogenic phenotype in HSCs. The results of the study are presented here.

MATERIALS AND METHODS

Bacterial Culture

Brucella abortus S2308 DsRed-expressing *B. abortus* S2308 or the isogenic *B. abortus virB10* polar mutants were grown overnight in 10 ml of tryptic soy broth (Merck, Buenos Aires, Argentina) with constant agitation at 37°C. Bacteria were harvested and the inocula were prepared as described previously (10).

To obtain heat-killed *B. abortus* (HKBA), bacteria were washed five times for 10 min each in sterile PBS, heat killed at 70°C for 20 min, aliquoted, and stored at −70°C until they were used. The total absence of *B. abortus* viability after heat killing was verified by the absence of bacterial growth on tryptose soy agar.

All live *Brucella* manipulations were performed in biosafety level 3 facilities located at the Instituto de Investigaciones Biomédicas en Retrovirus y SIDA (INBIRS).

Cell Culture

LX-2 cell line, a spontaneously immortalized human HSC line, was kindly provided by Dr. Scott L. Friedman (Mount Sinai School of Medicine, New York, NY). LX-2 cells were maintained in DMEM (Life Technologies–Invitrogen, Carlsbad, CA, USA) and supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2% (v/v) fetal bovine serum (FBS; Gibco–Invitrogen, Carlsbad, CA, USA). All cultures were grown at 37°C and 5% CO_2.

Cellular Infection

LX-2 cells were seeded in 24-well-plates and infected with *B. abortus* S2308, DsRed-expressing *B. abortus* S2308, or its isogenic mutants at multiplicities of infection (MOI) of 100 and 1000. After the bacterial suspension was dispensed, the plates were centrifuged for 10 min at 2,000 rpm and then incubated for 2 h at 37°C under a 5% CO_2 atmosphere. Cells were extensively washed with DMEM to remove extracellular bacteria and incubated in medium supplemented with 100 μg/ml gentamicin and 50 μg/ml streptomycin to kill extracellular bacteria. LX-2 cells were harvested at different times to determine cytokine production, MMP secretion, and collagen deposition.

Neutralization Experiments

Neutralization experiments were performed with 5 μM of Bay 11-7082, an inhibitory compound of the nuclear factor-κB (NF-κB), 50 μM of glybenclamide (glyburide), an inhibitor of the NLRP3 inflammasome, 50 μM of general caspase inhibitor Z-VAD-FMK, or 50 μM of caspase 1 inhibitor Y-VAD-FMK (all inhibitors were from Sigma-Aldrich). The cells were treated for 1 h with each inhibitor before infection.

AIM2 inflammasome complex formation was prevented using 3 μM of A151, a DNA sequence that inhibits in a competitive manner the immunostimulatory DNA. A151 (5-TTAGGGTTAG GGTTAGGGTTAGGG-3) and the control C151 (5-TTCAA ATTCAAATTCAAATTCAAA-3) constructs were synthesized with a phosphorothioate backbone. To determine the implication of IL-1β, neutralization experiments were performed by adding 50 ng/ml of ANAKINRA, the inhibitor of IL-1 receptor, and the natural antagonist IL-1Ra (R&D Systems). Recombinant human IL-1β (rIL-1β, R&D Systems) at a concentration of 50 ng/ml was used as a positive control.

mRNA Preparation and Quantitative PCR

RNA from LX-2 cells was isolated using the Quick-RNA MiniPrepKit (Zymo Research) and 1 μg of RNA was subjected to reverse transcription using Improm-II Reverse Transcriptase (Promega). PCR analysis was performed with StepOne real-time PCR detection system (Life Technology) using SYBR Green as fluorescent DNA binding dye. The primer sets used for amplification were as follows: β-actin sense: 5′-AACAGTCCGC CTAGAAGCAC-3′, β-actin antisense: 5′-CGTTGACATCCG TAAAGACC-3′; NLRP3 sense: 5′-CCACAAGATCGTGAGAA AACCC-3′; NLRP3 antisense: 5′-CGGTCCTATGTGCTC GTCA-3′; IL-1β sense: 5′-AGCTACGAATCTCCGACCAC-3′; IL-1β antisense: 5′-CGTTATCCCATGTGTCGAAGAA-3′; ASC sense: 5′-TGGATGCTCTGTACGGGAAG-3′; ASC antisense: 5′-CCAGGCTGGTGTGAAACTGAA-3′; Capase-1 sense: 5′-TTT CCGCAAGGTTCGATTTTCA-3′ Caspase-1 antisense: 5′-GGCATCTGCGCTCTACCATC-3′; AIM2 sense: 5′-TGGCAA AACGTCTTCAGGAGG-3′; AIM2 antisense: 5′-AGCTT GACTTAGTGGCTTTGG-3′.

The amplification cycle for Caspase-1, ASC, and β-actin was 95°C for 15 s, 58°C for 30 s, and 72°C for 60 s; the amplification cycle for IL-1β, NLRP3, and AIM2 was 95°C for 15 s, 61°C for 30 s, and 72°C for 60 s. All primer sets yielded a single product of the correct size. Relative expression levels were normalized against β-actin.

Immunofluorescence

LX-2 cells were infected with *B. abortus*, and after 24 h, cells were fixed in 4% paraformaldehyde for 10 min at room temperature, permeabilized with 0.3% Triton X-100 (Roche Diagnostics GmbH, Mannheim, Germany) for 10 min, and blocked with PBS containing 1% BSA for 1 h. Infected cells were stained with mouse anti-ASC (Santa Cruz Biotechnology) diluted in 0.1% PBS–Tween 20 overnight at 4°C. Cells then were incubated with rabbit anti-mouse Alexa Fluor 488 (Molecular Probes, Life Technologies) diluted in 0.1% PBS–Tween for 4 h at room temperature. 4,6-Diamidine-2-phenylindole (DAPI) was used for nuclear staining, and cells were stained for 30 min at room temperature. After washing in PBS, cells were mounted and then analyzed by fluorescence microscopy. Confocal images were analyzed using FV-1000 confocal microscope with an oil immersion Plan Apochromatic 60× NA1.42 objective (Olympus).

Zymography

Gelatinase activity was assayed by the method of Hibbs et al. with modifications, as described (11–13). Briefly, a total of 20 μl of cell culture supernatants from infected LX-2 cells cultured in the presence or not of the inhibitors Bay 11-7082, glyburide, Y-VAD-FMK, A151, control C151, and ANAKINRA at the concentrations mentioned above was mixed with 5 μl of 5× loading buffer [0.25 M Tris (pH 6.8), 50% glycerol, 5% SDS, and bromophenol blue crystals] and loaded onto 10% SDS-PAGE gels containing 1 mg/ml gelatin (Sigma-Aldrich, Buenos Aires, Argentina). After electrophoresis, gels were washed with a solution containing 50 mM Tris–HCl (pH 7.5) and 2.5% Triton X-100 (buffer A) for 30 min and with buffer A added with 5 mM $CaCl_2$ and 1 μM $ZnCl_2$ for 30 min and were later incubated with buffer A with an additional 10 mM $CaCl_2$ and 200 mM NaCl for 48 h at 37°C. This denaturation/renaturation step promotes MMP activity without the proteolytic cleavage of pro-MMP. Gelatin activity was visualized by the staining of the gels with 0.5% Coomassie blue. Unstained bands indicated the presence of gelatinase activity, and their positions in the gel indicate the molecular weights of the enzymes involved.

Measurement of Cytokine Concentrations

Secretion of TGF-β1 and IL-1β in the supernatants was quantified by ELISA (BD Biosciences).

Assessment of Collagen Deposition—Sirius Red Staining

Collagen deposition was quantified using Sirius red (Sigma-Aldrich, Argentina SA), a strong anionic dye that binds strongly to collagen molecules (14). Sirius red staining was performed as was described (15). Briefly, Sirius red was dissolved in saturated aqueous picric acid at a concentration of 0.1%. Bouin's fluid (for cell fixation) was prepared by mixing 15 ml saturated aqueous picric acid with 5 ml of 35% formaldehyde and 1 ml of glacial acetic acid. Cell layers were fixed with 1 ml of Bouin's fluid for 1 h. Afterwards, culture plates were washed three times with deionized water. Culture dishes were air dried before adding 1 ml of Sirius red dye reagent. Cells were stained for 18 h with mild shaking. The stained cell layers were extensively washed with 0.01 N hydrochloric acid to remove all unbound dye. The stained material was dissolved in 0.2 ml of 0.1 N sodium hydroxide by shaking for 30 min. The dye solution was transferred to microtiter plates, and OD was measured using a microplate reader (Thermo Scientific) at 550 nm against 0.1 N sodium hydroxide as a blank.

Lipoproteins and LPS

Brucella abortus lipidated outer membrane protein 19 (LOmp19) and unlipidated Omp19 (U-Omp19) were obtained as described (16). Both contained <0.25 endotoxin U/μg of protein as assessed by Limulus Amebocyte Lysates (Associates of Cape Cod). *B. abortus* S2308 LPS and *Escherichia coli* O111k58H2 LPS were provided by I. Moriyon. The synthetic lipohexapeptide (tripalmitoyl *S*-glyceryl-Cys-Ser- Lys4-OH [Pam3Cys]) was purchased from Boehringer Mannheim (Mannheim, Germany).

DNA From *B. abortus*

Brucella abortus DNA was purified using the kit Wizard® Genomic DNA (Promega) following the instructions of the manufacturer. *Brucella* DNA was measured spectrophotometrically. Transient transfections of LX-2 cells with 2 μg/ml of *B. abortus* DNA were carried out using Lipofectamine 2000 (Invitrogen), following the manufacturer's instructions. After the purification step, an aliquot of 100 μg of DNA was treated with DNase I (1 U/mg) DNA (Zymo Research) according to the instructions of the manufacturer.

Hepatic Fibrosis in a Mouse Model

Mouse strains used in this study included apoptosis-associated speck-like protein containing a CARD (ASC), Nod-like receptor (NLR) containing a pyrin domain 3 (NLRP3), absent in melanoma 2 (AIM2), and caspase-1 (Casp-1)/11 knock-out (KO) mice, as described previously (8), and C57BL/6 wild-type (WT) mice (provided by Federal University of Minas Gerais, Belo Horizonte, Brazil). Six- to eight-week-old mice were infected through the intraperitoneal route with 5×10^5 CFU of *B. abortus* S2308. Mice were born from breeding pairs that were housed under controlled temperature (22 ± 2°C) and artificial light under a 12-h cycle period. Mice were kept under specific pathogen-free conditions in positive-pressure cabinets and provided with sterile food and water *ad libitum*.

All animal procedures were performed according to the rules and standards for the use of laboratory animals of the National Institutes of Health. Animal experiments were approved by the Institutional Committee for the Care and Use of Laboratory Animals (CICUAL, permit number: 287/2015). Histological examination of liver was carried out at week 4 post-infection after routine fixation and paraffin embedding. Five-micrometer-thick sections were cut and stained with Masson's trichrome stain. Masson's trichrome staining was conducted according to the manufacturer's instructions (Sigma-Aldrich). Collagen-positive areas were visualized by light microscopy and quantified using Image Pro-Plus 6.0 software (Media Cybernetics, Inc.).

Statistical Analysis

Statistical analysis was performed with one-way ANOVA, followed by *post-hoc* Tukey test (a single-step multiple comparison statistical test that finds means that are significantly different from each other) using GraphPad Prism 4.0 software. Data were presented as mean ± SEM.

RESULTS

B. abortus Infection Induces IL-1β Secretion by LX-2 Cells via T4SS

It has been established that inflammasome components are present in HSCs and could regulate their function (3). To determine if *B. abortus* infection induces inflammasome activation, LX-2 cells were infected with *B. abortus* and the secretion of IL-1β was evaluated in culture supernatants by ELISA 24 h post-infection. *B. abortus* infection induces the secretion of IL-1β by LX-2 cells (**Figure 1A**). The T4SS encoded by *virB* genes was first involved in the capacity of *Brucella* to establish an intracellular replication niche in several cell types (17). In addition, this system has been involved in the induction of inflammatory response during *B. abortus* infection (18) and also in the inflammasome signaling activation (7). Therefore, experiments were conducted to determine if the T4SS could be involved in the secretion of IL-1β induced by *B. abortus* in LX-2 cells. To this end, LX-2 cells were infected with *B. abortus* and its

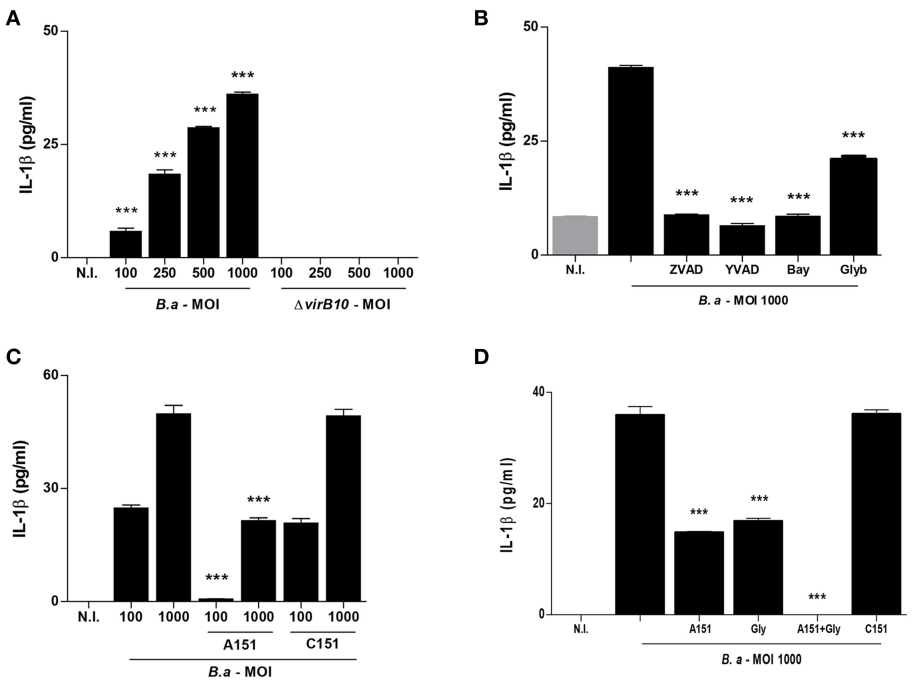


FIGURE 1 | *B. abortus* infection induces IL-1β in a VirB-dependent manner. LX-2 cells were infected with *B. abortus* (*B.a*) and its *vir*B10 isogenic mutant (Δ*vir*B10) at an MOI of 100–1000 and 24 h post-infection; IL-1β secretion was determined by ELISA in culture supernatants **(A)**. Effect of Z-VAD-FMK (ZVAD), Y-VAD-FMK (YVAD), Bay 11-7082 (Bay) and glybenclamide (Glyb) during *B. abortus* infection (MOI 1000) on IL-1β secretion **(B)**. Effect of A151 and the control 151 (C151) during *B. abortus* infection on IL-1β secretion **(C)**. Effect of Glyb plus A151 on IL-1βsecretion during *B. abortus* infection (MOI 1000). Data are given as the means ± SD from at least three individual experiments. ***$P < 0.001$ vs. cells infected with Δ*vir*B10 **(A)** or vs. infected and untreated cells **(B–D)**.

isogenic *B. abortus virB10* mutant, and IL-1β secretion induced by *B. abortus* was dependent on the expression of a functional T4SS, since the levels or IL-1β did not differ significantly between LX-2 cells infected with *B. abortus virB10* mutant and uninfected controls (**Figure 1A**). Taken together, our results indicated that *B. abortus* infection induces IL-1β secretion in a mechanism that is dependent on the presence of a functional T4SS.

IL-1β Secretion Induced by *B. abortus* Infection Is Dependent on Caspase-1 and NLRP3

Caspase-1 plays a fundamental role in innate immunity as the protease that activates the pro-inflammatory cytokines pro-IL-1β and pro-IL-18. Caspase-1 itself is activated in different inflammasome complexes; however, activation of the NLRP3 inflammasome has been frequently implicated in the development of fibrosis (19). To determine the role of caspase-1 and NLRP3 in IL-1β secretion by *B. abortus*-infected LX-2 cells, we performed the infection of LX-2 cells in the presence of specific pharmacological inhibitors. Inhibition of caspase-1 using the general caspase inhibitor Z-VAD-FMK or the specific caspase-1 inhibitor Ac-YVAD-cmk completely abrogated the secretion of IL-1β secretion induced by *B. abortus* infection of LX-2 cells (**Figure 1B**). When infection experiments were performed in the presence of Bay compound that inhibits NFκB, the secretion of IL-1β was significantly inhibited. However, glyburide, a compound that inhibits NLRP3, partially inhibits IL-1β secretion with respect to untreated cells (**Figure 1B**). Taken together, these results indicated that caspase-1 and NLRP3 are involved in the secretion of IL-1β by *B. abortus*-infected LX-2 cells.

IL-1β Secretion Induced by *B. abortus* Infection Is Also Dependent on AIM2

AIM2 inflammasome has been previously involved in the induction of IL-1β secretion in a T4SS-dependent manner during *B. abortus* infection of bone marrow derived macrophages and dendritic cells (6, 7). Since the inhibition of NLRP3 did not

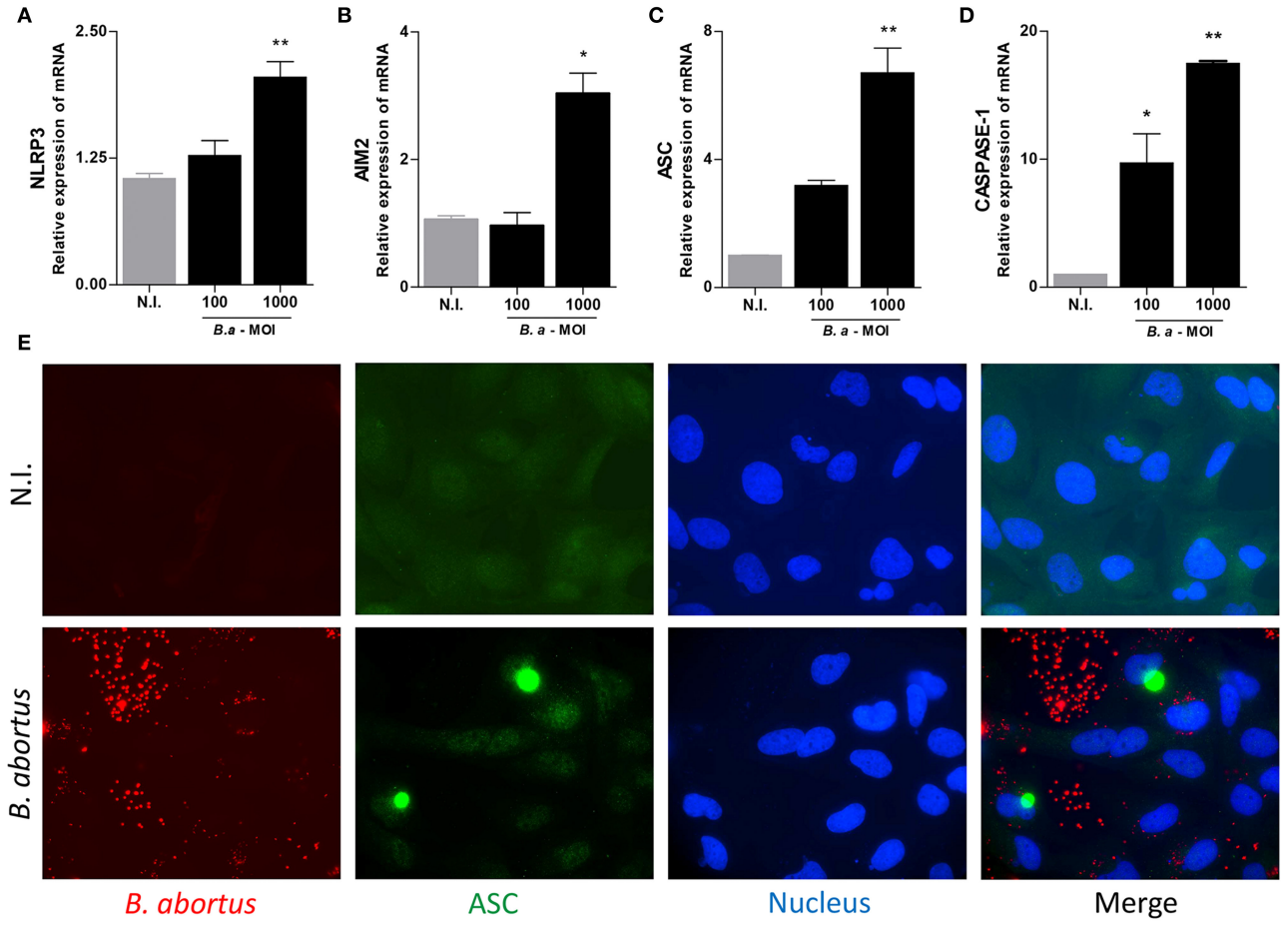


FIGURE 2 | *B. abortus* induces inflammasome in LX-2 cells. LX-2 cells were infected with *B. abortus* at MOI of 100 and 1000; at 24 h, post-infection levels of NLRP3 **(A)**, AIM2 **(B)**, ASC **(C)**, Caspase 1 **(D)**, and IL-1β **(E)** were determined by RT-qPCR. LX-2 cells were infected with Ds-Red *B. abortus* and ASC speck were revealed by immunofluorescence with a specific antibody labeled with Alexa 488. Nuclei were stained with DAPI. Data are given as the means ± SD from at least three individual experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. non-infected cells (N.I.).

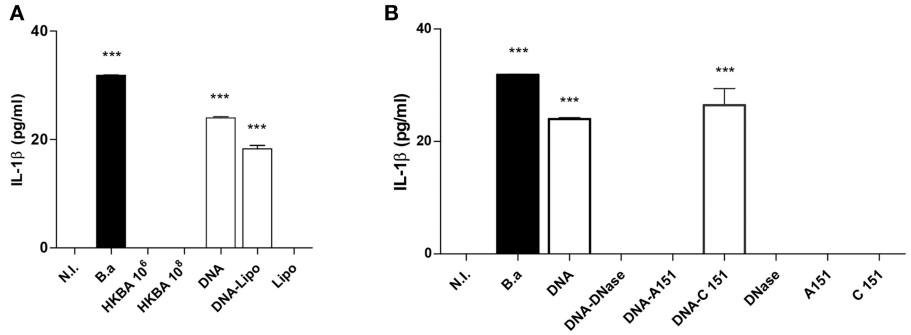


FIGURE 3 | DNA from *B. abortus* induces IL-1β. **(A)** LX-2 cells were infected with *B. abortus* (*B.a*) at MOI 1000 or treated with heat-killed *B. abortus* (HKBA) (1×10^6 and 1×10^8 bacteria/ml), 2 μg/ml of DNA, DNA and lipofectamine (DNA + Lipo), and lipofectamine alone as control (Lipo); IL-1β secretion was measured in culture supernatant after 24 h by ELISA. Determination of IL-1β in culture supernatants from LX-2 cells treated with DNA, DNA and A151, DNA and C151 as control, DNA treated with DNAse I, and DNAse I alone as control **(B)**. Data are given as the means ± SD from at least three individual experiments. ***$P < 0.001$ vs. non-infected cells (N.I.).

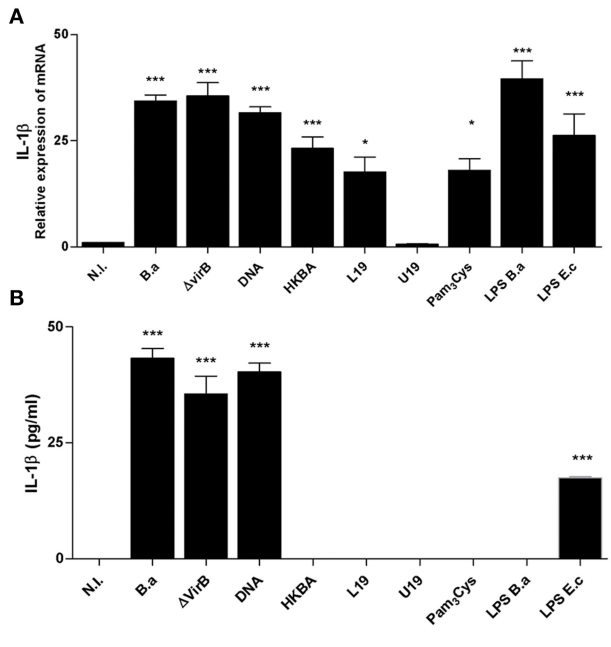


FIGURE 4 | L-Omp19 induces mRNA of IL-1β. **(A)** LX-2 cells were infected with *B. abortus* (*B.a*) and its *virB10* isogenic mutant (Δ*vir*B10) at MOI 1000, or incubated with DNA (2 μg/ml), heat-killed *B. abortus* (HKBA) (1×10^6 and 1×10^8 bacteria/ml), L-Omp19 (1,000 ng/ml), U-Omp19 (1,000 ng/ml), *B. abortus* LPS (1,000 ng/ml), Pam_3Cys (50 ng/ml), or *E. coli* LPS (100 ng/ml). Determination of IL-1β mRNA by RT-qPCR **(A)** and IL-1β secretion by ELISA **(B)**. Data are given as the means ± SD from at least three individual experiments. *$P < 0.05$; ***$P < 0.001$ vs. non-infected cells (N.I.).

completely abrogate the secretion of IL-1β in response to *B. abortus* infection, experiments were conducted to determine whether AIM2 inflammasome is also involved in caspase-1 activation. To this end, LX-2 cells were infected in the presence of A151, the oligodeoxinucleotide sequence that inhibits AIM2 or the sequence control C151. Our results indicated that AIM2 inflammasome contributes to IL-1β production by *B. abortus*-infected LX-2 cells, since the secretion of IL-1β was significantly inhibited when cells were treated with A151 with respect to untreated cells or cells treated with C151 (**Figure 1C**). When infection experiments were performed in the presence of A151 and glyburide administered in conjunction, the production of IL-1β was completely abrogated (**Figure 1D**). Taken together, these results indicated that NLRP3 and AIM2 inflammasomes are involved in the secretion of IL-1β by *B. abortus*-infected LX-2 cells.

B. abortus Infection Induces ASC, NLRP3, AIM2, and Caspase-1 mRNA Expression and ASC Speck Formation in LX-2 Cells

The basal AIM2 expression was sufficient to initiate inflammasome activation (20), but NLRP3 upregulation is necessary to initiate the activation of inflammasome (21, 22). Then, experiments were conducted to determine whether expression of inflammasome components could be upregulated during *B. abortus* infection. To this end, we determine the mRNA transcription of ASC, NLRP3, AIM2, and caspase-1 by RT-qPCR. Our results indicated that *B. abortus* infection induces an increase in ASC, NLRP3, AIM2, and caspase-1 mRNA transcription in LX-2 cells (**Figures 2A–D**). Most inflammasomes require oligomerization of ASC and thus the presence of ASC specks is a direct evidence of inflammasome activation. After infection with *B. abortus*, the formation of ASC specks was detected using specific antibodies by a fluorescence microscope. ASC specks were formed in *B. abortus*-infected LX-2 cells, but these structures were not detectable in non-infected cells. This indicates that *B. abortus* infection induces inflammasome assembly and consequently ASC speck formation (**Figure 2E**).

Brucella DNA Induces IL-1β Secretion in LX-2 Cells

IL-1β secretion was dependent on bacteria viability since stimulation of LX-2 cells with heat-killed *B. abortus* (HKBA) was unable to induce IL-1β (**Figure 3A**).

It has been recently demonstrated that *Brucella* DNA is involved in IL-1β secretion via activation of caspase-1 through the AIM2 inflammasome in macrophages and dendritic cells (6, 7). Therefore, we decided to test whether *Brucella* genomic DNA could be the putative ligand for AIM2 in the context of the inflammasome activation in LX-2 cells. To this end, *Brucella* DNA was transfected into LX-2 cells using lipofectamine or added to the culture medium to determine IL-1β secretion. *Brucella* DNA induced IL-1β secretion by LX-2 cells when it was added to the culture medium and also in transfected cells (**Figure 3A**). To determine if AIM2 inflammasome is involved in the secretion of IL-1β induced by *Brucella* DNA, experiments were performed in the presence of A151, the oligodeoxinucleotide that inhibits AIM2 or its oligodeoxinucleotide control C151. AIM2 is involved in the secretion of IL-1β induced by *Brucella* DNA, since A151 abrogated its secretion (**Figure 3B**). Additionally, DNase I treatment significantly reduced or abrogated *Brucella* DNA-induced IL-1β secretion (**Figure 3B**), demonstrating that bacterial DNA participates or is a major agonist that activates the inflammasome.

PAMPs Associated to Inflammasome Activation

For the production of IL-1β, PAMPs via TLRs and NLRs function in concert. PAMPs induce the expression of the precursor form of this cytokine (pro-IL-1β), and NLR-dependent CASP-1 activation induces its proteolytic processing and release (5). Hence, to assess the role of *Brucella* PAMPs, LX-2 cells were stimulated with HKBA and mRNA expression of IL-1β was determined by RT-qPCR. Our results indicated that HKBA was able to induce IL-1β expression at the mRNA level (**Figure 4A**) without IL-1β release, which demonstrated that viability is crucial for IL-1β protein production.

Previous observations indicated that LPS and lipoproteins from *B. abortus* are crucial for inflammatory responses induced by *B. abortus* in different models *in vivo* and *in vitro* (16, 23–26). Cells were then incubated with LPS from *B. abortus* and

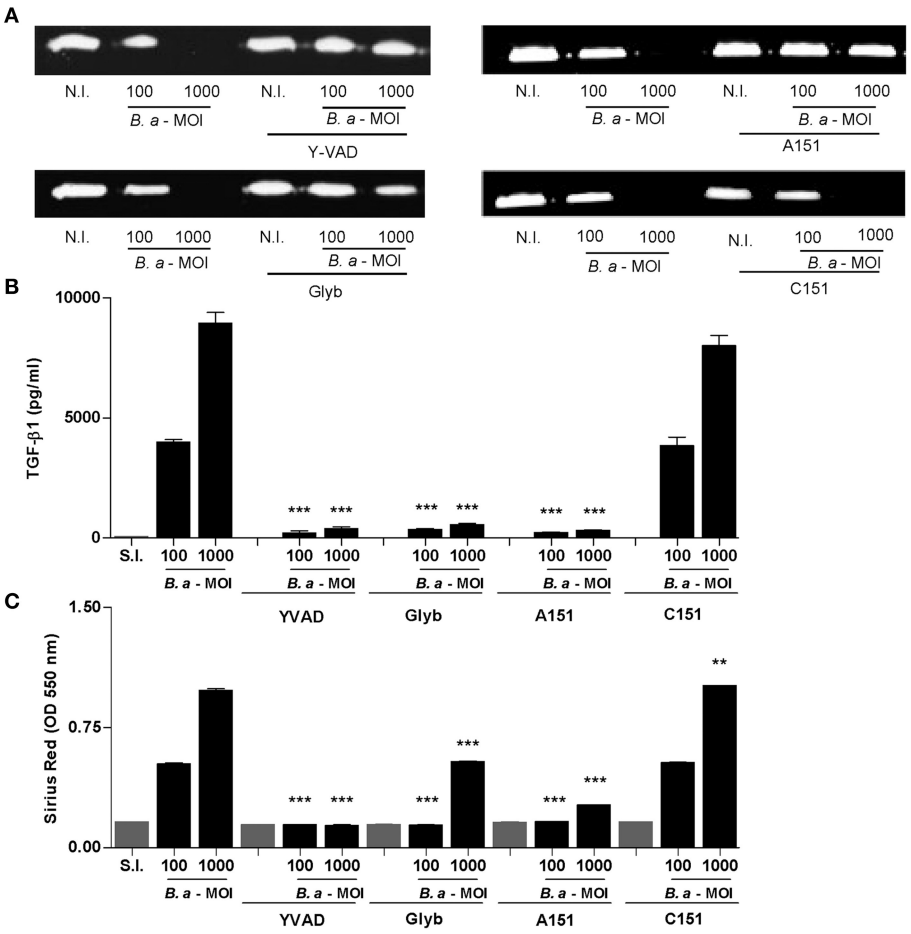


FIGURE 5 | Inflammasome is involved in profibrogienic response of LX-2 cells. LX-2 cells were infected with *B. abortus* at MOI of 100 and 1000 in the presence or not of Z-VAD-FMK (ZVAD), Y-VAD-FMK (YVAD), Bay 11-7082 (Bay), and glybenclamide (Glyb). At 24 h after infection, supernatants were harvested to analyze MMP-9 production by zymography **(A)** and TGF-β1 secretion by ELISA **(B)**. Quantification of collagen deposition was revealed by Sirius red staining by OD readings at 550 nm at 10 days post-infection **(C)**. Data are given as the means ± SD of duplicates. $^{**}P < 0.01$; $^{***}P < 0.001$ vs. non-infected cells (N.I.).

lipidated Omp19 (L-Omp19) as a *Brucella* lipoprotein model, and the expression of IL-1β mRNA was determined by RT-qPCR. Our results indicated that L-Omp19 and LPS induce an increase in IL-1β mRNA expression in LX-2 cells. IL-1β mRNA expression induced by Omp19 was dependent on the lipid moiety of the molecule because unlipidated Omp19 (U-Omp19) did not induce IL-1β mRNA expression. The requirement for lipidation was further supported by the fact that Pam_3Cys, a lipohexapeptide with an irrelevant peptide sequence, also induced the production of mRNA of IL-1β (**Figure 4A**). As expected, the presence of IL-1β at the protein level was not detected in supernatants of cultures of LX-2 cells treated with LPS or L-Omp19 (**Figure 4B**). Taken together, these results indicated that *B. abortus* lipoproteins and LPS induce mRNA of IL-1β in LX-2 cells.

The Inflammasome Pathway Is Involved in the Profibrogenic Response of LX-2 Cells Upon *B. abortus* Infection

It has been demonstrated that inflammasome activation has a variety of functional consequences for HSCs, including enhanced collagen 1 and TGF-β expression (4). Previously, we have demonstrated that upon infection of LX-2 cells, *B. abortus* inhibits MMP-9 secretion and induces concomitant collagen and TGF-β1 secretion (15). Therefore, experiments were conducted to determine the role of the inflammasome in the induction of a fibrogenic phenotype in LX-2 cells during *B. abortus* infection. To this end, the levels of secretion of MMP-9, TGF-β, and collagen deposition were determined in LX-2 cells infected with *B. abortus* in the presence of different inflammasome inhibitors. When infection experiments were performed in the presence of YVAD, glyburide, or A151, the effect of *B. abortus* infection on MMP-9 expression, collagen deposition, and TGF-β secretion on LX-2 cells was partially reversed with respect to untreated infected cells (**Figure 5**). These results indicated that NLRP3 and AIM2 inflammasome are involved in the induction of profibrogenic phenotype in LX-2 cells.

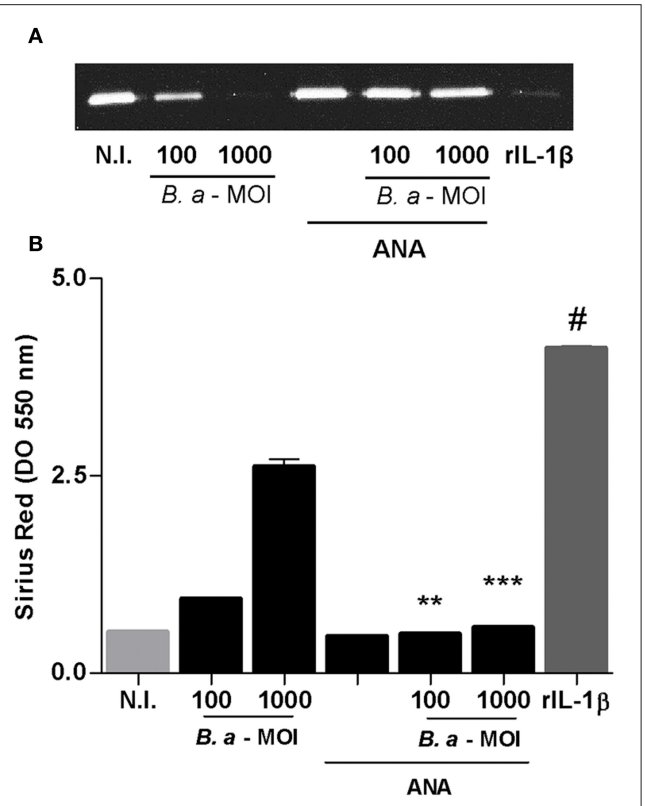


FIGURE 6 | IL-1β is involved in the induction of a profibrogenic phenotype. LX-2 cells were infected with *B. abortus* at MOI of 100 and 1000 in the presence or not of ANAKINRA (ANA). Determination of MMP-9 at 24 h post-infection in culture supernatants by zymography **(A)**. Recombinant human IL-1-β, (rIL-1β) (50 ng/ml) was used as a positive control. Quantification of collagen deposition was revealed by Sirius red staining by OD readings at 550 nm at 10 days post-infection **(B)**. Data are given as the means ± SD of duplicates. $^{**}P < 0.01$; $^{***}P < 0.001$ vs. untreated cells. $^{\#}P < 0.001$ vs. non-infected cells (N.I.).

IL-1β Is Involved in the Induction of a Profibrogenic Phenotype

Caspase-1 is required not only for IL-1β secretion but also for IL-18 secretion. To determine the role of IL-1β in the induction of a fibrotic phenotype, infection experiments were performed in the presence of the inhibitor of IL-1 receptor, the natural antagonist IL-1Ra (ANAKINRA). As shown in **Figure 6**, ANAKINRA abrogated the ability of *B. abortus* to inhibit MMP-9 and to induce collagen deposition. These results indicate that IL-1β could be a key cytokine during inflammasome activation involved in the fibrogenic phenotype triggered by *B. abortus* infection.

NLRP3 and AIM2 Influence Liver Fibrosis in Livers From *B. abortus*-Infected Mice

Finally, to verify the *in vivo* significance of our hypothesis, Casp-1, ASC, NLRP3, and AIM2 KO mice and WT mice, as control, were infected with *B. abortus*, and 4 weeks later, animals were sacrificed to determine the role of inflammasome in the liver fibrosis. Accordingly with our previous results, Masson's trichrome staining revealed the presence of fibrotic patch in livers from *B. abortus*-infected mice with respect to uninfected control (9, 15). In contrast, Casp-1, ASC, NLRP3, and AIM2 KO animals presented a significant reduction in the fibrotic patch (**Figure 7**). No fibrotic patch was observed in mice inoculated with saline (data not shown). These results indicated that inflammasomes NLRP2 and AIM2 play a key role in the modulation of fibrosis during *B. abortus* infection.

DISCUSSION

The liver is frequently affected in patients with active brucellosis, as revealed by the presence of histopathology lesions, such as granulomas, inflammatory infiltrates, and necrosis of liver parenchyma (27, 28).

Fibrosis is an intrinsic response to chronic persistent liver injury that results in a wound-healing process to mitigate the damage, but can also lead to scar formation. Inflammasome activation may play an important role in this process (19).

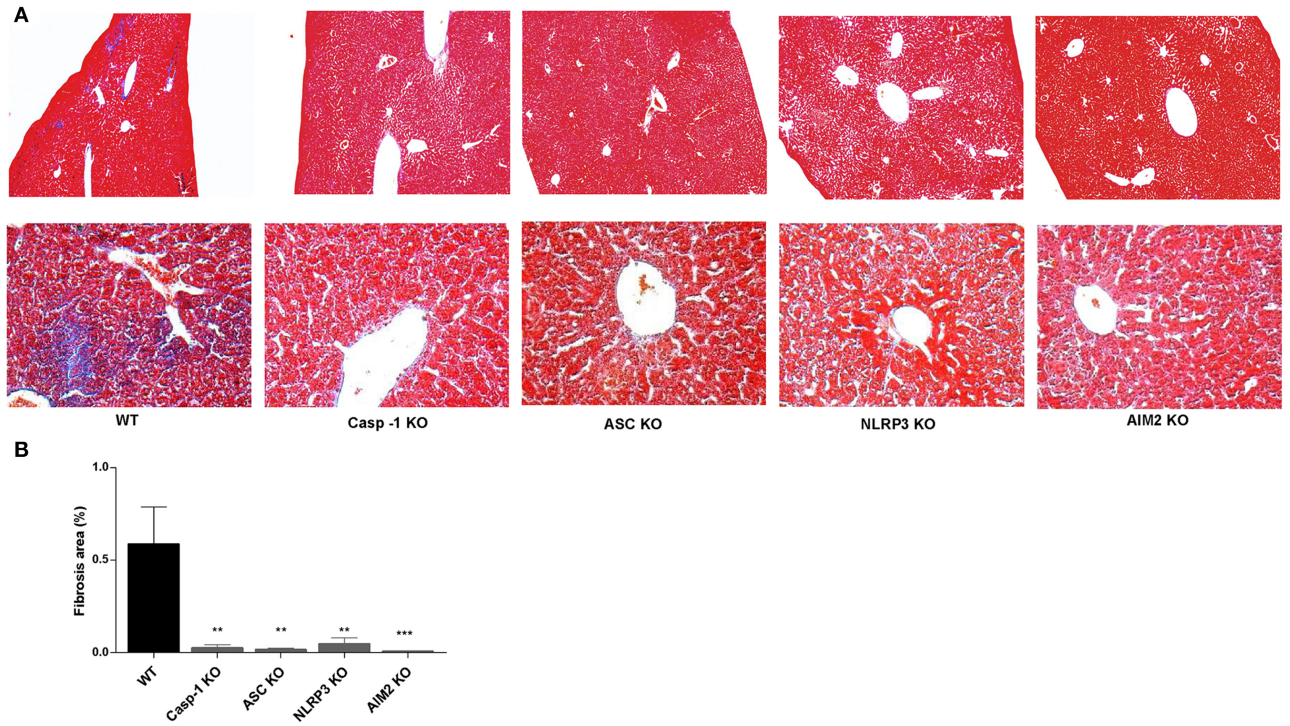


FIGURE 7 | NLRP3 and AIM2 influence liver fibrosis in livers from *B. abortus*-infected mice. Representative photomicrographs of liver sections from control mice, *B. abortus*-infected mice WT, Casp-1, ASC, NLRP3, and AM2 KO mice (n5) stained with Masson's trichrome staining. The top panels show images taken at the original magnification (×100), and the bottom panels show a detail of collagen patches (magnification, ×400) **(A)**. Collagen-positive areas were quantified using Image Pro-Plus 6.0 software **(B)**. Data are given as the means ± SD from at least three individual experiments. $^{**}P < 0.01$; $^{***}P < 0.001$ vs. wild type (WT).

In the present study, we demonstrated that *B. abortus* infection activates the inflammasome with concomitant secretion of IL-1β in HSC leading to upregulation of a profibrogenic phenotype.

Inflammasomes have emerged as critical signaling molecules of innate immune system involved in liver fibrosis. Inflammasomes are intracellular multiprotein complexes that act as regulators of inflammation and cell destiny. They respond to several danger signals by activating caspase-1 by the release of proinflammatory cytokines IL-1β and IL-18 (29). In particular, the NLRP3 inflammasome has been frequently implicated in the pathogenesis of chronic inflammatory liver diseases that causes liver fibrosis (30).

HSCs are the main cells involved in extracellular matrix deposition during liver fibrosis (31). In this process, the NLRP3 inflammasome has been involved in the functional changes in HSCs, including upregulation of the expression of collagen and TGF-β (4, 32), in findings that were confirmed by performing the knocking in NLRP3 (33).

Previous studies performed in dendritic cells, macrophages, and glial cells indicate that *Brucella* is sensed by ASC-dependent inflammasomes, mainly NLRP3 and AIM2, that induce caspase-1 activation with pro-inflammatory response (6, 8, 34, 35). Accordingly, in *Brucella*-infected HSCs, the secretion of IL-1β depends on NLRP3 and AIM2 inflammasomes. In this context, the participation of PAMPs in the activation of the inflammasome and the secretion of IL-1β must be discussed. The activation and release of IL-1β requires two distinct signals. The first signal can be triggered by various pathogen-associated molecular patterns via TLR activation, which induces the synthesis of pro–IL-1β. The second signal is provided by the activation of the inflammasome and caspase-1 leading to IL-1β processing. During *B. abortus* infection, the induction of IL-1β at mRNA level was independent of bacterial viability and induced at least by two structural bacterial components, including lipoproteins and LPS. However, the second signal requires bacterial viability and the presence of a functional T4SS and *B. abortus* DNA. The T4SS is encoded by *vir*B genes that play a main role in *Brucella* intracellular replication (17), and it has also been involved in the immune response to *Brucella* infection (7, 18). Bacteria utilize the T4SS to deliver effectors to eukaryotic cells. In LX-2 cells, *B. abortus vir*B10 mutant was unable to induce IL-1β secretion, and it could suggest that *Brucella* T4SS is involved in the transport of effector molecules that act via NLRP3 and/or AIM2 to activate inflammasomes. This is in agreement with our other finding in which we demonstrate that HKBA does not induce the secretion of IL-1β by LX-2 cells.

In vitro studies have shown that IL-1β promotes the proliferation and myofibroblast transdifferentiation of HSCs with substantial increased levels of their fibrogenic markers (36–38). Activated caspase-1 could also cleave pro-IL-18 into its active form IL-18, and this cytokine has also been involved in

fibrosis induction (38, 39). In a murine model of non-alcoholic steatohepatitis, fibrosis was not reversed by IL-1Ra treatment, indicating that other regulators of NLRP3 inflammasome are involved in fibrogenesis promotion (33). However, our experiments performed in *B. abortus*-infected HSCs in the presence of ANAKINRA, a version of the human interleukin-1 receptor antagonist, indicated that IL-1β secreted by HSCs has a main role in the induction of TGF-β with concomitant collagen deposition and inhibition of MMP-9 secretion. This role of IL-1β in the myofibroblast transdifferentiation with concomitant fibrosis is not exclusive for HSCs; accordingly, it has been previously described in endothelial and epithelial cell transdifferentiation and fibrosis (40–42).

These results and previous findings suggest that the interaction of *Brucella* with innate immunity *in vivo* may result in an increase of inflammatory response that results in liver fibrosis. Inflammasomes would dictate this fibrotic phenotype. Consequently, *B. abortus* infection induces fibrosis in mice that was reduced in mice lacking AIM2 or NLRP3.

Together, these results indicated that upon infection of HSCs, *B. abortus* triggers AIM2 and NLRP3 inflammasome activation with concomitant IL-1β secretion in a mechanism that is dependent on a functional T4SS and DNA. This IL-1β is implicated in the induction of fibrotic phenotype in HSC.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

This animal study was reviewed and approved by CICUAL-Facultad de Medicina, Universidad de Buenos Aires.

AUTHOR CONTRIBUTIONS

MD conceived and designed the experiments, supervised experiments, interpreted the data, and wrote the manuscript. PA, MG, and AP performed the experiments. PA analyzed the data and wrote sections of the manuscript. SO, JQ, and GG supported the work with key suggestions and helped with data interpretation. All authors reviewed the manuscript.

FUNDING

This work was supported by grants PICT 2014-1111 and PICT 2015-0316 from Agencia Nacional of Promoción Científica y Tecnológica (ANPCYT, Argentina) and by grant PIP 2015-2017-0200 from Consejo Nacional de Investigaciones científicas y técnicas (CONICET). Funding agencies had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

ACKNOWLEDGMENTS

We thank Horacio Salomón and the staff of the Instituto de Investigaciones Biomédicas en Retrovirus y Sida (INBIRS) for their assistance with biosafety level 3 laboratory uses. PA and AP are recipients of a fellowship from CONICET. JQ, GG, and MD are members of the Research Career of CONICET.

REFERENCES

1. Pappas G, Akritidis N, Bosilkovski M, Tsianos E. Brucellosis. *N Engl J Med*. (2005) 352:2325–36. doi: 10.1056/NEJMra050570
2. Madkour MM. Osteoarticular brucellosis. In: Madkour MM, editor. *Madkour's Brucellosis*. 2nd ed. Berlin: Springer-Verlag. (2001). p. 74–84. doi: 10.1007/978-3-642-59533-2_8
3. Alegre F, Pelegrin P, Feldstein AE. Inflammasomes in liver fibrosis. *Semin Liver Dis*. (2017) 37:119–27. doi: 10.1055/s-0037-1601350
4. Watanabe A, Sohail MA, Gomes DA, Hashmi A, Nagata J, Sutterwala FS, et al. Inflammasome-mediated regulation of hepatic stellate cells. *Am J Physiol Gastrointest Liver Physiol*. (2009) 296:G1248–57. doi: 10.1152/ajpgi.90223.2008
5. Miao EA, Andersen-Nissen E, Warren SE, Aderem A. TLR5 and Ipaf: dual sensors of bacterial flagellin in the innate immune system. *Semin Immunopathol*. (2007) 29:275–88. doi: 10.1007/s00281-007-0078-z
6. Costa Franco MMS, Marim FM, Alves-Silva J, Cerqueira D, Rungue M, Tavares IP, et al. AIM2 senses *Brucella abortus* DNA in dendritic cells to induce IL-1beta secretion, pyroptosis and resistance to bacterial infection in mice. *Microbes Infect*. (2018) 21:85–93. doi: 10.1016/j.micinf.2018.09.001
7. Gomes MT, Campos PC, Oliveira FS, Corsetti PP, Bortoluci KR, Cunha LD, et al. Critical role of ASC inflammasomes and bacterial type IV secretion system in caspase-1 activation and host innate resistance to *Brucella abortus* infection. *J Immunol*. (2013) 190:3629–38. doi: 10.4049/jimmunol.1202817
8. Miraglia MC, Costa Franco MM, Rodriguez AM, Bellozi PM, Ferrari CC, Farias MI, et al. Glial cell-elicited activation of brain microvasculature in response to *Brucella abortus* infection requires ASC inflammasome-dependent IL-1beta production. *J Immunol*. (2016) 196:3794–805. doi: 10.4049/jimmunol.1500908
9. Arriola Benitez PC, Rey Serantes D, Herrmann CK, Pesce Viglietti AI, Vanzulli S, Giambartolomei GH, et al. The effector protein BPE005 from *Brucella abortus* induces collagen deposition and matrix metalloproteinase 9 downmodulation via transforming growth factor beta1 in hepatic stellate cells. *Infect Immun*. (2016) 84:598–606. doi: 10.1128/IAI.01227-15
10. Scian R, Barrionuevo P, Rodriguez AM, Arriola Benitez PC, Garcia Samartino C, Fossati CA, et al. *Brucella abortus* invasion of synoviocytes inhibits apoptosis and induces bone resorption through RANKL expression. *Infect Immun*. (2013) 81:1940–51. doi: 10.1128/IAI.01366-12
11. Hibbs MS, Hasty KA, Seyer JM, Kang AH, Mainardi CL. Biochemical and immunological characterization of the secreted forms of human neutrophil gelatinase. *J Biol Chem*. (1985) 260:2493–500.
12. Scian R, Barrionuevo P, Giambartolomei GH, De Simone EA, Vanzulli SI, Fossati CA, et al. Potential role of fibroblast-like synoviocytes in joint damage induced by *Brucella abortus* infection through production and induction of matrix metalloproteinases. *Infect Immun*. (2011) 79:3619–32. doi: 10.1128/IAI.05408-11
13. Scian R, Barrionuevo P, Giambartolomei GH, Fossati CA, Baldi PC, Delpino MV. Granulocyte-macrophage colony-stimulating factor- and tumor necrosis factor alpha-mediated matrix metalloproteinase production by human osteoblasts and monocytes after infection with *Brucella abortus*. *Infect Immun*. (2011) 79:192–202. doi: 10.1128/IAI.00934-10
14. Tullberg-Reinert H, Jundt G. *In situ* measurement of collagen synthesis by human bone cells with a sirius red-based colorimetric microassay:

effects of transforming growth factor beta2 and ascorbic acid 2-phosphate. *Histochem Cell Biol*. (1999) 112:271–6. doi: 10.1007/s004180050447
15. Arriola Benitez PC, Scian R, Comerci DJ, Serantes DR, Vanzulli S, Fossati CA, et al. *Brucella abortus* induces collagen deposition and MMP-9 down-modulation in hepatic stellate cells via TGF-beta1 production. *Am J Pathol*. (2013) 183:1918–27. doi: 10.1016/j.ajpath.2013.08.006
16. Giambartolomei GH, Zwerdling A, Cassataro J, Bruno L, Fossati CA, Philipp MT. Lipoproteins, not lipopolysaccharide, are the key mediators of the proinflammatory response elicited by heat-killed *Brucella abortus*. *J Immunol*. (2004) 173:4635–42. doi: 10.4049/jimmunol.173.7.4635
17. Comerci DJ, Martinez-Lorenzo MJ, Sieira R, Gorvel JP, Ugalde RA. Essential role of the VirB machinery in the maturation of the *Brucella abortus*-containing vacuole. *Cell Microbiol*. (2001) 3:159–68. doi: 10.1046/j.1462-5822.2001.00102.x
18. Roux CM, Rolan HG, Santos RL, Beremand PD, Thomas TL, Adams LG, et al. *Brucella* requires a functional Type IV secretion system to elicit innate immune responses in mice. *Cell Microbiol*. (2007) 9:1851–69. doi: 10.1111/j.1462-5822.2007.00922.x
19. Szabo G, Csak T. Inflammasomes in liver diseases. *J Hepatol*. (2012) 57:642–54. doi: 10.1016/j.jhep.2012.03.035
20. Rathinam VA, Jiang Z, Waggoner SN, Sharma S, Cole LE, Waggoner L, et al. The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses. *Nat Immunol*. (2010) 11:395–402. doi: 10.1038/ni.1864
21. Franchi L, Eigenbrod T, Nunez G. Cutting edge: TNF-alpha mediates sensitization to ATP and silica via the NLRP3 inflammasome in the absence of microbial stimulation. *J Immunol*. (2009) 183:792–6. doi: 10.4049/jimmunol.0900173
22. Bauernfeind FG, Horvath G, Stutz A, Alnemri ES, MacDonald K, Speert D, et al. Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. *J Immunol*. (2009) 183:787–91. doi: 10.4049/jimmunol.0901363
23. Zwerdling A, Delpino MV, Barrionuevo P, Cassataro J, Pasquevich KA, Garcia Samartino C, et al. Brucella lipoproteins mimic dendritic cell maturation induced by *Brucella abortus*. *Microbes Infect*. (2008) 10:1346–54. doi: 10.1016/j.micinf.2008.07.035
24. Zwerdling A, Delpino MV, Pasquevich KA, Barrionuevo P, Cassataro J, Garcia Samartino C, et al. *Brucella abortus* activates human neutrophils. *Microbes Infect*. (2009) 11:689–97. doi: 10.1016/j.micinf.2009.04.010
25. Garcia Samartino C, Delpino MV, Pott Godoy C, Di Genaro MS, Pasquevich KA, Zwerdling A, et al. *Brucella abortus* induces the secretion of proinflammatory mediators from glial cells leading to astrocyte apoptosis. *Am J Pathol*. (2010) 176:1323–38. doi: 10.2353/ajpath.2010.090503
26. Oliveira SC, de Oliveira FS, Macedo GC, de Almeida LA, Carvalho NB. The role of innate immune receptors in the control of *Brucella abortus* infection: toll-like receptors and beyond. *Microbes Infect*. (2008) 10:1005–9. doi: 10.1016/j.micinf.2008.07.005
27. Akritidis N, Tzivras M, Delladetsima I, Stefanaki S, Moutsopoulos HM, Pappas G. The liver in brucellosis. *Clin Gastroenterol Hepatol*. (2007) 5:1109–12. doi: 10.1016/j.cgh.2006.08.010
28. Madkour MM. Gastrointestinal brucellosis. Madkour MM, editor. *Madkour's Brucellosis*. 2nd ed. Berlin: Springer-Verlag (2001). p. 150–8. doi: 10.1007/978-3-642-59533-2_13
29. Latz E, Xiao TS, Stutz A. Activation and regulation of the inflammasomes. *Nat Rev Immunol*. (2013) 13:397–411. doi: 10.1038/nri3452
30. Henao-Mejia J, Elinav E, Jin C, Hao L, Mehal WZ, Strowig T, et al. Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity. *Nature*. (2012) 482:179–85. doi: 10.1038/nature10809
31. Bataller R, Brenner DA. Liver fibrosis. *J Clin Invest*. (2005) 115:209–18. doi: 10.1172/JCI24282
32. Mederacke I, Hsu CC, Troeger JS, Huebener P, Mu X, Dapito DH, et al. Fate tracing reveals hepatic stellate cells as dominant contributors to liver fibrosis independent of its aetiology. *Nat Commun*. (2013) 4:2823. doi: 10.1038/ncomms3823
33. Wree A, McGeough MD, Pena CA, Schlattjan M, Li H, Inzaugarat ME, et al. NLRP3 inflammasome activation is required for fibrosis development in NAFLD. *J Mol Med*. (2014) 92:1069–82. doi: 10.1007/s00109-014-1170-1
34. Campos PC, Gomes MTR, Marinho FAV, Guimaraes ES, de Moura Lodi Cruz MGF, Oliveira SC. *Brucella abortus* nitric oxide metabolite regulates inflammasome activation and IL-1beta secretion in murine macrophages. *Eur J Immunol*. (2019) 49:1023–37. doi: 10.1002/eji.201848016
35. Costa Franco MM, Marim F, Guimaraes ES, Assis NRG, Cerqueira DM, Alves-Silva J, et al. *Brucella abortus* triggers a cGAS-independent STING pathway to induce host protection that involves guanylate-binding proteins and inflammasome activation. *J Immunol*. (2018) 200:607–22. doi: 10.4049/jimmunol.1700725
36. Yaping Z, Ying W, Luqin D, Ning T, Xuemei A, Xixian Y. Mechanism of interleukin-1beta-induced proliferation in rat hepatic stellate cells from different levels of signal transduction. *Apmis*. (2014) 122:392–8. doi: 10.1111/apm.12155
37. Reiter FP, Wimmer R, Wottke L, Artmann R, Nagel JM, Carranza MO, et al. Role of interleukin-1 and its antagonism of hepatic stellate cell proliferation and liver fibrosis in the Abcb4(-/-) mouse model. *World J Hepatol*. (2016) 8:401–10. doi: 10.4254/wjh.v8.i8.401
38. Artlett CM. Inflammasomes in wound healing and fibrosis. *J Pathol*. (2013) 229:157–67. doi: 10.1002/path.4116
39. Tanino A, Okura T, Nagao T, Kukida M, Pei Z, Enomoto D, et al. Interleukin-18 deficiency protects against renal interstitial fibrosis in aldosterone/salt-treated mice. *Clin Sci*. (2016) 130:1727–39. doi: 10.1042/CS20160183
40. Leaf IA, Nakagawa S, Johnson BG, Cha JJ, Mittelsteadt K, Guckian KM, et al. Pericyte MyD88 and IRAK4 control inflammatory and fibrotic responses to tissue injury. *J Clin Invest*. (2017) 127:321–34. doi: 10.1172/JCI87532
41. Fan JM, Huang XR, Ng YY, Nikolic-Paterson DJ, Mu W, Atkins RC, et al. Interleukin-1 induces tubular epithelial-myofibroblast transdifferentiation through a transforming growth factor-beta1-dependent mechanism in vitro. *Am J Kidney Dis*. (2001) 37:820–31. doi: 10.1016/S0272-6386(01)80132-3
42. Rieder F, Kessler SP, West GA, Bhilocha S, de la Motte C, Sadler TM, et al. Inflammation-induced endothelial-to-mesenchymal transition: a novel mechanism of intestinal fibrosis. *Am J Pathol*. (2011) 179:2660–73. doi: 10.1016/j.ajpath.2011.07.042

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: 18 February 2020
doi: 10.3389/fimmu.2020.00061

T Lymphocyte-Mediated Liver Immunopathology of Schistosomiasis

Bing Zheng[1,2], *Jianqiang Zhang*[1], *Hui Chen*[1], *Hao Nie*[1,2], *Heather Miller*[3], *Quan Gong*[1,2]* *and Chaohong Liu*[4]*

[1] Department of Immunology, School of Medicine, Yangtze University, Jingzhou, China, [2] Clinical Molecular Immunology Center, School of Medicine, Yangtze University, Jingzhou, China, [3] Department of Intracellular Pathogens, National Institute of Allergy and Infectious Diseases, Bethesda, MD, United States, [4] Department of Pathogen Biology, School of Basic Medicine, Tongji Medical College, Huazhong University of Science & Technology, Wuhan, China

OPEN ACCESS

Edited by:
Sergio C. Oliveira,
Federal University of Minas Gerais, Brazil

Reviewed by:
Sharvan Sehrawat,
Indian Institute of Science Education and Research Mohali, India
Rashika El Ridi,
Cairo University, Egypt
Miguel J. Stadecker,
Tufts University School of Medicine, United States

***Correspondence:**
Quan Gong
gongquan1998@163.com
Chaohong Liu
chaohongliu80@126.com

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

Received: 26 September 2019
Accepted: 10 January 2020
Published: 18 February 2020

Citation:
Zheng B, Zhang J, Chen H, Nie H, Miller H, Gong Q and Liu C (2020) T Lymphocyte-Mediated Liver Immunopathology of Schistosomiasis. Front. Immunol. 11:61. doi: 10.3389/fimmu.2020.00061

The parasitic worms, *Schistosoma mansoni* and *Schistosoma japonicum*, reside in the mesenteric veins, where they release eggs that induce a dramatic granulomatous response in the liver and intestines. Subsequently, infection may further develop into significant fibrosis and portal hypertension. Over the past several years, uncovering the mechanism of immunopathology in schistosomiasis has become a major research objective. It is known that T lymphocytes, especially $CD4^+$ T cells, are essential for immune responses against *Schistosoma* species. However, obtaining a clear understanding of how T lymphocytes regulate the pathological process is proving to be a daunting challenge. To date, $CD4^+$ T cell subsets have been classified into several distinct T helper (Th) phenotypes including Th1, Th2, Th17, T follicular helper cells (Tfh), Th9, and regulatory T cells (Tregs). In the case of schistosomiasis, the granulomatous inflammation and the chronic liver pathology are critically regulated by the Th1/Th2 responses. Animal studies suggest that there is a moderate Th1 response to parasite antigens during the acute stage, but then, egg-derived antigens induce a sustained and dominant Th2 response that mediates granuloma formation and liver fibrosis. In addition, the newly discovered Th17 cells also play a critical role in the hepatic immunopathology of schistosomiasis. Within the liver, Tregs are recruited to hepatic granulomas and exert an immunosuppressive role to limit the granulomatous inflammation and fibrosis. Moreover, recent studies have shown that Tfh and Th9 cells might also promote liver granulomas and fibrogenesis in the murine schistosomiasis. Thus, during infection, T-cell subsets undergo complicated cross-talk with antigen presenting cells that then defines their various roles in the local microenvironment for regulating the pathological progression of schistosomiasis. This current review summarizes a vast body of literature to elucidate the contribution of T lymphocytes and their associated cytokines in the immunopathology of schistosomiasis.

Keywords: T lymphocyte, schistosomiasis, immunopathology, liver fibrosis, soluble egg antigen

INTRODUCTION

Human schistosomiasis is a zoonotic parasitic disease caused by schistosomes, which are digenetic trematodes. In nature, many species of *Schistosoma* exist, but the main human pathogens are *Schistosoma japonicum*, *Schistosoma mansoni*, and *Schistosoma haematobium*. There is an estimate of more than 230 million people in tropical countries around the world infected with *Schistosoma* species (1). Infection occurs when cercariae, the free-living larval form of schistosomes, are released from freshwater snails and penetrate a human host's skin, where they may remain in the host epidermis for ∼72 h (2). Then, cercariae transform into schistosomula, the parasitic larvae form that enter the vasculature and travel via the pulmonary artery to the lungs, which they are then referred to as lung schistosomula (3). After exiting the lungs, schistosomula re-enter the venous circulation and finally migrate to the perivesicular venules (*S. haematobium*) or mesenteric venules (*S. japonicum*, *S. mansoni*), where they mature into worms and form copulating pairs. Paired adult worms migrate to the intestinal venous vasculature to release their eggs. Of the fertilized eggs released by the female, a portion is discharged via emunctory routes and hatch in freshwater, releasing free-living ciliated miracidia that invade snails and continue on the life cycle. However, a large portion of *S. haematobium* eggs may become trapped in the bladder and urogenital system, while the eggs of *S. japonicum* and *S. mansoni* become lodged in the intestinal wall and liver (4). The eggs then induce a local granulomas inflammatory response. The granulomas mainly consist of lymphocytes, macrophages, and eosinophils, which contain egg proteolytic enzymes to prevent tissue damage; however, the egg-induced granuloma also leads to chronic schistosomiasis. In this review, we will focus on *S. japonicum* and *S. mansoni*, both of which have been extensively studied and have also been used in mouse models to study liver immunopathology.

The basic pathological process of schistosomiasis includes acute and chronic phases of the disease. Acute schistosomiasis (sometimes referred to as Katayama fever) occurs most often in schistosome-endemic regions to naive individuals and to those who experience heavy reinfection. The major clinical manifestation of acute schistosomiasis includes a sudden onset of fever, fatigue, urticaria, eosinophilia, and abdominal tenderness. These symptoms occur weeks to months after schistosome infection because of worm maturation, egg production, and egg antigen-induced inflammatory granulomatous response (4, 5). Chronic schistosomiasis involves immune responses to the eggs within the liver and intestine, which causes formation of granuloma (6–9). Over time, the granulomatous response is gradually downregulated, leading to the progression of relatively tolerable chronic intestinal schistosomiasis. However, some patients will develop life-threatening hepatosplenic schistosomiasis accompanied by extensive hepatic and periportal fibrosis, portal hypertension, ascites, and gastrointestinal hemorrhage.

T lymphocytes are generally classified into $CD4^+$ T helper (Th) cells and $CD8^+$ cytotoxic T lymphocytes (CTLs). Th cells are important for host humoral and cellular immune responses against parasitic infections (6). The Th cells are further classified into several distinct Th phenotypes [Th1, Th2, Th17, follicular helper T cell (Tfh), Th9, and regulatory T cells (Tregs)] according to cytokine production and specialized functions. This review focuses on the role of various Th subsets and their associated cytokines in the immunopathogenesis of schistosomiasis.

THE ROLE OF Th1/Th2 RESPONSES IN THE IMMUNOPATHOLOGY OF SCHISTOSOMIASIS

An accumulation of evidence suggests that Th cells are involved in the immunopathogenesis of schistosomiasis. In athymic nude mice or mice without Th cells resulted in decreased granuloma size after infection by schistosomes (10, 11). The role of Th1/Th2 responses in schistosomiasis has been intensely investigated and reviewed (6, 9, 12). In the acute illness, parasite antigens elicit a moderate Th1 response, which is characterized by increased levels of proinflammatory cytokines, including tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), interleukin-1 (IL-1), and IL-6 (13). About 5–6 weeks post-infection, the schistosomula develop into mature worms. Female worms release fertilized eggs that stimulate the immune response via their soluble egg antigens (SEAs). SEA induces resident macrophages to secrete inflammatory cytokines and chemokines that stimulate the influx of lymphocytes, neutrophils, and monocytes, which initiates circumoval granulomatous inflammation (14). A clinical study comparing acutely and chronically infected patients showed that the level of TNF-α was elevated in the plasma of acutely infected patients and their peripheral blood mononuclear cells spontaneously secreted high levels of IL-1 and IL-6 and detectable levels of IFN-γ, while chronically infected patients produced little TNF-α or IFN-γ. Thus, higher levels of IFN-γ and proinflammatory cytokines in patients with acute illness show a dominant Th1 response (15). During the acute phase, the SEA-specifically activated $CD4^+$ Th cells release the Th1-type cytokines, IL-2 and IFN-γ, which mediate the establishment of early granulomas. Immunocytochemical examination *in situ* have confirmed the presence of Th1-type cytokine-producing cells within local microenvironments of the lesion in early granuloma formation (16). Although the granulomatous response is detrimental to the liver because of subsequent progression of hepatic fibrosis, the egg-induced granuloma is beneficial to the host. If the eggs are not sequestered effectively, continuously secreted egg antigens act as a stimulus and lead to uncontrolled inflammatory responses and permanent tissue damage. For instance, IL-4-deficient mice that cannot mount a normal Th2 response develop an unchecked Th1 response and die earlier than immunity intact mice when infected by *S. mansoni* (6). Similarly, in a Tamoxifen-induced IL-4 receptor α (IL-4Rα)-deficient mouse model, interrupting IL-4Rα-mediated signaling during the acute stage decreased protective Th2 responses, leading to severe disease and premature death (17). Therefore, moderate Th1 responses are involved in the acute schistosomiasis and early granuloma formation, whereas

excessive polarization toward the Th1 response is detrimental to the host. The Th1 response during the early stage of schistosomiasis is downregulated by IL-4 and IL-10. IL-10-/IL-4-deficient mice develop extremely polarized Th1-type cytokine IFN-γ responses that lead to 100% mortality during the acute illness (18).

The immune response and immunopathology of schistosomiasis are a consequence of $CD4^+$ T-cell sensitization to egg antigens. Some of the major components of egg are implicated in the Th response in schistosomiasis, including glycoprotein IPSE/α1, ω1 (19, 20), lacto-*N*-fucopentaose III (21), and *S. mansoni*-p40 (Sm-p40) (22), of which Sm-p40 is the most abundant egg component that can induce a strong Th1-polarized response (23). IPSE/α1 induces a mixed Th1/Th2 response and promote the development of hepatic granuloma (19), while lacto-*N*-fucopentaose III and ω1 directly act on dendritic cells (DCs) to enhance the Th2 response (21, 24). In addition, Th1-type responses could also be induced by the schistosome vaccine candidates MAP4 (25), egg-derived r38 (22, 26), rSmLy6B, rSmTSP6, and rSmTSP7 (27), and rSjCRT (28).

Compared to the Th1 response, the Th2 response exerts anti-inflammatory effects and regulates Th1-mediated immunopathology. SEA is considered to be the key factor in driving the dramatic transition from a Th1- to a Th2-dominated response. During the Th response transition, the interaction of CD40–CD40L, CD80/86–CD28, and B7-related protein 1-inducible costimulator (ICOS) are required for egg antigen-induced Th2 responses (29–31). In correlation with the Th2 transition, the cytokine profile is changed in that IFN-γ production is decreased, whereas Th2-type cytokines IL-4, IL-5, and IL-13 are increased (32). This has been shown in lymphocytes isolated from liver granulomas at 8 weeks, which secrete IL-4 and IL-5, but not IFN-γ. The mechanism underlying this switch to Th2 response has been deeply investigated. Lymphocytes from *S. mansoni* ova-infected signal transducer and activator of transcription (STAT6)-deficient mice produce minimal levels of Th2-type cytokines and enhanced IFN-γ production, which greatly reduces the size of pulmonary and hepatic granulomas (33). IL-4 signal is critical for the development of the Th2 response and animals treated with anti-IL-4 showed decreased Th2-type IL-4, IL-5, and IL-10 and increased Th1-type IL-2 and IFN-γ (34). IL-4 is recognized as the dominant cytokine for granuloma development and injection with neutralizing antibodies against IL-4 significantly suppresses splenic cell proliferation and hepatic granulomatous inflammation (35). Nevertheless, other studies showed that infected IL-4 knockout (KO) mice still have the ability to generate egg granulomas. Surprisingly, infected IL-4Rα-deficient mice exhibit only minimal hepatic granulomas and fibrosis, even though Th2-type cytokine production is similar to infected IL-4 KO mice, which demonstrates that the IL-4R signaling pathway, rather than IL-4, may be essential for egg granulomas (36). However, another Th2-type cytokine, IL-13, is known to be involved in granulomatous inflammation and fibrosis through the IL-4Rα signaling. Blocking IL-13 has been shown to be highly effective in treating an established and ongoing *S. mansoni* infection-induced fibrosis (37, 38). These results were further confirmed by studies using schistosome-infected IL-13 and IL-4-/IL-13-deficient mice (39). Furthermore, IL-13-deficient mice exhibit increased survival time, demonstrating the important role of IL-13 in the pathogenesis of schistosomiasis. *In vitro* studies revealed that IL-13 stimulates collagen production in fibroblasts and has a direct role in collagen homeostasis of normal human skin and keloid fibroblasts (40, 41). Overall, it is known that IL-4 and IL-13 play redundant roles in the schistosomiasis granulomatous response, but IL-13 is not dependent on profibrotic cytokines or affected by Th1/Th2 cytokines.

The source of Th2-type cytokines are not only produced by Th2 cells but also secreted by other innate lymphocytes, such as type 2 innate lymphoid cells (ILC2s). The three epithelial cell-derived cytokines, IL-33, IL-25, and thymic stromal lymphopoietin (TSLP), act as crucial initiators of Th2 responses to induce ILC2s to produce IL-13, therefore promoting Th2-type immunity (42–44). It has been reported that IL-33 treatment promotes a Th2 response together with increased liver immunopathology, and these effects are prevented by anti-IL-33 monoclonal antibodies. Furthermore, IL-33 is a requisite for IL-13-, but not IL-4, driven Th2 responses during the pathology (45). Subsequent studies have further confirmed that IL-33 is involved in initiating Th2 pathology after schistosome infection via regulating IL-13 expression in hepatic stellate cells (46) and inducing polarization of M2 macrophages (47). However, another study demonstrated an overlapping role of IL-33, IL-25, and TSLP in a schistosome-induced lung granuloma and liver fibrosis model. They showed that simultaneous disruption of IL-33, IL-25, and TSLP signaling inhibited the progression of Th2 cytokine-driven liver fibrosis but that individual disruption of each had no effect (48).

In addition to SEA-induced specific Th2 responses, parasite-derived cysteine peptidases are also responsible for Th2 responses. For instance, during acute schistosomiasis, injection of outbred mice with *S. mansoni* cathepsin B1 (SmCB1) or *S. mansoni* cathepsin L3 (SmCL3) develops a polarized Th2-type immune environment that is harmful for the development of *S. mansoni* larvae and leads to significant reduction in worm burden and liver egg counts (49). A similar study also showed the effectiveness of a cysteine-peptidase-based vaccine that protects hamsters from schistosomiasis *haematobium* by inducing Th2 immune responses (50). Therefore, the cysteine-peptidase-based vaccine has shown great potential to be further used in non-human primates, and even in humans, through inducing protective Th2 immune responses.

Although a strong Th2 response seems detrimental to the host, a Th2 response is indispensable for the survival of the host. Th2-deficient IL-$4^{-/-}$ mice are highly susceptible to infection and develop severe acute cachexia followed by death (51). Depletion of the Th2 response against the eggs results in tissue damage and increased host mortality and liver pathology due to proinflammatory Th1-type responses (52, 53). Clinical cases also demonstrate that more severe forms of hepatosplenic schistosomiasis is linked to low levels of Th2-type IL-5 and increased Th1-type IFN-γ and TNF-α (54). Therefore, Th2 immunity acts as a double-edged sword: on the one side, it protects the host to decrease the overall pathology and prevent

excessive granulomatous inflammation, but on the other side, it causes liver immunopathological damage. Thus, the Th2-response-mediated egg granuloma is a necessary evil for host survival. Therefore, maintaining the proper balance of Th1/Th2 responses is important to control the excessive pathology of schistosomiasis. Previous studies demonstrated that excessively polarized Th1- or Th2-type cytokine responses induce different but equally detrimental pathologies after infection (18). IL-4/IL-10 double-deficient mice develop highly polarized Th1-type cytokine responses, exhibited by rapid weight loss during egg production and 100% acute mortality by week 9 post-infection. In contrast, IL-12/IL-10 double-deficient mice with highly polarized Th2-type cytokine responses develop increased hepatic fibrosis and mortality during the chronic stages of infection (18). Both Th1 and Th2 phases are downregulated by endogenous IL-10, which is produced by macrophages and T cells (55). Thus, IL-10 acts as an important regulator to prevent excessive Th1 and Th2 responses during the development of schistosomiasis.

Th17/IL-17 EXACERBATE THE EGG-INDUCED LIVER IMMUNOPATHOLOGY

The first notion of Th17 cells playing a role in schistosomiasis egg-induced granuloma formation came from experiments using knockout mice unable to produce IL-23, which drives the production of IL-17 by Th17 cells. In these experiments, IL-12p40$^{-/-}$ mice, incapable of producing IL-12 or IL-23, were highly resistant to liver pathology, whereas IL-12p35$^{-/-}$ mice, able to produce IL-23 but not IL-12, developed severe granuloma lesions (56). Granuloma formation is associated with high levels of IL-17 and treatment with anti-IL-17 neutralizing antibodies significantly inhibited hepatic granulomatous inflammation (56). Therefore, the IL-17-producing CD4^{+} T-cell population driven by IL-23 was recognized as a separate lineage and designated as Th17 cells that contribute to severe immunopathology in schistosomiasis (56, 57).

The IL-23–IL-1–IL-17 axis plays an essential role in the development of severe forms of schistosomiasis (58). A study conducted on mice lacking the IL-23-specific subunit p19 revealed an impaired liver immunopathology together with a marked decrease in IL-17 in the granulomas, but not in the draining mesenteric lymph nodes (59). Subsequent studies conducted on high pathology-prone CBA mice and low pathology-prone C57BL/6 mice identified IL-23 and IL-1, derived from DCs stimulated by live schistosome eggs, as the critical host factors that drive IL-17 production (60, 61). *S. mansoni*-infected CBA mice possess more IL-17-producing cells in the spleen and granulomas when compared with C57BL/6 mice (62). It was later determined that egg antigens, but not adult worm antigens, preferentially induce the generation of Th17 cells. Lowering IL-17 levels by neutralizing anti-IL-17 antibodies can increase the parasite-specific antibody levels and supply partial protection against *S. japonicum* infection in mice (63). In addition, anti-IL-17 antibody markedly inhibited hepatic granulomatous inflammation and hepatocyte necrosis partly through reducing the proinflammatory cytokines/chemokines and infiltrating neutrophils (64). Similar results were obtained with *S. japonicum*-infected IL-17RA-deficient mice that displayed decreased granulomatous inflammation, hepatic fibrosis, improved liver function, and high survival (65). Rutitzky et al. investigated the role of IL-17 and IFN-γ in schistosomiasis immunopathology using mice lacking either one or both cytokines. They found that IL-17-deficient mice show significantly reduced immunopathology associated with the increased levels of IFN-γ, whereas IFN-γ-deficient mice displayed exacerbated immunopathology as well as increased levels of IL-17. Hence, IL-17 plays a powerful pathogenic role in severe immunopathology in murine schistosomiasis that normally is restrained by IFN-γ (66). It has also been reported that T-bet$^{-/-}$ mice have significantly increased egg-induced hepatic immunopathology, with the absence of IFN-γ and increased IL-23p19, IL-17, and TNF-α in granulomas. Thus, T-bet-dependent signaling negatively regulates Th17-mediated schistosomiasis immunopathology (67).

Recent reports found that ICOS is essential for pathogenic-induced Th17 cell development. This was discovered by Wang et al., who found that ICOSL KO mice had lower levels of Th17-associated cytokines (IL-17/IL-21), IL-13, and TGF-β1, which is correlated with improved survival rate, alleviated liver granulomatous inflammation, and hepatic fibrosis development (68). In addition, CD209a (C-type lectin receptor), expressed on DCs, also proved to be essential for the development of Th17 cell responses in murine schistosomiasis (61, 69). CD209a-deficient CBA mice had decreased Th17 responses and developed reduced egg-induced liver immunopathology (69, 70). Clinical studies also demonstrated the positive correlation between the percentage of Th17 cells and bladder pathology in *S. haematobium*-infected children (62).

The above findings demonstrate that Th17/IL-17 exacerbate the egg-induced liver immunopathology in schistosomiasis. However, it should be noted that a recent study found that acute schistosome infection induced a transient Th17 response to cathepsin B1 cysteine proteases secreted by the worms and that, this early, Th17 response may determine the pathogenic progression of the infection (71).

Although the role of Th17/IL-17 in schistosomiasis liver immunopathology has been defined, the source of IL-17 is not completely clear. Generally, IL-17-producing cells include Th17 cells, CTL cells, γδT cells, and natural killer T cells. Some researchers ignore the source of IL-17, and others found that Th17 cells are the major IL-17-producing cell population that contributes to pulmonary granuloma induced by *S. japonicum* (72). However, some studies showed that innate γδ T cells are the major IL-17-producing cells that contribute to the formation of granuloma in murine schistosomiasis (73, 74). The above discrepant findings may result from the different granuloma models. In addition, aforementioned ILC2s mediate Th2-type immunity through secreting IL-13. The ILC3s, as counterparts to Th17 cells, engage in Th17 immunity-mediated mucosal homeostasis and defense (75). However, the exact role of ILC3s has not been clarified yet in the pathology of schistosomiasis.

Thus far, numerous studies have promoted our understanding of the basic immunopathogenesis of schistosomiasis; however, it should be noted that schistosome-induced liver immunopathologies are associated with *Schistosoma* species and host. Examples of this include that the cellular composition of the *S. japonicum* egg-induced granulomas are mainly neutrophils, whereas *S. mansoni*-induced granulomas consist of a higher ratio of mononuclear cells and eosinophils, with lower numbers of neutrophils (76, 77). The differences between *S. japonicum*- and *S. mansoni*-induced hepatic granuloma could be attributed to the secreted specific leukocyte-associated chemokines at the site of inflammation (77). A host's genetic background also affects infection intensity and pathology of schistosomiasis (78, 79). Nevertheless, most researchers perform their studies using BALB/c and C57BL/6 strain mice. Actually, schistosome-infected BALB/c and C57BL/6 mice only develop mild hepatic granulomatous inflammation; however, CBA and C3H mice develop a severe pathology with larger size and poorly confined granulomas (80). As previously discussed, mouse strain-dependent schistosomiasis pathology may arise from the difference of Ag-specific Th responses (62), such as *S. mansoni*-infected CBA mice displaying exacerbated granulomatous lesions when compared to C57BL/6 mice because of high ratios of IL-17-producing cells in the granulomas (62).

Tfh PROMOTES LIVER PATHOLOGY OF SCHISTOSOMIASIS

Tfh cells are a specialized Th subset equipped to provide B cell help (81). Gene microarrays revealed that the transcriptional profile of Tfh cells is different from Th1, Th2, and Th17 cells (82). Tfh cells are mainly located in the periphery of B-cell follicles in secondary lymphoid organs and are identified by expression of various molecules, such as surface receptors CXCR5, programmed death 1 (PD-1), ICOS, the transcription factor B-cell lymphoma 6 (Bcl-6), and the cytokine IL-21 (83–85). Tfh cells were found to differentiate from Th2 cells in germinal centers responding to SEA (86). *S. japonicum* recombinant protein, SjGST-32, also has the ability to induce the formation of Tfh cells in BALB/c mice, which promotes humoral immune responses and long-lived memory B cells (87). Functional studies on Tfh cells showed that downregulation of their cellular development leads to immune deficiencies and that Tfh cells are closely associated with autoimmune and chronic inflammatory disease (85, 88). *S. mansoni* eggs induce differentiation of Tfh, which is highly dependent on Notch expression on T cells. Notch-deficient mice show impaired germinal center formation and decreased secretion of high-affinity antibodies (89). Tfh expansion and antibody production in response to schistosome infection are negatively regulated by B7-H1 (programmed death ligand 1) that is expressed on B cells (90). Immunization of B7-H1$^{-/-}$ mice with SEA leads to increased numbers of Tfh cells compared to wild-type mice (90). Recent studies have demonstrated that Tfh cells promote liver granulomas and fibrogenesis in mice infected with *S. japonicum* (91, 92). Wang et al. found that, in murine schistosomiasis, Tfh cells accumulate in the splenic germinal center and that the Tfh phenotypic molecule, Bcl-6, and the Tfh-type cytokine, IL-21, correlate with progression of liver fibrosis (92). In addition, clinical studies have shown that Tfh cells are involved in immune responses for both acute and chronic human schistosomiasis (93, 94). The frequency of circulating PD-1^{+}CXCR5^{+}CD4^{+} Tfh cells in the peripheral blood is increased in both acute and chronic schistosomiasis patients relative to healthy controls. However, the difference between acute and chronic schistosomiasis is that there is no correlation between percentages of PD-1^{+}CXCR5^{+}CD4^{+} Tfh cells, memory B cells, or the level of immunoglobulin G (IgG) specific to *S. japonicum* antigen in acute schistosomiasis patients; however, frequency of PD-1^{+}CXCR5^{+}CD4^{+} Tfh cells is positively correlated to the levels of IL-21 in sera and the levels of SEA-specific antibody in chronic schistosomiasis patients (93, 94). These findings demonstrate that Tfh cells might exhibit distinct mechanisms to regulate the immune response between acute and chronic schistosomiasis. In addition, IL-4-producing Tfh cells are presumed to be important for naturally acquired resistance to schistosome reinfection (95).

Th9 CELLS AND EGG-INDUCED HEPATIC GRANULOMATOUS AND FIBROSIS

Th9 cells are another unique subset of CD4^{+} T cells recently characterized by their production of cytokine IL-9 after activation. The specific transcription factors for Th9 cells include PU.1 and IRF-4 (96, 97). Before discovery of the Th9 cell, IL-9 was thought to be a Th2-specific cytokine. Until 2008, it was reported that IL-9 could be secreted exclusively by distinct IL-9^{+}IL-10^{+} Th cells lacking suppressive function (98). Th9 cells have been implicated in many diseases, such as allergic inflammation, autoimmune disorders, as well as helminth infections (96, 99, 100). Zhan et al. investigated the dynamics of splenic Th9 cells and IL-9 expression in liver and serum and found that the proportion of splenic Th9 cells and levels of IL-9 were significantly higher in *S. japonicum*-infected mice compared to uninfected controls. Moreover, dynamic changes of Th9, IL-9, and PU.1 levels were consistent with hepatic egg granulomatous inflammation (101). In agreement with these studies, Li et al. additionally showed that anti-IL-9 monoclonal antibody treatment significantly inhibits *S. japonicum*-induced hepatic granulomatous and fibrosing inflammation (102). The above findings indicate that Th9 cells may be involved in immunopathogenesis in murine schistosomiasis. However, a clinical investigation showed that the level of IL-9 in serum had no significant difference between acute and chronic schistosomiasis patients (103). Therefore, further studies are needed to clarify the defining roles of Th9 cells and IL-9 in the pathology of human schistosomiasis.

Tregs REGULATE THE GRANULOMATOUS INFLAMMATION AND FIBROSIS

In 1995, Sakaguchi et al. introduced regulatory T cells to the field of immunology (104). Tregs are a separate lineage of T cells that are responsible for maintaining immunological

homeostasis, suppressing potentially deleterious activities of Th cells, as well as mediating the magnitude of immunity against invading pathogens (105). It has been conceded that two main types of Tregs exist: one is termed "inducible" Tregs (iTregs), which responds to infectious challenge, and the other is termed "natural" Treg (nTregs), which is an endogenous Tregs (106). The forkhead box protein 3 (Foxp3) is a unique transcription factor that can be used to separate nTregs from iTregs that have similar regulatory properties (107). The nTregs develop from a normal process of maturation in the thymus and serve as an essential subset of T cells in the periphery. The specific markers for nTregs include CD25, the T-cell inhibitory co-receptor CTLA-4, and the glucocorticoid-inducible TNF receptor (108, 109). The iTregs originate from conventional $CD4^+$ T cells that are exposed to specific stimulatory factors, such as a cocktail of cytokines or drugs (110). Currently, the iTregs identified include IL-10-producing Tr1 cells, TGF-β-producing T helper type 3 (Th3) cells, and regulatory $CD4^+CD25^+Foxp3^-$ cells (111, 112). Both iTregs and nTregs exert suppressive/regulatory effects to restrict immune-mediated pathology.

So far, numerous studies have demonstrated that both nTregs and iTregs represent key players in the regulation of schistosomiasis pathology. During schistosome infection, Tregs suppress DC activation, mediate Th2 responses, and inhibit granuloma development and fibrosis. After infection with *S. mansoni*, the percentage of granuloma nTregs ($CD4^+$ $CD25^+$ $Foxp3^+$) has a significant increase at 8 and 16 weeks of the infection (113). Similarly, schistosome eggs show the ability to induce a significant $Foxp3^+$ Treg cell response, which suggests that SEA may be the most potent inducer for the generation of nTregs during infection (114). Except for SEA, schistosome-derived molecules, such as lysophosphatidylserine (lyso-PS), identified as the TLR2-activating molecule, can also actively induce the development of IL-10-producing Tregs (115). Although the percentage of Tregs are elevated either following infection or egg immunization, the natural ratio between nTregs and effector T cells is remarkably stable during the progress of the egg-induced inflammation, suggesting that the expansion of effector T cells maybe closely regulated by the nTregs response.

With the progression into the chronic stage of egg-induced inflammation, the phenotype of nTregs is changed so that the frequency of CD103-expressing nTregs is strongly increased. CD103 binds integrin β7 to form the complete integrin molecule αEβ7, which is an activation marker for nTregs. Thus, increasing the CD103-expressing nTregs is required for immunosuppression during chronic schistosomiasis (116). Although numerous studies have characterized nTregs during schistosome infection, the exact mechanism of how nTregs regulate immunopathology is not yet clear. To address the functional role of Tregs in schistosomiasis, many studies used the CD4 and CD25 sorting of Tregs; however, this makes it difficult to distinguish the function of either Tregs because $CD4^+CD25^+$ compartment includes both nTregs and iTregs. Nevertheless, Baumgart et al. established nTregs depleted mice to evaluate the role of nTregs in egg-induced inflammation. They found that both IFN-γ and IL-4 responses were increased following immunization, demonstrating that nTregs suppress both Th1 and Th2 responses (116), which is likely not associated with IL-10 (114).

In addition to nTregs, schistosomiasis infection also induces the production of iTregs (117, 118). It has been reported that IL-10, secreted from iTregs and Th2 cells, inhibits IL-12 production by CD40 agonist-stimulated DC and iTregs, thus suppressing development of egg-specific Th1 responses during schistosomiasis (118). Furthermore, IL-10-secreting iTregs isolated from the granuloma of chronically infected mice can inhibit $CD4^+$ T-cell proliferation (118), which is different from nTregs that do not appear to control the proliferation of T cells *in vivo* (116). Therefore, it is possible that both nTregs and iTregs have the ability to suppress both Th1 and Th2 responses. To investigate the importance of Tregs for controlling schistosomiasis liver pathology, adoptive transfer purified populations of CD25-depleted $CD4^+$ T cells into RAG-deficient mice (lack of mature T or B lymphocytes) lead to increased weight loss, liver damage, and mortality following infection, suggesting a strong capacity for Tregs to suppress liver pathology (117).

WHAT ABOUT THE ROLE OF $CD8^+$ T CELLS?

The role and mechanism of $CD8^+$ T cells in the process of schistosomiasis has been a somewhat neglected area of study. Early studies conducted in the pulmonary granuloma model demonstrated that $CD8^+$ T-cell deficiency increased granuloma formation by 70%, which was attributed to $CD8^+$ T-cell inhibition of Th2 maturation (119). However, a subsequent study found that granuloma formation and hepatic granulomatous reaction were unchanged in $CD8^+$ T-cell-deficient mice (120). Another study, using major histocompatibility complex (MHC) class II or I mutant mice to examine the role of $CD4^+$ and $CD8^+$ T cells in the pathology of schistosomiasis, found that schistosome-infected MHC I mutant mice developed normal granulomatous lesions, while in contrast, MHC II mutant mice failed to form egg granuloma (121). Therefore, $CD8^+$ T cells are not likely essential for regulating the immunopathology of schistosomiasis.

INNATE γδ T CELLS IN SCHISTOSOMIASIS

The previously discussed T lymphocyte, the αβ T cell, has TCR composed of two glycoprotein chains, α and β, while γδ T cells, another subset of lymphocytes, are $CD4^-CD8^-CD3^+$ T cells with TCR encoded by the γ and δ genes (122). Generally, γδ T cells consist of ∼5% of the circulating peripheral blood T cells, but in some infectious diseases, the enumeration of γδ T cells exceeds 30% of the peripheral blood T cells (123). During schistosomiasis, the response to schistosome antigen is primarily mediated by activated $CD4^+$ αβ T cells (33). However, it has also been reported that γδ T cells are recruited to egg-induced

granuloma in the murine schistosomiasis (124) and that the levels of γδ T cells are increased in schitosome-infected mice and patients (125, 126). However, an earlier study investigating the relative roles of αβ and γδ T cells in the immunopathology of schistosomiasis found that mutant mice lacking γδ T cells display vigorous formation of egg granulomas similar to normal mice, which demonstrates that granuloma formation is not dependent on γδT cells (127). In contrast with this study, another study identified two different γδ T cell subsets, including the Vγ1 γδ T cells that secrete IFN-γ only and the Vγ2 γδ T cells that secrete both IL-17A and IFN-γ (74). During *S. japonicum* infection, Vγ2 γδ T cells prevent hepatic fibrosis by recruiting neutrophils and secreting IL-17A (74). Thus, the role of innate γδ T cells in the pathology of schistosomiasis requires further investigation.

THE IMPACT OF OTHER IMMUNE CELLS ON CD4$^+$ T CELLS AND REGULATION ON IMMUNOPATHOLOGY OF SCHISTOSOMIASIS

Over the past four decades, immunoregulation has been deeply studied in the context of schistosomiasis. T-cell subsets are influenced by macrophages, B cells, DCs, and eosinophils, of which macrophages represent nearly 30% of the total granuloma cells. Macrophages, similar to Th1 and Th2 cells, can be classified into two major types, classically activated macrophages (CAM/M1) and alternatively activated macrophages (AAM/M2). CAM polarization, stimulated by inflammatory cytokines, such as IFN-γ, IL-12, and IL-18, serve a vital role in the response to intracellular pathogens like *Mycobacterium tuberculosis* (128), whereas AAM polarization is dependent on Th2-type cytokines, such as IL-4 and IL-13, that induce the expression of arginase-1 (Arg-1), Ym-1, and Fizzl, which is mainly involved in allergic, cellular, and humoral responses to parasites (129). Egg-induced inflammation and liver fibrosis promotes the Th2 response, which in turn increases AAM polarization in granulomas (130). In several models of Th1-polarized mice, such as IL-4/IL-10 deficient, IL-4/IL-13 deficient, and egg/IL-12-immunized or macrophage-specific IL-4 deficient (131, 132), schistosome infection failed to induce the expression of Arg-1, demonstrating that Th2 cytokines are necessary for the development of Arg-1-expressing AAM. Moreover, Th1-polarized mice showed an increased inducible nitric oxide synthase response together with smaller granulomas and elevated mortality (131, 132). Thus, CAM polarization is associated with Th1 responses and Arg-1-expressing AAM is associated with Th2 responses and liver fibrosis progression.

The function of B cells has also been intensely investigated in schistosomiasis. Several studies have demonstrated that the B-cell number in the lymph nodes and spleen significantly increase during the schistosome infection, suggesting that B cells are important for host responses against schistosome infection (133, 134). Mice immunized with radiation-attenuated cercariae of *S. mansoni* showed reduced protection against challenge infection in B-cell knockout mice compared to wild-type mice (135). IgE antibody against the worms, but not the eggs, has been closely associated with resistance to reinfection, and eosinophil-mediated antibody-dependent cellular cytotoxicity is the main immune mechanism to kill early schistosome larvae (136). Evidence shows that SEA-specific IgG1 is the main isotype released from the early granulomas and that, during the chronic infection stage, granulomas secrete a mixture of IgG1, IgG2, IgG3, and IgA (137). The antibodies produced within intragranulomas mainly function to downregulate the granuloma formation, and it has been found that immune complexes from chronic schistosomiasis patients can inhibit granuloma formation *in vitro* (138). In addition, research has been done on the regulatory role of B cells in schistosomiasis immunopathology and T-cell effector functions. A previous study has proven that B cells are important in promoting a strong Th2-type response to helminths (139). After SEA stimulation, mesenteric lymph cells from B-cell-deficient mice produce more Th1-type cytokines and less Th2-type cytokines compared to wild-type mice (140, 141). A recent study has shown that the absence of IL-4α signaling in B cells leads to increased mortality and pathology in *S. mansoni*-infected mice, which is attributed to decreased Th2 responses (142). These findings demonstrate that B cells are critical in directing and mediating Th cells toward Th2 responses. In addition, in a study about *S. mansoni*-mediated protection against experimental ovalbumin-induced allergic airway inflammation, the investigators found that splenic marginal zone CD1d$^+$ B cells from schistosome-infected mice had the ability to secret IL-10 and induce generation of FoxP3$^+$ Treg cells. Although B cells are essential for Th2 responses and Treg cell generation, B cells are not responsible for granuloma formation (140). However, a contrasting study showed that B-cell deficiency had no effect on Th2 responses but augmented tissue pathology (143). Therefore, further study is needed to clarify the role of B cells in immunopathology of schistosomiasis.

DCs are important antigen-presenting cells that have superior activity to stimulate naive T-cell activation and mediate the polarization of CD4$^+$ T cells in response to invading pathogens. DCs determine Th differentiation through secreted polarizing cytokines. For example, *M. tuberculosis* infection induces DCs to produce IL-12 to elicit a strong IFN-γ-producing Th1 response, which leads to macrophage activation and killing of intracellular bacteria (144). Depletion of DCs in CD11c-diphtheria toxin receptor mice demonstrated that DCs are required to initiate Th2 responses in the *S. mansoni* infection (145). However, DC subsets, such as conventional CD11c$^+$ DCs and some specialized DCs do not produce IL-4, which is important for Th2 polarization. Ma et al. uncovered a novel subset of CD11c$^+$CD49b$^+$FcεRI$^+$ DCs that can produce IL-4 and subsequently promote Th2 differentiation in an IL-4-dependent manner (146). However, contradictory findings revealed that conventional DCs are critical subsets for Th2 effector cell development during acute *S. mansoni* infection (147). In fact, more researchers support the notion that DCs do not need to produce IL-4 to promote Th2 development (148–150) because IL-4-deficient DCs still show

the ability to induce excellent Th2 responses (148). In addition, a recent study revealed an unrecognized role of type I IFN in the Th2 response, whereby IFN-I signaling is implicated in not only activating DCs but also enhancing DCs effective migration and antigen presentation during the *S. mansoni*-induced Th2 response (151). Thus, the exact mechanism by which DCs induce Th2 responses needs to be further clarified.

SUMMARY AND CONCLUDING COMMENTS

Schistosomiasis is a disease with profound impact on human health. At least 230 million people worldwide are affected by the parasitic disease (1). In the near future, we may face a situation where there is no available drug to treat schistosomiasis, as praziquantel is still the only effective drug being used to treat the disease and praziquantel resistance has been reported in endemic areas and in the laboratory (152, 153). Therefore, it is urgent to develop some alternative drugs to control schistosomiasis, including the use of vaccines (154–156). Schistosome infection can lead to acute febrile illness and chronic life-threatening hepatosplenic disease. As summarized in **Figure 1**, during the progression of the disease, Th cells are activated and differentiated into distinct effector subsets, including Th1, Th2, Th17, Tfh, Th9, and Treg cells. Acute schistosomiasis is recognized as a Th1-dominated disease. Recent studies showed that Th17 and Tfh are also involved in the immune response of acute cases (71, 94). Chronic disease of hepatic granuloma formation and fibrosis are upregulated by Th2 and Th17 cells, mainly secreting IL-4 and IL-17, respectively (33, 63), and downregulated by Th1 and Treg cells (80, 113). The plasticity of Th cells are affected by the local microenvironment (e.g., cytokines and antigens of schistosomes) and regulated by various immune cells (e.g., macrophages, B cells, and DCs) through a complicated interacting network. Although we have acquired sufficient knowledge about the immunopathology of schistosomiasis, the effective strategies to restrain the development of granulomas and subsequent fibrosis are still lacking. A vaccine designed to skew the immune response toward the Th1 phenotype would be useful to prevent the development of fibrosis (80). However, murine studies have shown that extreme immune deviation toward either Th1 or Th2 results in increased pathology and premature death (18, 157). Most importantly, maintaining the balance of various effector T cells would be critical to prevent excessive pathology. While many researchers are focused on the Tregs-associated suppression and the powerful force of IL-10, successful immunotherapies will only be developed for schistosomiasis if we have a broader view

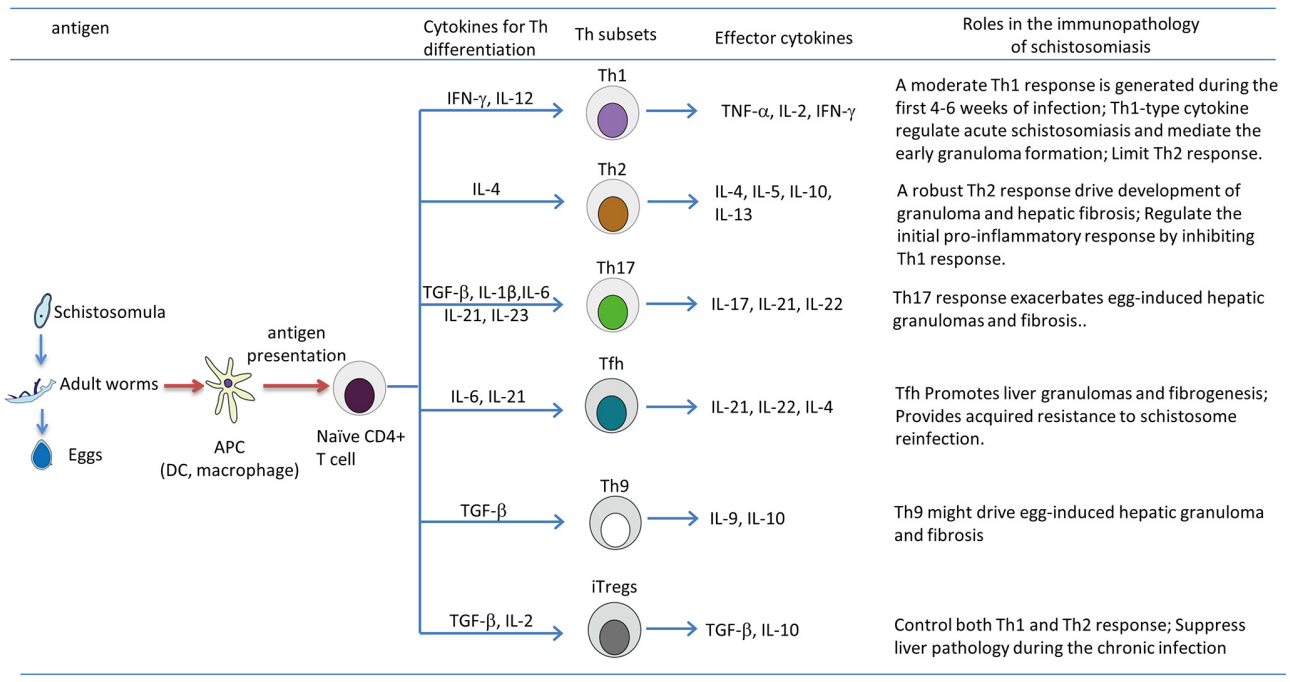


FIGURE 1 | T helper (Th) subsets differentiation and their roles in immunopathology of the murine schistosomiasis. During the progression of schistosomiasis, the schistosome antigen from schistosomula, adult worms, or eggs are captured and processed by antigen-presenting cells, such as dendritic cells (DCs) and macrophages; this leads to the production of various cytokines, and processed antigens are presented to naive $CD4^+$ T cell in the form of major histocompatibility complex (MHC) class II-peptide complexes, then activated $CD4^+$ T cells differentiate into Th1, Th2, Th17, Tfh, Th9, and Tregs, which are strictly regulated by the local microenvironment (e.g., cytokines and antigens of schistosomes) and cross-talk with various immune cells (e.g., macrophages, B cells, and DCs) through a complicated interacting network. It is generally thought that Th2, Th17 responses and Tfh, Th9 cells positively drive the development of pathology of chronic schistosomiasis, whereas the Th1 response counteracts Th2-mediated chronic pathology, and Tregs exert a suppressive effect on the immunopathology of schistosomiasis by inhibiting both Th1 and Th2 responses.

and deeper understanding of the mechanism of T-cell-mediated liver immunopathology.

AUTHOR CONTRIBUTIONS

CL organized the article. BZ wrote the draft. JZ, HC, HN and HM participated in the conception, discussion and revision of the draft. QG edited the language and figure.

FUNDING

This work was supported by National Natural Science Foundation of China (81861138002, 81722002, and 81271872), Joint Foundation of the Health Commission of Hubei Province (WJ2018H173), and Central Funds Guiding the Local Science and Technology Development of Hubei Province (2019ZYYD066).

REFERENCES

1. Vos T, Flaxman AD, Naghavi M, Lozano R, Michaud C, Ezzati M, et al. Years lived with disability (YLDs) for 1160 sequelae of 289 diseases and injuries 1990–2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet.* (2012) 380:2163–96. doi: 10.1016/S0140-6736(12)61729-2
2. He Y-X, Chen L, Ramaswamy K. *Schistosoma mansoni*, *S. haematobium*, and *S. japonicum*: early events associated with penetration and migration of schistosomula through human skin. *Exp Parasitol.* (2002) 102:99–108. doi: 10.1016/S0014-4894(03)00024-9
3. Gobert GN, Chai M, McManus DP. Biology of the schistosome lung-stage schistosomulum. *Parasitology.* (2007) 134:453–60. doi: 10.1017/S0031182006001648
4. Colley DG, Bustinduy AL, Secor WE, King CH. Human schistosomiasis. *Lancet.* (2014) 383:2253–64. doi: 10.1016/S0140-6736(13)61949-2
5. Ross AG, Vickers D, Olds GR, Shah SM, McManus DP. Katayama syndrome. *Lancet Infect Dis.* (2007) 7:218–24. doi: 10.1016/S1473-3099(07)70053-1
6. Pearce EJ, MacDonald AS. The immunobiology of schistosomiasis. *Nat Rev Immunol.* (2002) 2:499–511. doi: 10.1038/nri843
7. Wilson MS, Mentink-Kane MM, Pesce JT, Ramalingam TR, Thompson R, Wynn TA. Immunopathology of schistosomiasis. *Immunol Cell Biol.* (2007) 85:148–54. doi: 10.1038/sj.icb.7100014
8. Burke ML, Jones MK, Gobert GN, Li YS, Ellis MK, McManus DP. Immunopathogenesis of human schistosomiasis. *Parasite Immunol.* (2009) 31:163–76. doi: 10.1111/j.1365-3024.2009.01098.x
9. Lundy SK, Lukacs NW. Chronic schistosome infection leads to modulation of granuloma formation and systemic immune suppression. *Front Immunol.* (2013) 4:39. doi: 10.3389/fimmu.2013.00039
10. Amsden AF, Boros DL, Hood AT. Etiology of the liver granulomatous response in *Schistosoma mansoni*-infected athymic nude mice. *Infect Immun.* (1980) 27:75–80. doi: 10.1128/IAI.27.1.75-80.1980
11. Mathew RC, Boros DL. Anti-L3T4 antibody treatment suppresses hepatic granuloma formation and abrogates antigen-induced interleukin-2 production in *Schistosoma mansoni* infection. *Infect Immun.* (1986) 54:820–6. doi: 10.1128/IAI.54.3.820-826.1986
12. Boros DL. T helper cell populations, cytokine dynamics, and pathology of the schistosome egg granuloma. *Microbes Infect.* (1999) 1:511–6. doi: 10.1016/S1286-4579(99)80090-2
13. Hiatt RA, Sotomayor ZR, Sanchez G, Zambrana M, Knight WB. Factors in the pathogenesis of acute schistosomiasis mansoni. *J Infect Dis.* (1979) 139:659–66. doi: 10.1093/infdis/139.6.659
14. Burke ML, McManus DP, Ramm GA, Duke M, Li Y, Jones MK, et al. Temporal expression of chemokines dictates the hepatic inflammatory infiltrate in a murine model of schistosomiasis. *PLoS Negl Trop Dis.* (2010) 4:e598. doi: 10.1371/journal.pntd.0000598
15. de Jesus AR, Silva A, Santana LB, Magalhaes A, de Jesus AA, de Almeida RP, et al. Clinical and immunologic evaluation of 31 patients with acute schistosomiasis mansoni. *J Infect Dis.* (2002) 185:98–105. doi: 10.1086/324668
16. Bogen SA, Flores Villanueva PO, McCusker ME, Fogelman I, Garifallou M, el-Attar ES, et al. *In situ* analysis of cytokine responses in experimental murine schistosomiasis. *Lab Invest.* (1995) 73:252–8.
17. Nono JK, Ndlovu H, Aziz NA, Mpotje T, Hlaka L, Brombacher F. Host regulation of liver fibroproliferative pathology during experimental schistosomiasis via interleukin-4 receptor alpha. *PLoS Negl Trop Dis.* (2017) 11:e0005861. doi: 10.1371/journal.pntd.0005861
18. Hoffmann KF, Cheever AW, Wynn TA. IL-10 and the dangers of immune polarization: excessive type 1 and type 2 cytokine responses induce distinct forms of lethal immunopathology in murine schistosomiasis. *J Immunol.* (2000) 164:6406–16. doi: 10.4049/jimmunol.164.12.6406
19. Fahel JS, Macedo GC, Pinheiro CS, Caliari MV, Oliveira SC. IPSE/alpha-1 of *Schistosoma mansoni* egg induces enlargement of granuloma but does not alter Th2 balance after infection. *Parasite Immunol.* (2010) 32:345–53. doi: 10.1111/j.1365-3024.2009.01192.x
20. Dunne DW, Jones FM, Doenhoff MJ. The purification, characterization, serological activity and hepatotoxic properties of two cationic glycoproteins (alpha 1 and omega 1) from *Schistosoma mansoni* eggs. *Parasitology.* (1991) 103:225–36. doi: 10.1017/S0031182000059503
21. Wang Y, Da'Dara AA, Thomas PG, Harn DA. Dendritic cells activated by an anti-inflammatory agent induce $CD4^+$ T helper type 2 responses without impairing $CD8^+$ memory and effector cytotoxic T-lymphocyte responses. *Immunology.* (2010) 129:406–17. doi: 10.1111/j.1365-2567.2009.03193.x
22. Cai Y, Langley JG, Smith DI, Boros DL. A cloned major *Schistosoma mansoni* egg antigen with homologies to small heat shock proteins elicits Th1 responsiveness. *Infect Immun.* (1996) 64:1750–5. doi: 10.1128/IAI.64.5.1750-1755.1996
23. Hernandez HJ, Edson CM, Harn DA, Ianelli CJ, Stadecker MJ. Schistosoma mansoni: genetic restriction and cytokine profile of the $CD4^+$ T helper cell response to dominant epitope peptide of major egg antigen Sm-p40. *Exp Parasitol.* (1998) 90:122–30. doi: 10.1006/expr.1998.4309
24. Everts B, Perona-Wright G, Smits HH, Hokke CH, van der Ham AJ, Fitzsimmons CM, et al. Omega-1, a glycoprotein secreted by Schistosoma mansoni eggs, drives Th2 responses. *J Exp Med.* (2009) 206:1673–80. doi: 10.1084/jem.20082460
25. Reis EAG, Mauadi Carmo TA, Athanazio R, Reis MG, Harn DA Jr. *Schistosoma mansoni* triose phosphate isomerase peptide MAP4 is able to trigger naive donor immune response towards a type-1 cytokine profile. *Scand J Immunol.* (2008) 68:169–76. doi: 10.1111/j.1365-3083.2008.02131.x
26. Chen Y, Boros DL. The *Schistosoma mansoni* egg-derived r38 peptide-induced Th1 response affects the synchronous pulmonary but not the asynchronous hepatic granuloma growth. *Parasite Immunol.* (2001) 23:43–50. doi: 10.1046/j.1365-3024.2001.00354.x
27. Egesa M, Lubyayi L, Tukahebwa EM, Bagaya BS, Chalmers IW, Wilson S, et al. *Schistosoma mansoni* schistosomula antigens induce Th1/Pro-inflammatory cytokine responses. *Parasite Immunol.* (2018) 40:e12592. doi: 10.1111/pim.12592
28. Ma LZ, Li DD, Yuan CX, Zhang XQ, Ta N, Zhao XC, et al. SjCRT, a recombinant *Schistosoma japonicum* calreticulin, induces maturation of dendritic cells and a Th1-polarized immune response in mice. *Parasites Vectors.* (2017) 10:570. doi: 10.1186/s13071-017-2516-7
29. MacDonald AS, Patton EA, La Flamme AC, Araujo MI, Huxtable CR, Bauman B, et al. Impaired Th2 development and increased mortality during *Schistosoma mansoni* infection in the absence of CD40/CD154 interaction. *J Immunol.* (2002) 168:4643–9. doi: 10.4049/jimmunol.168.9.4643
30. Hernandez HJ, Sharpe AH, Stadecker MJ. Experimental murine schistosomiasis in the absence of B7 costimulatory molecules: reversal of elicited T cell cytokine profile and partial inhibition of egg granuloma formation. *J Immunol.* (1999) 162:2884–9.

31. Rutitzky LI, Ozkaynak E, Rottman JB, Stadecker MJ. Disruption of the ICOS-B7RP-1 costimulatory pathway leads to enhanced hepatic immunopathology and increased gamma interferon production by CD4 T cells in murine schistosomiasis. *Infect Immun.* (2003) 71:4040–4. doi: 10.1128/IAI.71.7.4040-4044.2003
32. Pearce EJ, Caspar P, Grzych JM, Lewis FA, Sher A. Downregulation of Th1 cytokine production accompanies induction of Th2 responses by a parasitic helminth, *Schistosoma mansoni*. *J Exp Med.* (1991) 173:159–66. doi: 10.1084/jem.173.1.159
33. Kaplan MH, Whitfield JR, Boros DL, Grusby MJ. Th2 cells are required for the *Schistosoma mansoni* egg-induced granulomatous response. *J Immunol.* (1998) 160:1850–6.
34. Cheever AW, Williams ME, Wynn TA, Finkelman FD, Seder RA, Cox TM, et al. Anti-IL-4 treatment of Schistosoma mansoni-infected mice inhibits development of T cells and non-B, non-T cells expressing Th2 cytokines while decreasing egg-induced hepatic fibrosis. *J Immunol.* (1994) 153:753–9.
35. Yamashita T, Boros DL. IL-4 influences IL-2 production and granulomatous inflammation in murine schistosomiasis mansoni. *J Immunol.* (1992) 149:3659–64.
36. Jankovic D, Kullberg MC, Noben-Trauth N, Caspar P, Ward JM, Cheever AW, et al. Schistosome-infected IL-4 receptor knockout (KO) mice, in contrast to IL-4 KO mice, fail to develop granulomatous pathology while maintaining the same lymphokine expression profile. *J Immunol.* (1999) 163:337–42.
37. Wynn TA. IL-13 effector functions. *Annu Rev Immunol.* (2003) 21:425–56. doi: 10.1146/annurev.immunol.21.120601.141142
38. Chiaramonte MG, Cheever AW, Malley JD, Donaldson DD, Wynn TA. Studies of murine schistosomiasis reveal interleukin-13 blockade as a treatment for established and progressive liver fibrosis. *Hepatology.* (2001) 34:273–82. doi: 10.1053/jhep.2001.26376
39. Fallon PG, Richardson EJ, McKenzie GJ, McKenzie AN. Schistosome infection of transgenic mice defines distinct and contrasting pathogenic roles for IL-4 and IL-13: IL-13 is a profibrotic agent. *J Immunol.* (2000) 164:2585–91. doi: 10.4049/jimmunol.164.5.2585
40. Chiaramonte MG, Donaldson DD, Cheever AW, Wynn TA. An IL-13 inhibitor blocks the development of hepatic fibrosis during a T-helper type 2-dominated inflammatory response. *J Clin Invest.* (1999) 104:777–85. doi: 10.1172/JCI7325
41. Oriente A, Fedarko NS, Pacocha SE, Huang SK, Lichtenstein LM, Essayan DM. Interleukin-13 modulates collagen homeostasis in human skin and keloid fibroblasts. *J Pharmacol Exp Ther.* (2000) 292:988–94.
42. Fort MM, Cheung J, Yen D, Li J, Zurawski SM, Lo S, et al. IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies *in vivo*. *Immunity.* (2001) 15:985–95. doi: 10.1016/S1074-7613(01)00243-6
43. Soumelis V, Reche PA, Kanzler H, Yuan W, Edward G, Homey B, et al. Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. *Nat Immunol.* (2002) 3:673–80. doi: 10.1038/ni805
44. 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
45. Yu Y, Deng W, Lei J. Interleukin-33 promotes Th2 immune responses in infected mice with *Schistosoma japonicum*. *Parasitol Res.* (2015) 114:2911–8. doi: 10.1007/s00436-015-4492-1
46. He X, Xie J, Wang Y, Fan X, Su Q, Sun Y, et al. Down-regulation of microRNA-203–3p initiates type 2 pathology during schistosome infection via elevation of interleukin-33. *PLoS Pathog.* (2018) 14:e1006957. doi: 10.1371/journal.ppat.1006957
47. Peng H, Zhang Q, Li X, Liu Z, Shen J, Sun R, et al. IL-33 contributes to *Schistosoma japonicum*-induced hepatic pathology through induction of M2 macrophages. *Sci Rep.* (2016) 6:29844. doi: 10.1038/srep29844
48. Vannella KM, Ramalingam TR, Borthwick LA, Barron L, Hart KM, Thompson RW, et al. Combinatorial targeting of TSLP, IL-25, and IL-33 in type 2 cytokine–driven inflammation and fibrosis. *Sci Transl Med.* (2016) 8:337ra65. doi: 10.1126/scitranslmed.aaf1938
49. Tallima H, Dvorak J, Kareem S, Abou El Dahab M, Abdel Aziz N, Dalton JP, et al. Protective immune responses against *Schistosoma mansoni* infection by immunization with functionally active gut-derived cysteine peptidases alone and in combination with glyceraldehyde 3-phosphate dehydrogenase. *PLoS Negl Trop Dis.* (2017) 11:e0005443. doi: 10.1371/journal.pntd.0005443
50. Tallima H, Dalton JP, El Ridi R. Induction of protective immune responses against *Schistosomiasis haematobium* in hamsters and mice using cysteine peptidase-based vaccine. *Front Immunol.* (2015) 6:130. doi: 10.3389/fimmu.2015.00130
51. Brunet LR, Finkelman FD, Cheever AW, Kopf MA, Pearce EJ. IL-4 protects against TNF-alpha-mediated cachexia and death during acute schistosomiasis. *J Immunol.* (1997) 159:777–85.
52. Fallon PG, Smith P, Dunne DW. Type 1 and type 2 cytokine-producing mouse $CD4^+$ and $CD8^+$ T cells in acute *Schistosoma mansoni* infection. *Eur J Immunol.* (1998) 28:1408–16. doi: 10.1002/(SICI)1521-4141(199804)28:04<1408::AID-IMMU1408>3.0.CO;2-H
53. La Flamme AC, Patton EA, Bauman B, Pearce EJ. IL-4 plays a crucial role in regulating oxidative damage in the liver during schistosomiasis. *J Immunol.* (2001) 166:1903–11. doi: 10.4049/jimmunol.166.3.1903
54. Mwatha JK, Kimani G, Kamau T, Mbugua GG, Ouma JH, Mumo J, et al. High levels of TNF, soluble TNF receptors, soluble ICAM-1, and IFN-gamma, but low levels of IL-5, are associated with hepatosplenic disease in human schistosomiasis mansoni. *J Immunol.* (1998) 160:1992–9.
55. Boros DL, Whitfield JR. Endogenous IL-10 regulates IFN-gamma and IL-5 cytokine production and the granulomatous response in *Schistosomiasis mansoni*-infected mice. *Immunology.* (1998) 94:481–7. doi: 10.1046/j.1365-2567.1998.00544.x
56. Rutitzky LI, Lopes da Rosa JR, Stadecker MJ. Severe CD4 T cell-mediated immunopathology in murine schistosomiasis is dependent on IL-12p40 and correlates with high levels of IL-17. *J Immunol.* (2005) 175:3920–6. doi: 10.4049/jimmunol.175.6.3920
57. Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, et al. Interleukin 17-producing $CD4^+$ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol.* (2005) 6:1123–32. doi: 10.1038/ni1254
58. Shainheit MG, Lasocki KW, Finger E, Larkin BM, Smith PM, Sharpe AH, et al. The pathogenic Th17 cell response to major schistosome egg antigen is sequentially dependent on IL-23 and IL-1 beta. *J Immunol.* (2011) 187:5328–35. doi: 10.4049/jimmunol.1101445
59. Rutitzky LI, Bazzone L, Shainheit MG, Joyce-Shaikh B, Cua DJ, Stadecker MJ. IL-23 is required for the development of severe egg-induced immunopathology in schistosomiasis and for lesional expression of IL-17. *J Immunol.* (2008) 180:2486–95. doi: 10.4049/jimmunol.180.4.2486
60. Shainheit MG, Smith PM, Bazzone LE, Wang AC, Rutitzky LI, Stadecker MJ. Dendritic cell IL-23 and IL-1 production in response to schistosome eggs induces Th17 cells in a mouse strain prone to severe immunopathology. *J Immunol.* (2008) 181:8559–67. doi: 10.4049/jimmunol.181.12.8559
61. Ponichtera HE, Shainheit MG, Liu BYC, Raychowdhury R, Larkin BM, Russo JM, et al. CD209a expression on dendritic cells is critical for the development of pathogenic Th17 cell responses in murine schistosomiasis. *J Immunol.* (2014) 192:4655–65. doi: 10.4049/jimmunol.1400121
62. Mbow M, Larkin BM, Meurs L, Wammes LJ, de Jong SE, Labuda LA, et al. T-helper 17 cells are associated with pathology in human schistosomiasis. *J Infect Dis.* (2013) 207:186–95. doi: 10.1093/infdis/jis654
63. Wen X, He L, Chi Y, Zhou S, Hoellwarth J, Zhang C, et al. Dynamics of Th17 cells and their role in *Schistosoma japonicum* infection in C57BL/6 mice. *PLoS Negl Trop Dis.* (2011) 5:e1399. doi: 10.1371/journal.pntd.0001399
64. Zhang Y, Chen L, Gao W, Hou X, Gu Y, Gui L, et al. IL-17 neutralization significantly ameliorates hepatic granulomatous inflammation and liver damage in *Schistosoma japonicum* infected mice. *Eur J Immunol.* (2012) 42:1523–35. doi: 10.1002/eji.201141933
65. Zhang YX, Huang DK, Gao WD, Yan J, Zhou WL, Hou X, et al. Lack of IL-17 signaling decreases liver fibrosis in murine schistosomiasis japonica. *Int Immunol.* (2015) 27:317–25. doi: 10.1093/intimm/dxv017
66. Rutitzky LI, Stadecker MJ. Exacerbated egg-induced immunopathology in murine *Schistosoma mansoni* infection is primarily mediated by IL-17 and restrained by IFN-gamma. *Eur J Immunol.* (2011) 41:2677–87. doi: 10.1002/eji.201041327
67. Rutitzky LI, Smith PM, Stadecker MJ. T-bet protects against exacerbation of schistosome egg-induced immunopathology by

regulating Th17-mediated inflammation. *Eur J Immunol.* (2009) 39:2470–81. doi: 10.1002/eji.200939325
68. Wang B, Liang S, Wang Y, Zhu X-Q, Gong W, Zhang H-Q, et al. Th17 down-regulation is involved in reduced progression of schistosomiasis fibrosis in ICOSL KO mice. *PLoS Negl Trop Dis.* (2015) 9:e3434. doi: 10.1371/journal.pntd.0003434
69. Kalantari P, Morales Y, Miller EA, Jaramillo LD, Ponichtera HE, Wuethrich MA, et al. CD209a synergizes with dectin-2 and mincle to drive severe Th17 cell-mediated schistosome egg-induced immunopathology. *Cell Rep.* (2018) 22:1288–300. doi: 10.1016/j.celrep.2018.01.001
70. Kalantari P, Bunnell SC, Stadecker MJ. The C-type lectin receptor-driven, Th17 cell-mediated severe pathology in schistosomiasis: not all immune responses to helminth parasites are Th2 dominated. *Front Immunol.* (2019) 10:26. doi: 10.3389/fimmu.2019.00026
71. Soloviova K, Fox EC, Dalton JP, Caffrey CR, Davies SJ. A secreted schistosome cathepsin B1 cysteine protease and acute schistosome infection induce a transient T helper 17 response. *PLoS Negl Trop Dis.* (2019) 13:e7070. doi: 10.1371/journal.pntd.0007070
72. Chen D, Xie H, Luo X, Yu X, Fu X, Gu H, et al. Roles of Th17 cells in pulmonary granulomas induced by *Schistosoma japonicum* in C57BL/6 mice. *Cell Immunol.* (2013) 285:149–57. doi: 10.1016/j.cellimm.2013.09.008
73. Chen D, Luo X, Xie H, Gao Z, Fang H, Huang J. Characteristics of IL-17 induction by *Schistosoma japonicum* infection in C57BL/6 mouse liver. *Immunology.* (2013) 139:523–32. doi: 10.1111/imm.12105
74. Zheng L, Hu Y, Wang Y, Huang X, Xu Y, Shen Y, et al. Recruitment of neutrophils mediated by Vgamma2 gammadelta T cells deteriorates liver fibrosis induced by *Schistosoma japonicum* infection in C57BL/6 mice. *Infect Immun.* (2017) 85:e01020-16. doi: 10.1128/IAI.01020-16
75. Eberl G, Di Santo JP, Vivier E. The brave new world of innate lymphoid cells. *Nat Immunol.* (2015) 16:1–5. doi: 10.1038/ni.3059
76. Von Lichtenberg F, Erickson DG, Sadun EH. Comparative histopathology of schistosome granulomas in the hamster. *Am J Pathol.* (1973) 72:149–78.
77. Chuah C, Jones MK, Burke ML, McManus DP, Gobert GN. Cellular and chemokine-mediated regulation in schistosome-induced hepatic pathology. *Trends Parasitol.* (2014) 30:141–50. doi: 10.1016/j.pt.2013.12.009
78. Cheever AW, Duvall RH, Hallack TA Jr, Minker RG, Malley JD, Malley KG. Variation of hepatic fibrosis and granuloma size among mouse strains infected with *Schistosoma mansoni*. *Am J Trop Med Hyg.* (1987) 37:85–97. doi: 10.4269/ajtmh.1987.37.85
79. Larkin BM, Smith PM, Ponichtera HE, Shainheit MG, Rutitzky LI, Stadecker MJ. Induction and regulation of pathogenic Th17 cell responses in schistosomiasis. *Semin Immunopathol.* (2012) 34:873–88. doi: 10.1007/s00281-012-0341-9
80. Stadecker MJ, Asahi H, Finger E, Hernandez HJ, Rutitzky LI, Sun J. The immunobiology of Th1 polarization in high-pathology schistosomiasis. *Immunol Rev.* (2004) 201:168–79. doi: 10.1111/j.0105-2896.2004.00197.x
81. King C. New insights into the differentiation and function of T follicular helper cells. *Nat Rev Immunol.* (2009) 9:757–66. doi: 10.1038/nri2644
82. Chtanova T, Tangye SG, Newton R, Frank N, Hodge MR, Rolph MS, et al. T follicular helper cells express a distinctive transcriptional profile, reflecting their role as non-Th1/Th2 effector cells that provide help for B cells. *J Immunol.* (2004) 173:68–78. doi: 10.4049/jimmunol.173.1.68
83. Breitfeld D, Ohl L, Kremmer E, Ellwart J, Sallusto F, Lipp M, et al. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J Exp Med.* (2000) 192:1545–52. doi: 10.1084/jem.192.11.1545
84. Crotty S. Follicular helper CD4 T cells (TFH). *Annu Rev Immunol.* (2011) 29:621–63. doi: 10.1146/annurev-immunol-031210-101400
85. King C, Tangye SG, Mackay CR. T follicular helper (TFH) cells in normal and dysregulated immune responses. *Annu Rev Immunol.* (2008) 26:741–66. doi: 10.1146/annurev.immunol.26.021607.090344
86. Zaretsky AG, Taylor JJ, King IL, Marshall FA, Mohrs M, Pearce EJ. T follicular helper cells differentiate from Th2 cells in response to helminth antigens. *J Exp Med.* (2009) 206:991–99. doi: 10.1084/jem.20090303
87. Zhang JY, Gao WJ, Guo QR, Huang BB, Wang B, Xia GL, et al. Helminth protein vaccine induced follicular T helper cell for enhancement of humoral immunity against *Schistosoma japonicum*. *Biomed Res Int.* (2013) 2013:798164. doi: 10.1155/2013/798164
88. Tripodo C, Petta S, Guarnotta C, Pipitone R, Cabibi D, Colombo MP, et al. Liver follicular helper T-cells predict the achievement of virological response following interferon-based treatment in HCV-infected patients. *Antiviral Ther.* (2012) 17:111–18. doi: 10.3851/IMP1957
89. Auderset F, Schuster S, Fasnacht N, Coutaz M, Charmoy M, Koch U, et al. Notch signaling regulates follicular helper T cell differentiation. *J Immunol.* (2013) 191:2344–50. doi: 10.4049/jimmunol.1300643
90. Hams E, McCarron MJ, Amu S, Yagita H, Azuma M, Chen LP, et al. Blockade of B7-H1 (programmed death ligand 1) enhances humoral immunity by positively regulating the generation of T follicular helper cells. *J Immunol.* (2011) 186:5648–55. doi: 10.4049/jimmunol.1003161
91. Chen X, Yang X, Li Y, Zhu J, Zhou S, Xu Z, et al. Follicular helper T cells promote liver pathology in mice during *Schistosoma japonicum* infection. *PLoS Pathog.* (2014) 10:e1004097. doi: 10.1371/journal.ppat.1004097
92. Wang YY, Lin C, Cao Y, Duan ZL, Guan ZX, Xu J, et al. Up-regulation of interleukin-21 contributes to liver pathology of schistosomiasis by driving GC immune responses and activating HSCs in mice. *Sci Rep.* (2017) 7:16682. doi: 10.1038/s41598-017-16783-7
93. Zhang YM, Jiang YY, Wang YJ, Liu H, Shen YJ, Yuan ZY, et al. Higher frequency of circulating PD-1(high) $CXCR5^{+}CD4^{+}$ Tfh cells in patients with chronic schistosomiasis. *Int J Biol Sci.* (2015) 11:1049–55. doi: 10.7150/ijbs.12023
94. Zhang YM, Wang YJ, Jiang YY, Pan W, Liu H, Yin JH, et al. T follicular helper cells in patients with acute schistosomiasis. *Parasites Vectors.* (2016) 9:321. doi: 10.1186/s13071-016-1602-6
95. Fairfax K, Nascimento M, Huang SCC, Everts B, Pearce EJ. Th2 responses in schistosomiasis. *Semin Immunopathol.* (2012) 34:863–71. doi: 10.1007/s00281-012-0354-4
96. Kaplan MH. Th9 cells: differentiation and disease. *Immunol Rev.* (2013) 252:104–15. doi: 10.1111/imr.12028
97. Zhao P, Xiao X, Ghobrial RM, Li XC. IL-9 and T(h)9 cells: progress and challenges. *Int Immunol.* (2013) 25:547–51. doi: 10.1093/intimm/dxt039
98. Dardalhon V, Awasthi A, Kwon H, Galileos G, Gao W, Sobel RA, et al. IL-4 inhibits TGF-beta-induced $Foxp3^{+}$ T cells and, together with TGF-beta, generates IL-9^{+} IL-10^{+} $Foxp3^{-}$ effector T cells. *Nat Immunol.* (2008) 9:1347–55. doi: 10.1038/ni.1677
99. Anuradha R, George PJ, Hanna LE, Chandrasekaran V, Kumaran P, Nutman TB, et al. IL-4-, TGF-beta-, and IL-1-dependent expansion of parasite antigen-specific Th9 cells is associated with clinical pathology in human lymphatic filariasis. *J Immunol.* (2013) 191:2466–73. doi: 10.4049/jimmunol.1300911
100. Li H, Nourbakhsh B, Cullimore M, Zhang G-X, Rostami A. IL-9 is important for T-cell activation and differentiation in autoimmune inflammation of the central nervous system. *Eur J Immunol.* (2011) 41:2197–206. doi: 10.1002/eji.201041125
101. Zhan TZ, Zhang TT, Wang YY, Wang XL, Lin C, Ma HH, et al. Dynamics of Th9 cells and their potential role in immunopathogenesis of murine schistosomiasis. *Parasites Vectors.* (2017) 10:305. doi: 10.1186/s13071-017-2242-1
102. Li L, Xie HY, Wang M, Qu JL, Cha HF, Yang Q, et al. Characteristics of IL-9 induced by *Schistosoma japonicum* infection in C57BL/6 mouse liver. *Sci Rep*. (2017) 7:2343. doi: 10.1038/s41598-017-02422-8
103. Matos Sa Barreto AV, Nunes de lacerda GA, de Castro Figueiredo AL, Nunes Diniz GT, Souza Gomes EC, Coutinho Domingues AL, et al. Evaluation of serum levels of IL-9 and IL-17 in human *Schistosoma mansoni* infection and their relationship with periportal fibrosis. *Immunobiology.* (2016) 221:1351–54. doi: 10.1016/j.imbio.2016.07.014
104. Sakaguchi S, Wing K, Miyara M. Regulatory T cells–a brief history and perspective. *Eur J Immunol.* (2007) 37:S116–23. doi: 10.1002/eji.200737593
105. Belkaid Y, Rouse BT. Natural regulatory T cells in infectious disease. *Nat Immunol.* (2005) 6:353–60. doi: 10.1038/ni1181
106. Jonuleit H, Schmitt E. The regulatory T cell family: distinct subsets and their interrelations. *J Immunol.* (2003) 171:6323–7. doi: 10.4049/jimmunol.171.12.6323
107. Fontenot JD, Rasmussen JP, Williams LM, Dooley JL, Farr AG, Rudensky AY. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity.* (2005) 22:329–41. doi: 10.1016/j.immuni.2005.01.016

108. Piccirillo CA, Shevach EM. Naturally-occurring $CD4^+CD25^+$ immunoregulatory T cells: central players in the arena of peripheral tolerance. *Semin Immunol.* (2004) 16:81–8. doi: 10.1016/j.smim.2003.12.003
109. Fontenot JD, Rudensky AY. A well adapted regulatory contrivance: regulatory T cell development and the forkhead family transcription factor Foxp3. *Nat Immunol.* (2005) 6:331–7. doi: 10.1038/ni1179
110. Barrat FJ, Cua DJ, Boonstra A, Richards DF, Crain C, Savelkoul HF, et al. *In vitro* generation of interleukin 10-producing regulatory $CD4^+$ T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines. *J Exp Med.* (2002) 195:603–16. doi: 10.1084/jem.20011629
111. Bluestone JA, Abbas AK. Natural versus adaptive regulatory T cells. *Nat Rev Immunol.* (2003) 3:253–7. doi: 10.1038/nri1032
112. O'Garra A, Vieira PL, Vieira P, Goldfeld AE. IL-10-producing and naturally occurring $CD4^+$ Tregs: limiting collateral damage. *J Clin Invest.* (2004) 114:1372–8. doi: 10.1172/JCI23215
113. Singh KP, Gerard HC, Hudson AP, Reddy TR, Boros DL. Retroviral Foxp3 gene transfer ameliorates liver granuloma pathology in *Schistosoma mansoni* infected mice. *Immunology.* (2005) 114:410–7. doi: 10.1111/j.1365-2567.2004.02083.x
114. Taylor JJ, Mohrs M, Pearce EJ. Regulatory T cell responses develop in parallel to Th responses and control the magnitude and phenotype of the Th effector population. *J Immunol.* (2006) 176:5839–47. doi: 10.4049/jimmunol.176.10.5839
115. van der Kleij D, Latz E, Brouwers JFHM, Kruize YCM, Schmitz M, Kurt-Jones EA, et al. A novel host-parasite lipid cross-talk. Schistosomal lyso-phosphatidylserine activates toll-like receptor 2 and affects immune polarization. *J Biol Chem.* (2002) 277:48122–9. doi: 10.1074/jbc.M206941200
116. Baumgart M, Tompkins F, Leng J, Hesse M. Naturally occurring $CD4^+Foxp3^+$ regulatory T cells are an essential, IL-10-independent part of the immunoregulatory network in *Schistosoma mansoni* egg-induced inflammation. *J Immunol.* (2006) 176:5374–87. doi: 10.4049/jimmunol.176.9.5374
117. Hesse M, Piccirillo CA, Belkaid Y, Prufer J, Mentink-Kane M, Leusink M, et al. The pathogenesis of schistosomiasis is controlled by cooperating IL-10-producing innate effector and regulatory T cells. *J Immunol.* (2004) 172:3157–66. doi: 10.4049/jimmunol.172.5.3157
118. McKee AS, Pearce EJ. $CD25^+CD4^+$ cells contribute to Th2 polarization during helminth infection by suppressing Th1 response development. *J Immunol.* (2004) 173:1224–31. doi: 10.4049/jimmunol.173.2.1224
119. Chensue SW, Warmington KS, Hershey SD, Terebuh PD, Othman M, Kunkel SL. Evolving T cell responses in murine schistosomiasis. Th2 cells mediate secondary granulomatous hypersensitivity and are regulated by $CD8^+$ T cells *in vivo*. *J Immunol.* (1993) 151:1391–400.
120. Yap G, Cheever A, Caspar P, Jankovic D, Sher A. Unimpaired down-modulation of the hepatic granulomatous response in CD8 T-cell- and gamma interferon-deficient mice chronically infected with *Schistosoma mansoni*. *Infect Immun.* (1997) 65:2583–6. doi: 10.1128/IAI.65.7.2583-2586.1997
121. Hernandez HJ, Wang Y, Tzellas N, Stadecker MJ. Expression of class II, but not class I, major histocompatibility complex molecules is required for granuloma formation in infection with *Schistosoma mansoni*. *Eur J Immunol.* (1997) 27:1170–6. doi: 10.1002/eji.1830270518
122. Hayday AC. [gamma][delta] cells: a right time and a right place for a conserved third way of protection. *Annu Rev Immunol.* (2000) 18:975–1026. doi: 10.1146/annurev.immunol.18.1.975
123. Bank I, Marcu-Malina V. Quantitative peripheral blood perturbations of gammadelta T cells in human disease and their clinical implications. *Clin Rev Allergy Immunol.* (2014) 47:311–33. doi: 10.1007/s12016-013-8391-x
124. Sandor M, Sperling AI, Cook GA, Weinstock JV, Lynch RG, Bluestone JA. Two waves of gamma delta T cells expressing different V delta genes are recruited into schistosome-induced liver granulomas. *J Immunol.* (1995) 155:275–84.
125. Schwartz E, Rosenthal E, Bank I. Gamma delta T cells in non-immune patients during primary schistosomal infection. *Immun Inflamm Dis.* (2014) 2:56–61. doi: 10.1002/iid3.18
126. Yu X, Luo X, Xie H, Chen D, Li L, Wu F, et al. Characteristics of gammadelta T cells in *Schistosoma japonicum*-infected mouse mesenteric lymph nodes. *Parasitol Res.* (2014) 113:3393–401. doi: 10.1007/s00436-014-4004-8
127. Iacomini J, Ricklan DE, Stadecker MJ. T cells expressing the γδ T cell receptor are not required for egg granuloma formation in schistosomiasis. *Eur J Immunol.* (1995) 25:884–88. doi: 10.1002/eji.1830250404
128. Holscher C, Atkinson RA, Arendse B, Brown N, Myburgh E, Alber G, et al. A protective and agonistic function of IL-12p40 in mycobacterial infection. *J Immunol.* (2001) 167:6957–66. doi: 10.4049/jimmunol.167.12.6957
129. Gordon S. Alternative activation of macrophages. *Nat Rev Immunol.* (2003) 3:23–35. doi: 10.1038/nri978
130. Hesse M, Cheever AW, Jankovic D, Wynn TA. NOS-2 mediates the protective anti-inflammatory and antifibrotic effects of the Th1-inducing adjuvant, IL-12, in a Th2 model of granulomatous disease. *Am J Pathol.* (2000) 157:945–55. doi: 10.1016/S0002-9440(10)64607-X
131. Hesse M, Modolell M, La Flamme AC, Schito M, Fuentes JM, Cheever AW, et al. Differential regulation of nitric oxide synthase-2 and arginase-1 by type 1/type 2 cytokines *in vivo*: granulomatous pathology is shaped by the pattern of L-arginine metabolism. *J Immunol.* (2001) 167:6533–44. doi: 10.4049/jimmunol.167.11.6533
132. Herbert DBR, Holscher C, Mohrs M, Arendse B, Schwegmann A, Radwanska M, et al. Alternative macrophage activation is essential for survival during schistosomiasis and downmodulates T helper 1 responses and immunopathology. *Immunity.* (2004) 20:623–35. doi: 10.1016/S1074-7613(04)00107-4
133. Chensue SW, Boros DL. Modulation of granulomatous hypersensitivity. I. Characterization of T lymphocytes involved in the adoptive suppression of granuloma formation in *Schistosoma mansoni*-infected mice. *J Immunol.* (1979) 123:1409–14.
134. el-Cheikh MC, Bonomo AC, Rossi MI, Pinho Mde F, Borojevic R. Experimental murine schistosomiasis mansoni: modulation of the B-1 lymphocyte distribution and phenotype expression. *Immunobiology.* (1998) 199:51–62. doi: 10.1016/S0171-2985(98)80063-6
135. Jankovic D, Wynn TA, Kullberg MC, Hieny S, Caspar P, James S, et al. Optimal vaccination against Schistosoma mansoni requires the induction of both B cell- and IFN-gamma-dependent effector mechanisms. *J Immunol.* (1999) 162:345–51.
136. Dunne DW, Butterworth AE, Fulford AJ, Kariuki HC, Langley JG, Ouma JH, et al. Immunity after treatment of human schistosomiasis: association between IgE antibodies to adult worm antigens and resistance to reinfection. *Eur J Immunol.* (1992) 22:1483–94. doi: 10.1002/eji.1830220622
137. Boros DL, Amsden AF, Hood AT. Modulation of granulomatous hypersensitivity. IV. Immunoglobulin and antibody production by vigorous and immunomodulated liver granulomas of *Schistosoma mansoni*-infected mice. *J Immunol.* (1982) 128:1050–3.
138. Goes AM, Gazzinelli G, Rocha R, Katz N, Doughty BL. Granulomatous hypersensitivity to *Schistosoma mansoni* egg antigens in human schistosomiasis. III. *In vitro* granuloma modulation induced by immune complexes. *Am J Trop Med Hyg.* (1991) 44:434–43. doi: 10.4269/ajtmh.1991.44.434
139. Harris N, Gause WC. To B or not to B: B cells and the Th2-type immune response to helminths. *Trends Immunol.* (2011) 32:80–8. doi: 10.1016/j.it.2010.11.005
140. Hernandez HJ, Wang Y, Stadecker MJ. In infection with *Schistosoma mansoni*, B cells are required for T helper type 2 cell responses but not for granuloma formation. *J Immunol.* (1997) 158:4832–7.
141. Ferru I, Roye O, Delacre M, Auriault C, Wolowczuk I. Infection of B-cell-deficient mice by the parasite *Schistosoma mansoni*: demonstration of the participation of B cells in granuloma modulation. *Scand J Immunol.* (1998) 48:233–40. doi: 10.1046/j.1365-3083.1998.00376.x
142. Hurdayal R, Ndlovu HH, Revaz-Breton M, Parihar SP, Nono JK, Govender M, et al. IL-4-producing B cells regulate T helper cell dichotomy in type 1- and type 2-controlled diseases. *Proc Natl Acad Sci USA.* (2017) 114:E8430–9. doi: 10.1073/pnas.1708125114
143. Jankovic D, Cheever AW, Kullberg MC, Wynn TA, Yap G, Caspar P, et al. $CD4^+$ T cell-mediated granulomatous pathology in schistosomiasis

is downregulated by a B cell-dependent mechanism requiring Fc receptor signaling. *J Exp Med.* (1998) 187:619–29. doi: 10.1084/jem.187.4.619
144. Yamane H, Paul WE. Early signaling events that underlie fate decisions of naive $CD4^+$ T cells toward distinct T-helper cell subsets. *Immunol Rev.* (2013) 252:12–23. doi: 10.1111/imr.12032
145. Phythian-Adams AT, Cook PC, Lundie RJ, Jones LH, Smith KA, Barr TA, et al. CD11c depletion severely disrupts Th2 induction and development *in vivo*. *J Exp Med.* (2010) 207:2089–96. doi: 10.1084/jem.20100734
146. Ma YL, Huang FJ, Cong L, Gong WC, Bai HM, Li J, et al. IL-4-producing dendritic cells induced during *Schistosoma japonica* infection promote Th2 cells via IL-4-dependent pathway. *J Immunol.* (2015) 195:3769–80. doi: 10.4049/jimmunol.1403240
147. Lundie RJ, Webb LM, Marley AK, Phythian-Adams AT, Cook PC, Jackson-Jones LH, et al. A central role for hepatic conventional dendritic cells in supporting Th2 responses during helminth infection. *Immunol Cell Biol.* (2016) 94:400–10. doi: 10.1038/icb.2015.114
148. MacDonald AS, Pearce EJ. Cutting edge: polarized Th cell response induction by transferred antigen-pulsed dendritic cells is dependent on IL-4 or IL-12 production by recipient cells. *J Immunol.* (2002) 168:3127–30. doi: 10.4049/jimmunol.168.7.3127
149. Whelan M, Harnett MM, Houston KM, Patel V, Harnett W, Rigley KP. A filarial nematode-secreted product signals dendritic cells to acquire a phenotype that drives development of Th2 cells. *J Immunol.* (2000) 164:6453–60. doi: 10.4049/jimmunol.164.12.6453
150. MacDonald AS, Straw AD, Bauman B, Pearce EJ. CD8- dendritic cell activation status plays an integral role in influencing Th2 response development. *J Immunol.* (2001) 167:1982–8. doi: 10.4049/jimmunol.167.4.1982
151. Webb LM, Lundie RJ, Borger JG, Brown SL, Connor LM, Cartwright AN, et al. Type I interferon is required for T helper (Th) 2 induction by dendritic cells. *EMBO J.* (2017) 36:2404–18. doi: 10.15252/embj.201695345
152. Ismail M, Metwally A, Farghaly A, Bruce J, Tao LF, Bennett JL. Characterization of isolates of *Schistosoma mansoni* from Egyptian villagers that tolerate high doses of praziquantel. *Am J Trop Med Hyg.* (1996) 55:214–8. doi: 10.4269/ajtmh.1996.55.214
153. Couto FF, Coelho PM, Araujo N, Kusel JR, Katz N, Jannotti-Passos LK, et al. *Schistosoma mansoni*: a method for inducing resistance to praziquantel using infected *Biomphalaria glabrata* snails. *Mem Inst Oswaldo Cruz.* (2011) 106:153–7. doi: 10.1590/S0074-02762011000200006
154. Doenhoff MJ, Cioli D, Utzinger J. Praziquantel: mechanisms of action, resistance and new derivatives for schistosomiasis. *Curr Opin Infect Dis.* (2008) 21:659–67. doi: 10.1097/QCO.0b013e328318978f
155. Thomas CM, Timson D. The mechanism of action of praziquantel: can new drugs exploit similar mechanisms? *Curr Med Chem.* (2018). doi: 10.2174/0929867325666180926145537. [Epub ahead of print].
156. Bergquist R, Utzinger J, McManus DP. Trick or treat: the role of vaccines in integrated schistosomiasis control. *PLoS Negl Trop Dis.* (2008) 2:e244. doi: 10.1371/journal.pntd.0000244
157. Rutitzky LI, Hernandez HJ, Stadecker MJ. Th1-polarizing immunization with egg antigens correlates with severe exacerbation of immunopathology and death in schistosome infection. *Proc Natl Acad Sci USA.* (2001) 98:13243–8. doi: 10.1073/pnas.231258498

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: 05 May 2020
doi: 10.3389/fimmu.2020.00795

NLRP6 Plays an Important Role in Early Hepatic Immunopathology Caused by *Schistosoma mansoni* Infection

*Rodrigo C. O. Sanches[1†], Cláudia Souza[1†], Fabio Vitarelli Marinho[1], Fábio Silva Mambelli[2], Suellen B. Morais[1], Erika S. Guimarães[1] and Sergio Costa Oliveira[1,3]**

[1] Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil, [2] Departamento de Genética, Ecologia e Evolução, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil, [3] Instituto Nacional de Ciência e Tecnologia em Doenças Tropicais (INCT-DT), CNPq MCT, Salvador, Brazil

OPEN ACCESS

Edited by:
Michael Harrison Hsieh,
Children's National Hospital,
United States

Reviewed by:
Malcolm Jones,
The University of Queensland,
Australia
Zhipeng Xu,
Nanjing Medical University, China

***Correspondence:**
Sergio Costa Oliveira
scozeus1@gmail.com

[†] These authors have contributed equally to this work

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

Received: 05 December 2019
Accepted: 07 April 2020
Published: 05 May 2020

Citation:
Sanches RCO, Souza C, Marinho FV, Mambelli FS, Morais SB, Guimarães ES and Oliveira SC (2020) NLRP6 Plays an Important Role in Early Hepatic Immunopathology Caused by Schistosoma mansoni Infection. Front. Immunol. 11:795. doi: 10.3389/fimmu.2020.00795

Schistosomiasis is a debilitating parasitic disease that affects more than 200 million people worldwide and causes approximately 280,000 deaths per year. Inside the definitive host, eggs released by *Schistosoma mansoni* lodge in the intestine and especially in the liver where they induce a granulomatous inflammatory process, which can lead to fibrosis. The molecular mechanisms initiating or promoting hepatic granuloma formation remain poorly understood. Inflammasome activation has been described as an important pathway to induce pathology mediated by NLRP3 receptor. Recently, other components of the inflammasome pathway, such as NLRP6, have been related to liver diseases and fibrotic processes. Nevertheless, the contribution of these components in schistosomiasis-associated pathology is still unknown. In the present study, using dendritic cells, we demonstrated that NLRP6 sensor is important for IL-1β production and caspase-1 activation in response to soluble egg antigens (SEA). Furthermore, the lack of NLRP6 has been shown to significantly reduce periovular inflammation, collagen deposition in hepatic granulomas and mRNA levels of α-SMA and IL-13. Livers of *Nlrp6*$^{-/-}$ mice showed reduced levels of CXCL1/KC, CCL2, CCL3, IL-5, and IL-10 as well as Myeloperoxidase (MPO) and Eosinophilic Peroxidase (EPO) enzymatic activity. Consistently, the frequency of macrophage and neutrophil populations were lower in the liver of NLRP6 knockout mice, after 6 weeks of infection. Finally, it was further demonstrated that the onset of hepatic granuloma and collagen deposition were also compromised in *Caspase-1*$^{-/-}$, *IL-1R*$^{-/-}$ and *Gsdmd*$^{-/-}$ mice. Our findings suggest that the NLRP6 inflammasome is an important component for schistosomiasis-associated pathology.

Keywords: *Schistosoma mansoni*, immunopathology, inflammasome, NLRP6, fibrosis

INTRODUCTION

Schistosomiasis is a debilitating parasitic disease which affects 78 countries worldwide. This disease leads to approximately 200,000 deaths annually and severely compromises the life quality from those affected. Among causative species, *Schistosoma haematobium*, *Schistosoma japonicum,* and *Schistosoma mansoni* stand out as those of major importance to human health (1–3). Infection occurs through the direct contact of the host with the parasite's larval form. After parasite penetration and sexual development, egg laying begins. The release of eggs in the feces and its hatching in the environment closes the parasite's life cycle (4). However, a significant amount of these eggs is trapped in some of the host's organs, such as liver and intestine, where they induce a granulomatous inflammatory reaction (5, 6). Hepatic granulomatous inflammation arises from the egg-secreted antigens, which perform hepatotoxic and immunological activities capable of recruiting immune cells to the organ and forming periovular granuloma. The composition of the granuloma includes macrophages, eosinophils, neutrophils, T and B lymphocytes and especially fibroblasts, responsible for the fibrotic characteristic of the structure (7, 8).

Although the process of hepatic granuloma formation is extensively studied, all cellular events and key participants have not been fully established yet. The role of intracellular immune receptors in granuloma formation, for instance, was first described in a seminal study conducted by Ritter and colleagues (9). However, a better understanding of cytosolic sensors during *S. mansoni* infection is required. These intracellular receptors are those responsible for activating the inflammasome pathway. This pathway induces the formation of an intracellular protein complex typically consisting of Nucleotide-binding oligomerization domain (NOD), leucine-rich repeat (LRR)-containing protein (NLR) family members, an adapter molecule known as ASC, and the cysteine protease caspase-1 as an effector molecule. Activation of this pathway leads to cleavage of immature forms of IL-1β and IL-18 into their mature forms. It might also induce cell death by pyroptosis. The inflammasome activation takes place in both immune and non-immune cells and is essentially triggered by pathogen-associated molecular patterns (PAMPs) and Danger-associated molecular patterns (DAMP) (10, 11).

It is known that the inflammasome pathway plays an important role during chronic liver diseases (12). Besides fighting pathogens such as bacteria (13) and viruses (14), inflammasome also participates in aggravating sterile liver inflammations such as Alcoholic Liver Disease (ALD) (15) and Non-alcoholic Steatohepatitis (NASH) (16). NLRP3 is the most widely studied receptor in this context since it is activated by several types of insults (17). On the other hand, the participation of other NLR family receptors in hepatic pathological processes, such as NLRP6, is still elusive.

The inflammasome pathway plays an essential role in schistosomiasis-associated liver pathology. It has been demonstrated that NLRP3 is critical for granuloma formation and hepatic stellate cells (HSCs) activation (18) in *S. japonicum* infections. Regarding *S. mansoni* infection this same sensor has been shown to be involved in the adaptive immune response and also granuloma formation (9). Recent studies have reported that NLRP3 and NLRP6 expression are simultaneously modulated in some processes, including those occurring in the liver (19, 20). Additionally, the role of NLRP6 in fibrotic diseases has already been described (21, 22). Thus, we decided to investigate whether the NLRP6 sensor plays a role in the course of *S. mansoni* infection and liver pathology. In this study, we demonstrate that lack of NLRP6 modulates the formation of hepatic granuloma, influencing local chemokine and cytokine production as well as macrophage and neutrophil recruitment into the liver. Also, this receptor is important for promoting collagen deposition.

MATERIALS AND METHODS

Ethics Statement

This study was carried out in accordance with Brazilian laws #6638 and #9605 in Animal Experiments. The protocol was approved by the Committee on Ethics of Animal Experiments of the Universidade Federal de Minas Gerais (UFMG) (Permit Number: #367/2017).

Mice and Parasite

Wild-type C57BL/6 mice were purchased from the Universidade Federal de Minas Gerais (UFMG). *Nlrp3*$^{-/-}$, *Nlrp6*$^{-/-}$, *Casp-1*$^{-/-}$, *IL-1R*$^{-/-}$, and *Gsdmd*$^{-/-}$ were described previously (23–26). The animals were maintained at UFMG and used at 6–10 week of age. *Schistosoma mansoni* (LE strain) cercariae at Fundação Oswaldo Cruz – Centro de Pesquisas René Rachou (CPqRR-Brazil) were routinely obtained from infected *Biomphalaria glabrata* snails exposed to light, inducing the shedding of parasites.

Eggs, SEA, and SWAP

Eggs were obtained from 50-day-infected Swiss mice livers. Briefly, the liver was blender processed in cold saline (2% NaCl) for 2 min. Next, the material was decanted into a glass goblet for 35 min at low temperature. Part of the decanting supernatant was discarded and the remaining solution was washed with cold saline. Decantation-washing was repeated until reaching a translucent solution. Eggs were recovered by filtration. For the preparation of Soluble Egg Antigens (SEA), eggs were disrupted for 40 min at low temperature in PBS and then the homogenate was centrifuged at 100,000 × *g* for 1 h at 4°C. The resulting supernatant was frozen at −80°C. Soluble adult worm antigen (SWAP) was obtained by mechanical maceration of worms in cold PBS. After centrifugation (13,000 × *g* – 7 min), the supernatant was collected and stored at −80°C. The protein concentration of SEA and SWAP was determined using BCA™ protein assay kit (Thermo Fisher Scientific, Waltham, MA, United States).

BMDC Generation and Activation

To obtain bone marrow-derived dendritic cells (BMDCs), bone marrow cells were cultured in RPMI with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin and 20 ng/mL murine

recombinant GM-CSF (Peprotech, Riberão Preto, Brazil). Petri dishes containing 1×10^7 cells were incubated at 37°C in 5% CO_2. At day 3 of incubation, 5 mL of fresh complete medium with GM-CSF was added, and 5 mL of medium was replaced with fresh supplemented medium containing GM-CSF on days 5 and 7. At day 10, non-adherent cells were harvested and seeded in 24-well plates (5×10^5 cells/well). Stimulation of BMDCs was performed by priming cells with 1 μg/ml of Pam_3Cys (Sigma-Aldrich, St. Louis, MO, United States) for 5 h and then stimulating with 50 μg/mL of SEA for 17 and 24 h. As positive control for inflammasome activation, cells were primed with 1 μg/ml of Pam_3Cys (5 h) or 1 μg/ml of LPS (4 h) and stimulated with ATP (5 mM) (50 min) or Nigericin (20 μM) (50 min). Culture supernatants were collected and cells were lysed with M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific) supplemented with 1:100 protease inhibitor mixture (Sigma-Aldrich).

Western Blotting

Cell lysates and supernatants from DCs culture were subjected to SDS-PAGE analysis and western blotting. The proteins were resolved on a 15% SDS-PAGE gel, and transferred to nitrocellulose membranes (Amersham Biosciences, Uppsala, Sweden). Membranes were blocked for 1 h in TBS (0.1% Tween-20; 5% non-fat dry milk) and incubated with primary antibodies at 4°C, overnight. Primary antibody used was mouse monoclonal against the p20 subunit of caspase-1 (Adipogen, San Diego, CA, United States). Monoclonal antibody against β-actin (Cell Signaling Technology, Danvers, MA, United States) was used as a loading control blot (1:1,000). The membranes were washed three times for 10 min in TBS with 0.1% Tween 20. Next, membranes were incubated for 1 h at room temperature with the suitable HRP-conjugated secondary antibody (1:1,000). Immunoreactive bands were visualized using Luminol chemiluminescent HRP substrate (Millipore).

Splenocyte Culture

Spleen cells were obtained from macerated spleens of individual C57BL/6 and *Nlrp6*$^{-/-}$ mice after 6 weeks of infection with *S. mansoni* cercariae (n = 5/group). Cells were washed with PBS and the erythrocytes were lysed with a hemolytic solution (155 mM NH4Cl, 10 mM KHCO3, pH 7.2). Cells were adjusted to 1×10^6/well in complete RPMI medium (10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin). Spleen cells were cultured in 96-well plates with medium and stimulated with SWAP (200 μg/mL), SEA (20 μg/mL), Eggs (50/well) or concanavalin A (ConA) (5 μg/mL). Culture supernatants were collected after 24 h for IL-5 and after 72 h for IFN-γ, IL-10, and IL-13 measurements by ELISA.

Liver Processing

Right lobe of liver from 6-week-infected C57BL/6 and *Nlrp6*$^{-/-}$ mice was collected and 1 mL of cytokine extraction solution (0.4 M NaCl, 0.05% Tween 20, 0.5% BSA, 0.1 mM PMSF, 0.1 mM benzethoniumchloride, 10 mM EDTA and 20 KI aprotinin) was added to each 100 mg of tissue. Ultra-Turrax homogenizer-dispenser was used to homogenize solutions containing the organs. Next, the samples were centrifuged at 10,000 × g for 10 min at 4°C. Non-parenchymal cells from left lobe were used for flow cytometry analysis. Tissue was removed without perfusion, cut into small pieces, incubated in RPMI medium containing 30 μg/ml of Liberase TM (Roche) and 20 U/mL of DNAse I (GE) for 40 min, and passed through a 70 μm pore-size cell strainer. After centrifugation, the cells were resuspended in PBS containing 2% fetal bovine serum (FBS) and 5 mM EDTA. Low-speed centrifugation (50 × g – 5 min) was used to remove parenchymal cells. Erythrocytes were lysed with a hemolytic solution (155 mM NH_4Cl, 10 mM $KHCO_3$, pH 7.2). The remaining non-parenchymal cells were resuspended in RPMI culture medium.

Cytokine Measurements

Cytokine/Chemokine production was evaluated using the Duoset ELISA kit (R&D Diagnostic, Minneapolis, MN, United States) according to the manufacturer's instructions.

EPO and MPO Activity Assays

Eosinophilic Peroxidase and Myeloperoxidase assays were performed as described by Cançado et al. (27). Right lobe of the liver was homogenized, red blood cells subjected to hypotonic lysis and the remaining liver cells subjected to detergent lysis and freeze-thaw cycles. The enzymatic assay was performed using the suitable substrates and the result was measured on a microplate reader at the appropriate wavelength (492 nm for EPO and 450 nm for MPO). The result was expressed in absorbance units.

Flow Cytometry Analysis

Spleen and non-parenchymal liver cells were stained for CD11b, CD11c, Ly6G, F4/80, CD3, and CD4. Briefly, cells were incubated for 20 min with anti-mouse CD16/32 (BD Biosciences) in FACS buffer (PBS, 1% FBS, 1 mM NaN3) and were stained for surface markers for 20 min using: APC-Cy7-conjugated anti-mouse CD11b (1:200, M1/70; BD Biosciences), FITC-conjugated anti-mouse CD11c (1:100, HL3; BD Biosciences), PE-conjugated anti-mouse Ly6G (1:200, 1A8; BD Biosciences), biotinylated anti-mouse F4/80 (1:200, BM8; BD Biosciences), PE-Cy7-conjugated anti-mouse CD3 (1:100, BD Biosciences) and APC-conjugated anti-mouse CD4 (1:200, BD Biosciences). The appropriate isotype controls were used. Next, cells were washed and incubated for 20 min at 4°C in the dark with PerCP-Cy5.5 conjugated streptavidin (1:200 BD Biosciences). Lastly, cells were washed and resuspended in PBS. Attune Flow Cytometer (Applied Biosystems, Waltham, MA, United States) was used for collecting approximately 100,000 events and data were analyzed using FlowJo software (Tree Star, Ashland, OR, United States).

In order to evaluate macrophage polarization, non-parenchymal liver cells were stained as described above using APC-Cy7-conjugated anti-mouse CD11b (1:200, M1/70; BD Biosciences), biotinylated anti-mouse F4/80 (1:200, BM8; BD Biosciences), BB700-conjugated anti-mouse CD197 (1:200, 4B12

BD Biosciences), FITC-conjugated anti-mouse CD80 (1:200, 16-10A1, BD Biosciences), PE-conjugated anti-mouse CD163 (1:200, TNKUPJ, eBioscience) and APC-conjugated anti-mouse CD206 (1:200, MR5D3, BD Biosciences).

Quantitative Real-Time PCR

Liver middle lobe of 6-week-infected C57BL/6 and *Nlrp6*$^{-/-}$ mice was used for RNA extraction. The tissue was homogenized in TRIzol (Invitrogen) and total RNA was isolated in accordance with the manufacturer's instructions. Reverse transcription of total RNA was performed and quantitative real-time RT-PCR was conducted in a final volume of 20 μL containing SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, United States), oligo-dT cDNA as the PCR template and 2.5 μM of primers. The PCR reaction was performed with QuantStudio3 real-time PCR instrument (Applied Biosystems). Primers were used to amplify a specific fragment (100–120 bp) corresponding to specific gene targets as follows: *18S* Forward (5′-CGTTCC ACCAACTAAGAACG-3′) and *18S* Reverse (5′-CTCAACACGG GAAACCTCAC-3′; α-*SMA* Forward (5′-GTCCCAGACATCAG GGAGTAA-3′) and α-*SMA* Reverse (5′-TCGGATACTTCA GCGTCAG-3′); *IL-13* Forward (5′-CCTGGCTCTTGCTTGCC-3′) and *IL-13* Reverse (5′-GGTCTTGTGTGATGTTGCTCA-3′); *IL-1*β Forward (5′-TGACCTGGGCTGTCCAGATG-3′) and *IL-1*β Reverse (5′-CTGTCCATTGAGGTGGAGAG-3′); *Casp-1* Forward (5′-GGAAGCAATTTATCAACTCAGTG-3′) and *Casp-1* Reverse (5′-GCCTTGTCCATAGCAGTAATG-3′).

Mice Infection and Parasite Burden

Six-to-eight-week-old wild-type and knockout mice were anesthetized with 5% ketamine, 2% xylazine and 0.9% NaCl and then infected with 100 cercariae (LE strain) through exposure of percutaneous abdominal skin, for 1 h. After 6 weeks of infection, mice were euthanized and perfused from the portal veins, the recovered worms were counted and the mean difference between groups of mice was evaluated.

Pathological Parameters

Number of eggs was obtained from liver median lobe. The tissue was weighed and digested in an aqueous solution of KOH (5%) for 16 h at 37°C. After, eggs were washed in saline and centrifuged twice at 270 × *g* for 10 min and counted using a light microscope. The number of calculated eggs was corrected by considering the mass of the tissue, resulting in number of eggs per gram of liver. The left lobe was fixed with 10% buffered formaldehyde in PBS. Histological sections were performed using microtome at 6 μm and stained on a slide with Hematoxylin-Eosin (HE) and Masson blue. For measurement of granuloma size and collagen deposition, a JVC TK-1270/RBG camera, attached to the microscope (10 × objective lens), was used to obtain the images. Analysis were carried out using ImageJ software (U.S. National Institutes of Health, Bethesda, MD, United States)[1]. Granuloma size was measured, in μm^2, for all granulomas found in liver sections.

[1]http://rsbweb.nih.gov/ij/index.html

Statistical Analysis

The statistical tests were performed using Student's *t-test*, one-way and two-way ANOVA followed by Bonferroni adjustments for comparison between groups. *P*-values obtained were considered significant if they were <0.05. Statistical analysis was performed using GraphPad Prism 6 (La Jolla, CA, United States).

RESULTS

IL-1β Production and Caspase-1 Activation Are Partially Dependent on NLRP6 in SEA/Eggs-Stimulated Dendritic Cells

During *S. mansoni* infection, hepatic dendritic cells (DCs) are the main cells responsible for promoting the shift from Th1 to Th2 immune profile, which is triggered by egg antigens (28). Ritter and colleagues (2010) reported that SEA induces inflammasome activation in DCs and described the involvement of NLRP3 (9). Thus, given the relevance of these cells, we decided to use bone marrow-derived dendritic cells (BMDCs) in order to investigate whether the NLRP6 sensor is involved in IL-1β secretion in response to different parasite antigens. Using Pam_3Cys (P_3Cys) as the first signal, we observed that eggs and their soluble antigens (SEA) induce high levels of IL-1β and this production was partially influenced by NLRP6 (**Figure 1A**). In contrast, IL-1β production induced by soluble adult worm antigens (SWAP) was much lower compared to SEA (**Figure 1A**). Additionally, TNF-α levels were not altered comparing both WT and NLRP6 knockout (KO) mice (**Figure 1B**). Since SEA was sufficient to induce IL-1β production, we used this stimulus to evaluate the role of NLRP6 in caspase-1 (casp-1) activation. **Figures 1C,D** demonstrate that SEA induces activation of casp-1 in WT DCs and this process was clearly inhibited in *Nlrp6*$^{-/-}$ DCs.

NLRP6 Influences Granuloma Formation and Collagen Deposition in the Liver

Since the liver is one of the main entrapment tissues for parasite's eggs and once the participation of NLRP6 in the egg and SEA-induced immune response has been observed, we wondered if this sensor could play any role in liver pathology. First, we observed that in livers of 4- and 6-week infected animals the levels of *IL-1*β and *caspase-1* mRNA did not significantly change between *Nlrp6*$^{-/-}$ and WT mice (**Supplementary Figure S1**). Initially, we observed that the number of eggs per gram of tissue was not altered between WT and knockout mice (**Figure 2A**). Consistently, the worm burden recovery was the same comparing both groups (**Supplementary Figure S2**). On the other hand, NLRP6 has been shown to influence the periovular inflammatory response, contributing significantly to granuloma formation (**Figure 2B**). In addition, collagen deposition within the granulomatous structure was reduced in *Nlrp6*$^{-/-}$ mice when compared to WT (**Figures 2B,C,H**). We also evaluated levels of cytokines and fibrotic markers within the tissue, such as the cytokines IL-5, IL-10, IL-13, and the protein Alpha-smooth muscle actin (α-SMA). IL-5 and IL-10 cytokines

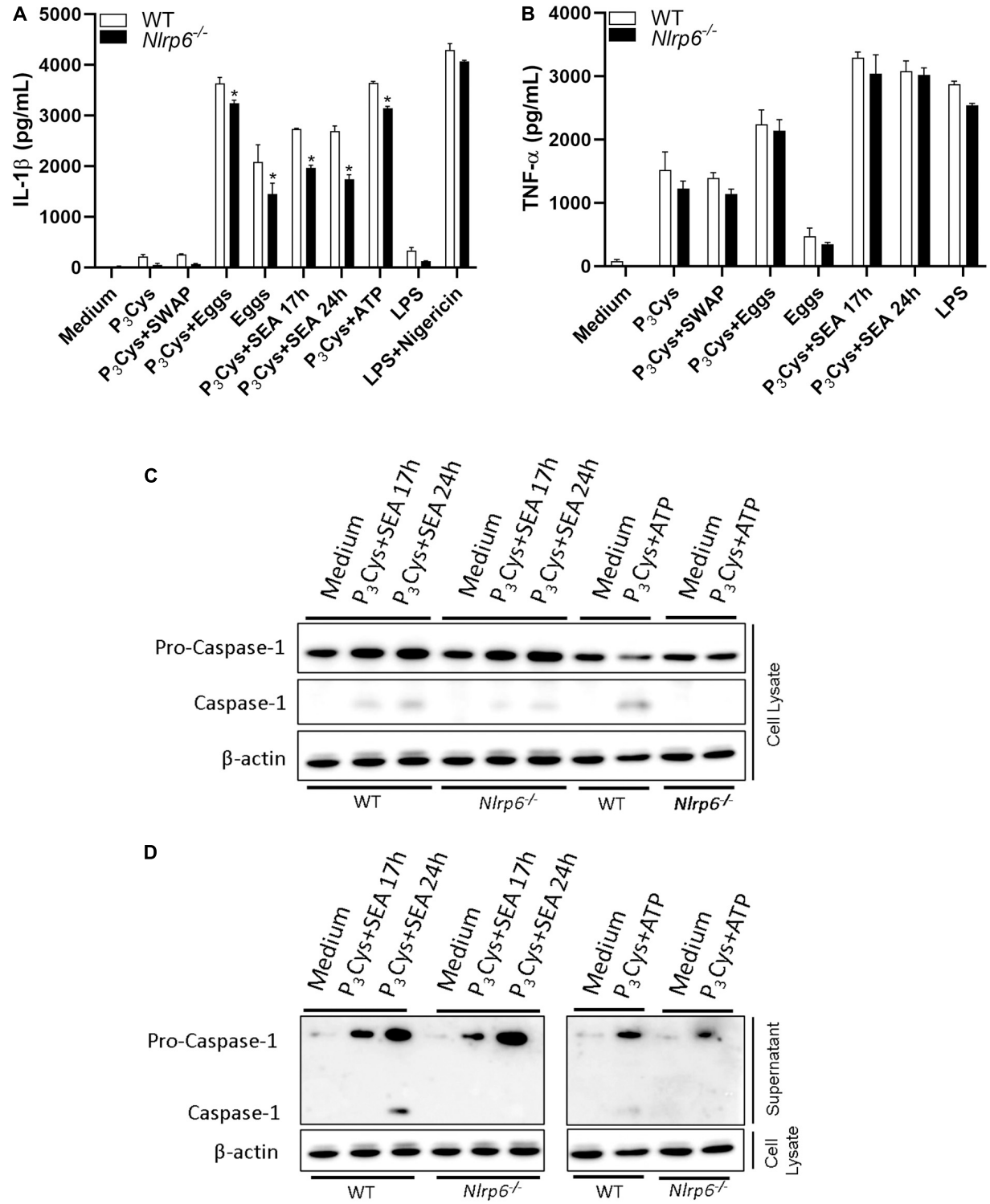


FIGURE 1 | NLRP6 regulates IL-1β production and Casp-1 activation induced by SEA. WT or *Nlrp6*$^{-/-}$ deficient BMDCs were primed with P_3Cys (1 μg/ml – 5 h) and stimulated with SEA (50 μg/mL – 17 h, 24 h), Eggs (100 eggs/well – 24 h), SWAP (200 μg/mL – 24 h) or ATP (5 mM – 50 min). For nigericin control (20 μM), cells were primed with LPS (1 μg/ml – 4 h) and stimulate for 50 min. **(A)** IL-1β and **(B)** TNF-α were measured by ELISA. Casp-1 activation was analyzed by western blot in **(C)** cell lysate and **(D)** supernatant using antibody against p20 subunit. An asterisk denotes statistically significant differences between NLRP6 versus WT animals ($p < 0.05$).

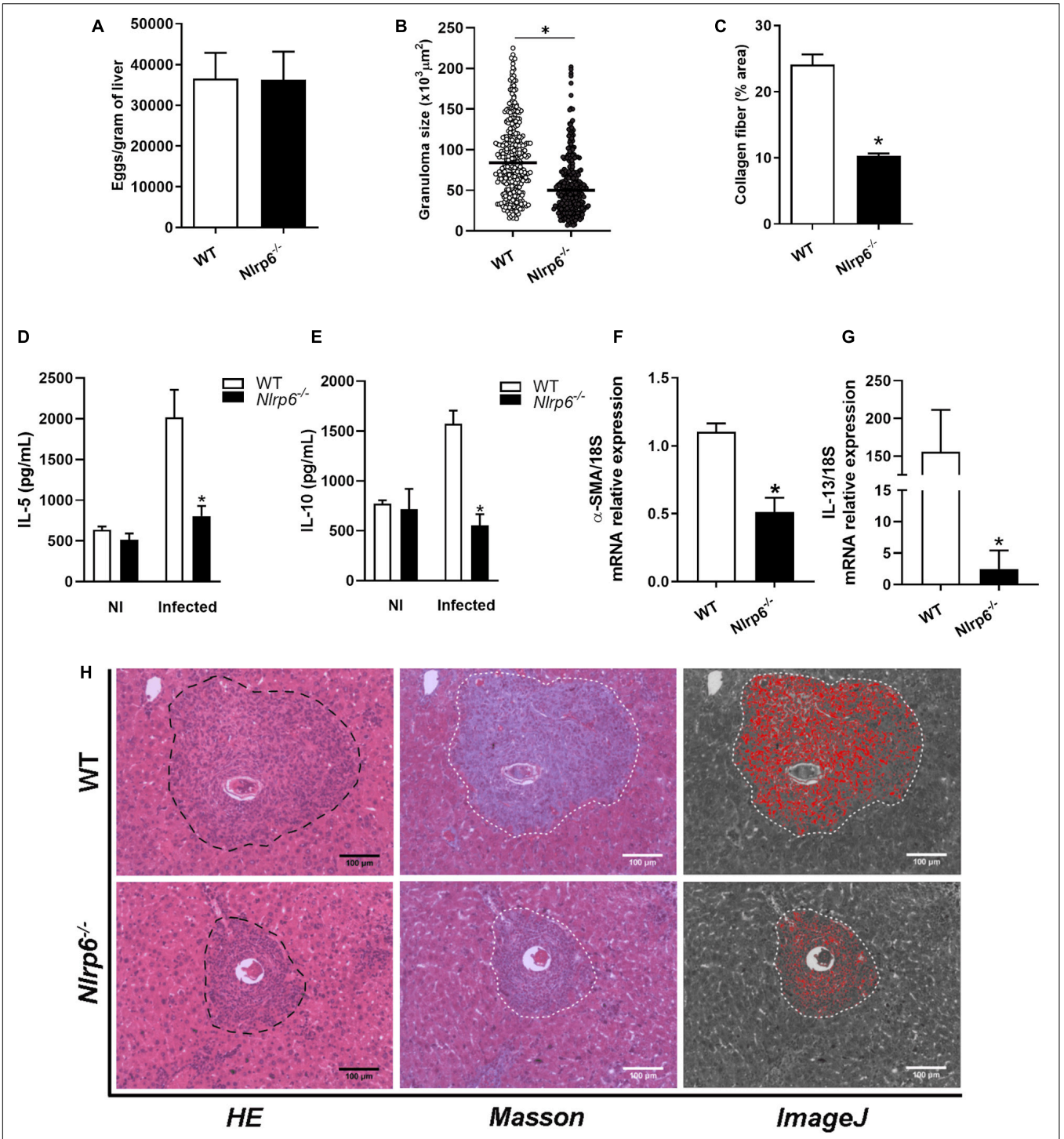


FIGURE 2 | *Schistosoma*-induced liver pathology is influenced by NLRP6 sensor. Pathological and molecular parameters were evaluated, after 6 weeks of infection in WT and *Nlrp6*$^{-/-}$ mice. **(A)** Number of eggs per gram of liver, **(B)** Granuloma size (μm^2) and **(C)** Collagen deposition. **(D)** IL-5 and **(E)** IL-10 cytokine levels were detected. **(F)** α-SMA and **(G)** IL-13 transcripts were also measured. **(H)** Representative images of granulomas detected in hematoxylin-eosin, masson blue and the output from ImageJ software, respectively. An asterisk denotes statistically significant differences between NLRP6 versus WT animals ($p < 0.05$). The bar represents 100 μm. NI stands for Non-infected mice.

levels, as well as, α-SMA and IL-13 mRNA measurements were reduced in *Nlrp6*$^{-/-}$ group when compared to WT (**Figures 2D–G**). These data demonstrate that NLRP6 contributes to the pathology caused by *S. mansoni*.

NLRP6 Influences IL-10 and IFN-γ Production by Spleen Cells Activated With Egg Antigens

After determining the impact of NLRP6 on inflammasome activation and granuloma formation in response to egg antigens, we decided to investigate the frequency of dendritic cells and CD4$^+$ T lymphocytes in spleen cells derived from *S. mansoni* infected mice. Cells were obtained following the gate strategy described in **Supplementary Figure S3**. We observed that 6-week-infected *Nlrp6*$^{-/-}$ and WT mice presented no significant difference regarding CD11b$^+$CD11c$^+$ (dendritic cells) and CD3$^+$CD4$^+$ (CD4$^+$ lymphocytes) cell populations (**Figures 3A,B**). Interestingly, when spleen cells were stimulated with eggs or SEA, the cytokine production was altered. Both antigens induced increased levels of IL-10 and IFN-γ in splenocyte culture supernatants from *Nlrp6*$^{-/-}$ compared to WT mice (**Figures 3E,F**). Additionally, NLRP6 appears to have no effect on IL-5 and IL-13 production (**Figures 3C,D**). Curiously, when *Nlrp6*$^{-/-}$ and WT spleen cells from *S. mansoni* infected mice were stimulated with SWAP, no significant difference on IFN-γ, IL-10, IL-5, and IL-13 levels was observed (**Supplementary Figure S4**). These data emphasize the relevance of NLRP6 during egg antigen response regulating IL-10 and IFN-γ production. Furthermore, enhanced IL-10 production in *Nlrp6*$^{-/-}$ may be related to reduced granuloma formation and fibrosis.

NLRP6 Mediates Innate Immune Cells Recruitment in *Schistosoma*-Infected Liver

Since lack of NLRP6 has been shown to modulate hepatic granuloma formation, we decided to investigate how this sensor influences liver pathology. Initially, we evaluated the level of chemokines (CCL2, CCL3, CCL11, and CXCL1) in livers of *Schistosoma*-infected mice. These chemokines have already been described as related to granuloma formation. In *Schistosoma*-infected *Nlrp6*$^{-/-}$ mice only the production of CCL11 was not reduced in comparison to WT mice (**Figure 4D**). CCL2, CXCL1, and CCL3 were diminished in *Nlrp6*$^{-/-}$ mice compared to WT animals (**Figures 4A–C**). Additionally, we observed that the enzymes MPO and EPO were also reduced in *Nlrp6*$^{-/-}$ mice when compared to WT (**Figures 4E,F**). Our next step was to evaluate which non-parenchymal cell populations could be altered in *Schistosoma*-infected *Nlrp6*$^{-/-}$ mice. Cells were obtained following the gate strategy described in **Supplementary Figure S5**. Neutrophils and macrophages were the major cell populations reduced in *Nlrp6*$^{-/-}$ mice when compared to WT (**Figures 4G,H**). The frequency of dendritic cells and CD4$^+$ T lymphocytes remained unaltered in either mouse groups (**Figures 4I,J**). We also found that the lack of NLRP6 does not affect macrophage polarization, even though there is a strong tendency of reduction in anti-inflammatory macrophages (CD206$^+$CD163$^+$) in *Nlrp6*$^{-/-}$ compared to WT mice (**Supplementary Figure S6**). Therefore, the NLRP6 sensor possibly induces the formation of hepatic granuloma by favoring chemokine production and the recruitment of immune cells to the liver.

Inflammasome Pathway Is Broadly Relevant to Granuloma Formation

The NLR family receptors perform their functions depending on tissue and cell type, as already demonstrated for NLRP3 and NLRP6 (29, 30). For this reason, we decided to evaluate whether other inflammasome pathway-related molecules, such as Casp-1, GSDMD, and IL-1R, played a role in the formation of hepatic granuloma. Similarly to what we observed here for *Nlrp6*$^{-/-}$ mice, a significant reduction in periovular inflammatory response in *Casp-1*$^{-/-}$, *Gsdmd*$^{-/-}$, and *IL-1R*$^{-/-}$ mice was observed. In addition, a reduction in collagen deposition was observed in the granulomas of *Casp-1*$^{-/-}$, *Gsdmd*$^{-/-}$, and *IL-1R*$^{-/-}$ mice when compared to WT (**Figures 5B–D**). This was accompanied by no alteration in the number of eggs in the tissue (**Figure 5A**), and worm burden recovery (**Supplementary Figure S2**) when compared to WT mice.

DISCUSSION

The role of the inflammasome pathway in the pathogenesis of chronic liver diseases has been investigated in the last few years (31, 32). Hepatic injuries from different sources are capable of leading to inflammasome activation, as described for drug-induced damage (33), ischemia-reperfusion (34), alcoholic and non-alcoholic fatty liver disease (15, 16), and viral hepatitis (14). Inflammasome triggers or amplify liver diseases by releasing pro-inflammatory cytokines such as IL-1β, IL-1α, IL-18, and also through other inflammatory mediators such as High Mobility Group Box 1 (HMGB1) (35). Release of such cytokines and DAMPs occur, in part, due to Gasdermin-D cleavage and subsequent pyroptosis of the cell (36). The fibrotic process resulting from chronic liver diseases has also involved the inflammasome pathway. IL-1β and danger signals induce Hepatic Stellate Cells (HSC) to transdifferentiate and perform extracellular matrix remodeling function. In addition, HSCs can internalize pre-formed inflammasome complexes released by other pyroptosis dying cells (37–39).

The participation of the NLRP3 receptor in chronic liver diseases such as non-alcoholic fatty liver disease (NAFLD) is well described (16, 17). On the other hand, the role of the NLRP6 sensor in these liver pathologies is still elusive. Recently, Xiao and colleagues (2018) reported that in the NAFLD model induced by methionine-choline deficient (MCD) diet, NLRP3 and NLRP6 expression is highly detected in the liver. After *Lycium barbarum* polysaccharides (LBP) treatment, NAFLD condition improves and the expression of both NLR receptors decreases (20). Besides, a previous study with NAFLD obese patients demonstrates that when hepatic portal fibrosis is present, the expression of *NLRP6* mRNA in adipose tissues is higher compared to cases when

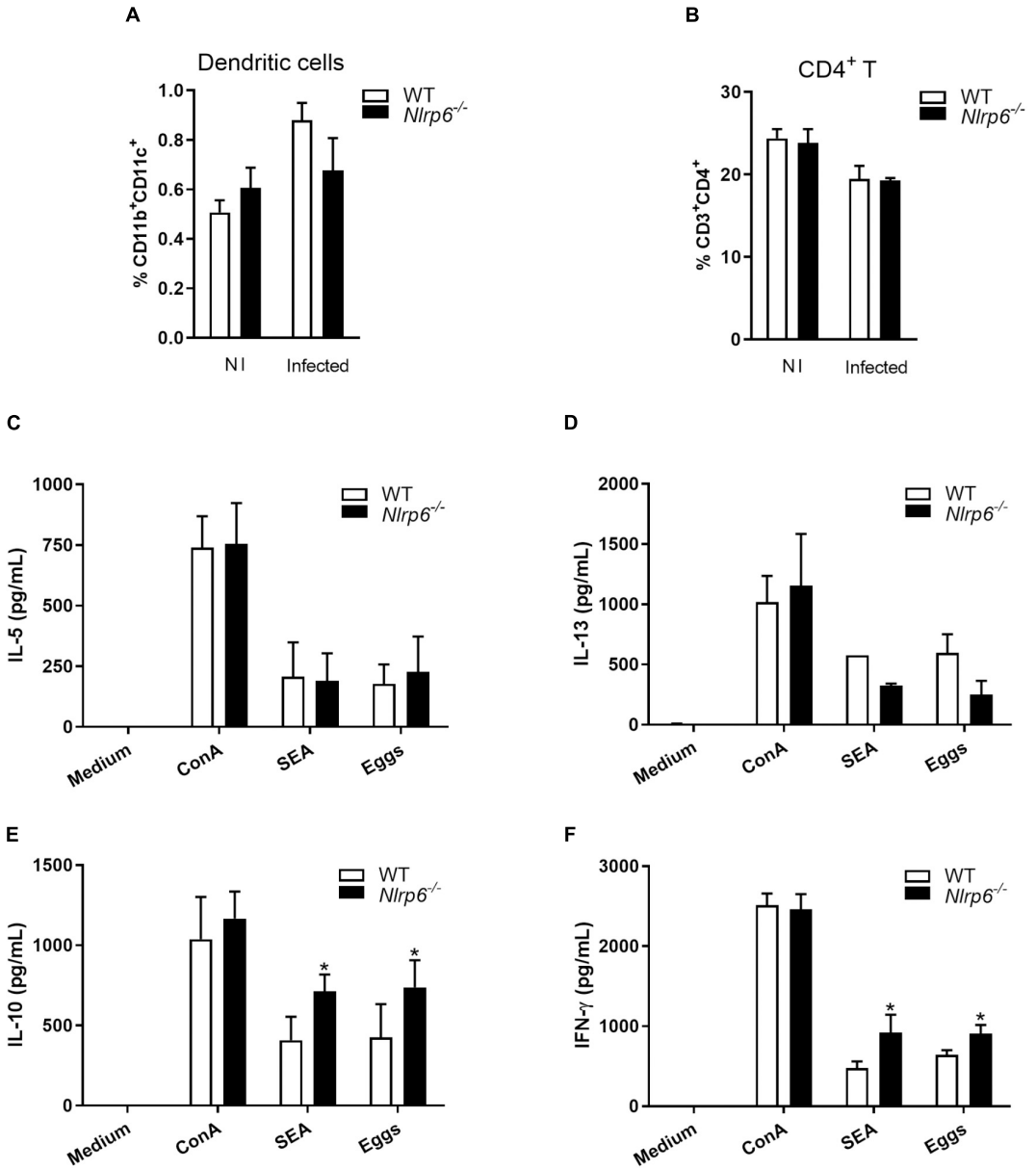


FIGURE 3 | Cytokine profile induced by SEA/Eggs in *Nlrp6*$^{-/-}$ mice. Six-weeks post infection the percentage of **(A)** dendritic cells (CD11b^{+}CD11c^{+}) and **(B)** CD4^{+} T lymphocytes (CD3^{+}CD4^{+}) in spleens from non-infected (NI) and infected animals were analyzed by flow cytometry. Spleen cells of infected mice were restimulated with ConA (5 μg/mL), SEA (20 μg/mL) or Eggs (50/well). Cytokine levels were measured by ELISA in cell supernatants from antigen restimulated cells, **(C)** IL-5, **(D)** IL-13, **(E)** IL-10 and **(F)** IFN-γ. An asterisk denotes statistically significant differences between NLRP6 versus WT animals ($p < 0.05$).

hepatic portal fibrosis is not observed (21). These findings suggest that NLRP6 might play important role in chronic liver disease and fibrosis. All those findings intrigued us to evaluate the role of this sensor in *S. mansoni* infection.

Schistosoma mansoni and *S. japonicum* infections are sources of injury and are able to induce chronic liver disease. The long survival period of *Schistosoma* worms within the human host implies recurrent inflammation and wound-healing cycles in the liver, triggered by egg antigens which can result in fibrosis, portal hypertension and hepatosplenomegaly (4, 8). Among non-parenchymal liver cells, DCs stand out as key cells during this pathological process. Broadly responsive to egg antigens, DCs are essential for promoting systemic shift in the immune response profile (from Th1 to Th2), which is crucial for host survival upon infection (28, 40). In addition, the first report of inflammasome pathway activation by *S. mansoni* antigens involved dendritic cells responding to SEA. For these reasons, we initially decided to evaluate the role of NLRP6 in DCs. Our findings suggest that NLRP6 is important for the formation of the inflammasome complex, since it influences IL-1β secretion

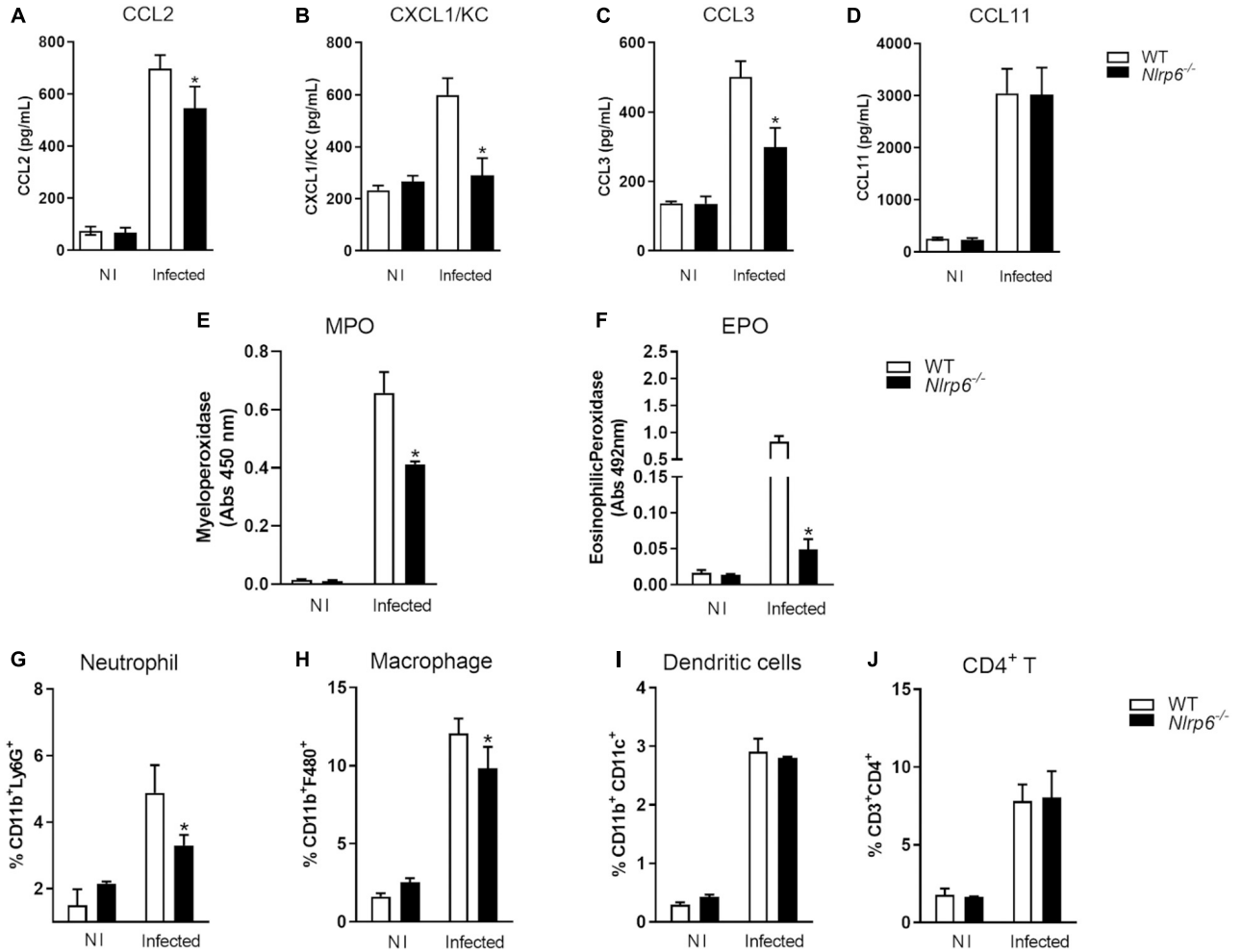


FIGURE 4 | NLRP6 regulates chemokine production and immune cell recruitment into the liver. After 6 weeks of infection, the liver was used to measure chemokine levels, MPO and EPO activities and the frequency of immune cells. Levels of **(A)** CCL2, **(B)** CXCL1 **(C)** CCL3 and **(D)** CCL11 were measured by ELISA. **(E,F)** MPO and EPO activities were also detected. The percentage of **(G)** $CD11b^+Ly6G^+$, **(H)** $CD11b^+F4/80$, **(I)** $CD11b^+CD11c^+$ and **(J)** $CD3^+CD4^+$ T lymphocyte populations were measured by flow cytometry. An asterisk denotes statistically significant differences between NLRP6 versus WT animals ($p < 0.05$). NI stands for Non-infected mice.

and caspase-1 activation in response to SEA. Previous studies demonstrate that NLRP6 structurally has the ability to form the inflammasome complex and does so in response to gram-positive bacteria cell wall components, activating both caspase-1 and caspase-11 in the same complex (41, 42).

The activation of intracellular receptors was not expected to occur in response to multicellular parasites such as *S. mansoni*. Surprisingly, Ritter and colleagues (2010) demonstrated that SEA triggers NLRP3 inflammasome pathway in DCs (9). Following this seminal study, NLRP3 inflammasome role has been investigated, especially in *S. japonicum* infection. In this context, NLRP3 has been shown to be pivotal for inducing hepatic granuloma formation and collagen deposition in the granulomatous structure (18, 43). In this study, we confirmed that NLRP3 is pivotal for hepatic granuloma formation, but we did not find any alteration in collagen deposition in *Nlrp3*$^{-/-}$ mice when compared to WT (**Supplementary Figure S7**). This observation may be related to the early time point infection of our model (6 weeks), which also could explain the distribution of collagen throughout the granuloma structure and not only peripherally, as typically observed in later granulomas. Surprisingly, we have demonstrated for the first time that NLRP6 also plays an important role in *S. mansoni*-induced pathology. We found that this sensor influences the formation of hepatic granuloma, altering local chemokine (CCL2, CCL3, and CXCL1) and cytokine (IL-5, IL-10, and IL-13) production, macrophage and neutrophil recruitment into the liver, and also is important for promoting collagen deposition. In *Schistosoma* egg-induced pathology, chemokine production is essential to modulate granuloma formation (44, 45). CCL3-deficient mice, for instance, showed size reduced granuloma, lower fibrosis and lower EPO activity in the liver (46). Although classically responsible for neutrophil recruitment, CXCL1 also impacts on the recruitment of HSCs, which are responsible for collagen

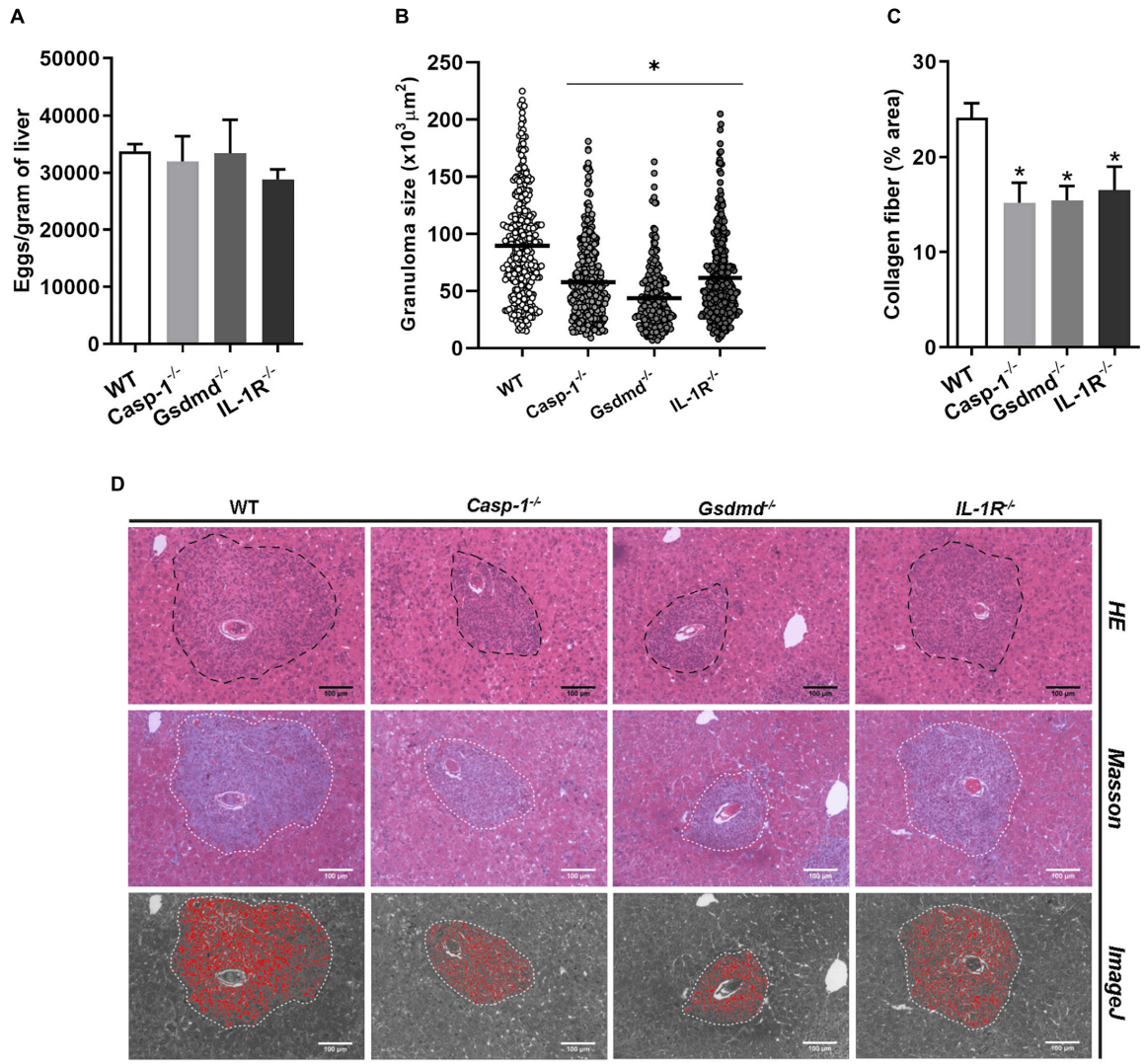


FIGURE 5 | Inflammasome activation influences granuloma formation and collagen deposition. Pathological parameters were analyzed for other inflammasome components such as Casp-1, GSDMD and IL-1R in mouse livers. **(A)** Number of eggs per gram of liver, **(B)** Granuloma size and **(C)** collagen deposition were measured in WT and Casp-1, GSDMD and IL-1R deficient animals. **(D)** Representative images of granulomas detected in hematoxylin and eosin, masson blue and the output from ImageJ software, respectively. An asterisk denotes statistically significant differences between deficient mice versus WT animals ($p < 0.05$). The bar represents 100 μm.

deposition in the granuloma structure (47). Following injury, hepatic resident cells produce CCL2, important for monocyte and macrophage recruitment (48).

NLRP6 is known as an atypical sensor with wide functional capability, performing activities integrated and/or independent on the inflammasome complex (30). Our findings demonstrate that Casp-1 activation and IL-1β production, in response to SEA, can be regulated by NLRP6. Therefore, our findings suggest that the role of this receptor in hepatic granuloma formation is due to the inflammasome activation, since *Nlrp3*$^{-/-}$ mice have demonstrated a similar phenotype. It is clear that the inflammasome pathway is important for *Schistosoma*-induced liver pathology, once we have observed granuloma reduction and lower collagen deposition in *Casp-1*$^{-/-}$, *Gsdmd*$^{-/-}$, and *IL-1R*$^{-/-}$ mice when compared to WT. Previous liver disease studies support our observations for *Casp-1*$^{-/-}$ and *IL-1R*$^{-/-}$ mice. In a high fat diet-induced NASH model, deficient Casp-1 animals showed improvement in hepatic steatosis, inflammation and fibrogenesis (49). Similarly, IL-1R signaling has been shown to be critical for the progression of steatohepatitis and hepatic fibrosis in hypercholesterolemic mice (50). The role of Gasdermin- D and pyroptosis has also been described in chronic liver diseases (51, 52). During human NAFLD/NASH, GSDMD and its N-terminal peptide (GSDMD-N) are upregulated, besides MCD-fed *Gsdmd*$^{-/-}$ mice showed decreased severity of steatosis and inflammation comparing to WT (52). During *Schistosoma* infection, Liu and colleagues (2019) demonstrated that *S. japonicum* induces expression of the GSDMD-N in the

liver, and that this expression is modulated by NLRP3 sensor (53). In addition, it has been reported that SEA from *S. japonicum* eggs induces pyroptosis in HSCs (54). However, no *in vivo* mouse study has been reported correlating GSDMD deficiency and granuloma formation and fibrosis induced by *S. mansoni* infection as demonstrated here.

In summary, the data presented here demonstrate that lack of NLRP6 modulates activation of the inflammasome pathway in response to *S. mansoni* egg antigens. In addition, NLRP6 and the inflammasome components are important in liver pathology induced by *S. mansoni* infection. Taken together, these data reinforce the relevance of understanding the inflammasome signaling pathway, given its potential to influence the severe pathological conditions induced by this disease.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by the Committee on Ethics of Animal Experiments of the Federal University of Minas Gerais (Permit Number: #367/2017) and carried out in accordance with Brazilian laws #6638 and #9605 in Animal Experiments.

AUTHOR CONTRIBUTIONS

RS and SO designed the project and experiments, and wrote the manuscript. RS, CS, FVM, FSM, EG, and SM carried out most of the experiments. RS carried out statistical analysis and prepared the figures. SO submitted this manuscript. All authors reviewed the manuscript.

FUNDING

This study was funded by Conselho Nacional de Desenvolvimento Científico e Tecnológico grants #302660/2015-1 and 465229/2014-0, and Fundação de Amparo à Pesquisa do Estado de São Paulo grant # 2017/24832-6.

ACKNOWLEDGMENTS

We thank Dr. Cristina T. Fonseca from Fundação Oswaldo Cruz – Centro de Pesquisas René Rachou (CPqRR-Brazil) for providing *S. mansoni* cercariae to carry out the experiments.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00795/full#supplementary-material

REFERENCES

1. Hotez PJ, Alvarado M, Basáñez M-G, Bolliger I, Bourne R, Boussinesq M, et al. The global burden of disease study 2010: interpretation and implications for the neglected tropical diseases. *PLoS Negl Trop Dis*. (2014) 8:e2865. doi: 10.1371/journal.pntd.0002865
2. Steinmann P, Keiser J, Bos R, Tanner M, Utzinger J. Schistosomiasis and water resources development: systematic review, meta-analysis, and estimates of people at risk. *Lancet Infect Dis*. (2006) 6:411–25. doi: 10.1016/S1473-3099(06)70521-7
3. Utzinger J, Raso G, Brooker S, De Savigny D, Tanner M, Ørnbjerg N, et al. Schistosomiasis and neglected tropical diseases: towards integrated and sustainable control and a word of caution. *Parasitology*. (2009) 136:1859–74. doi: 10.1017/S0031182009991600
4. Colley DG, Bustinduy AL, Secor WE, King CH. Human schistosomiasis. *Lancet*. (2014) 383:2253–64. doi: 10.1016/S0140-6736(13)61949-2
5. Hams E, Aviello G, Fallon PG. The *Schistosoma* granuloma: friend or foe? *Front Immunol*. (2013) 4:89. doi: 10.3389/fimmu.2013.00089
6. Moore DV, Sandground JH. The relative egg producing capacity of *Schistosoma Mansoni* and *Schistosoma Japonicum* 1, 2. *Am J Trop Med Hyg*. (1956) 5:831–40. doi: 10.4269/ajtmh.1956.5.831
7. Almadi MA, Aljebreen AM, Sanai FM, Marcus V, AlMeghaiseeb ES, Ghosh S. New insights into gastrointestinal and hepatic granulomatous disorders. *Nat Rev Gastroenterol Hepatol*. (2011) 8:455. doi: 10.1038/nrgastro.2011.115
8. Wilson MS, Mentink−Kane MM, Pesce JT, Ramalingam TR, Thompson R, Wynn TA. Immunopathology of schistosomiasis. *Immunol Cell Biol*. (2007) 85:148–54. doi: 10.1038/sj.icb.7100014
9. Ritter M, Gross O, Kays S, Ruland J, Nimmerjahn F, Saijo S, et al. *Schistosoma mansoni* triggers Dectin-2, which activates the Nlrp3 inflammasome and alters adaptive immune responses. *Proc Natl Acad Sci USA*. (2010) 107:20459–64. doi: 10.1073/pnas.1010337107
10. Broz P, Dixit VM. Inflammasomes: mechanism of assembly, regulation and signalling. *Nat Rev Immunol*. (2016) 16:407. doi: 10.1038/nri.2016.58
11. Martinon F, Burns K, Tschopp J. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-β. *Mol Cell*. (2002) 10:417–26. doi: 10.1016/s1097-2765(02)00599-3
12. Luan J, Ju D. Inflammasome: a double-edged sword in liver diseases. *Front Immunol*. (2018) 9:2201. doi: 10.3389/fimmu.2018.02201
13. Maltez VI, Tubbs AL, Cook KD, Aachoui Y, Falcone EL, Holland SM, et al. Inflammasomes coordinate pyroptosis and natural killer cell cytotoxicity to clear infection by a ubiquitous environmental bacterium. *Immunity*. (2015) 43:987–97. doi: 10.1016/j.immuni.2015.10.010
14. Zalinger ZB, Elliott R, Weiss SR. Role of the inflammasome-related cytokines Il-1 and Il-18 during infection with murine coronavirus. *J Neurovirol*. (2017) 23:845–54. doi: 10.1007/s13365-017-0574-4
15. Petrasek J, Bala S, Csak T, Lippai D, Kodys K, Menashy V, et al. IL-1 receptor antagonist ameliorates inflammasome-dependent alcoholic steatohepatitis in mice. *J Clin Invest*. (2012) 122:3476–89. doi: 10.1172/JCI60777
16. Wree A, McGeough MD, Peña CA, Schlattjan M, Li H, Inzaugarat ME, et al. NLRP3 inflammasome activation is required for fibrosis development in NAFLD. *J Mol Med (Berl)*. (2014) 92:1069–82. doi: 10.1007/s00109-014-1170-1
17. Wu X, Dong L, Lin X, Li J. Relevance of the NLRP3 inflammasome in the pathogenesis of chronic liver disease. *Front Immunol*. (2017) 8:1728. doi: 10.3389/fimmu.2017.01728
18. Lu Y-Q, Zhong S, Meng N, Fan Y-P, Tang W-X. NLRP3 inflammasome activation results in liver inflammation and fibrosis in mice infected with *Schistosoma japonicum* in a Syk-dependent manner. *Sci Rep*. (2017) 7:8120. doi: 10.1038/s41598-017-08689-1
19. Chen H, Li Y, Gu J, Yin L, Bian F, Su L, et al. TLR4-MyD88 pathway promotes the imbalanced activation of NLRP3/NLRP6 via caspase-8 stimulation after

alkali burn injury. *Exp Eye Res*. (2018) 176:59–68. doi: 10.1016/j.exer.2018.07.001

20. Xiao J, Wang F, Liong EC, So K-F, Tipoe GL. *Lycium barbarum polysaccharides improve hepatic injury through NFkappa-B and NLRP3/6 pathways in a methionine choline deficient diet steatohepatitis mouse model. Int J Biol Macromol*. (2018) 120:1480–9. doi: 10.1016/j.ijbiomac.2018.09.151
21. Mehta R, Neupane A, Wang L, Goodman Z, Baranova A, Younossi ZM. Expression of NALPs in adipose and the fibrotic progression of non-alcoholic fatty liver disease in obese subjects. *BMC Gastroenterol*. (2014) 14:208. doi: 10.1186/s12876-014-0208-8
22. Zhu Y, Ni T, Deng W, Lin J, Zheng L, Zhang C, et al. Effects of NLRP6 on the proliferation and activation of human hepatic stellate cells. *Exp Cell Res*. (2018) 370:383–8. doi: 10.1016/j.yexcr.2018.06.040
23. Kayagaki N, Stowe IB, Lee BL, O'Rourke K, Anderson K, Warming S, et al. Caspase-11 cleaves gasdermin D for non-canonical inflammasome signalling. *Nature*. (2015) 526:666. doi: 10.1038/nature15541
24. Lara-Tejero M, Sutterwala FS, Ogura Y, Grant EP, Bertin J, Coyle AJ, et al. Role of the caspase-1 inflammasome in *Salmonella typhimurium* pathogenesis. *J Exp Med*. (2006) 203:1407–12. doi: 10.1084/jem.20060206
25. Mayer-Barber KD, Barber DL, Shenderov K, White SD, Wilson MS, Cheever A, et al. Cutting edge: caspase-1 independent IL-1β production is critical for host resistance to *Mycobacterium tuberculosis* and does not require TLR signaling in vivo. *J Immunol*. (2010) 184:3326–30. doi: 10.4049/jimmunol.0904189
26. Vandanmagsar B, Youm Y-H, Ravussin A, Galgani JE, Stadler K, Mynatt RL, et al. The NLRP3 inflammasome instigates obesity-induced inflammation and insulin resistance. *Nat Med*. (2011) 17:179. doi: 10.1038/nm.2279
27. Cançado GGL, Fiuza JA, de Paiva NCN, de Carvalho Dhom Lemos L, Ricci ND, Gazzinelli-Guimaraes PH, et al. Hookworm products ameliorate dextran sodium sulfate-induced colitis in BALB/c mice. *Inflamm Bowel Dis*. (2011) 17:2275–86. doi: 10.1002/ibd.21629
28. Kaisar MM, Ritter M, del Fresno C, Jónasdóttir HS, van der Ham AJ, Pelgrom LR, et al. Dectin-1/2–induced autocrine PGE2 signaling licenses dendritic cells to prime Th2 responses. *PLoS Biol*. (2018) 16:e2005504. doi: 10.1371/journal.pbio.2005504
29. Bruchard M, Rebé C, Derangère V, Togbé D, Ryffel B, Boidot R, et al. The receptor NLRP3 is a transcriptional regulator of T H 2 differentiation. *Nat Immunol*. (2015) 16:859. doi: 10.1038/ni.3202
30. Levy M, Shapiro H, Thaiss CA, Elinav E. NLRP6: a multifaceted innate immune sensor. *Trends Immunol*. (2017) 38:248–60. doi: 10.1016/j.it.2017.01.001
31. Alegre F, Pelegrin P, Feldstein AE. Inflammasomes in liver fibrosis. *Semin Liver Dis*. (2017) 37:119–27. doi: 10.1055/s-0037-1601350
32. Szabo G, Csak T. Inflammasomes in liver diseases. *J Hepatol*. (2012) 57:642–54. doi: 10.1016/j.jhep.2012.03.035
33. Chen C-J, Kono H, Golenbock D, Reed G, Akira S, Rock KL. Identification of a key pathway required for the sterile inflammatory response triggered by dying cells. *Nat Med*. (2007) 13:851. doi: 10.1038/nm1603
34. Zhu P, Duan L, Chen J, Xiong A, Xu Q, Zhang H, et al. Gene silencing of NALP3 protects against liver ischemia–reperfusion injury in mice. *Hum Gene Ther*. (2010) 22:853–64. doi: 10.1089/hum.2010.145
35. Yang D, Postnikov YV, Li Y, Tewary P, de la Rosa G, Wei F, et al. High-mobility group nucleosome-binding protein 1 acts as an alarmin and is critical for lipopolysaccharide-induced immune responses. *J Exp Med*. (2012) 209:157–71. doi: 10.1084/jem.20101354
36. Miao EA, Rajan JV, Aderem A. Caspase−1−induced pyroptotic cell death. *Immunol Rev*. (2011) 243:206–14. doi: 10.1111/j.1600-065X.2011.01044.x
37. Franklin BS, Bossaller L, De Nardo D, Ratter JM, Stutz A, Engels G, et al. The adaptor ASC has extracellular and'prionoid'activities that propagate inflammation. *Nat Immunol*. (2014) 15:727. doi: 10.1038/ni.2913
38. Reiter FP, Wimmer R, Wottke L, Artmann R, Nagel JM, Carranza MO, et al. Role of interleukin-1 and its antagonism of hepatic stellate cell proliferation and liver fibrosis in the Abcb4-/-mouse model. *World J Hepatol*. (2016) 8:401. doi: 10.4254/wjh.v8.i8.401
39. Tang N, Zhang Y-P, Ying W, Yao X-X. Interleukin-1β upregulates matrix metalloproteinase-13 gene expression via c-Jun N-terminal kinase and p38 MAPK pathways in rat hepatic stellate cells. *Mol Med Rep*. (2013) 8:1861–5. doi: 10.3892/mmr.2013.1719
40. Brunet LR, Finkelman FD, Cheever AW, Kopf MA, Pearce EJ. IL-4 protects against TNF-alpha-mediated cachexia and death during acute schistosomiasis. *J Immunol*. (1997) 159:777–85.
41. Hara H, Seregin SS, Yang D, Fukase K, Chamaillard M, Alnemri ES, et al. The NLRP6 inflammasome recognizes lipoteichoic acid and regulates gram-positive pathogen infection. *Cell*. (2018) 175:1651–1664.e14. doi: 10.1016/j.cell.2018.09.047
42. Shen C, Lu A, Xie WJ, Ruan J, Negro R, Egelman EH, et al. Molecular mechanism for NLRP6 inflammasome assembly and activation. *Proc Natl Acad Sci USA*. (2019) 116:2052–7. doi: 10.1073/pnas.1817221116
43. Meng N, Xia M, Lu YQ, Wang M, Boini KM, Li PL, et al. Activation of NLRP3 inflammasomes in mouse hepatic stellate cells during *Schistosoma J*. infection. *Oncotarget*. (2016) 7:39316–31. doi: 10.18632/oncotarget.10044
44. Burke ML, McManus DP, Ramm GA, Duke M, Li Y, Jones MK, et al. Temporal expression of chemokines dictates the hepatic inflammatory infiltrate in a murine model of schistosomiasis. *PLoS Negl Trop Dis*. (2010) 4:e598. doi: 10.1371/journal.pntd.0000598
45. Chuah C, Jones MK, Burke ML, McManus DP, Gobert GN. Cellular and chemokine-mediated regulation in schistosome-induced hepatic pathology. *Trends Parasitol*. (2014) 30:141–50. doi: 10.1016/j.pt.2013.12.009
46. Souza AL, Roffê E, Pinho V, Souza DG, Silva AF, Russo RC, et al. Potential role of the chemokine macrophage inflammatory protein 1α in human and experimental schistosomiasis. *Infect Immun*. (2005) 73:2515–23. doi: 10.1128/IAI.73.4.2515-2523.2005
47. Saiman Y, Friedman SL. The role of chemokines in acute liver injury. *Front Physiol*. (2012) 3:213. doi: 10.3389/fphys.2012.00213
48. Warmington KS, Boring L, Ruth JH, Sonstein J, Hogaboam CM, Curtis JL, et al. Effect of CC chemokine receptor 2 (CCR2) knockout on type-2 (schistosomal antigen-elicited) pulmonary granuloma formation: analysis of cellular recruitment and cytokine responses. *Am J Pathol*. (1999) 154:1407–16. doi: 10.1016/S0002-9440(10)65394-1
49. Dixon LJ, Flask CA, Papouchado BG, Feldstein AE, Nagy LE. Caspase-1 as a central regulator of high fat diet-induced non-alcoholic steatohepatitis. *PLoS One*. (2013) 8:e56100. doi: 10.1371/journal.pone.0056100
50. Kamari Y, Shaish A, Vax E, Shemesh S, Kandel-Kfir M, Arbel Y, et al. Lack of interleukin-1α or interleukin-1β inhibits transformation of steatosis to steatohepatitis and liver fibrosis in hypercholesterolemic mice. *J Hepatol*. (2011) 55:1086–94. doi: 10.1016/j.jhep.2011.01.048
51. Guo H, Xie M, Zhou C, Zheng M. The relevance of pyroptosis in the pathogenesis of liver diseases. *Life Sci*. (2019) 223:69–73. doi: 10.1016/j.lfs.2019.02.060
52. Xu B, Jiang M, Chu Y, Wang W, Chen D, Li X, et al. Gasdermin D plays a key role as a pyroptosis executor of non-alcoholic steatohepatitis in humans and mice. *J Hepatol*. (2018) 68:773–82. doi: 10.1016/j.jhep.2017.11.040
53. Liu X, Zhang Y-R, Cai C, Ni X-Q, Zhu Q, Ren J-L, et al. Taurine alleviates *Schistosoma*-induced liver injury by inhibiting the TXNIP/NLRP3 inflammasome signal pathway and pyroptosis. *Infect Immun*. (2019) 87:12. doi: 10.1128/IAI.00732-19
54. Kong D-L, Kong F-Y, Liu X-Y, Yan C, Cui J, Tang R-X, et al. Soluble egg antigen of *Schistosoma japonicum* induces pyroptosis in hepatic stellate cells by modulating ROS production. *Parasit Vectors*. (2019) 12:1–12. doi: 10.1186/s13071-019-3729-8

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: 22 May 2020
doi: 10.3389/fimmu.2020.00846

Perforin Acts as an Immune Regulator to Prevent the Progression of NAFLD

Qian Wang[1,2†], Dehai Li[1,2†], Jing Zhu[1,2†], Mingyue Zhang[1,2], Hua Zhang[1,2], Guangchao Cao[1,2], Leqing Zhu[1,2], Qiping Shi[3], Jianlei Hao[1,2], Qiong Wen[1,2], Zonghua Liu[1,2], Hengwen Yang[1,2*] and Zhinan Yin[1,2*]*

[1] Zhuhai Precision Medical Center, Zhuhai People's Hospital (Zhuhai Hospital Affiliated with Jinan University), Jinan University, Zhuhai, China, [2] The Biomedical Translational Research Institute, Faculty of Medical Science, Jinan University, Guangzhou, China, [3] The First Affiliated Hospital of Jinan University, Guangzhou, China

OPEN ACCESS

Edited by:
Gary Splitter,
University of Wisconsin-Madison,
United States

Reviewed by:
Nadia Maria Terrazzini,
University of Brighton,
United Kingdom
Erik Petersen,
East Tennessee State University,
United States

***Correspondence:**
Qian Wang
wangqian@jnu.edu.cn
Hengwen Yang
hengwenyang@jnu.edu.cn
Zhinan Yin
zhinan.yin@yale.edu

[†] These authors share first authorship

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

Received: 15 October 2019
Accepted: 14 April 2020
Published: 22 May 2020

Citation:
Wang Q, Li D, Zhu J, Zhang M, Zhang H, Cao G, Zhu L, Shi Q, Hao J, Wen Q, Liu Z, Yang H and Yin Z (2020) Perforin Acts as an Immune Regulator to Prevent the Progression of NAFLD. Front. Immunol. 11:846. doi: 10.3389/fimmu.2020.00846

Non-alcoholic fatty liver disease (NAFLD) is one of the main causes of cirrhosis and major risk factors for hepatocellular carcinoma and liver-related death. Despite substantial clinical and basic research, the pathogenesis of obesity-related NAFLD remains poorly understood. In this study, we show that perforin can act as an immune regulator to prevent the progression of NAFLD. Aged perforin-deficient ($Prf^{-/-}$) mice have increased lipid accumulation in the liver compared to WT mice. With high-fat diet (HFD) challenge, $Prf^{-/-}$ mice have increased liver weight, more severe liver damage, and increased liver inflammation when compared with WT controls. Mechanistic studies revealed that perforin specifically regulates intrinsic IFN-γ production in CD4 T cells, not CD8 T cells. We found that CD4 T cell depletion reduces liver injury and ameliorates the inflammation and metabolic morbidities in $Prf^{-/-}$ mice. Furthermore, improved liver characteristics in HFD $Prf^{-/-}$ and IFN-$\gamma R^{-/-}$ double knockout mice confirmed that IFN-γ is a key factor for mediating perforin regulation of NAFLD progression. Overall, our findings reveal the important regulatory role perforin plays in the progression of obesity-related NAFLD and highlight novel strategies for treating NAFLD.

Keywords: perforin, NAFLD, CD4 T cells, IFN-γ, inflammation

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) pathogenesis is tightly linked to obesity and therefore is an emerging healthcare problem worldwide (1, 2). NAFLD, along with related inflammation, progressive subtype non-alcoholic steatohepatitis (NASH), fibrosis, and ultimately hepatocellular carcinoma, is becoming one of the leading causes of liver-related morbidity and mortality worldwide (3–5). The pathogenesis of NAFLD remains incompletely understood. It is appreciated that multiple concurrent intrahepatic and extrahepatic events contribute to development and progression of NAFLD, including cell senescence, insulin resistance, and immune system dysfunction (6, 7). Cellular senescence refers to the irreversible arrest of cell growth that occurs when cells are exposed to various stresses (8–10). Recent experimental evidence suggests that hepatocyte senescence is linked to the fibrosis that develops as NAFLD progresses; hepatocyte expression of p21, the universal cell cycle inhibitor, is positively correlated with fibrosis stage in liver sections from 70 NAFLD patients (11). Dysregulated lipid metabolism plays a key role in initiation and progression of hepatic steatosis and is frequently associated with inflammation of

the liver (12, 13). Elevated inflammation promotes the development of insulin resistance, which in turn further promotes ectopic fat accumulation in the liver, thus forming a vicious cycle (14, 15). Inflammation and fibrogenesis are regulated by complex immunologic pathways that may present possible new therapeutic targets in the liver for NAFLD (7).

Perforin, which is primarily released by $CD8^+$ T cells and natural killer (NK) cells, helps eliminate infected or dangerous cells and induce apoptosis (16, 17). Following degranulation, pores formed by perforin enable granzyme entry into cells and subsequent caspase activation. Perforin-mediated cytotoxicity is also involved in the homeostatic regulation of CD4 and CD8 T cells *in vivo* (18, 19). Recent reports revealed that perforin-mediated exocytosis (but not death-receptor-mediated apoptosis) is essential for immune surveillance of senescent cells, and disruption of this pathway as a result of disease or inflammation can lead to the accumulation of senescent cells in the liver (20). Interestingly, a recent study showed that mice on a high-fat diet (HFD) lacking perforin developed more severe obesity, glucose tolerance, and insulin resistance and had higher triglyceride levels in the liver when compared with wild-type (WT) controls (21). However, the precise role of perforin in the context of HFD-induced NAFLD has not been systematically researched yet.

We show that perforin acts as an important immune regulator to prevent NAFLD progression. Aged $Prf^{-/-}$ mice had more severe liver injury and lipid accumulation than did WT control mice. In the condition of HFD-induced NAFLD, we also found that $Prf^{-/-}$ mice developed more severe hepatic steatosis with more macrophage and IFN-γ, producing $CD4^+$ T cell infiltration of the liver. Depletion of $CD4^+$ T cells in $Prf^{-/-}$ mice almost completely rescued the observed phenotypes, suggesting an important regulatory role for $CD4^+$ T cells. Moreover, when IFN-γ receptor signaling is ablated by using perforin and IFN-γ receptor double knockout mice, both liver injury and lipid accumulation were dramatically diminished, indicating that IFN-γ signaling plays a pivotal role in mediating NAFLD pathogenesis.

Overall, our studies reveal that perforin acts as an important immune regulator for NAFLD progression. This finding expands our understanding of inflammation in regulating NAFLD and may have therapeutic implications for NAFLD in the future.

MATERIALS AND METHODS

Mice

$Prf^{-/-}$ and IFN-$\gamma R^{-/-}$ mice were purchased from the Jackson Laboratory. C57BL/6J mice were purchased from Guangdong Medical Laboratory Animal Center (Guangzhou, China). All mice were males and received either a normal control diet (SFD) or HFD (60 kcal % fat; Research Diets) beginning at an age of 6–8 weeks old. All mice were maintained under specified pathogen-free conditions at Jinan University (Guangzhou, China). Animal procedures were approved by and performed in accordance with the Jinan University's Institutional Laboratory Animal Care and Use Committee guidelines.

Isolation of Liver Mononuclear Cells

The protocol used for isolating murine liver mononuclear cells (MNCs) was as described previously (22). Liver tissue was obtained from mice, and the tissue was dissociated to procure MNCs. To obtain liver MNCs, murine livers were pressed through a 200-gauge stainless steel mesh and suspended in either RPMI-1640 medium or PBS. The cells were then centrifuged at 50 g for 1 min. The cell suspension was collected and centrifuged again at 974 g for 10 min. The cell pellet containing MNCs was then resuspended in 40% Percoll (GE Healthcare, Uppsala, Sweden), after which the cell suspension was overlaid on 70% Percoll and centrifuged at 1,260 g for 30 min. The resulting cell pellets were collected from the interphase following two additional washings in PBS or RPMI-1640 medium.

Serum Biochemistry

Mice were fasted overnight. Then, whole blood was collected, and serum alanine aminotransferase (ALT) and cholesterol levels were determined using an automatic biochemistry analyzer (7600-020, Hitachi, Japan).

Cytokine Detection With ELISA

Mice were fasted overnight, and 0.1 g of liver tissue was harvested from the mice in 1 ml of PBS. Liver tissue was then homogenized by hand and centrifuged at 3,000 rpm for 10 min, after which the supernatant was carefully collected. All steps were performed at 4°C. IL-6, IFN-γ, and TNF-α levels in liver supernatants were determined using a commercially available mouse enzyme-linked immunosorbent assay (ELISA) kit (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions.

Flow Cytometry Analysis

Non-parenchymal cells were transferred to a new well and treated with 1:1000 GolgiPlug, 1 ng/ml ionomycin, and 50 ng/ml PMA for 4–6 h. Intracellular and cell surface staining was performed as described in the fixation/permeabilization kit (554714; BD) protocol. Cells were stained with the surface markers PEcy7-anti-mouse CD3, PE-anti-mouse NK1.1, FITC-anti-mouse CD4, and PerCPCY5.5-anti-mouse CD8 for 15 min at 4°C. Cells were stained for cytokines with BV421 anti–mouse IFN-γ and APC-IL-17A for 30 min at 4°C, washed with PBS, and analyzed using FACS verse flow cytometry (BD). Data were analyzed using FlowJo (TreeStar).

$CD4^+$ T Cell Depletion

To deplete $CD4^+$ T cells, 200-μg doses of anti-CD4 monoclonal antibody (clone: GK1.5; Sungene Biotech) per mouse were intraperitoneally injected weekly during HFD challenge. Sterile-filtered PBS was used as a control.

Histological Examination

Liver tissue was harvested and fixed in 4% (w/v) paraformaldehyde, and 4 mm-thick sections that had been affinized and rehydrated were stained with hematoxylin and eosin (H&E). Hepatic lipid content was determined using frozen sections embedded in Tissue-Tek O.C.T. compound and stained with Oil Red O (Sigma-Aldrich, St. Louis, MO, USA). Images were acquired on a Leica DM3000 microscope.

Immunofluorescence

Liver tissue was harvested, fixed in 4% (w/v) paraformaldehyde, and cut in 4 mm-thick sections. Liver sections were then perfused with 30 ml of 4% paraformaldehyde for fixation. Sections were then incubated with the following dilutions of mouse-specific primary antibodies: 1:200 anti-F4/80 (ab16911, Abcam) and 1:200 iNOS antibody (GTX74171, Gentex). For visualization, 1:200 fluorescent Alexa Fluor 594 and FITC 488 secondary antibodies (Invitrogen Vector) were used for both individual staining and co-staining at room temperature for 2 h. After washing, tissue sections were fixed with Vectashield containing DAPI for visualization. A laser cofocal microscopy (TCS SP8, Leica) was used to capture images and conduct further analysis. For the microscopy images displaying M1 (iNOS+ F4/80+) or total macrophages (F4/80+), 4 slides per mouse liver tissue were prepared and 4 fields were captured from each slide. The quantification of M1 or total macrophages was conducted in these 16 fields and designated as one biological independent sample, and the percentage of M1 in total macrophages was calculated and shown.

Tissue Triglyceride Quantification

The protocol for quantifying hepatic triglyceride (TG) levels was carried out as described previously (23). Briefly, 20–30 mg of liver tissue was homogenized in 500 μl of PBS and mixed with chloroform/methanol 2:1 (vol/vol). The organic phase was transferred, air-dried overnight, and resuspended in 1% Triton X-100 in absolute ethanol. The concentration of TGs was then quantified using a serum triglyceride determination kit (Sigma, Triglyceride Reagent T2449 and Free Glycerol Reagent F6428).

RNA Extraction and Quantitative Real-Time PCR

Total liver RNA was isolated using TRIzol Reagent (DP424, Tiangen, China). cDNA synthesis was performed using a Prime Script RT Reagent Kit (Takara, Shiga, Japan). Levels of mRNA expression were quantified by real-time PCR (RT-PCR). RT-PCR was performed using TB Green (Takara). Primer sequences are shown in the **Table 1**.

TABLE 1 | Primers for real-time RT-PCR.

Hprt forward	5′-CGTCGTGATTAGCGATGATGAAC-3′
Hprt reverse	5′-TCACTAATGACACAAACGTGATTC-3′
Fabp4 forward	5′-GACGACAGGAAGGTGAAGAG-3′
Fabp4 reverse	5′-ACATTCCACCACCAGCTTGT-3′
Cebpα forward	5′-AAGAACAGCAACGAGTACCGG-3′
Cebpα reverse	5′-CATTGTCACTGGTCAGCTCCA-3′
SREBP-1C forward	5′-GATCAAAGAGGAGCCAGTGC-3′
SREBP-1C reverse	5′-TAGATGGTGGCTGCTGAGTG-3′
PPARγ forward	5′-GCCCTTTGGTGACTTTATGG-3′
PPARγ reverse	5′-CAGCAGGTTGTCTTGGATGT-3′
PPARα forward	5′-TCGGACTCGGTCTTCTTGAT-3′
PPARα reverse	5′-TCTTCCCAAAGCTCCTTCAA-3′
Cox-1 forward	5′-CTCACAGTGCGGTCCAAC-3′
Cox-1 reverse	5′-CCAGCACCTGGTACTTAA-3′
AOX forward	5′-TCGGGCAAGTGAGGCGCATT-3′
AOX reverse	5′-AGCAACAGCATTGGGGCGGA-3′
Cpt1α forward	5′-CCCAAGTATCCACAGGGTCA-3′
Cpt1α reverse	5′-TTTGAATCGGCTCCTAATGG-3′
Lipe forward	5′-GTGGAGGCACATTTAGTTCT-3′
Lipe reverse	5′-GTGACCTGTTTGTTTGTTCT-3′
Lpl forward	5-TAGATGAGGCCAACCTGTCC-3′
Lpl reverse	5-CTGCGTAGTCGGGGTACATT-3′
CD36 forward	5′-AGATGACGTGGCAAAGAACAG-3′
CD36 reverse	5′-CCTTGGCTAGATAACGAACTCTG-3′
Scd1 forward	5′-TTCTTGCGATACACTCTGGTGC-3′
Scd1 reverse	5′-CGGGATTGAATGTTCTTGTCGT-3′
Cidea forward	5′-TGACATTCATGGGATTGCAGAC-3′
Cidea reverse	5′-GGCCAGTTGTGATGACTAAGAC-3′
Chrebpβ forward	5′-TCTGCAGATCGCGTGGAG-3′
Chrebpβ reverse	5′-CTTGTCCCGGCATAGCAAC-3′
Fasn forward	5′-CCTTGGCTAGATAACGAACTCTG–3′
Fasn reverse	5′-ATCCATAGAGCCCAGCCTTCCATC–3′

Statistical Analysis

Data are presented as the mean ± SEM. Statistical significance between two groups was evaluated using a two-tailed unpaired Student's *t*-test. Values of $P < 0.05$ were considered to be statistically significant. The data shown in each panel of these figures were collected from a single experiment; each experiment was repeated for at least three times and showed consistent results. Moreover, the statistical analysis was conducted on each single experiment.

RESULTS

Perforin Deficiency Accelerates Liver Injury and Enhances Lipid Accumulation in 14 Month-Old Mice

NAFLD is common in the elderly, in whom it carries a more substantial burden of hepatic (non-alcoholic steatohepatitis, cirrhosis, and hepatocellular carcinoma) and extra-hepatic manifestations and complications (cardiovascular disease, extrahepatic neoplasms) than in younger age groups (24). Aged $Prf^{-/-}$ mice have been reported to have accumulation of senescent cells and development of chronic systemic and local inflammation in the liver (25, 26). We hypothesized that aged $Prf^{-/-}$ mice might also have more severe hepatic morbidities since inflammation correlates with liver dysfunction. To test this hypothesis, we first determined liver weights and liver injury (ALT) levels in aged WT and $Prf^{-/-}$ mice at 14 months of age. As expected, the aged $Prf^{-/-}$ mice showed significantly increased liver weight (**Figure 1A**), elevated liver damage, and increased lipid accumulation as shown by levels of ALT that trended as increased and significantly increased liver TG levels (**Figure 1B**). Furthermore, liver histological analysis revealed more severe hepatic steatosis and significantly increased accumulation of lipid in aged $Prf^{-/-}$ mice compared with WT mice (**Figure 1C**). These results indicated that the perforin deficiency aggravates liver injury and steatosis in aged mice.

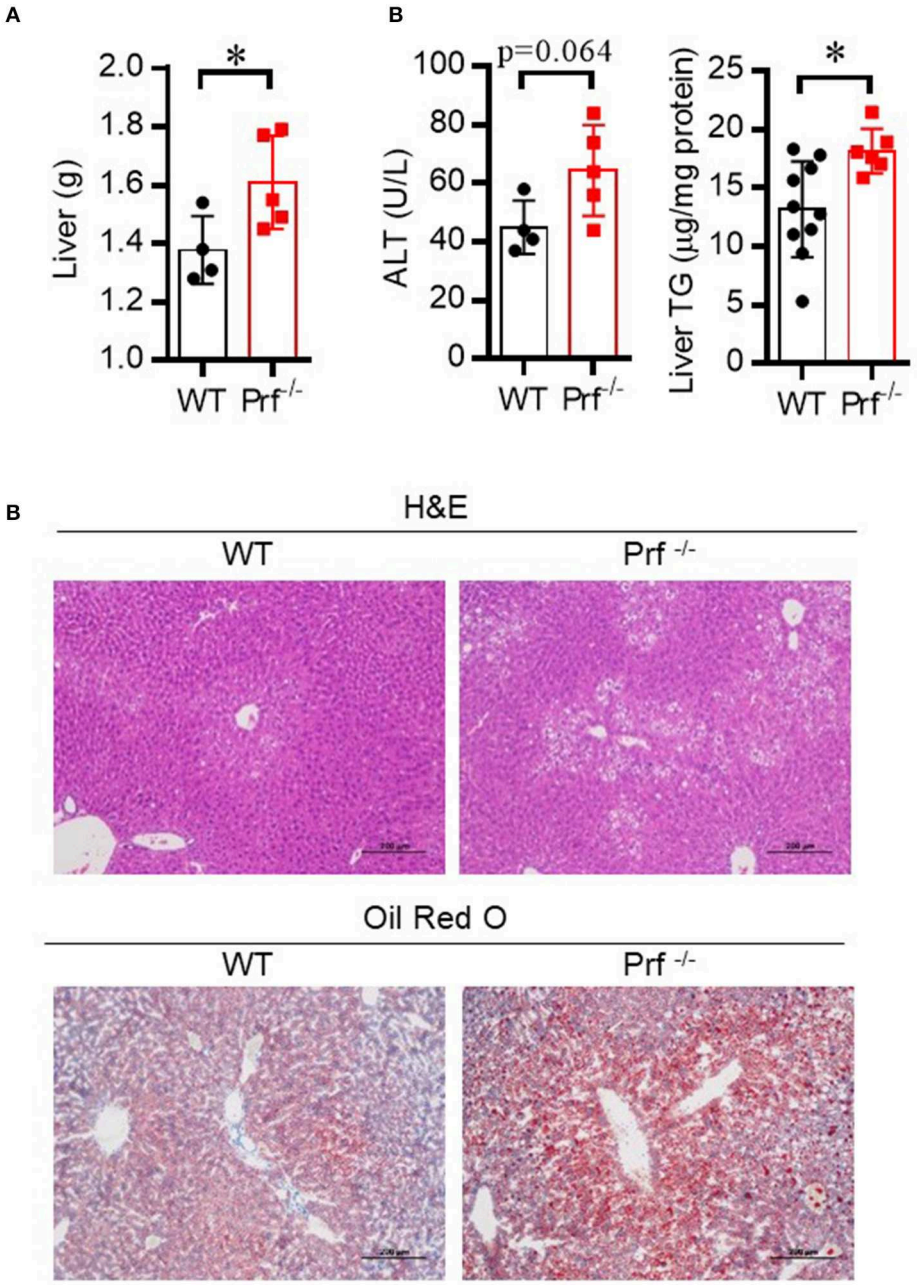


FIGURE 1 | Perforin deficiency accelerates liver injury and enhances lipid accumulation in 14 month-old mice. **(A)** Liver weight was determined for WT and Prf$^{-/-}$ mice on a normal chow diet ($n = 4$–5). **(B)** Serum levels of ALT (left, $n = 4$–5) and liver TG content (right, $n = 6$–10) were measured. **(C)** Representative images of liver sections stained with H&E and Oil red O in WT and Prf$^{-/-}$ mice at the age of 14 months. ALT, alanine aminotransferase; WT, wild-type; TG, triglyceride. The data shown in each panel of the figures were collected from a single experiment, and each experiment was repeated for at least three times and rendered consistent results. Means ± SEM, *$p < 0.05$.

Perforin Deficiency Aggravates HFD-Induced Liver Injury and Steatohepatitis

The role of perforin in NAFLD was then investigated using an HFD-induced NAFLD model. Prf$^{-/-}$ and WT mice at 6–8 weeks of age were fed on SFD or HFD for 10 weeks to induce NAFLD. As expected, HFD challenge was associated with elevated body weight and ALT activation in WT mice (**Figures 2A,B**). The increase in body weight in response to HFD challenge was comparable between WT and Prf$^{-/-}$ mice; however, Prf$^{-/-}$ mice had significantly enlarged livers (**Figure 2A**). Additionally, the Prf$^{-/-}$ mice had more severe liver damage as indicated

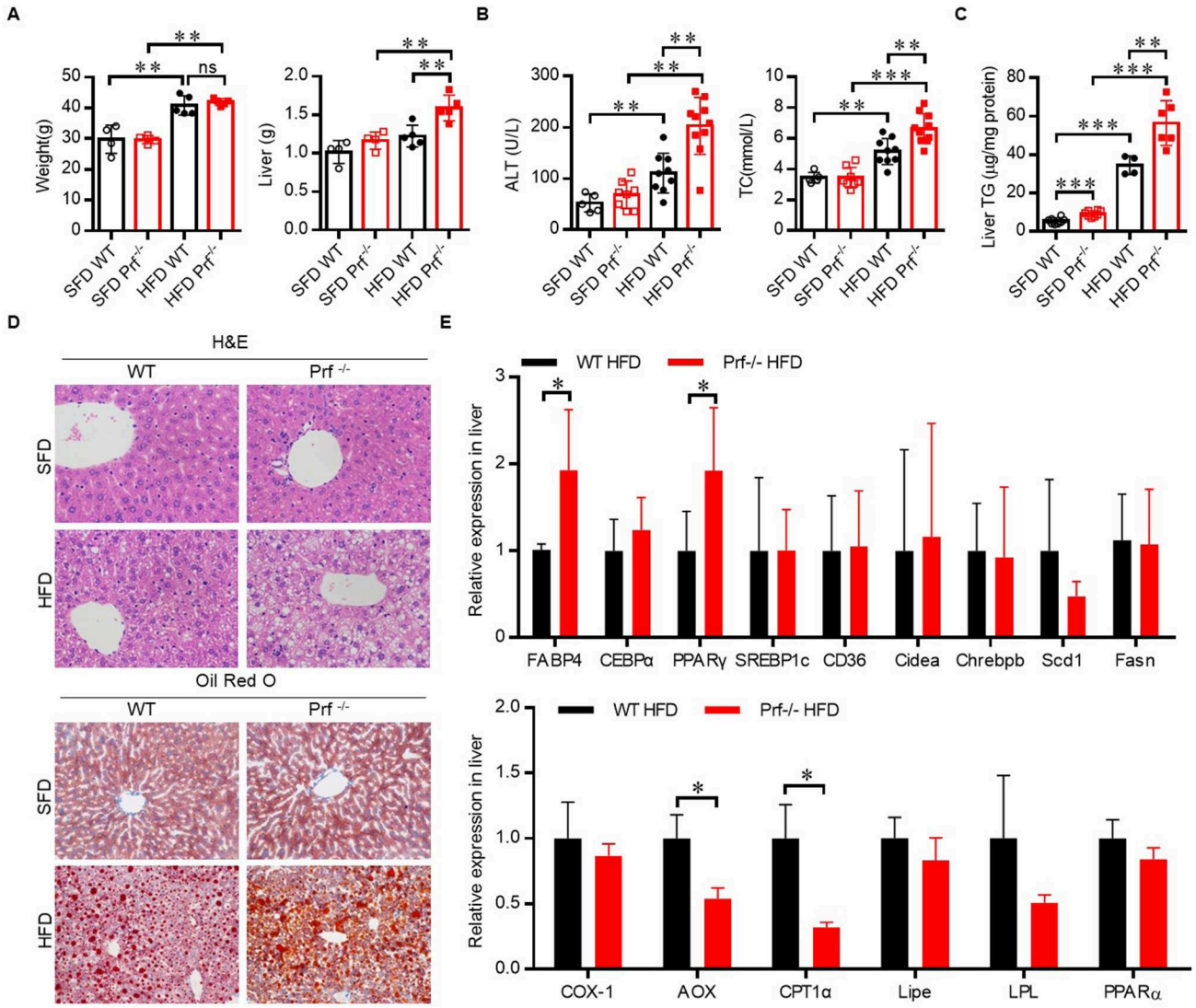


FIGURE 2 | Perforin deficiency aggravates HFD-induced liver injury and steatohepatitis. WT and Prf$^{-/-}$ mice were fed SFD or HFD for 10 weeks at 6–8 weeks of age. **(A)** Body weight (left panel) and liver weights (right panel) (n = 4–5), **(B)** measurements of serum ALT (left panel) and cholesterol levels (right panel) (n = 5–10), and **(C)** measurements of liver TG content (n = 4–8). **(D)** Representative images of liver sections stained with H&E and Oil red O. **(E)** Expression levels of lipogenic genes (top) and fatty acid oxidation genes (bottom) in the liver, relative to Hprt expression levels (n = 4–9). The data shown in each panel of the figures were collected from a single experiment, and each experiment was repeated for at least three times and rendered consistent results. Means ± SEM, $^{*}p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$.

by higher ALT levels. The livers of HFD Prf$^{-/-}$ mice exhibited significantly increased lipid accumulation (**Figure 2C**). Histological analysis of livers indicated that HFD Prf$^{-/-}$ mice had more severe hepatic steatosis and lipid accumulation when compared with WT controls (**Figure 2D**). Moreover, RT-PCR analysis of liver samples from HFD mice showed that the expression levels of genes involved in lipid production such as fatty acid binding protein 4 (Fabp4) and peroxisome proliferator-activated receptor gamma (PPARγ) were significantly increased, whereas expression levels of lipid catabolism-related genes such as carnitine palmitoyl transferase 1 (CPT-1α) and aldehyde oxidase (AOX-1) were significantly decreased in HFD Prf$^{-/-}$ mice (**Figure 2E**). These results indicated that perforin deficiency with HFD challenge aggravated liver injury and steatohepatitis.

Perforin Deficiency Promotes an Inflammatory Response in the Liver After HFD Challenge

Pro-inflammatory T cells promote M1 macrophage activation and intensively contribute to HFD-induced NAFLD (27). To explore the mechanism that drives more severe NAFLD in Prf$^{-/-}$ mice, we analyzed the composition of the immune cell infiltrate in the liver by flow cytometry. Perforin deficiency did not alter the infiltration of CD4, CD8, NK, NK1.1+ T cells,

or total macrophages in the liver (**Figures 3A–C**). However, the cell number of CD11c+ macrophages was significantly increased (**Figure 3D**). We next evaluated inflammatory cytokine production by these immune cell subsets. Interestingly, we observed that IFN-γ production from CD4 T, but not CD8 T cells, NK cells, or NK1.1+ T cells, was significant increased, while IL-17 was barely detectable and largely unaffected in all subsets (**Figures 3E–H**). We characterized the cell number in each category and found no significant difference of CD4 T cells, CD8 T cells, and NK and NK1.1+T cells. The cell numbers of CD11c+ macrophages (M1) and IFN-γ+CD4 T cells were significantly increased in perforin KO liver, which equivalent as the percentage analysis (**Figure 3I**).

We also determined the levels of pro-inflammatory cytokines secreted by the liver in HFD-challenged $Prf^{-/-}$ and WT mice. As expected, $Prf^{-/-}$ livers produced more IL-6, TNF-α, and IFN-γ compared with livers from WT controls (**Figure 4A**). Immunofluorescence analysis showed that perforin deficiency robustly promoted the enrichment of M1 macrophage in the liver, which was consistent with the previous percentage and cell number analysis (**Figure 4B**). These findings suggest that upon HFD challenge, the IFN-γ level and M1 macrophage-mediated inflammation are enhanced in $Prf^{-/-}$ mice.

Perforin Regulates Fatty Liver Disease Through CD4 T Cells in the Liver

To determine whether increased IFN-γ production from CD4 T cells in $Prf^{-/-}$ mice was associated with the exacerbated liver phenotypes that develop after HFD challenge, we depleted CD4 T cells in $Prf^{-/-}$ mice and then fed the mice with HFD. As expected, CD4 T cell depletion predisposed $Prf^{-/-}$ mice to decreased liver weights, lipid accumulation, and diminished liver damage (**Figures 5A–C**). Notably, levels of the pro-inflammatory cytokine TNF-α, as well as macrophage accumulation, were also significantly decreased following CD4 T cell depletion in $Prf^{-/-}$ mice; so was the IFN-γ level, though CD4 T cells are not the only cells producing IFN-γ (**Figures 5D,E**). Furthermore, the mRNA expression levels of genes involved in lipogenesis such as Fabp4, CEBPα, PPARγ, SREBP1c, Chrebpβ, and Scd1 were decreased following CD4 T cell depletion in $Prf^{-/-}$ mice, whereas lipolysis-related genes such as AOX1, CPT1α, and LPL were unchanged following CD4 T cell depletion in $Prf^{-/-}$ mice (**Figure 5F**). These findings indicate that CD4 T cells play a critical role in perforin-mediated regulation of NAFLD progression.

Hepatic Steatosis in $Prf^{-/-}$ Mice Is Dependent on IFN-γ-Mediated Inflammation

Since the level of IFN-γ was significantly increased in the livers of $Prf^{-/-}$ mice, we next explored whether CD4 T cells contribute to exacerbated NAFLD in these mice via IFN-γ activity. Therefore, we crossed $Prf^{-/-}$ mice with IFN-γ receptor-deficient mice to get double knockout mice ($IFN\text{-}\gamma R^{-/-}$ and $Prf^{-/-}$). Following HFD challenge, $IFN\text{-}\gamma R^{-/-}$ and $Prf^{-/-}$ mice gained similar amounts of body weight but had significantly decreased liver weights when compared to $Prf^{-/-}$ mice (**Figures 6A,B**). Notably, $IFN\text{-}\gamma R^{-/-}$ and $Prf^{-/-}$ mice showed significantly rescued NAFLD symptoms, including diminished hepatic steatosis, cellular ballooning, and lipid accumulation (**Figure 6C**). We also found that $IFN\text{-}\gamma R^{-/-}$& $Prf^{-/-}$ mice had reduced serum ALT, cholesterol, and liver TG levels, as well as diminished pro-inflammatory cytokine production, while the level of IFN-γ was no significantly changed after IFN-γ receptor deficiency (**Figure 6D**). Moreover, the cell number of pro-inflammatory ($F4/80^{+}iNOS^{+}$) macrophage in the livers of $IFN\text{-}\gamma R^{-/-}Prf^{-/-}$ mice was also dramatically decreased when compared to $Prf^{-/-}$ mice (**Figure 6E**). Taken together, these findings strongly support an important role for elevated IFN-γ in promoting NAFLD progression in the context of perforin deficiency, given that ablation of IFN-γ signaling had a protective effect on the liver in an NAFLD mouse model.

CD4 T Cells Demonstrate Intrinsically Elevated IFN-γ Production in $Prf^{-/-}$ Mice

To define the functional properties of CD4 T cells from $Prf^{-/-}$ mice, total spleen lymphocytes from WT and $Prf^{-/-}$ mice were stimulated *in vitro* with anti CD3/anti-CD28 in the presence of Golgi-Stop. CD4 but not CD8 T cells from $Prf^{-/-}$ mice showed increased levels of IFN-γ production upon CD3/CD28 stimulation (**Figures 7A,B**). To further study whether elevated IFN-γ production by CD4 T cells in $Prf^{-/-}$ mice was an intrinsic property of these mice, naïve CD4 T cells were sorted from WT and $Prf^{-/-}$ spleens and directly differentiated into Th1 cells. Interestingly, naïve CD4 T cells from $Prf^{-/-}$ mice showed an elevated ability to differentiate into Th1 cells (**Figure 7C**). These findings support the conclusion that CD4 T cells undergo an intrinsic functional change in $Prf^{-/-}$ mice.

DISCUSSION

HFD-induced NAFLD is a well-established mouse model for studying the pathophysiological mechanisms of human fatty liver disease. NAFLD is a prevalent liver disease worldwide that can have severe complications such as liver fibrosis and even development of hepatocellular carcinoma, for which there are no effective therapeutic approaches (28). Numerous factors such as leptin, TNF-α, and IL-6 are involved in the initiation and progression of hepatic steatosis and related metabolic dysfunction (29–31). However, the precise role of perforin, a cytotoxic factor released by T cells, has not been precisely studied in the context of HFD-induced NAFLD. Here we described an important protective role for perforin in regulating NAFLD progression. We found that perforin regulates intrinsic IFN-γ production in CD4 T cells, which influences pro-inflammatory macrophage accumulation to affect the progression of NAFLD.

One major finding of this study is the discovery of the protective role perforin plays in regulating NAFLD progression. Perforin is a ∼67-kDa pore-forming protein that is stored in the secretory vesicles (granules) of CTLs and NK cells (32). Perforin is known to have potent and extensive functions in mediating targeted killing together with various other factors

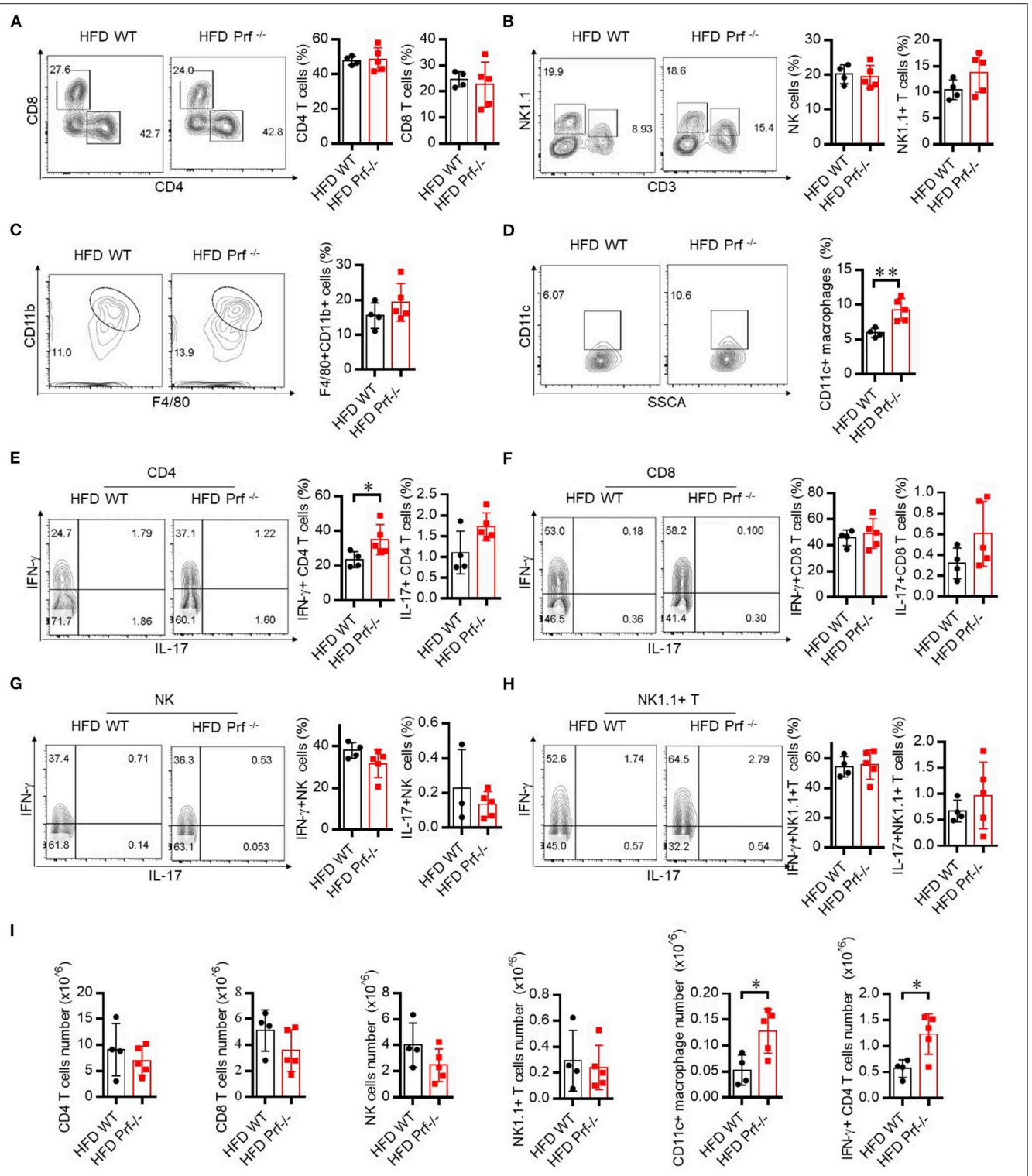


FIGURE 3 | Perforin deficiency drives inflammatory reactions in liver after HFD challenge. WT and Prf$^{-/-}$ mice were fed HFD for 10 weeks. Flow cytometry analysis of the representative histogram (left) and frequency (right) of liver CD4 T cells, CD8 T cells **(A)**, NK cells, NK1.1+ T cells **(B)**, macrophages **(C)**, and CD11c+ macrophages **(D)** and intracellular staining in the liver for IFN-γ and IL-17 in CD4 T cells **(E)**, CD8 T cells **(F)**, NK cells **(G)**, and NK1.1+ T cells **(H)**. **(I)** The total number of CD4 T cells, CD8 T cells, NK cells, and NK1.1+ T cells, CD11c+ macrophages and IFN-γ+CD4 T cells. $n = 4$–5 mice per group. The data shown in each panel of the figures were collected from a single experiment, and each experiment was repeated for at least three times and rendered consistent results. Means ± SEM; $^{*}p < 0.05$.

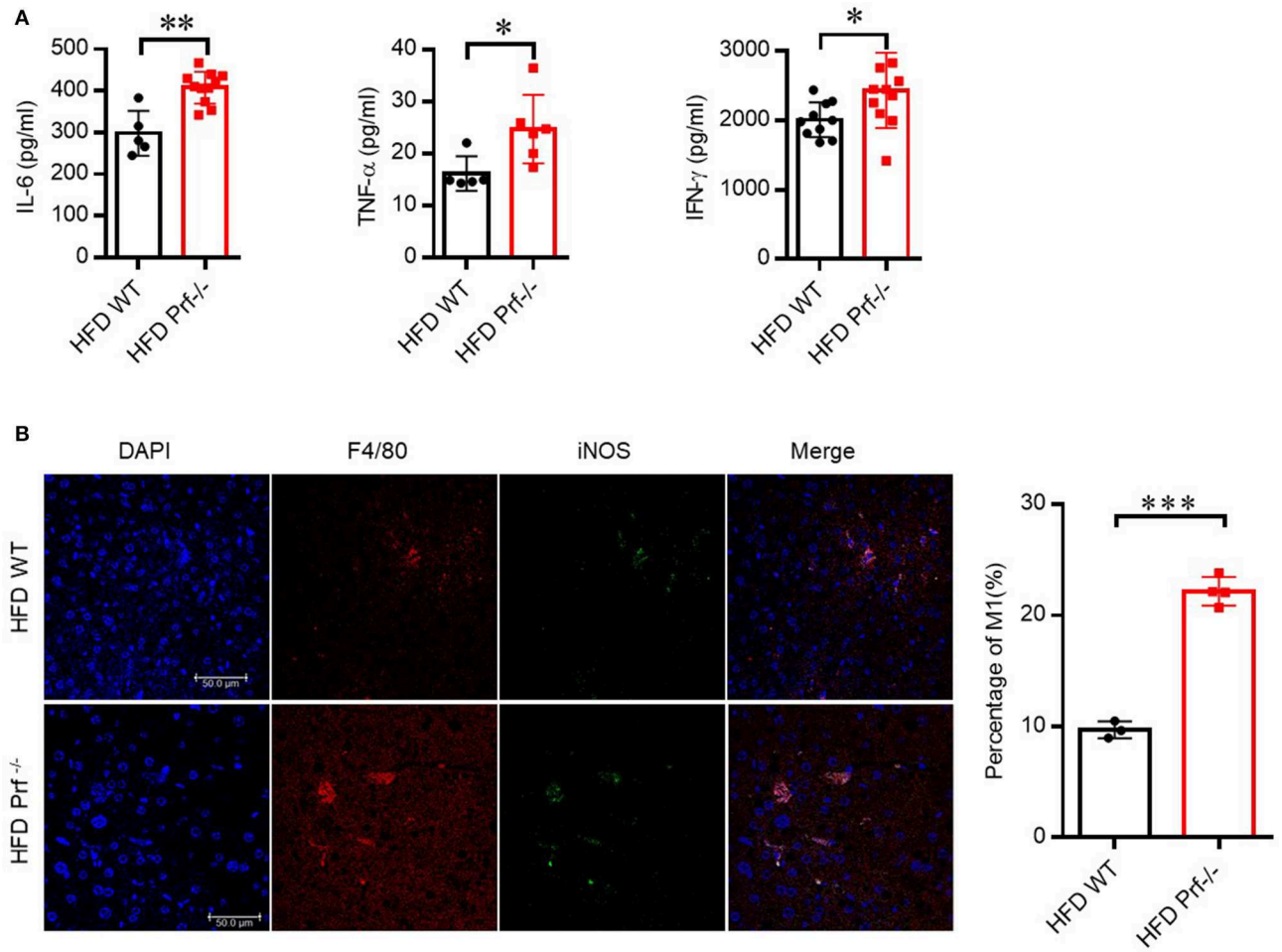


FIGURE 4 | Perforin deficiency drives the accumulation of M1 macrophages and releases of pro-inflammatory cytokines in the liver with HFD challenge. WT and Prf$^{-/-}$ mice were fed HFD for 10 weeks. **(A)** IL-6 (left panel, $n = 5$–11), TNF-α (middle panel, $n = 5$–6), and IFN-γ (right panel, $n = 10$–11) levels in liver supernatants. **(B)** Representative immunofluorescence images from liver with DAPI (blue), F4/80 (red), iNOS (green), and co-localization (merged image) (left) and the percentage of M1/M (refers to iNOS+F4/80+cells/F4/80+ cells) was calculated (right). The data shown in each panel of the figures were collected from a single experiment, and each experiment was repeated for at least three times and rendered consistent results. Means ± SEM, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

secreted by immune cells (19). Previous studies have shown that perforin-deficient mice are sensitive to obesity-induced insulin resistance as a result of restricted T cell expansion and activation in adipose tissue. Perforin has also been reported to play critical roles in promoting inflammation-mediated diseases, including type 1 diabetes (33), cerebral malaria (34), and viral myocarditis (35). A recent study revealed that perforin expressed in CD8 T cells regulates innate and adaptive immunity in the liver and exerts a protective effect in MCD (methionine/choline-deficient diet) diet-induced NASH models (36). Interestingly, MCD diet-induced non-obese NAFLD displays characteristics distinct from those of obesity-induced NAFLD. The precise role of perforin in liver metabolic disorders such as obesity-induced fatty liver disease has not been systematically researched yet. Using 14 month-old Prf$^{-/-}$ mice fed either normal chow or HFD, we demonstrated that perforin played a critical protective role in obesity-induced NAFLD.

In our mouse experiments, we chose male mice fed on HFD (60% fat) for 10 weeks to induce NAFLD and found that Prf$^{-/-}$ mice had more liver weight and liver TG accumulation in hepatocytes. These data are seemingly in contrast to a recent paper published by Cuff et al. which showed that after 24 weeks of obesogenic diet [22.6% fat, 23.0% protein, and 40.2% carbohydrate (w/w) supplement with sweetened condensed milk (Nestle) *ad libitum*], there was no difference in hepatomegaly and liver weight between the wild-type and perforin knockout female mice; otherwise, the fibrosis was significantly lower, and perforin KO mice suffer from less severe NAFLD mediated by NK cells (37). These conflicting findings may be due to the different diets, gender, and feeding time. In Cuff et al.'s paper, they chose 24 weeks as the timepoint so that they could compare the development of fibrosis, which is not usually pronounced at 10 weeks in NAFLD. Several reports indicated that free access to condensed milk induced an increase in serum AST activity

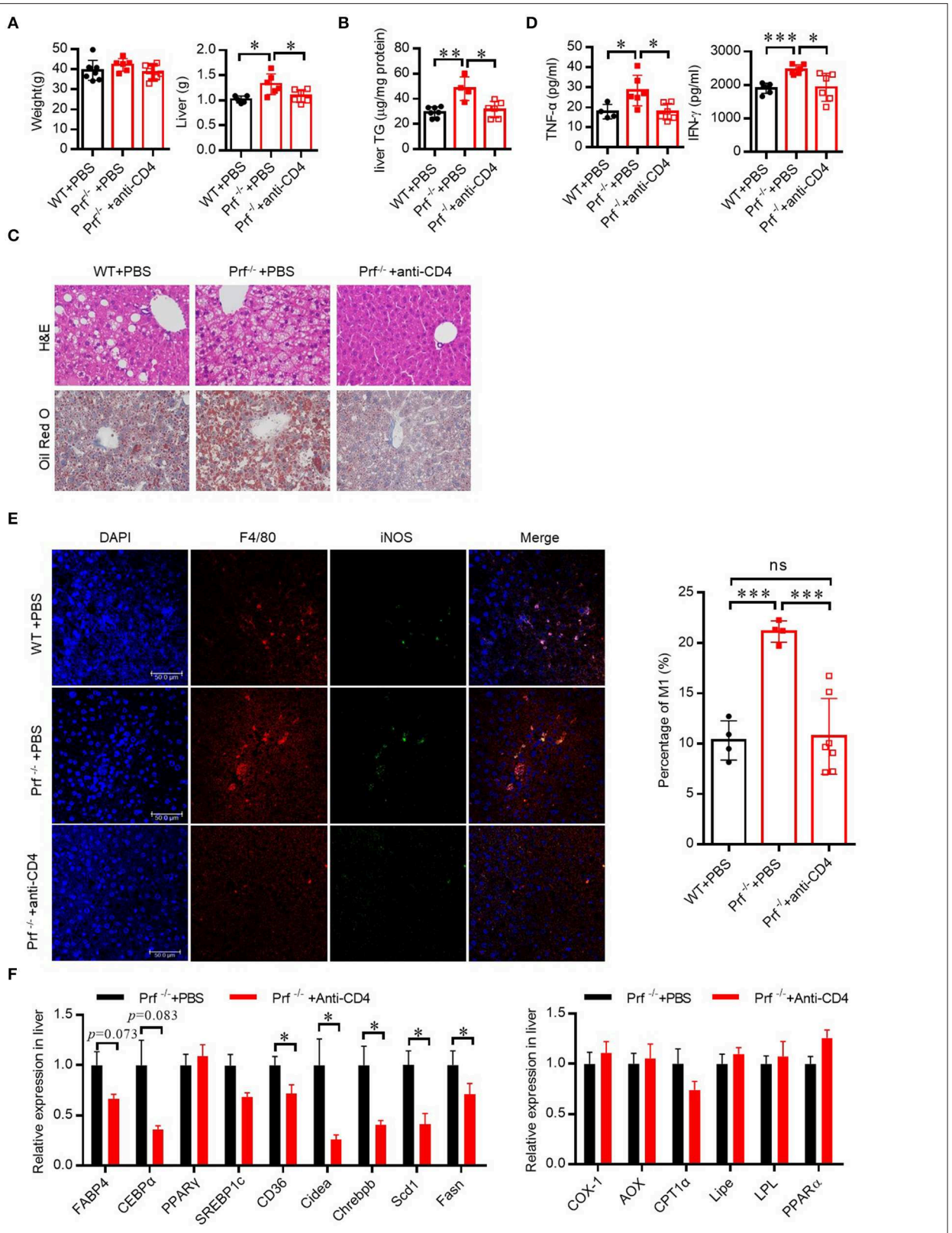


FIGURE 5 | Perforin regulates fatty liver disease through CD4 T cells in the liver. WT and Prf$^{-/-}$ mice were injected with PBS, or Prf$^{-/-}$ mice were injected with an anti-CD4 antibody weekly during 10 weeks of HFD challenge. **(A)** Body and liver weight were measured (n = 6–8). **(B)** Measurements of liver TG content (n = 4–7). **(C)**

(Continued)

FIGURE 5 | Representative images of liver sections stained with H&E (top) and Oil red O (bottom). **(D)** Serum TNF-α and IFN-γ levels were determined ($n = 4–6$). **(E)** Representative immunofluorescence images from liver with DAPI (blue), F4/80 (red), iNOS (green), and co-localization (merged image) (left) and the percentage of M1/M (refers to iNOS+F4/80+cells/F4/80+ cells) was calculated (right). **(F)** Expression levels of lipogenic genes (left) and fatty acid oxidation genes (right) in the liver relative to Hprt expression levels ($n = 6–11$). The data shown in each panel of the figures were collected from a single experiment, and each experiment was repeated for at least three times and rendered consistent results. Means ± SEM, $^{*}p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$.

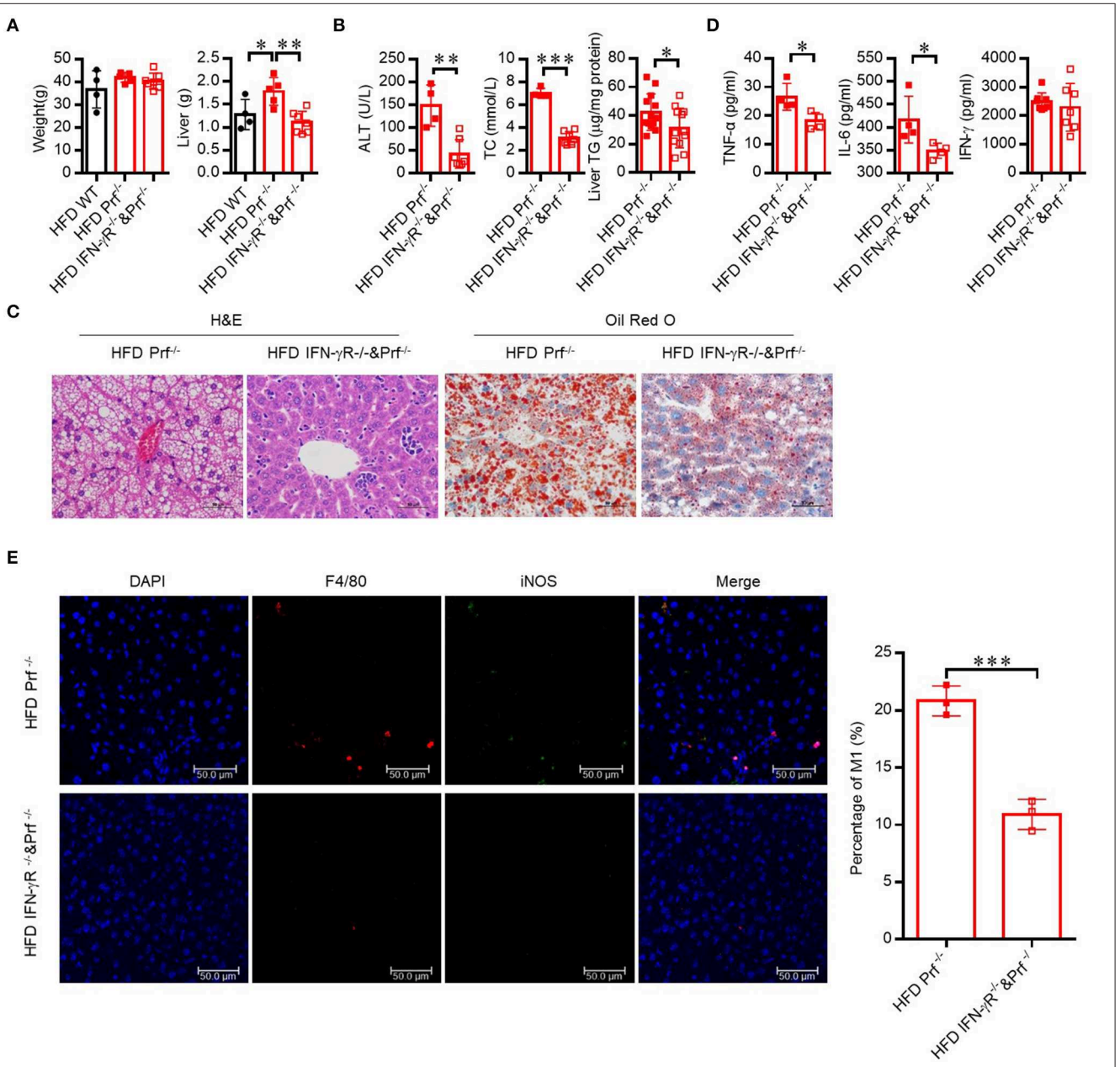


FIGURE 6 | Hepatic steatosis in $Prf^{-/-}$ mice is dependent on IFN-γ mediated inflammation. $Prf^{-/-}$ and IFN-$\gamma R^{-/-}$ and $Prf^{-/-}$ mice were fed HFD for 10 weeks. **(A)** Body and liver weight were measured ($n = 4–7$). **(B)** Serum levels of ALT (left panel, $n = 4–7$), cholesterol (middle panel, $n = 4–7$), and liver TG (right panel) were measured ($n = 12–13$). **(C)** Representative images of liver sections stained with H&E (left) and Oil red O (right). **(D)** TNF-α, IL-6, and IFN-γ levels were detected in liver culture supernatants ($n = 4$). **(E)** Representative immunohistochemistry of DAPI (blue), F4/80 (red), iNOS (green), and co-localization (merged image) in liver, and the percentage of M1/M (refers to iNOS+F4/80+cells/ F4/80+ cells) was calculated (right). The data shown in each panel of the figures were collected from a single experiment, and each experiment was repeated for at least three times and rendered consistent results. Means ± SEM, $^{*}p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$.

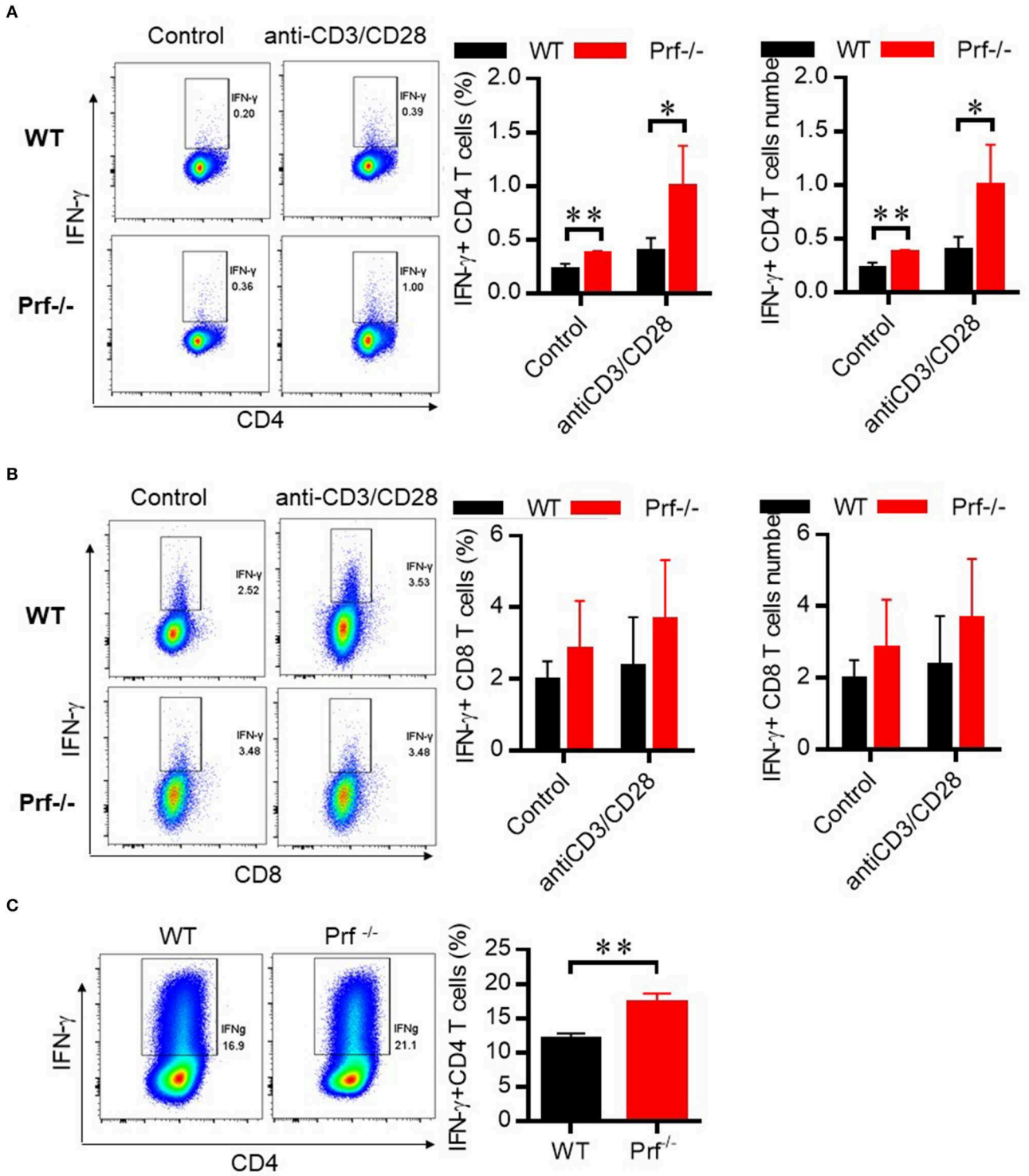


FIGURE 7 | CD4 T cells in Prf$^{-/-}$ mice have an intrinsically elevated ability of IFN-γ production. CD4 T cells **(A)** and CD8 T cells **(B)** from WT and Prf$^{-/-}$ mouse spleens were cultured in media supplemented with GolgiStop in the presence or absence of plate-bound anti-CD3 plus anti-CD28 for 4–6 h and then stained for intracellular IFN-γ (left, percentage; right, cell number, n = 5–6). **(C)** Naive CD4+ T cells were sorted from the spleen of WT or KO mice and differentiated to Th1 cells for 3 days. The IFN-γ production in these cells was detected by flow cytometry. Representative FACS plots and total cell number of IFN-γ+CD4+T cells were shown. The data shown in each panel of the figures were collected from a single experiment, and each experiment was repeated for at least three times and rendered consistent results. Means ± SEM, $^{*}p < 0.05$; $^{**}p < 0.01$.

and type I collagen deposition in the liver (38). NAFLD refers to a spectrum of liver diseases, including non-alcoholic fatty liver, which is characterized by steatosis with no or minor inflammation, and NASH, which is associated with inflammation and ballooning with or without fibrosis, and it may progress to liver cirrhosis and hepatocellular carcinoma (39, 40). The livers from mice fed a high-fat diet lacked fibrosis and showed mild steatosis and focal hepatocellular necrosis and apoptosis (41, 42). These contradictory findings suggest that perforin might have different actions at different stages during the pathogenesis of NAFLD and NASH.

Compared with WT controls, SFD-fed $Prf^{-/-}$ mice showed increased liver TG levels at an early age (**Figure 2C**), which suggests that perforin may regulate early liver lipid accumulation independent of diet. After 10 weeks of HFD challenge, $Prf^{-/-}$ mice had more IFN-γ-producing CD4 T cells in the liver. Further studies revealed that $Prf^{-/-}$ mice had intrinsically increased IFN-γ-producing ability in CD4 T cells. However, it is still unclear how perforin, a cytotoxic factor that helps mediate target cell death, could stimulate CD4 T cells to produce IFN-γ. Further studies are needed to better understand this phenomenon.

The promotion of hepatic steatosis resulting from perforin deficiency was associated with a strong increase in hepatic macrophage accumulation and inflammation as evaluated by the expression of TNF-α, IL-6, and iNOS. Traditionally, macrophages are divided into pro-inflammatory (M1) and wound-healing (M2) classes. M1 macrophages, which are induced by IFN-γ and LPS and express pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β, are implicated in the pathogenesis of chronic liver inflammation. M2 macrophages, which are induced by IL-4, IL-10, and IL-13 and produce IL-10, TGF-β, PDGF, and EGF, have anti-inflammatory effects and promote wound healing (43–45). It is well established that macrophages play an important role during NAFLD pathogenesis. Previous studies found that depletion of macrophages with clodronate could significantly reverse NAFLD in mice (3, 46). Liver immune homeostasis is largely regulated within the hepatic sinusoid, where resident macrophages (Kupffer cells) are located as part of the liver reticuloendothelial system (also known as the mononuclear phagocyte system). This system forms a highly active, dynamic, and complex network, constituting the primary line of defense against invading microorganisms along with the involvement of other immune cells such as neutrophils. In different stages of liver disease, resident Kupffer cells and freshly recruited monocyte-derived macrophages play a key role in the regulation of inflammation, fibrogenesis, and fibrolysis (47). In our study, $Prf^{-/-}$ mice had more macrophages, especially M1-type macrophage accumulation in liver after HFD challenge when compared with WT controls. However, we did not determine the mechanism of M1-type macrophage accumulation in the liver in this study. Is the increased accumulation due to the proliferation of resident Kupffer cells, or recruitment from peripheral circulatory systems, or the polarization of monocytes? We speculated that the increase in M1-type macrophage accumulation in the liver might be the result of monocyte polarization, since liver injury and lipid accumulation were almost non-existent in IFN-$\gamma R^{-/-}$ &$Prf^{-/-}$ mice with decreased M1-type macrophage accumulation in the liver when compared with $Prf^{-/-}$ mice. Further studies are necessary to better understand the mechanism behind this observation.

Depletion of CD4 T cells in $Prf^{-/-}$ mice rendered these mice less sensitive to NAFLD, with similar levels of liver TG and macrophage accumulation detected when compared with WT controls. This finding highlights the indispensable role of CD4 T cells, especially Th1 cells, in NAFLD progression. In clinical studies, it was reported that the peripheral CD4 compartment in obese children displayed a Th1-prone phenotype, and pediatric patients with NASH also showed increased expression of IFN-γ in the liver. Dysregulated lipid metabolism in NAFLD was reported to cause a selective loss of intrahepatic CD4+ lymphocytes, leading to accelerated hepatocarcinogenesis (48). In this study, we demonstrated that the protective effect of perforin in HFD-induced NAFLD was almost completely dependent on Th1 cells, which is consistent with the existing literature.

In conclusion, we demonstrated that perforin acts as an important immune regulator in NAFLD progression through regulating INF-γ-producing CD4 T cells to decrease macrophage accumulation in the liver. Based on these findings, therapeutic strategies targeting perforin might be a promising approach for the development of novel strategies to prevent or treat hepatic steatosis and related metabolic disorders in the liver.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by Laboratory Animal Ethics Committee Jinan University.

AUTHOR CONTRIBUTIONS

DL and QWa designed the project, performed experiments, and collected and analyzed the data. QWa wrote the manuscript. JZ and MZ worked on the mouse model. GC and JH helped modify and revise the article. QWe, HZ, and ZL maintained and genotyped the mice. LZ and QS performed RT-PCR. ZY, HY, and QWa supervised and coordinated the work, designed the overall research study, and helped write the manuscript. All authors have read, discussed, and approved the final manuscript.

FUNDING

This work was supported by the National Natural Science Foundation of China (the Key Program, 31830021), and the State Bureau of Foreign Experts Affairs (111 project, B16021) to ZY and HY. This work was also supported by the Science and Technology Department of the Guangdong Province of China (2018B030312008) to HY, the National Natural Science Foundation of China (grant 31800721 to QWa), the China Postdoctoral Fund (grant 2018M633278 to QWa), the National Natural Science Foundation of China (81630025, 31970830 to

JH), the Traditional Chinese Medicine Bureau of Guangdong Province (2018071 to JH), the Guangzhou Municipal Science and Technology Bureau (201904010090 to JH) and the Health Commission of Guangdong Province (A2019520 to JH).

ACKNOWLEDGMENTS

We thank Tucker Sarah for helping us correct typos and grammar errors and polish the text.

REFERENCES

1. Vernon G, Baranova A, Younossi ZM. Systematic review: the epidemiology and natural history of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis in adults. *Aliment Pharm Ther.* (2011) 34:274–85. doi: 10.1111/j.1365-2036.2011.04724.x
2. Temple JL, Cordero P, Li J, Nguyen V, Oben JA. A guide to non-alcoholic fatty liver disease in childhood and adolescence. *Int J Mol Sci.* (2016) 17:947. doi: 10.3390/ijms17060947
3. Kazankov K, Jorgensen SMD, Thomsen KL, Moller HJ, Vilstrup H, George J, et al. The role of macrophages in nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. *Nat Rev Gastroenterol Hepatol.* (2019) 16:145–59. doi: 10.1038/s41575-018-0082-x
4. Cha JY, Kim DH, Chun KH. The role of hepatic macrophages in nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. *Lab Anim Res.* (2018) 34:133–39. doi: 10.5625/lar.2018.34.4.133
5. Osayande AS, Kale N. Nonalcoholic fatty liver disease: identifying patients at risk of inflammation or fibrosis. *Am Family Phys.* (2017) 95:796–97.
6. Hardy T, Oakley F, Anstee QM, Day CP. Nonalcoholic fatty liver disease: pathogenesis and disease spectrum. *Annu Rev Pathol Mech.* (2016) 11:451–96. doi: 10.1146/annurev-pathol-012615-044224
7. Koyama Y, Brenner DA. Liver inflammation and fibrosis. *J Clin Invest.* (2017) 127:55–64. doi: 10.1172/JCI88881
8. Campisi J. Aging, cellular senescence, and cancer. *Annu Rev Physiol.* (2013) 75:685–705. doi: 10.1146/annurev-physiol-030212-183653
9. Provinciali M, Cardelli M, Marchegiani F, Pierpaoli E. Impact of cellular senescence in aging and cancer. *Curr Pharm Des.* (2013) 19:1699–709. doi: 10.2174/1381612811319090017
10. Herranz N, Gil J, Mechanisms and functions of cellular senescence. *J Clin Invest.* (2018) 128:1238–46. doi: 10.1172/JCI95148
11. Aravinthan A, Scarpini C, Tachtatzis P, Verma S, Penrhyn-Lowe S, Harvey R, et al. Hepatocyte senescence predicts progression in non-alcohol-related fatty liver disease. *J Hepatol.* (2013) 58:549–56. doi: 10.1016/j.jhep.2012.10.031
12. Tacke F. Targeting hepatic macrophages to treat liver diseases. *J Hepatol.* (2017) 66:1300–12. doi: 10.1016/j.jhep.2017.02.026
13. Cai DS, Yuan MS, Frantz DF, Melendez PA, Hansen L, Lee J, et al. Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB. *Nat Med.* (2005) 11:183–90. doi: 10.1038/nm1166
14. Glass CK, Olefsky JM. Inflammation and lipid signaling in the etiology of insulin resistance. *Cell Metab.* (2012) 15:635–45. doi: 10.1016/j.cmet.2012.04.001
15. Seki E, Schwabe RF. Hepatic inflammation and fibrosis: functional links and key pathways. *Hepatology.* (2015) 61:1066–79. doi: 10.1002/hep.27332
16. Badovinac VP, Tvinnereim AR, Harty JT. Regulation of antigen-specific CD8(+) T cell homeostasis by perforin and interferon-gamma. *Science.* (2000) 290:1354–7. doi: 10.1126/science.290.5495.1354
17. Bolitho P, Voskoboinik I, Trapani JA, Smyth MJ. Apoptosis induced by the lymphocyte effector molecule perforin. *Curr Opin Immunol.* (2007) 19:339–47. doi: 10.1016/j.coi.2007.04.007
18. Voskoboinik I, Smyth MJ, Trapani JA. Perforin-mediated target-cell death and immune homeostasis. *Nat Rev Immunol.* (2006) 6:940–52. doi: 10.1038/nri1983
19. Voskoboinik I, Whisstock JC, Trapani JA. Perforin and granzymes: function, dysfunction and human pathology. *Nat Rev Immunol.* (2015) 15:388–400. doi: 10.1038/nri3839
20. Spicer BA, Conroy PJ, Law RHP, Voskoboinik I, Whisstock JC. Perforin-A key (shaped) weapon in the immunological arsenal. *Semin Cell Dev Biol.* (2017) 72:117–23. doi: 10.1016/j.semcdb.2017.07.033
21. Revelo XS, Tsai S, Lei H, Luck H, Ghazarian M, Tsui H, et al. Perforin is a novel immune regulator of obesity-related insulin resistance. *Diabetes.* (2015) 64:90–103. doi: 10.2337/db13-1524
22. Zhang S, Liang R, Luo W, Liu C, Wu X, Gao Y, et al. High susceptibility to liver injury in IL-27 p28 conditional knockout mice involves intrinsic interferon-gamma dysregulation of CD4+ T cells. *Hepatology.* (2013) 57:1620–31. doi: 10.1002/hep.26166
23. Shan B, Wang X, Wu Y, Xu C, Xia Z, Dai J, et al. The metabolic ER stress sensor IRE1alpha suppresses alternative activation of macrophages and impairs energy expenditure in obesity. *Nat Immunol.* (2017) 18:519–29. doi: 10.1038/ni.3709
24. Bertolotti M, Lonardo A, Mussi C, Baldelli E, Pellegrini E, Ballestri S, et al. Nonalcoholic fatty liver disease and aging: epidemiology to management. *World J Gastroenterol.* (2014) 20:14185–204. doi: 10.3748/wjg.v20.i39.14185
25. Ovadya Y, Landsberger T, Leins H, Vadai E, Gal H, Biran A, et al. Impaired immune surveillance accelerates accumulation of senescent cells and aging. *Nat Commun.* (2018) 9:5435. doi: 10.1038/s41467-018-07825-3
26. Sagiv A, Biran A, Yon M, Simon J, Lowe SW, Krizhanovsky V. Granule exocytosis mediates immune surveillance of senescent cells. *Oncogene.* (2013) 32:1971–7. doi: 10.1038/onc.2012.206
27. Alisi A, Carpino G, Oliveira FL, Panera N, Nobili V, Gaudio E. The role of tissue macrophage-mediated inflammation on NAFLD pathogenesis and its clinical implications. *Mediat Inflamm.* (2017) 2017:8162421. doi: 10.1155/2017/8162421
28. Friedman SL, Neuschwander-Tetri BA, Rinella M, Sanyal AJ. Mechanisms of NAFLD development and therapeutic strategies. *Nat Med.* (2018) 24:908–22. doi: 10.1038/s41591-018-0104-9
29. Stojsavljevic S, Gomercic Palcic M, Virovic Jukic L, Smircic Duvnjak L, Duvnjak M. Adipokines and proinflammatory cytokines, the key mediators in the pathogenesis of nonalcoholic fatty liver disease. *World J Gastroenterol.* (2014) 20:18070–91. doi: 10.3748/wjg.v20.i48.18070
30. Angulo P, Alba LM, Petrovic LM, Adams LA, Lindor KD, Jensen MD. Leptin, insulin resistance, and liver fibrosis in human nonalcoholic fatty liver disease. *J Hepatol.* (2004) 41:943–9. doi: 10.1016/j.jhep.2004.08.020
31. Canbakan B, Tahan V, Balci H, Hatemi I, Erer B, Ozbay G, et al. Leptin in nonalcoholic fatty liver disease. *Ann Hepatol.* (2008) 7:249–54. doi: 10.1016/S1665-2681(19)31856-3
32. Walch M, Latinovic-Golic S, Velic A, Sundstrom H, Dumrese C, Wagner CA, et al. Perforin enhances the granulysin-induced lysis of Listeria innocua in human dendritic cells. *BMC Immunol.* (2007) 8:14. doi: 10.1186/1471-2172-8-14
33. Kagi D, Odermatt B, Ohashi PS, Zinkernagel RM, Hengartner H. Development of insulitis without diabetes in transgenic mice lacking perforin-dependent cytotoxicity. *J Exp Med.* (1996) 183:2143–52. doi: 10.1084/jem.183.5.2143
34. Nitcheu J, Bonduelle O, Combadiere C, Tefit M, Seilhean D, Mazier D, et al. Perforin-dependent brain-infiltrating cytotoxic CD8+ T lymphocytes mediate experimental cerebral malaria pathogenesis. *J Immunol.* (2003) 170:2221–8. doi: 10.4049/jimmunol.170.4.2221
35. Koike H, Kanda T, Sumino H, Yokoyama T, Arai M, Motooka M, et al. Reduction of viral myocarditis in mice lacking perforin. *Res Commun Mol Pathol Pharmacol.* (2001) 110:229–37.
36. Wang T, Sun G, Wang Y, Li S, Zhao X, Zhang C, et al. The immunoregulatory effects of CD8 T-cell-derived perforin on diet-induced nonalcoholic steatohepatitis. *FASEB J.* (2019) 33:8490–503. doi: 10.1096/fj.201802534RR
37. Cuff AO, Sillito F, Dertschnig S, Hall A, Luong TV, Chakraverty R, et al. The obese liver environment mediates conversion of NK cells to a less cytotoxic ILC1-like phenotype. *Front Immunol.* (2019) 10:2180. doi: 10.3389/fimmu.2019.02180

38. Brown GT, Kleiner DE. Histopathology of nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. *Metab Clin Exp.* (2016) 65:1080. doi: 10.1016/j.metabol.2015.11.008
39. Karagozian R, Derdak Z, Baffy G. Obesity-associated mechanisms of hepatocarcinogenesis. *Metab Clin Exp.* (2014) 63:607–17. doi: 10.1016/j.metabol.2014.01.011
40. Starley BQ, Calcagno CJ, Harrison SA. Nonalcoholic fatty liver disease and hepatocellular carcinoma: a weighty connection. *Hepatology.* (2010) 51, 1820–32. doi: 10.1002/hep.23594
41. Hebbard L, George J. Animal models of nonalcoholic fatty liver disease. *Nat Rev Gastro Hepat.* (2011) 8:34–44. doi: 10.1038/nrgastro.2010.191
42. Masi LN, Martins AR, Crisma AR, do Amaral CL, Davanso MR, Serdan TDA, et al. Combination of a high-fat diet with sweetened condensed milk exacerbates inflammation and insulin resistance induced by each separately in mice. *Sci Rep.* (2017) 7:3937. doi: 10.1038/s41598-017-04308-1
43. Murray PJ. Macrophage polarization. *Ann Rev Physiol.* (2017) 79:541–66. doi: 10.1146/annurev-physiol-022516-034339
44. Saradna A, Do DC, Kumar S, Fu QL, Gao P. Macrophage polarization and allergic asthma. *Transl Res.* (2018) 191:1–14. doi: 10.1016/j.trsl.2017.09.002
45. Sica A, Erreni M, Allavena P, Porta C. Macrophage polarization in pathology. *CMLS.* (2015) 72:4111–26. doi: 10.1007/s00018-015-1995-y
46. Sica A, Invernizzi P, Mantovani A. Macrophage plasticity and polarization in liver homeostasis and pathology. *Hepatology.* (2014) 59:2034–42. doi: 10.1002/hep.26754
47. Freitas-Lopes MA, Mafra K, David BA, Carvalho-Gontijo R, Menezes GB. Differential location and distribution of hepatic immune cells. *Cells.* (2017) 6:E48. doi: 10.3390/cells6040048
48. Ma C, Kesarwala AH, Eggert T, Medina-Echeverz J, Kleiner DE, Jin P, et al. NAFLD causes selective CD4(+) T lymphocyte loss and promotes hepatocarcinogenesis. *Nature.* (2016) 531:253–7. doi: 10.1038/nature16969

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 June 2020
doi: 10.3389/fimmu.2020.00906

The Genus *Alistipes*: Gut Bacteria With Emerging Implications to Inflammation, Cancer, and Mental Health

Bianca J. Parker[1], *Pamela A. Wearsch*[1], *Alida C. M. Veloo*[2] *and Alex Rodriguez-Palacios*[3,4]*

[1] Department of Pathology, Case Western Reserve University School of Medicine, Cleveland, OH, United States, [2] Department of Medical Microbiology, University Medical Centre Groningen, University of Groningen, Groningen, Netherlands, [3] Division of Gastroenterology and Liver Disease, Case Western Reserve University School of Medicine, Cleveland, OH, United States, [4] Germ-Free and Gut Microbiome, Digestive Health Research Institute, Case Western Reserve University, Cleveland, OH, United States

OPEN ACCESS

Edited by:
M. Victoria Delpino, CONICET Institute of Immunology, Genetics and Metabolism (INIGEM), Argentina

Reviewed by:
Valerio Iebba, Istituto Pasteur Italia Cenci Bolognetti Foundation, Italy
Alexander R. Moschen, Innsbruck Medical University, Austria

***Correspondence:**
Alex Rodriguez-Palacios
axr503@case.edu

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

Received: 21 December 2019
Accepted: 20 April 2020
Published: 09 June 2020

Citation:
Parker BJ, Wearsch PA, Veloo ACM and Rodriguez-Palacios A (2020) The Genus Alistipes: Gut Bacteria With Emerging Implications to Inflammation, Cancer, and Mental Health. Front. Immunol. 11:906. doi: 10.3389/fimmu.2020.00906

Alistipes is a relatively new genus of bacteria isolated primarily from medical clinical samples, although at a low rate compared to other genus members of the *Bacteroidetes* phylum, which are highly relevant in dysbiosis and disease. According to the taxonomy database at The National Center for Biotechnology Information, the genus consists of 13 species: *Alistipes finegoldii*, *Alistipes putredinis*, *Alistipes onderdonkii*, *Alistipes shahii*, *Alistipes indistinctus*, *Alistipes senegalensis*, *Alistipes timonensis*, *Alistipes obesi*, *Alistipes ihumii*, *Alistipes inops, Alistipes megaguti, Alistipes provencensis,* and *Alistipes massiliensis. Alistipes communis* and *A. dispar*, and the subspecies *A. Onderdonkii* subspecies vulgaris (vs. *onderdonkii* subsp.) are the newest strains featured outside that list. Although typically isolated from the human gut microbiome various species of this genus have been isolated from patients suffering from appendicitis, and abdominal and rectal abscess. It is possible that as *Alistipes* spp. emerge, their identification in clinical samples may be underrepresented as novel MS-TOF methods may not be fully capable to discriminate distinct species as separate since it will require the upgrading of MS-TOF identification databases. In terms of pathogenicity, there is contrasting evidence indicating that *Alistipes* may have protective effects against some diseases, including liver fibrosis, colitis, cancer immunotherapy, and cardiovascular disease. In contrast, other studies indicate *Alistipes* is pathogenic in colorectal cancer and is associated with mental signs of depression. Gut dysbiosis seems to play a role in determining the compositional abundance of *Alistipes* in the feces (*e.g.*, in non-alcoholic steatohepatitis, hepatic encephalopathy, and liver fibrosis). Since *Alistipes* is a relatively recent sub-branch genus of the *Bacteroidetes* phylum, and since *Bacteroidetes* are commonly associated with chronic intestinal inflammation, this narrative review illustrates emerging immunological and mechanistic implications by which *Alistipes* spp. correlate with human health.

Keywords: *Alistipes finegoldii*, inflammatory bowel diseases, *A. putredinis*, *A. onderdonkii*, *A. shahii*, *A. indistinctus*, *A. senegalensis*, *A. timonensis*

INTRODUCTION

The human gut microbiome is acquired at birth and as individual go through life, their gut becomes a home to trillions of microorganisms, namely bacteria. With the use of 16S rRNA gene sequencing a diverse profile of bacterial phyla has been identified, with the two most common being *Bacteroidetes* and *Firmicutes*. Collectively, the composition of the gut microbiota amounts to a massive number of cells and functions that equate (even surpass) to that of an additional organ (1). The human microbiota plays a critical role in regulation of the immune response, protection against pathogens, aiding in digestion, as well as in neurologic signaling and vascularization (2). Notably, the microbiota has been found to be involved in health and disease. The imbalance, or dysbiosis, of the intestinal microbiota has been attributed to numerous diseases such as cancer (3), cardiovascular disease (4), inflammatory bowel disease (5), and nervous system disorders (6). Investigators have examined various genera and species that make up the microbiome trying to decipher their role as a collective trying to identify individual microbial species with the ability to modulate diseases.

Taxonomically, *Alistipes* (a. li. sti'pes) is a genus described in 2003 after being discovered in tissue samples of children with appendicitis (7). *Alistipes* are anaerobic bacteria found mostly in the healthy human gastrointestinal (GI) tract microbiota (8). According to the taxonomy database at The National Center for Biotechnology Information (NCBI, txid239759), as of April 2020, *Alistipes* consists of 13 species: *Alistipes finegoldii*, *Alistipes putredinis*, *Alistipes onderdonkii*, *Alistipes shahii*, *Alistipes indistinctus*, *Alistipes senegalensis*, *Alistipes timonensis*, *Alistipes obesi*, *Alistipes ihumii*, *Alistipes inops, Alistipes megaguti, Alistipes provencensis,* and *Alistipes massiliensis* (the latter strain reported as "unpublished by Lacroix et al., as of March 4, 2004, NCBI:txid265312" remains unpublished under that name). However, a case study in 2017 from the same group of scientists reported a novel species called *Tidjanibacter massiliensis* Marseille-P3084, isolated from the colon of a person with irritable bowel syndrome, with 92.1% sequence homology to *A. putredinis* (9), but the strain has not been officially recognized or deposited in public strain biorepositories. To date it is unclear if the *Tidjanibacter* is the same *Alistipes* isolate, or what occurred with the publication of the *Alistipes massiliensis* for which there is no genome or 16S RNA gene sequences available in NCBI. Three newer species were added to the the "List of Prokaryotic names with Standing in Nomenclature" database in January 2020: *A. communis* (strain 5CBH24; DSM 108979; JCM 32850), *A. dispar* (5CPEGH6; DSM 108978; JCM 32848), and the sub-speciation scheme for *A. Onderdonkii* as subsp. *vulgaris* subsp. nov. and the subsp. *onderdonkii* subsp. nov. (https://lpsn.dsmz.de/genus/alistipes, which listed only 11 species on May 12, 2020) (10).

Anecdotally, the first species of *Alistipes* to be discovered was *A. finegoldii* (fi.ne.gol'di.i) which was then named after Sydney M. Finegold (born in 1921; died on September 27, 2018, age 97), a contemporary American researcher in anaerobic bacteriology and infectious diseases clinician who held several Emeritus academic positions at the UCLA and the Wardsworth VA Hospital in Los Angeles since the year 2000 (7, 11). Several recent articles commemorate Dr. Finegold's career in anaerobic microbiology, who greatly contributed to our understanding of anaerobes, which led to the species of *Alistipes* being named after him (12). With a remarkable productivity, Dr. Finegold left a legacy with 819 publications on various aspects of medicine and anaerobic microbiology (13). Alongside *A. finegoldii*, in 2003, *Bacteroides putredinis* was reclassified as *A. putredinis* based on 16S rRNA gene sequencing and biochemical features that showed this species did not belong to the genus *Bacteroides*, but to the genus *Alistipes* (7). Dr. Finegold's charisma is illustrated with the species *A. onderdoinklii*, which was named to honour Andrew B. Onderdonk, a contemporary American microbiologist, Professor of Pathology at Harvard Medical School, for his contribution to intestinal and anaerobic microbiology.

In recent years, several studies have investigated the alterations in bacterial abundance for *Alistipes* in human patients and mouse models during disease. Studies have shown that *Alistipes* dysbiosis can be either beneficial, or harmful. *Alistipes* has been implicated in liver fibrosis (14), colorectal cancer (15), cardiovascular disease (16), and mood disorders (17), among other potential diseases. Additionally, the unique way of fermenting amino acids, putrefaction, has implicated *Alistipes* to play a critical role in inflammation and disease (18). The objective of this review is to expound upon the relationship between *Alistipes* and several non-communicable diseases and to highlight the implications of *Alistipes* dysbiosis.

PHENOTYPING, CULTURE, AND SPECIATION

Alistipes is classified as gram-negative, rod-shaped, anaerobic, and non-spore forming. The species within this genus are non-motile except for *A. obesi*. The genus consists of 12-published species (*A. massiliensis* is not yet published, see above). *Alistipes finegoldii* (type strain AHN 2437^T; CCUG 46020) has circular colonies (0.3–1.0 mm; raised, gray to opaque on sheep blood agar). Despite supplementation, its growth is difficult in liquid media. *Alistipes finegoldii* is bile-resistant and esculin-negative. This bacterium is catalase-negative and nitrogen-reductase negative; and it cannot liquefy gelatin in liquefaction test. This bacterium can hydrolyze tryptophan to indole. In peptone yeast glucose (PYG) broth the major acid produced is succinic acid with a minor amount of acetic and propionic acid produced. The main fatty acid is 13-metyltetradecanoic acid (iso-C15:0). These strains of *A. finegoldii* are resistant to vancomycin, kanamycin, and colistin, as it is expected for *Bacteroides,* according to the Wadsworth manual (19) (named after the hospital where Dr. Finegold worked as a physician and scientist). For the primary identification of anaerobic bacteria, the further use of a bile susceptibility test combined with the antimicrobial resistance helps in differentiating *Alistipes* species as illustrated for some species below (for comparison, *Bacteroides* are resistant to bile) (20), although the precise definition of a species is a subject of constant debate (21). The phenotypic characteristics

TABLE 1 | Summary of first 10 *Alistipes* species and their characteristics.

	A. finegoldii: human appendiceal tissue	***A. putredinis:*** feces, appendicitis, abdom/rectal abscess foot rot sheep, farm soil	***A. onderdonkii:*** abdominal abscess	***A. shahii:*** appendix tissue, urine	***A. indistinctus:*** human feces	***A. timonensis:*** feces, healthy patients Senegal	***A. senegalensis:*** feces, healthy patients Senegal	***A. obesi:*** fecal flora of a French patient suffering from obesity	***A. ihumii:*** fecal flora of female French patient; severe anorexia nervosa	***A. inops:*** human feces
Gram stain	−	−	−	−	−	−	−	−	−	−
Motile	−	−	−	−	−	−	−	+	−	−
Bile resistant	+	−	+	+	−	/	/	/	/	/
Pigment	+	−	+	+	−	+	+	/	/	/
Esculin	−	/	/	/	/	/	/	/	/	/
Gelatin	+	/	/	/	/	/	/	/	/	/
Catalase	−	+	−	−	+	+	+	+	−	−
Nitrogen red.	−	−	−	−	−	/	/	−	−	−
Indole	+	+	+	+	−	+	+	−	−	+
Fermentative	F	NF	F	F	/	NF	F	NF	/	NF
Urease	/	−	−	−	−	/	/	−	−	−

Antibiotic Resistance. A. finegoldii, vancomycin, kanamycin, and colistin. A. putredinis, his strain is sensitive to clindamycin, cefoxitin, chloramphenicol, erythromycin, and metronidazole and moderately resistant to tetracycline and doxycycline. A. timonensis, susceptible to penicillin G, amoxicillin + clavulanic acid, imipenem and clindamycin; A. timonensis is susceptible to metronidazole. A. senegalensis, susceptible to penicillin G, amoxicillin + clavulanic acid, imipenem and clindamycin; A. senegalensis is resistant to metronidazole. A. obesi, susceptible to imipenem, ciprofloxacin, metronidazole, nitrofurantoin, and rifampicin, but resistant to penicillin G, amoxicillin, amoxicillin-clavulanic acid, erythromycin, vancomycin, gentamicin, doxycycline, ceftriaxone, and trimethoprim/sulfamethoxazole. A. ihummi, susceptible to amoxicillin, imipenem, and clindamycin, but resistant to vancomycin. /, not reported; F, fermentative; NF, non-fermentative.

of 10 *Alistipes* spp. in culture media are summarized in **Table 1**.

From a clinical perspective, it is reasonable to expect that bile-resistant strains are more likely to be present or abundant than bile-susceptible strains in the terminal ileum where most bile reabsorption takes place in several mammalian species including humans (22) and where chronic inflammatory bowel conditions are more likely to occur due to alterations in bile-mediated T-cell immunoreactivity (23). However, with the recent discovery of this genus, and the emerging complexity of the genus speciation, it will be necessary to determine if the resistance to bile acids indeed determines the *Alistipes* abundance regionally within the GI, or clinically in diseases characterized by alterations in bile production, which originates in the liver, or their recirculation pathways through the small intestine. Also, clinically relevant, some strains produce beta-lactamases (7), which enhance their antimicrobial resistance to beta-lactam antibiotics including penicillin and cephalosporins.

The species *A. putredinis* (type strain ATCC 29800^T) has been isolated from various specimens such as feces, appendiceal tissue of a patient with acute appendicitis, abdominal and rectal abscesses, foot rot in sheep, and even farm soil (7). This species has circular to slightly irregular convex colonies (pinpoint to 0.5 mm; smooth, translucent to gray). This is a non-pigment producing species and is not 20% bile-resistant. It is catalase-positive and nitrate reductase- and urease-negative. This bacterium can hydrolyze tryptophan to indole. This species is non-fermentative. In 6-day old PYG broth only trace amount of acid was produced, however, in 24-h chopped meat carbohydrate broth succinic acid was produced as a major acid product with minor amounts of acetic, isobutyric, isovaleric, and propionic acid. This strain is sensitive to clindamycin, cefoxitin, chloramphenicol, erythromycin, and metronidazole and moderately resistant to tetracycline and doxycycline (7).

Alistipes onderdonkii (type strain WAL 8169^T; CCUG 48946^T; ATCC BAA-1178^T) and *A. shahii* (type strain WAL 8301^T; CCUG 48947^T; ATCC BAA-1179^T) were isolated from abdominal abscess and appendix tissue respectively, as well as urine (24). They have circular colonies (0.5–0.8 mm and 0.5–1 mm, respectively; gray to opaque on blood agar) and are pigment producing, non-fluorescing when grown on rabbit blood agar. Both species are resistant to 20% bile and hydrolyze tryptophan to indole. They are catalase-, nitrogen reductase-, and urease-negative. Mannose and raffinose are fermented when using the API rapid ID 32A system. Succinic acid is the major metabolic end-product with minor production of acetic and propionic acid. The primary long-chain fatty acid is iso-C15:0 (24). Details for *A. onderdonkii* subspeciation as *vulgaris* subsp. nov. (3BBH6T = JCM 32839T = DSM 108977T, isolated from human feces) for can be found at https://lpsn.dsmz.de/genus/alistipes.

Alistipes indistinctus (type strain YIT 12060^T; DSM 22520^T; JCM 16068^T) was the next species to be discovered. It was isolated from human feces. This species is slightly different, since its shape is more coccoid than rod-shaped, as well as

its inability to hydrolyze tryptophan to indole. Its colonies are circular (0.1–0.5 mm slight opaque and gray on modified GAM agar). This bacterium is susceptible to 20% bile, catalase-positive, and urease- and nitrogen reductase-negative. The major products in PYG broth are succinic and acetic acid. The major cellular fatty acid is iso-C15:0 (25).

Two other species, *A. senegalensis* (type strain JC50^T; CSUR P156; DSM 25460) and *A. timonensis* (type strain *JC136^T; CSUR P148; DSM 25383)*, were initially isolated from the fecal flora of healthy patients in Senegal. They have circular colonies (0.2–0.3 mm) and are pigment producing. They are catalase and can hydrolyze tryptophan to indole. *A. senegalensis* does ferment mannose, and *A. timonensis* does not. These strains of bacteria are susceptible to penicillin G, amoxicillin plus clavulanic acid, imipenem, and clindamycin. Furthermore, *A. senegalensis* strains are resistant to metronidazole and *A. timonensis* strains are susceptible to metronidazole (21, 26).

Alistipes obesi (type strain ph8^T; CSUR; P186;DSM 25724) was isolated from the fecal microbiota of a French patient suffering from obesity (27). It is a pigment producing species with colonies that are 0.5 mm in diameter; translucent and light gray on blood enriched Columbia agar. It is catalase-positive, nitrogen reductase-, and urease-negative as well as non-fermentative. This bacterium cannot hydrolyze tryptophan to indole. These strains are susceptible to imipenem, ciprofloxacin, metronidazole, nitrofurantoin, and rifampicin, but resistant to penicillin G, amoxicillin, amoxicillin-clavulanic acid, erythromycin, vancomycin, gentamicin, doxycycline, ceftriaxone, and trimethoprim/sulfamethoxazole (27).

Alistipes ihumii (type strain AP11^T; CSUR P204; DSM 26107) was isolated from the fecal microbiota of a female French patient suffering from severe anorexia nervosa. This species forms colonies that are 0.2 mm and translucent on blood-enriched Columbia agar. This bacterium is non-fermentative. It is negative for urease, nitrate reduction, and catalase activity. This bacterium cannot hydrolyze tryptophan to indole. *A. ihumii* strains are susceptible to amoxicillin, imipenem, and clindamycin, but resistant to vancomycin (28).

The species *Alistipes inops* (type strain 627^T; 5DSM 28863^T; VKM B-2859^T) was isolated from human feces. The colonies are circular (0.18–0.30 mm; light gray, with a pale brown center). It has scanty growth on liquid media. It is positive for indole production and negative for catalase, nitrate reductase, and urease. This bacterium is non-fermentative. The major metabolic end products in PYG broth are succinic and acetic acid. The major fatty acid is iso C15:0 (8).

Recently, between November 2019–January 2020, four, new species of *Alistipes* were discovered, *A. megaguti*, *A. provencensis*, *A. communis* (type strain 5CBH24; DSM 108979; JCM 32850, commonly in human feces) and *A. dispar* (5CPEGH6; DSM 108978; JCM 32848, from human feces). *A. megaguti* (type strain Marseille-P5997^T) was isolated from a fresh fecal sample of a young healthy female. This species forms colonies that are 0.2–0.9 mm in diameter on blood-enriched Columbia agar. *A. megaguti* are catalase, urease, and oxidase negative. *A. provencensis* (type strain Marseille-P2431^T) was isolated from a male patient with hypertension and diabetes. This species forms colonies that are 0.4–0.64 mm in diameter on blood-enriched Columbia agar. This species is oxidase and urease negative, and catalase positive (29).

The phenotypic identification of *Alistipes* species is feasible (**Table 1**), yet challenging. A more suitable way to identify *Alistipes* spp. is via NGS (next generation sequencing-based microbiome studies) (30). However, most studies for the classification of new species, which cannot be fully sequenced have been based on Sanger sequencing of the full gene or parts of the 16S rRNA gene. For reference, the specificity of various primers employed for the identification of *Alistipes* species are listed and referenced in **Table 2**, while the details in nucleotide sequence homology and genetic distances within the genus are depicted in **Figure 1**. Compared to culture techniques, the NGS method is more powerful for bacterial identification. However, it does not provide information on the metabolic functions of the organism. Another form of bacterial identification is through metagenomic studies. In this approach, bacterial DNA is processed and sequenced along with host DNA. A new clinical approach that is faster and more effective than Sanger sequencing for bacterial classification is based on mass-spectrometry. Specifically, new rapid high-through put strategies based on matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) have become widely adapted and common in clinical microbiological settings, which require the constant update of databases to incorporate the spectral of new bacterial strains, primarily of clinical relevance.

MASS SPECTROSCOPY FOR IDENTIFICATION OF BACTERIAL SPECIES

Since the innovative use of MALDI-TOF MS to expedite the identification of microbes in clinical microbiology, there has been a major increase in our understanding of the presence and prevalence of *Alistipes* spp. in clinical medicine. MALDI-TOF MS is a method based on protein spectra, enabling identification of an unknown bacterium within minutes. Due to the introduction of this method in diagnostic microbiology laboratories, fastidious organisms which could previously only be identified using molecular methods, are now identified with ease. With the advancement of MALDI-TOF MS, there has been collaborative initiatives to improve microbial identification, including the European Network of Rapid Identification of Anaerobes (ENRIA), which is multi-national consortium of laboratories in Europe with the goal of unifying the MALDI-TOF MS database for the rapid identification of clinical anaerobes. According to the ENRIA validation study, conducted in 2018 (38), at present 5 *Alistipes* species are represented in the MALDI-TOF MS database: *A. finegoldii*, *A. onderdonkii, A. shahii, A. indistinctus,* and *A. putredinis*.

TABLE 2 | Referent partial Sanger 16S rRNA gene sequences and primers for *Alistipes* spp.

Species (strain)	Accession	F-primer 5′-3′
A. finegoldii (30) CIP 107999	AY643083 AY643084 1,446 bp	fD1 ccgaattcgtcgacaacAGAGTTTGATCCTGGCTCAG* rP2-cccgggatccaagcttACGGCTACCTTGTTACGACTT*
A. putredinis (31) ATCC 29800	NR_025909 1,468 bp	
A. onderdonkii (24) WAL 8169	NR_043318 1,440 bp	Position Sequence for most *Bacteroides* subgroup
A. shahii (24) WAL 8301	AY974072 1,433 bp	104–121 GTTACTCACCCGTGCGCC[a] 288–307 ACCTTCCTCTCAGAACCCCT [b]
A. indistinctus (25) YIT 12060	NR_112896 1,482 bp	344–358 ACTGCTGCCTCCCGT 519–536 GWATTACCGCGGCKGCTG
A. timonensis (32) JC136	NR_125589 1,423 bp	553–572 TAAACCCAATAAATCCGGAT[c] 786–803 CTACCAGGGTATCTAATC
A. senegalensis (32) JC50	NR_118219 1,423 bp	907–926 CCGTCAATTCMTTTRAGTTT[c] 1092–1114 GGGTTGCGCTCGTTATGGCACTT [c]
A. obesi (32) ph8	NR_133025 1,388 bp	1225–1242 CCATTGTAACACGTGTGT GGCGGTGTGTRC [c]
A. ihumii (28) AP11	NR_144706 1,494 bp	1487–1505 CCTTGTTACGACTTAGCCC *Standard IUB codes for bases and ambiguity*
A. inops (8) *627*	NR_145882 1,237 bp	Inset table, See details in Paster et al. (31). Primer sequence specificity slightly varying across gene positions [a]*Bacteroides* + *Prevotella* except *Prevotella nigrescens, P. intermedia*. No porphyromonas. [b]*Bacteroides* + *Prevotella*, but also for *Parabacteroides distasonis*, butyric acid-producing *Odoribacter splanchnicus* comb. nov. (33, 34), and periodontitis-associated *Tannerella forsythia* (formerly, *Bacteroides forsythus*), and biofilm-associated *Rikenella microfusus* (35). [c]Most bacteria with some exceptions for *P. saccharolytica* (35) and periodontitis associated *Porphyromona endodontalis* (36), *A. putredinis*, *O. splanchnicus*, and *R. microfusus*
A. megaguti (29) Marseille-P5997	fig|2364787.3.rna.19 Complete 1,534 bp** LS999984 1,525 bp	
A. provencensis (29) Marseille-P2431	LT223566 1,491 bp	
Tidjanibacter massiliensis	LT598563 1,492 bp	
A. Onderdonkii subsp vulgaris	LC468789 1456bp	LC468802*** 558bp
A. dispar	LC468801 1464bp	LC46889*** 558bp
A. communis	LC468799 1484bp	LC468808*** 558bp

**Primers (upper case) used by Weisburg et al. (37) and designed for most eubacteria. f, forward; r, reverse; D, distal; P, proximal. Linker sequences containing restriction sites for cloning are designated in lower case. The "f" series of linkers contain EcoRI and SalI sites, and the "r" series contain HindIJI, BamHI, and XmaI recognition sequences. Reverse primers produce sequences complimentary to the rRNA. Primers rPI, rP2, and rP3 are identical except for the 17th base from the 3′ end. The P2 primer was determined as the sequence providing the greatest diversity of bacteria. FASTA files available for download at https://www.ncbi.nlm.nih.gov/search/all/?term=NR__125589 **complete sequence extracted from complete genome available in PATRIC https://docs.patricbrc.org.*

****Phylogenetic analysis for speciation can also be conducted for Alistipes using the heat shock protein 60 gene (hsp60) as reported by Sakamoto in 2020. Accession numbers for the whole genome sequences of strains 3BBH6T, 5CBH24T, 5CPEGH6T, 5CPYCFAH4, 5NYCFAH2 and 6CPBBH3 are AP019734-AP019739, respectively.*

To contextualize the current relevance of the genus in clinical diagnostics, we conducted a screening of the number of *Alistipes* isolates recovered from human clinical samples at the University Medical Center Groningen, Groningen, the Netherlands, within the 7 years prior to submission of this manuscript. Keeping in mind that only species represented in MALDI-TOF MS databases have been identified using this method, it is noteworthy to mention that only 11 ($n = 11$) clinical isolates belonged to the *Alistipes* genus. Of relevance, these isolates were identified only as either *A. onderdonkii* ($n = 7$) or *A. finegoldii* ($n = 4$). The *Alistipes* isolates were recovered from pus, blood, ascites fluid or from an unspecified specimen. Considering that there have been at least 13 different species identified as distinct according to the NCBI lineage database (as of November 2019), it is interesting that only few species ($n = 5$) have been identified in clinical settings screened by ENRIA. The lack of identification of the other species (e.g., *A. senegalensis*, *A. timonensis*, *A. obesi*, *A. ihumii*, and *A. inops*) in this population indicated that either: (i) *Alistipes* may have specific patterns of geographically-restricted distribution, (ii) that their prevalence in the environment and patients is rather low, (iii) that they are comparatively more difficult to be cultured from clinical specimens using media that apparently facilitates the recovery of the other *Alistipes* spp. (namely, *A onderdonkii* and *A. finegoldii*), (iv) that the MS profile used

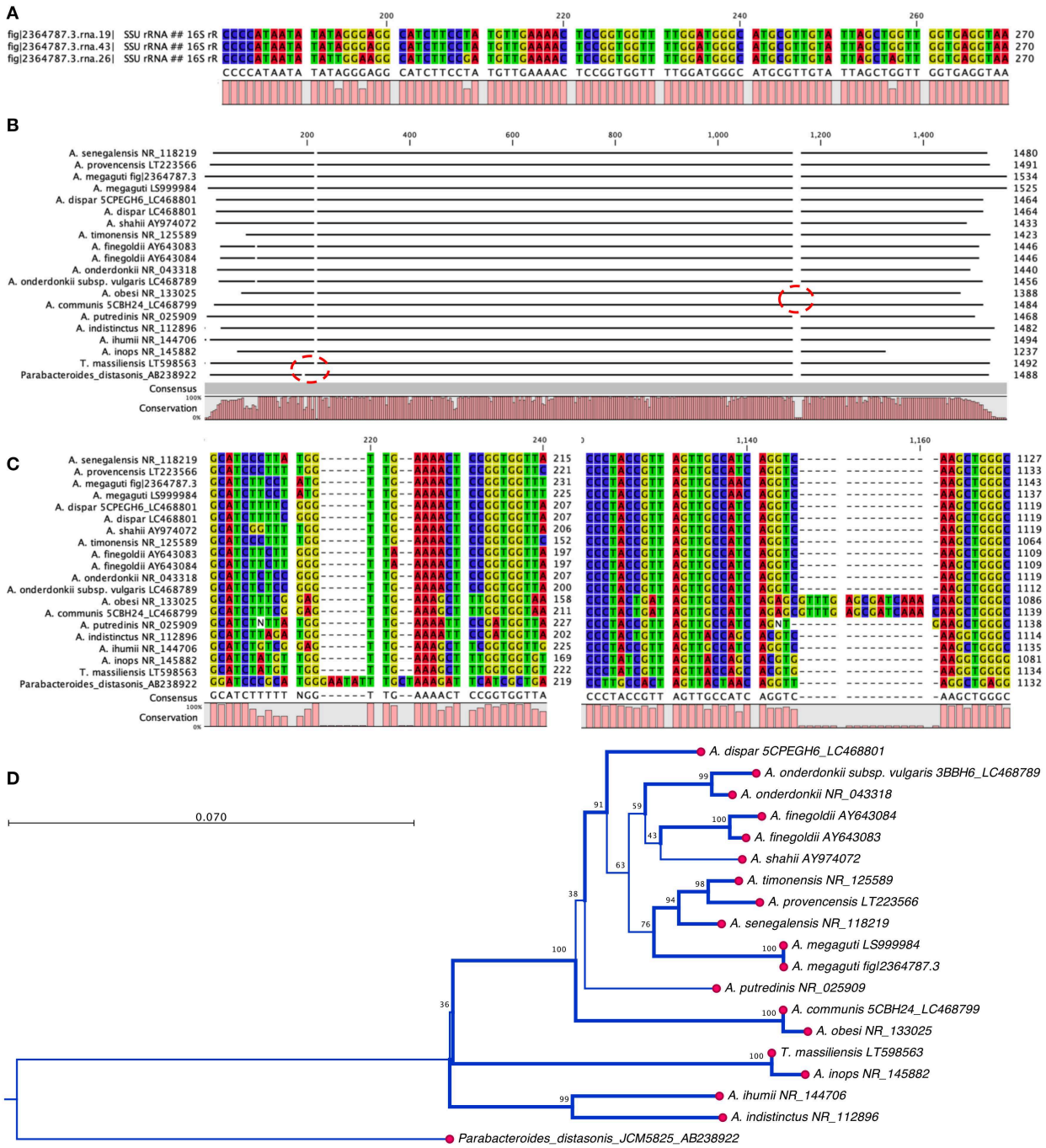


FIGURE 1 | Nucleotide and phylogram of 16S rRNA gene DNA sequences from thirteen strains described in the genus *Alistipes*. **(A)** *Alsitipes megaguti* complete 16S rRNA gene sequences derived from PATRIC complete genome illustrates slight differences between three gene copies contained within three operons in a single genome. Only the most divergent parts of the genes are shown. **(B)** Overview of complete alignment for the 13 strains. Notice the *A. obesi* insertion at around 1,100 bp position. **(C)** Detail of sequence insertion in the *A. obesi* starting at position 1,137 bp position. **(D)** Overview of the distance tree with 10,000 bootstrap branch values depicted if value >90%. Branching set at 60% threshold. Notice that differences may occur in part due to uneven partial gene sequences. Analysis conducted with CLC genomics viewer. Notice position of *T. massiliensis* vs. *A. inops* and *A. putredinis*.

to differentiate species is still suboptimal to discriminate such species as separate, or (v) that the original species descriptions may not be reproducible descriptions in the context of culture and mass spectrometry profiles.

ECOLOGY IN DISEASES

According to the NCBI, the full lineage of *Alistipes* is Bacteria; *Bacteroidetes*; *Bacteroidia*; *Bacteroidales*; *Rikenellaceae* (NCBI:

txid239759). Therefore, *Alistipes* is a genus member of the *Rikenellaceae* family (NCBI:t xid171550), which is a small family within the *Bacteroidales* order that is composed of eight genera: *Acetobacteroides*, *Alistipes*, *Anaerocella*, *Millionella*, *Mucinivorans*, *Rikenella*, *Ruminofilibacter*, and *Tidjanibacter*. These genera are commonly found in the intestinal tract of various animals and humans, where they are believed to have symbiotic relationships with the host. One of the most commonly detected species from the genus *Alistipes,* that can be isolated from human feces, is *A. onderdonkii* (39).

From an ecological perspective, *Alistipes* is found primarily in the gut of healthy humans (8). However, *Alistipes* has also been isolated from the blood stream, as well as appendicular, abdominal, perirectal and brain abscesses highlighting their potential opportunistic pathogenic role in human diseases. *Alistipes* has been found in other body fluids, namely, urine and peritoneal fluid (8). Of contrasting interest within this genus, *A. ihumii* has been isolated from the feces of a patient suffering from anorexia nervosa, while *A. obesi* was isolated from a patient suffering from morbid obesity. Combining the later observations with the fact that *Alistipes finegoldii* has been considered a growth promoter in broiler chickens (40), and *A. putredinis* has been observed to increase with cruciferous vegetable intake in humans (41), it is reasonable to assume that different *Alistipes* species may have different roles in nutrition and health, depending on the host, and the bodily system influenced. Although the NCBI taxonomy database has a disclaimer stating that it is not an authoritative source for nomenclature or classification, the NCBI recommend the database users to consult the relevant scientific literature for the most reliable information, which is summarized below and in **Table 3**.

ALISTIPES IN LIVER DISEASE AND SHORT-CHAIN FATTY ACIDS

Hepatocellular carcinoma (HCC) is the second deadliest form of cancer worldwide (55).

HCC is often developed from advanced liver fibrosis that is caused by cirrhosis, non-alcoholic fatty liver disease (NAFLD), or non-alcoholic steatohepatitis (NASH). These liver diseases have been associated with the "microbiota-liver axis," indicating that dysbiosis as one of the potential causes (56). In studies done on the microbiota composition and liver fibrosis. It is seen that throughout the advancement of the fibrosis that *Alistipes* is decreased.

For example, in patients with compensated and decompensated liver cirrhosis (LC), a paired-end metagenomic sequence of the gut microbiome from fresh stool samples of healthy volunteers and patients with various types of LC. This study showed a decrease of *A. shahii* and *A. putredinis* when compared to healthy controls. This finding of an increase in the abundance of *Alistipes* in healthy control patients in comparison to patients with LC has also been seen in studies focusing on feces and biopsies of LC patients (57). Moreover, a decrease of *A. indistinctus* was observed as the disease progressed from compensated to decompensated (43). Once an individual has decompensated liver cirrhosis, the patient begins to generate a multitude of severe complications, such as hepatic encephalopathy. Another study showed that when comparing stool microbiome between patients suffering from decompensated liver cirrhosis with acute hepatic encephalopathy, *Alistipes* has a protective role and the decrease in its abundance correlates to an increase of hepatic encephalopathy recurrence (44). Thus, a decrease in *Alistipes* spp. correlates with the progression of liver cirrhosis into the decompensated state.

Additionally, the reduction in *Alistipes* abundance in patients with liver fibrosis can be seen in other fibrotic diseases such NASH and NAFLD. Rau et al. (14) showed that patients with NAFLD with substantial fibrosis had a reduction in fecal concentration of acetate and propionate, with no significant difference in butyrate concentration. When comparing healthy controls with patients suffering from NASH, there is a significant reduction in *A. finegoldii* with the normalized count mean reduced from 542 to 19 (fold-change of −1.829). Moreover, in NAFLD patients with significant fibrosis, a major reduction in *A. onderdonkii* was observed from 285 to 31 (fold change of −2.566). It is noteworthy that *Alistipes* spp. decreases in these advanced fibrotic patients as well as fecal acetate and propionate levels. This correlates with a study done by Polansky et al. (58) where they showed that in the cecal microbiota of chickens that *Alistipes* is a propionate producer expressing methylmalonyl-CoA epimerase, in which the gene for this enzyme is located on an operon with the acetyl-CoA carboxylase gene. It has also been shown that *Alistipes* is an acetate producer (59). Due to previous studies suggesting that short chain fatty acids (SCFAs) have anti-inflammatory mechanisms, it can be suggested that this decrease in *Alistipes* contributes to the decrease in SCFA and therefore contributes to the advanced fibrosis seen in these NAFLD patients.

Moreover, a study performed on mice with HCC showed the potential anti-inflammatory effects of a health-beneficial bacteria probiotic. It was believed that T-regs would be induced in patients with HCC thus suppressing the Th17 expression. A probiotic entitled Prohep was used, and after 38 days a reduction in tumor growth and Th17 cells was observed. Moreover, there was an increase in Treg/Tr1 cells, an anti-inflammatory cell subset. *Alistipes* was shown to increase in abundance in the mice cohort receiving the probiotic (45, 60). At the species level it is shown that *A. shahii* was one of the species that was significantly increased in the probiotic group. They hypothesize that *A. shahii* was playing a role in tumor suppression similar to what was is seen in cancer immunotherapy (61). Additionally, the investigators noted a significant increase in acetate and propionate metabolic potential in the probiotic group correlating with an increase in SCFAs-producing bacteria, such as *Alistipes* contributing to the suppression of Th17 cells in the gut, ultimately reducing the recruitment of Th17 cells to the liver (45). Also, in past studies it has been shown that SCFAs derived from fermented dietary fibers increase the levels of propionate found within the portal vein ultimately preventing cancer cell proliferation in liver tissue (62). Ultimately, *Alistipes*

TABLE 3 | Summary of studies reporting the experimental or observational associations between *Alistipes* spp. and various non-communicable diseases (2003–2019).

Disease	Study model	Study design	*Alistipes* effects
Inflammation Colitis (42) (Protective)*	Mice: BALB/c	Oral DSS-colitis. 16S microbiome and oral infection.	Pglyrp1-4 (antibacterial immunomodulator gene) KO mice have less *Alistipes* spp. Oral *A. finegoldii* attenuates antibiotic > DSS-colitis in WT mice
Inflammation Liver cirrhosis (43) (Protective)	Humans: China	Meta-omics-based study analyzing urine and stool samples from health controls, compensated and decompensated LC patients	In both compensated and decompensated LC patients *A. shahii* and *A. putredinis* decrease and during progression from compensated to decompensated *A. indistinctus* further decreased
Inflammation Acute hepatic encephalopathy (44) (Protective)	Humans: Taiwan	Longitudinal cohort before treatment, 2–3 d after, and 2–3 mo after	*Alistipes* decreases during AHE compared to healthy controls, compensated, and decompensated cirrhosis. Also associated with recurrence at 1 year
Inflammation NASH/NAFLD (14) (Protective)	Humans: Germany	16S rRNA sequencing of stool	*A. finegoldii* decreased in NASH when compared to healthy controls. *A. onderdonkii* was reduced in patients with NAFLD
Inflammation Hepatocellular carcinoma, HCC (45) (Protective)	Mice: Male C57BL6/N	Metagenomic study using the stool of control mice vs. mice with tumor. The mice were fed probiotic [mixture (1:1:1) of *Lactobacillus rhamnosus* GG, *Escherichia coli* Nissle 1917 and heat inactive VSL#3]	*Alistipes* spp. and *A. shahii* increased in mice receiving the probiotic, in which there was modulation in pro-inflammatory cytokines in tumor microenvironment
Cancer Colorectal cancer (46) (Pathogenic)	Mice: C57BL/6J	qPCR/16S rRNA seq. to identify *Alistipes* in $Lcn2^{-/-}$, $Il10^{-/-}$ mice. $Il10^{-/-}$ mice gavaged with *A. finegoldii* to determine tumor localization.	*Alistipes* promotes colorectal cancer via IL-6/STAT3 pathway causing right sided tumorigenesis
Cardiovascular Atrial fibrillation (16) (Protective)	Humans: China	Metagenomic and metabolomic analyses of fecal samples extracted from patients with non-vascular atrial fibrillation or HC	*Alistipes* decreases in abundance in atrial fibrillation group when compared to HC
Cardiovascular Hypertension (47, 48) (Pathogenic)	Humans: America	Metagenomics of DNA extracted from fecal samples. High blood pressure (HBP) vs. controls.	*A. finegoldii* and *A. indistinctus* increase in HBP; LPS increase, Th17 cells, and inflammation; in HBP decrease in butyrate-producing cells; increase plasma intestinal fatty acid binding protein (I-FABP); and gut epithelial tight junction Zonulin
Cardiovascular Congestive heart failure (49) (Protective)	Humans: China	Metagenomics of DNA extracted from fecal samples	*Alistipes* decreased together with *Faecalibacterium* and *Oscillibacter*, while *Ruminococcus*, *Acinetobacter,* and *Veillonella* increased
Cardiovascular atherosclerosis cardiovascular disease (50) (Protective)	Humans: China	Metagenomics of DNA extracted from fecal samples	*Alistipes shahii* decreases together with *Bacteroides* spp., *Prevotella copri*, in ACVD
Mental health Anxiety (17) (Pathogenic)	Mice: BALB/c	Stress induced by grid floor housing. DGGE,16S Microbiome, Triple test, tail suspension test, and burrowing	*Alistipes* spp. increased after 2 weeks on grid floor housing. There was also significant change in the mice behavior
Mental health Myalgic encephal. Chronic fatigue (51) (Pathogenic)	Humans: Belgium Norway	16S rRNA sequencing of stool.	3.8-fold increase of *Alistipes* spp. in Norwegian patients vs. Norwegian controls
Mental health depression (52) (Pathogenic)	Humans: China	Pyrosequencing of DNA from feces of either healthy controls of patients with active- and responded-major depressive disorder (MMD).	*Alistipes* increases in both A-MDD and R-MDD groups when compared to HC; due to being indole positive decrease in serotonin availability. *Faecalibacterium* decreases in MMD
Mental health Autism spectrum disorder (53) (Protective)	Humans: Italy	Pyrosequencing using 16S rRNA from stool samples of 40 autistic and 40 neurotypical pediatric patients	*Alistipes* decreased with *Bilophila*, *Dialister, Parabacteroides, Veillonella*. *Collinsella, Dorea Corynebacterium,* and *Lactobacillus* increased
Mental health PDD-NOS (autism) (54) (Pathogenic)	Humans: Italian children	bTEFAP analysis on DNA and cDNA samples from each patient and pyrosequencing of 16S rDNA and rRNA	*Alistipes* was found at the highest abundance in AD and PDD-NO; possibly due to high production of propionic acid which has been shown to have neurobiological effects in rats

**Protective; overall interpretation of results with respect to outcome for each disease.*

can be seen as a potential SCFA producer and their decrease contributes to these hepatic fibrotic conditions due to a decrease in anti-inflammatory cytokines and inability to suppress Th17 cells. Further studies will be important in the near future to better define the role of this genus in liver pathologies and health.

ALISTIPES IN CARDIOVASCULAR DISEASE, HYPERTENSION, AND THE EPITHELIUM

Cardiovascular disease (CVD) is the leading cause of mortality and morbidity in both developing and developed countries (5). With CVDs projected to rise in the future as the global population ages, the evaluation of the relationship with the gut microbiota has been investigated more extensively. *Alistipes* has been linked with CVD risk factors such as hypertension, as well as several CVDs such as atrial fibrillation (AF), congestive heart failure (CHF), and atherosclerosis cardiovascular disease (ACVD) (50).

In hypertension, it is believed that *Alistipes* contributes to inflammation and epithelium alterations. Kim et al. (48) demonstrated the relationship between the gut barrier dysfunction in patients with hypertension and the gut microbiota in humans. They used shotgun metagenomic analysis studying fecal samples from 22 patients with high blood pressure and 18 control patients. The data revealed an increase in *A. finegoldii* and *A. indistinctus* that was positively correlated with systolic blood pressure. Additionally, it was shown that *A. finegoldii*, known to trigger intestinal inflammation, had an increased number and functional genes in the high blood pressure cohort. Overall, the increase in *A. finegoldii* was observed to be positively correlated with systolic blood pressure (SBP), suggesting that this species is a potential driver for gut barrier dysfunction and inflammation in patients with high blood pressure. Also, it is believed that the hypotensive phenotype of inflammation is caused by the lipopolysaccharide (LPS) in *Alistipes*, which is known to be pro-inflammatory, leading to an increase in Th17 cells expressing CD161 and CCR6/integrin Beta7, as well as the decrease in butyrate-producing bacteria, which are known to be anti-inflammatory (47). Despite such argument, it is important to highlight that several other types of anaerobic bacteria, including species within the *Bacteroides* genus, may also contribute to the complexity of disease attributes conferred to date to the *Alistipes* genus. Further studies will be critical to elucidate more precise mechanisms.

Various studies have indicated that *Alistipes* plays a protective role in CVDs. Additionally, *Alistipes* has been associated directly with CVDs such as atrial fibrillation (AF). Atrial fibrillation is the most common arrhythmia and is prevalent in patients with hypertension, heart failure, and obesity (63). Atrial fibrillation is characterized on an EKG by the absence of P waves with irregular R-R intervals due to irregular beating of the atria leading to irregular conduction of impulses to the ventricles (64). In a study by Zuo et al. (16), conducted to quantify the relationship between the gut microbiome and AF, a whole-metagenomic shotgun sequence performed on 100 stool samples from Chinese participants, showed a drastic decrease in *Alistipes* spp. in the intestinal tract of patients with AF. However, bacteria that drastically increased during AF, such as *Streptococcus*, were proposed in the study to be the cause of the decline in *Alistipes,* suggesting a potential antagonistic effect between *Alistipes* and *Streptococcus*. This trend was commonly seen in other heart conditions, such as ACVD (50) and CHF (49). Evidence for the involvement of *Alistipes* in CVD is contradictory; that is, it is unclear if associations are protective or beneficial or pathogenic. Because most CVDs share common pathophysiological characteristics, such as endothelial dysfunction (16), it is possible that the role of *Alistipes* may depend on disease mechanisms shared across several CDVs. Therefore, more studies on the gut-heart axis could lead to the future understanding of microbiome-related diseases and potential therapies.

ALISTIPES IN GUT INFLAMMATION AND OTHER *BACTEROIDETES*

Due to the diverse microbial community in the gastrointestinal tract, there is a strong correlation between dysbiosis and inflammatory bowel disease (IBD). The most common IBDs within the human population are Crohn's disease (CD) and ulcerative colitis (UC). UC is a chronic inflammatory disease that primarily targets the colon. It has been suggested that *A. finegoldii* is a protective species against colitis since *A. finegoldii* is decreased in mice with colitis. Due to this fact, a study was performed in which microbiota-depleted mice were treated with oral DSS to induce colitis. When gavaged with *A. finegoldii*, the severity of colitis was similar to that of the WT mice (42). Mice developed colitis when *A. finegoldii* was added with *Bacteroides eggerthii*, a colitis- inducing bacteria, but the severity of the colitis was significantly decreased compared to that of mice with *B. eggerthii* alone or added with other bacteria such as *Parabacteroides distasonis*, or *Prevotella falsenii*. This further indicates that *A. finegoldii* is a colitis-attenuating bacteria (42). Of contrasting clinical interest, A. finegoldii has been isolated in association with other Bacteroides from gut-wall cavernous fistulous tract (CavFT) microlesions in severe Crohn's disease. The causal-effect and their prevalence in such lesions are currently under investigation in our laboratory (65–67).

A study by Butera et al. (68) showed 8 weeks old NOD2 knockout mice had an enrichment of *Alistipes*, anti-inflammatory cytokines (TGF-beta and IL-10), and $CD4^+LAP^+FoxP3^-$ regulatory T cells. A possible connection for these observations comes from studies with curcumin, a spice that has been shown to modulate bowel inflammation by increasing $CD4^+LAP^+FoxP3^-$ cells via IL-10 (69). To test to see the severity of the NOD2 knock-out in mice with colitis, they induced colitis via intra-rectal administration of 2,4,6-Trinitrobenzene sulfonic acid (TNBS) and used the expression of anti-inflammatory cytokine mRNA to determine the severity. It was found that the NOD2 knock-out mice had less severe colitis than the wild-type. Also, it was noted that the severity of the colitis was related to the different proportion of $CD4^+LAP^+FoxP3^-$ cells observed prior to the TNBS treatment. Moreover, previous studies have shown a common trend of *Alistipes* abundance in NOD2 knock-out murine microbiota profiles (70). Of interest, *Alistipes* has been observed increasing among patients taking probiotics in an anti-inflammatory effect background (45). To date, it remains unclear what mechanisms of interaction exist between this genus and the other microorganisms in the gut, including food and probiotic strains, and the intramural fistulizing

lessions we reported in surgical patients with CD. Metagenomic studies of fecal samples from the mouse model of spontaneous CD ileitis (SAMP1/YitFc, characterized by a fully penetrant 3D- stereomicroscopic pattern of segmental cobblestone ileitis resembling human CD) revealed an enrichment of Alistipes compared to the parental AKR/J mouse colony (cohabiting for >10 years in the same room), suggesting that Alistipes spp. could be associated with the promotion of segmental ileitis (65, 71, 72).

ALISTIPES AND CANCER VIA BENEFICIAL IMMUNOMODULATION

Cancer, like cardiovascular disease, causes high rates of mortality and morbidity worldwide. Colorectal cancer (CRC) is one of the most common types of cancer typically targeting older individuals, as well as African Americans. CRC is also a form of cancer that has been linked to dysbiosis of the gut microbiota. *Alistipes* has been found to contribute to the pathogenesis, acting as a potential pathogen. Moschen et al. (15) showed that *A. finegoldii* promotes right sided colorectal cancer via the IL-6/STAT 3 pathway. Lipocalin 2 (LCN 2), an anti-microbial protein that binds to siderophores ultimately reduces iron availability (73). In patients with IBD LCN 2 is found in high concentrations in mucosal and fecal samples. Essentially, this can reduce the prevalence of *Alistipes*, as iron is a regulatory factor for the growth of *A. finegoldii*. However, Moschen et al. (15) went on to show that *A. finegoldii* caused intestinal inflammation after 1 week of being orally administered in WT, LCN 2 KO, and IL-10 KO C57BL/6J mice. Therefore, the paper concluded that *Alistipes* thrives in an inflamed environment that lacks LCN 2 and promotes inflammation and tumor formation. Moreover, they showed that *Alistipes finegoldii* was found in higher abundance in the ceca than other locations within the large intestine.

Despite the pathogenic effects observed for *Alistipes* in CRC, this genus has been shown to have a beneficial role in cancer immunotherapy by modulating the tumor microenvironment. One of the main hallmarks of cancers is to evade the immune system. Therefore, one form of anti-cancer treatment has been to manipulate the tumor microenvironment. An example of immunotherapy is to manipulate the microenvironment by inducing tumor necrosis factor (TNF) production by tumor-associated myeloid cells which ultimately leads to tumor eradication. One way to do this is to use a combination of intra-tumoral CpG-oligodeoxynucleotides (ODN) to activate TLR9, and inhibitory IL-10R antibodies. This immunotherapy typically halts tumor growth and induces TNF-dependent hemorrhagic necrosis by tumor-associated myeloid cells leading to tumor suppression.

In a study performed by Iida et al. (61) C57Bl/6 mice were injected subcutaneously with MC38 colon carcinoma cells and pre-treated with antibiotics (vancomycin, imipenem, and neomycin). The study initially determined whether antibiotics affected cancer immunotherapy. The authors found that antibiotics led to a decrease in efficiency of the tumor eradication due to a reduction in TNF production. The investigators then determined if those results depended on the bacterial load in the intestinal tract. Therefore, germ free (GF) mice with the MC38 tumors received anti-IL-10R/CpG-ODN treatment. Treated GF mice produced a significantly lower amount of TNF than specific pathogen free (SPF) mice. This suggests that the tumor-associated innate myeloid cells are primed by microbiota for inflammatory cytokine production in response to anti-IL-10R/CpG-ODN and that the reduced bacterial load from either the antibiotic treatment or germ-free status reduces this response and the TNF-dependent early-tumor necrosis. To better understand the role of antibiotics and the role of the gut microbiota, MC38 tumor bearing mice were gavaged with LPS and the TNF expression was reestablished. When they examined the microbiota involved via fecal microbiota composition, there was a positive correlation between *Alistipes* genus and a role for TLR4-priming/TNF production. Iida et al. (61) believed the TNF restoration was due to the role of pro-inflammatory gram-negative bacteria, *A. shahii*, binding to TLR4, priming the expression of TNF production. To further prove their hypothesis, authors then showed a delay in the recovery of *A. shahii* following antibiotic treatment, which also paralleled an ∼4-week phase of TNF restoration after the antibiotic administration. Furthermore, they showed that when mice that were pre-treated with antibiotic and gavaged with *A. shahii,* the tumor-associated myeloid cells function to produce TNF was restored. Clinically relevant, the study indicated that when there is a reduction in *Alistipes*, there is a parallel reduction in optimal responses to cancer immunotherapy (61).

Others have also identified a role for *Alistipes* in cancer immunotherapy. For instance, non-small cell lung carcinoma (NSCLC) has a poor prognosis and no current therapy. Recently checkpoint inhibitors for PD-1 have been proposed as a potential immunotherapy for NSCLC. However, a major number of patients still persist with poor prognosis. Nivolumab is a fully human Immunoglobulin G4 monoclonal antibody against PD-1, therefore it blocks T cells from binding to the ligand PD-1L, typically expressed by the tumor, preventing T cell exhaustion. A study to find a potential correlation between gut microbiota and patients with NSCLC responding favorably to Nivolumab was conducted that presented evidence that *A. putredinis* was increased in patients who responded well to Nivolumab correlating with the study mentioned above (74).

MENTAL HEALTH

Although *Alistipes* can be found commonly in the intestinal tract, it has been shown to have a significant effect on diseases with localization outside of the gut such depression, anxiety, chronic fatigue syndrome, autism, cirrhosis, and aging. Dysbiosis within the intestine can affect the gut-brain axis and be used to explain the relationship between the gut microbiota, depression, and other mood disorders such as anxiety.

In a study conducted with BALB/c mice placed in a stressful environment, induced by grid-floor housing, there was a significant increase in *Alistipes* abundance (17). Furthermore, *Alistipes* concentration was also found to be increased, almost 4-fold, in Norwegian patients suffering from chronic fatigue syndrome (51). These findings correlate with the evidence of an

increase in *Alistipes* for patients suffering from depression, since patients with depression typically struggle with fatigue and stress (75). It is believed that this increase in *Alistipes* disrupts the gut-brain axis because *Alistipes* is an indole-positive organism, and, thus decreases serotonin availability. Tryptophan is a precursor for serotonin, and a decrease in serotonin is associated with depression (52). Moreover, *Alistipes* has been shown to express glutamate decarboxylase, an enzyme that metabolizes glutamate into γ-aminobutyric acid (GABA) in chickens (58). This increase in *Alistipes* abundance could also possibly be related to the increase in GABA. However, studies should be done to show if the GABA is being secreted into the gut lumen (58).

Additionally, there are associations between the brain and gut amongst patients suffering from autism spectrum disorder. It is often found that individuals suffering from autism have frequent GI symptoms, speculating that it could be due to a dysbiosis within the microbiota. Strati et al. (53) found that there was a decrease in *Alistipes* in patients with autism spectrum disorder. However, another study done on a different form of autism, PDD-NOS, showed a significant abundance of *Alistipes* in children (54). It has been speculated that this could be from the production of propionic acid, which has shown to have neurobiological effects in rats (76). There is need for more studies on *Alistipes* and its effects on the gut-brain axis, since there is contradictory evidence regarding its protective/pathogenic role in both systems.

SULFONOLIPIDS AND BIOCHEMICAL MARKERS

Alistipes is a genus of bacteria with numerous immunological and biochemical pathways that are associated with the diseases mentioned above. One important implication is the promotion of CRC by *Alistipes* via IL-6/STAT 3 pathway. Thus, future studies could consider the use of *Alistipes* species as potential biomarkers for CRC, using our understanding based on microbiome DNA based data and the integration of biochemical concepts on disease pathogenesis. A potential method to accomplish this would be to look for sulfonolipids, a unique class of sphingolipids with a sulfonic acid group in the sphingoid base (77). Walker et al. (78) showed that when C57BL/6N mice are fed high fat diets with either safflower oil or lard fat for 3 weeks there is an increase in sulfonolipids, as well as, body weight when compared to mice fed the normal chow diet. A metagenome analysis was performed and screened for bacterial genes involved in sulfonolipid biosynthesis in the cecum of these mice. All species of *Alistipes* were found to produce sulfonolipids except *A. inops* (information on the most recent species A. *megaguti, A. provencensis,* and sulfonolipid production remain unknown). To further prove that sulfonolipids are a product of bacteria and a marker of *Alistipes,* scientists performed a mono-colonization study of germ-free (GF) mice with *Alistipes* spp. and detected a significant emergence of sulfonolipids in the cecum of the mono-colonized mice that was previously absent in the GF mice. Therefore, with common risk factors of CRC being high fat diets, obesity, and age (79), in addition to the increased abundance of *Alistipes* in these colonic conditions, there is the intriguing proposition for the potential of using sulfonolipids as markers for patients at risk for developing CRC, particularly within ethnicities, such as African-Americans with family histories of CRC. Additionally, studies should be conducted to verify if *Alistipes* abundance is increased in polyps, pre-cancerous vs. cancerous, being polyps another risk factor for CRC.

Moreover, *Alistipes* has the highest number of putrefaction pathways amongst commensal bacteria. Putrefaction is the fermentation of undigested proteins in the GI tract by the gut microbiota typically leading to bacterial production of harmful metabolites (80). These products have been reported as deleterious and associated with CRC (81). Such products include ammonia, H_2S, cresol, indole, and phenol (82). In a study done to identify the main putrefaction pathways used by the gut bacteria, Kaur et al. (18), found that *Alistipes* contributed to histidine degradation/THF production, indole production, and phenol production. Histidine degradation/THF production have been found to release excess ammonia, which when absorbed, damages the colonic cells (83). Ammonia has also been found to increase intestinal cell proliferation and assist in the growth of cancer cells in CRC (84). Findings of excess ammonia and other *Alistipes* produced putrefaction products could be of use to clinicians when patients are at risk for developing CRC.

CONCLUSION, LIMITATIONS, AND FUTURE DIRECTIONS

Alistipes is a relatively new genus of bacteria isolated from clinical samples, although at a low rate compared to others within the *Bacteroidetes* phylum. At the protein level, a genome-wide protein phylogenetic analysis shown in **Figure 2** illustrates that this genus may have unique functional properties that may enable them to have unique physiological roles, compared to other members within the *Bacteroidetes*, and/or also affect our ability to isolate these species *in vitro*. Therein, it is possible that the identification of this genus in clinical samples may be underrepresented as novel MS-TOF methods may not be fully capable to discriminate distinct species as separate. Immunologically, *Alistipes* has been seen to contribute to disease in both clinical and preclinical studies. Intriguingly, other studies have shown their presence is correlated with the promotion of healthy phenotypes such as *Alistipes* protective roles in diseases such as colitis, autism spectrum disorder, and various liver and cardiovascular fibrotic disorders. Despite *Alistipes* role in healthy phenotypes, *Alistipes* contrastingly has been shown to have a pathogenic role in diseases such as anxiety, myalgic encephalomyelitis/chronic fatigue syndrome, depression, PDD-NOS, and CRC.

Collectively, this review represents a summary of studies where *Alistipes* has been experimentally tested after inoculation into animal models, or where *Alistipes* has been found among other abnormally present species in human or animal microbiome studies. Thus, the perspective here presented, indicates that the genus, depending on the study discussed, could

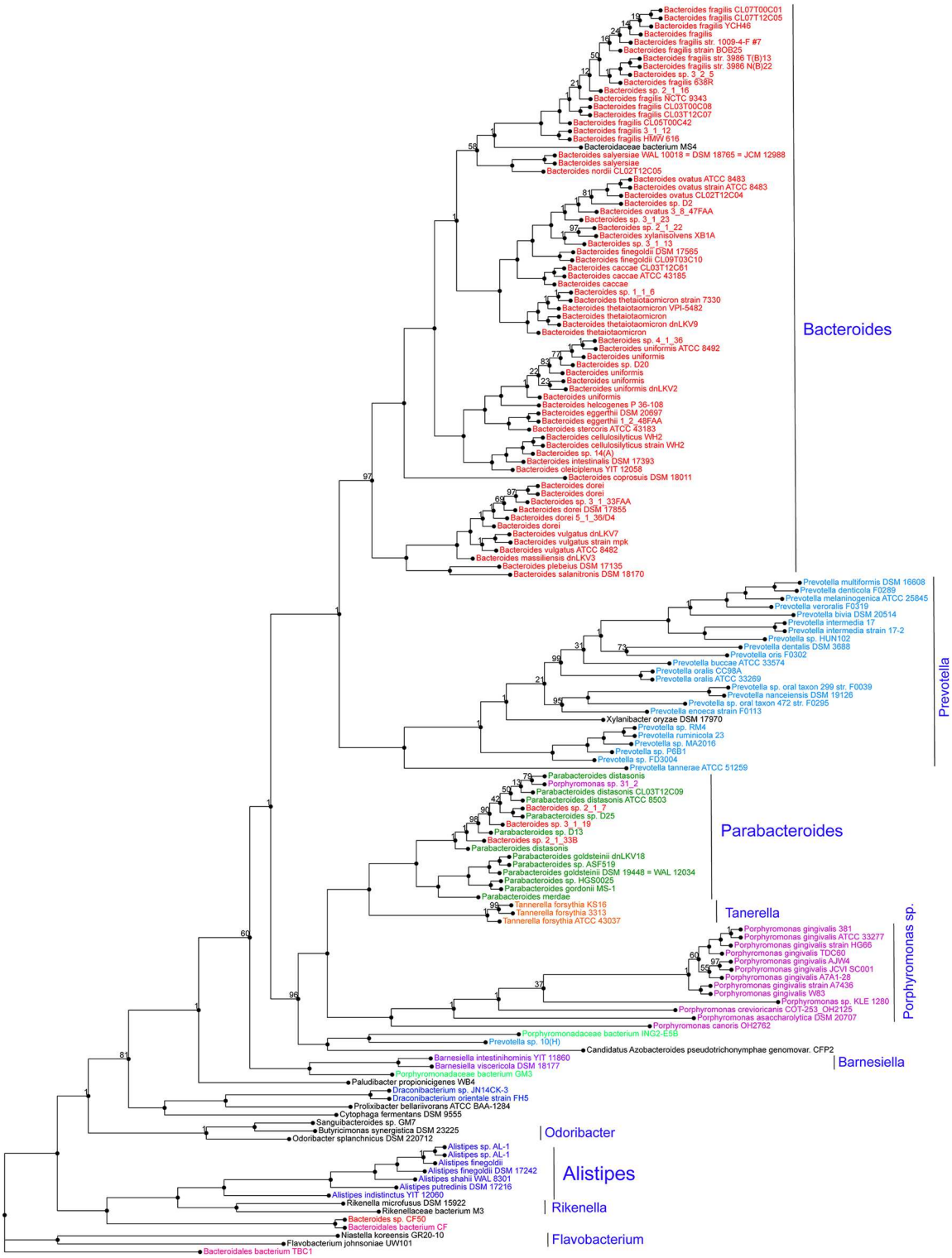


FIGURE 2 | Protein phylogram of 155 complete genomes of the *Bacteroidetes* phylum to illustrate the potential functional distinction of the genus *Alistipes* within the group. The pipeline for genomic phylograms is described in detail as follow by PATRIC, the Pathosystems Resource Integration Center, https://docs.patricbrc.org. In short, the order-level pre-built trees in PATRIC are constructed by an automated pipeline that begins with amino acid sequence files for each genome. For each order-level tree the genomes from that order are used along with a small set of potential outgroup genomes. Branch values are not bootstrap values, which can be overly optimistic for long genomes. Instead, trees are built from random samples of 50% of the homology groups used for the main tree (gene-wise jackknifing). One hundred of these 50% gene-wise jackknife trees are made using FastTree, and the support values shown indicate the number of times a particular branch was observed in the support trees. As of May 19, 2020, there were 140 Alistipes genomes available (11287 unique contigs), of which 10 are complete.

have a leading role in the modulation of diseases, or alternatively could just have either a bystander role, or a co-inducer role (with other gut microbes) of the observed clinical phenotypes. Animal studies will be further needed to decipher the mechanisms that may explain disease modulation alone and as symbiont by this genus across a multitude of complex multimodal diseases, which will benefit from the targeted study of subtype phenotypes, as we have analytically illustrated (9).

The use of germ-free animals and models will be beneficial to understand the role of this genus in disease and health and the interaction with the host immune defense tolerance, such as there should be studies investigating the roles on the SCFAs produced by *Alistipes* and their effect on the various liver diseases and *Alistipes* direct role on T-cell differentiation.

AUTHOR CONTRIBUTIONS

BP, PW, AV, and AR-P conceived the performed review and wrote the manuscript. All authors discussed and edited the manuscript and approved its final version.

FUNDING

This project was primarily supported by NIH grant R21 DK118373 to AR-P, entitled Identification of pathogenic bacteria in Crohn's disease. We acknowledge the Biorepository Core of the NIH Silvio O. Conte Cleveland Digestive Disease Research Core Center (P30DK097948) for supporting the above-mentioned R21 project. Partial support also originated for AR-P from a career development award from the Crohn's and Colitis Foundation and National Institutes of Health award 2P01DK091222-06 (Germ-Free and Gut Microbiome Core to Dr. Fabio Cominelli, Case Western Reserve University and AR-P).

ACKNOWLEDGMENTS

BP was especially grateful to the Department of Pathology for the scientific and academic opportunities, their mentoring, and their support during the preparation of this manuscript. This project was part of a Master's of Science program in Pathology at Case Western Reserve University and she is currently admitted to the medical school at Wayne State University School of Medicine.

REFERENCES

1. Goodrich JK, Davenport ER, Clark AG, Ley RE. The relationship between the human genome and microbiome comes into view. *Annu Rev Genet.* (2017) 51:413–33. doi: 10.1146/annurev-genet-110711-155532
2. Lynch SV, Pedersen O. The human intestinal microbiome in health and disease. *N Engl J Med.* (2016) 375:2369–79. doi: 10.1056/NEJMra1600266
3. Vivarelli S, Salemi R, Candido S, Falzone L, Santagati M, Stefani S, et al. Gut microbiota and cancer: from pathogenesis to therapy. *Cancers.* (2019) 11:38. doi: 10.3390/cancers11010038
4. Halfvarson J, Brislawn CJ, Lamendella R, Vázquez-Baeza Y, Walters WA, Bramer LM, et al. Dynamics of the human gut microbiome in inflammatory bowel disease. *Nat Microbiol.* (2017) 2:17004. doi: 10.1038/nmicrobiol.2017.4
5. Yoshida N, Yamashita T, Hirata KI. Gut microbiome and cardiovascular diseases. *Diseases.* (2018) 6:56. doi: 10.3390/diseases6030056
6. Petra AI, Panagiotidou S, Hatziagelaki E, Stewart JM., Conti P, Theoharides TC. Gut-microbiota-brain axis and its effect on neuropsychiatric disorders with suspected immune dysregulation. *Clin Ther.* (2015) 37:984–95. doi: 10.1016/j.clinthera.2015.04.002
7. Rautio M, Eerola E, Väisänen-Tunkelrott ML, Molitoris D, Lawson P, Collins MD, et al. Reclassification of *Bacteroides putredinis* (Weinberg et al., 1937) in a new genus *Alistipes* gen. nov., as *Alistipes putredinis* comb. nov., and description of *Alistipes finegoldii* sp. nov., from human sources. *Syst Appl Microbiol.* (2003) 26:182–8. doi: 10.1078/072320203322346029
8. Shkoporov AN, Chaplin AV, Khokhlova EV, Shcherbakova VA, Motuzova OV, Bozhenko VK, et al. *Alistipes inops* sp. nov. and *Coprobacter secundus* sp. nov., isolated from human faeces. *Int J Syst Evol Microbiol.* (2015) 65:4580–8. doi: 10.1099/ijsem.0.000617
9. Basson AR, Cominelli F, Rodriguez-Palacios A. Patterns of 'analytical irreproducibility' in multimodal diseases. *bioRxiv [preprint]*:bioRxiv 2020.03.22.002469. (2020). doi: 10.1101/2020.03.22.002469
10. Sakamoto M, Ikeyama N, Ogata Y, Suda W, Iino T, Hattori M, et al. *Alistipes communis* sp. nov., *Alistipes dispar* sp. nov. and *Alistipes onderdonkii* subsp. *vulgaris* subsp. nov., isolated from human faeces, and creation of *Alistipes onderdonkii* subsp. *onderdonkii* subsp. nov. *Int J Syst Evol Microbiol.* (2020) 70:473–80. doi: 10.1099/ijsem.0.003778
11. *In Memoriam: Sydney "Sid" Finegold, MD, FIDSA 1921–2018.* Infectious Disease Society of America News Archives (2018).
12. Sydney M. Finegold. Biographical Feature: Sydney M. Finegold. *J Clin Microbiol.* (2012) 50:2546–7. doi: 10.1128/JCM.01295-12
13. Abbreviated Curriculum Vitae: Sydney M. Finegold MD. *Clin Infect. Dis.* (2002) 35:287. doi: 10.1086/341912
14. Rau M, Rehman A, Dittrich M, Groen AK, Hermanns HM, Seyfried F, et al. Fecal SCFAs and SCFA-producing bacteria in gut microbiome of human NAFLD as a putative link to systemic T-cell activation and advanced disease. *United Euro. Gastroenterol J.* (2018) 6:1496–1507. doi: 10.1177/2050640618804444
15. Moschen AR, Gerner RR, Wang J, Klepsch V, Adolph TE, Reider SJ, et al. Lipocalin 2 protects from inflammation and tumorigenesis associated with gut microbiota alterations. *Cell Host Microbe.* (2016) 19:455–69. doi: 10.1016/j.chom.2016.03.007
16. Zuo K, Li J, Li K, Hu C, Gao Y, Chen M, et al. Disordered gut microbiota and alterations in metabolic patterns are associated with atrial fibrillation. *Gigascience.* (2019) 8:giz058. doi: 10.1093/gigascience/giz058
17. Bangsgaard Bendtsen KM, Krych L, Sørensen DB, Pang W, Nielsen DS, Josefsen K, et al. Gut microbiota composition is correlated to grid floor induced stress and behavior in the BALB/c mouse. *PLoS ONE.* (2012) 7:e46231. doi: 10.1371/journal.pone.0046231
18. Kaur H, Das C, Mande SS. Analysis of putrefaction pathways in bacteria and its implication in colorectal cancer. *Front Microbiol.* (2017) 8:2166. doi: 10.3389/fmicb.2017.02166
19. Jousimies-Somer H. *Wadsworth-KTL Anaerobic Bacteriology Manual.* Star Pub. (2002).
20. Wexler HM. Bacteroides: the good, the bad, and the nitty-gritty. *Clin Microbiol Rev.* (2007) 20:593–621. doi: 10.1128/CMR.00008-07
21. Mishra AK, Gimenez G, Lagier JC, Robert C, Raoult D, Fournier PE. Genome sequence and description of *Alistipes senegalensis* sp. nov. *Stand Genomic Sci.* (2012) 6:1–16. doi: 10.4056/sigs.3076460
22. Urdaneta V, Casadesús J. Interactions between bacteria and bile salts in the gastrointestinal and hepatobiliary tracts. *Front Med.* (2017) 4:163. doi: 10.3389/fmed.2017.00163
23. Cao W, Kayama H, Chen ML, Delmas A, Sun A, Kim SY, et al. The xenobiotic transporter Mdr1 enforces T cell homeostasis in the presence of intestinal bile acids. *Immunity.* (2017) 47:1182–96.e10. doi: 10.1016/j.immuni.2017.11.012
24. Song Y, Könönen E, Rautio M, Liu C, Bryk A, Eerola E, et al. *Alistipes onderdonkii* sp. nov. and *Alistipes shahii* sp. nov., of human origin. *Int J Syst Evol Microbiol.* (2006) 56:1985–90. doi: 10.1099/ijs.0.64318-0
25. Nagai F, Morotomi M, Watanabe Y, Sakon H, Tanaka, R. *Alistipes indistinctus* sp. nov. and *Odoribacter laneus* sp. nov., common members of the human

intestinal microbiota isolated from faeces. *Int J Syst Evol Microbiol.* (2010) 60:1296–302. doi: 10.1099/ijs.0.014571-0
26. Lagier JC, Armougom F, Mishra AK, Nguyen TT, Raoult D, Fournier PE. Non-contiguous finished genome sequence and description of *Alistipes timonensis* sp. nov. *Stand Genomic Sci.* (2012) 6:315–24. doi: 10.4056/sigs.2685971
27. Hugon P, Ramasamy D, Lagier JC, Rivet R, Couderc C, Raoult D, et al. Non contiguous-finished genome sequence and description of *Alistipes obesi* sp. nov. *Stand Genomic Sci.* (2013) 7:427–39. doi: 10.4056/sigs.3336746
28. Pfleiderer A, Mishra AK, Lagier JC, Robert C, Caputo A, Raoult D. et al. Non-contiguous finished genome sequence and description of *Alistipes ihumii* sp. nov. *Stand Genomic Sci*. (2014) 9:1221–35. doi: 10.4056/sigs.4698398
29. Bellali S, Naud S, Ndongo S, Lo CI, Anani H, Raoult D, *Corynebacterium pacaense* sp. nov., *Alistipes megaguti* sp. nov., *Alistipes provencensis* sp. nov., 3 new bacteria isolated from fresh human stool specimens. *New Microbes New Infect*. (2019) 32:100593. doi: 10.1016/j.nmni.2019.100593
30. Fenner L, Roux V, Ananian P, Raoult D. *Alistipes finegoldii* in blood cultures from colon cancer patients. *Emerg Infect Dis*. (2007) 13:1260–2. doi: 10.3201/eid1308.060662
31. Paster BJ, Dewhirst FE, Olsen I, Fraser GJ. Phylogeny of *Bacteroides*, *Prevotella*, and *Porphyromonas* spp. and related bacteria. *J Bacteriol.* (1994) 176:725–32. doi: 10.1128/JB.176.3.725-732.1994
32. Lagier JC, Armougom F, Million M, Hugon P, Pagnier I, Robert C, et al. Microbial culturomics: paradigm shift in the human gut microbiome study. *Clin Microbiol Infect*. (2012) 18:1185–93. doi: 10.1111/1469-0691.12023
33. Hardham JM, King KW, Dreier K, Wong J, Strietzel C, Eversole RR, et al. Transfer of *Bacteroides splanchnicus* to Odoribacter gen. nov. as *Odoribacter splanchnicus* comb. nov., and description of *Odoribacter denticanis* sp. nov., isolated from the crevicular spaces of canine periodontitis patients. *Int J Syst Evol Microbiol.* (2008) 58:103–9. doi: 10.1099/ijs.0.63458-0
34. Werner H, Rintelen G, Kunstek-Santos H. A new butyric acid-producing bacteroides species: B. splanchnicus n. sp. (author's transl). *Zentralbl Bakteriol Orig A*. (1975) 231:133–44.
35. Downes J, Tanner AC, Dewhirst FE, Wade WG. Prevotella saccharolytica sp. nov., isolated from the human oral cavity. *Int J Syst Evol Microbiol.* (2010) 60:2458–61. doi: 10.1099/ijs.0.014720-0
36. van Winkelhoff AJ, van Steenbergen TJ, de Graaff J. Porphyromonas (Bacteroides) endodontalis: its role in endodontal infections. *J Endod*. (1992) 18:431–4. doi: 10.1016/S0099-2399(06)80843-5
37. Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol*. (1991) 173:697–703. doi: 10.1128/JB.173.2.697-703.1991
38. Veloo ACM, Jean-Pierre H, Justesen US, Morris T, Urban E, Wybo I, et al. Validation of MALDI-TOF MS Biotyper database optimized for anaerobic bacteria: The ENRIA project. *Anaerobe.* (2018) 54:224–230. doi: 10.1016/j.anaerobe.2018.03.007
39. Kulagina EV, Efimov BA, Maximov PY, Kafarskaia LI, Chaplin AV, Shkoporov AN. Species composition of Bacteroidales order bacteria in the feces of healthy people of various ages. *Biosci Biotechnol Biochem*. (2012) 76:169–71. doi: 10.1271/bbb.110434
40. Torok VA, Hughes RJ, Mikkelsen LL, Perez-Maldonado R, Balding K, MacAlpine R, et al. Identification and characterization of potential performance-related gut microbiotas in broiler chickens across various feeding trials. *Appl Environ Microbiol*. (2011) 77:5868–78. doi: 10.1128/AEM.00165-11
41. Li F, Hullar MA, Schwarz Y, Lampe JW. Human gut bacterial communities are altered by addition of cruciferous vegetables to a controlled fruit- and vegetable-free diet. *J Nutr*. (2009) 139:1685–91. doi: 10.3945/jn.109.108191
42. Dziarski R, Park SY, Kashyap DR, Dowd SE, Gupta, D. Pglyrp-regulated gut microflora *Prevotella falsenii*, *Parabacteroides distasonis* and *Bacteroides eggerthii* enhance and *Alistipes finegoldii* attenuates colitis in mice. *PLoS ONE.* (2016) 11:e0146162. doi: 10.1371/journal.pone.0146162
43. Shao L, Ling Z, Chen D, Liu Y, Yang F, Li, L. Disorganized gut microbiome contributed to liver cirrhosis progression: a meta-omics-based study. *Front Microbiol*. (2018) 9:3166. doi: 10.3389/fmicb.2018.03166
44. Sung CM, Lin YF, Chen KF, Ke HM, Huang HY, Gong YN, et al. Predicting clinical outcomes of cirrhosis patients with hepatic encephalopathy from the fecal microbiome. *Cell Mol Gastroenterol Hepatol*. (2019) 8:301–18.e2. doi: 10.1016/j.jcmgh.2019.04.008
45. Li J, Sung CY, Lee N, Ni Y, Pihlajamäki J, Panagiotou G, et al. Probiotics modulated gut microbiota suppresses hepatocellular carcinoma growth in mice. *Proc Natl Acad Sci USA*. (2016) 113:E1306–15. doi: 10.1073/pnas.1518189113
46. Feng Q, Liang S, Jia H, Stadlmayr A, Tang L, Lan Z, et al. Gut microbiome development along the colorectal adenoma-carcinoma sequence. *Nat Commun*. (2015) 6:6528. doi: 10.1038/ncomms7528
47. Jama HA, Beale A, Shihata WA, Marques FZ. The effect of diet on hypertensive pathology: is there a link via gut microbiota-driven immunometabolism? *Cardiovasc Res*. (2019) 115:1435–47. doi: 10.1093/cvr/cvz091
48. Kim S, Goel R, Kumar A, Qi Y, Lobaton G, Hosaka K, et al. Imbalance of gut microbiome and intestinal epithelial barrier dysfunction in patients with high blood pressure. *Clin Sci*. (2018) 132:701–18. doi: 10.1042/CS20180087
49. Cui X, Ye L, Li J, Jin L, Wang W, Li S, et al. Metagenomic and metabolomic analyses unveil dysbiosis of gut microbiota in chronic heart failure patients. *Sci Rep*. (2018) 8:635. doi: 10.1038/s41598-017-18756-2
50. Jie Z, Xia H, Zhong SL, Feng Q, Li S, Liang S, et al. The gut microbiome in atherosclerotic cardiovascular disease. *Nat Commun.* (2017) 8:845. doi: 10.1038/s41467-017-00900-1
51. Frémont M, Coomans D, Massart S, De Meirleir, K. High-throughput 16S rRNA gene sequencing reveals alterations of intestinal microbiota in myalgic encephalomyelitis/chronic fatigue syndrome patients. *Anaerobe.* (2013) 22:50–6. doi: 10.1016/j.anaerobe.2013.06.002
52. Jiang H, Ling Z, Zhang Y, Mao H, Ma Z, Yin Y, et al. Altered fecal microbiota composition in patients with major depressive disorder. *Brain Behav Immun.* (2015) 48:186–94. doi: 10.1016/j.bbi.2015.03.016
53. Strati F, Cavalieri D, Albanese D, De Felice C, Donati C, Hayek J, et al. New evidences on the altered gut microbiota in autism spectrum disorders. *Microbiome.* (2017) 5:24. doi: 10.1186/s40168-017-0242-1
54. De Angelis M, Piccolo M, Vannini L, Siragusa S, De Giacomo A, Serrazzanetti DI, et al. Fecal microbiota and metabolome of children with autism and pervasive developmental disorder not otherwise specified. *PLoS ONE.* (2013) 8:e76993. doi: 10.1371/journal.pone.0076993
55. Tang A, Hallouch O, Chernyak V, Kamaya A, Sirlin CB. Epidemiology of hepatocellular carcinoma: target population for surveillance and diagnosis. *Abdom Radiol*. (2018) 43:13–25. doi: 10.1007/s00261-017-1209-1
56. Campion D, Giovo I, Ponzo P, Saracco GM, Balzola F, Alessandria, C. Dietary approach and gut microbiota modulation for chronic hepatic encephalopathy in cirrhosis. *World J Hepatol.* (2019) 11:489–512. doi: 10.4254/wjh.v11.i6.489
57. Iebba V, Guerrieri F, Di Gregorio V, Levrero M, Gagliardi A, Santangelo F, et al. Combining amplicon sequencing and metabolomics in cirrhotic patients highlights distinctive microbiota features involved in bacterial translocation, systemic inflammation and hepatic encephalopathy. *Sci Rep.* (2018) 8:8210. doi: 10.1038/s41598-018-26509-y
58. Polansky O, Sekelova Z, Faldynova M, Sebkova A, Sisak F, Rychlik, I. Important metabolic pathways and biological processes expressed by chicken cecal microbiota. *Appl Environ Microbiol*. (2015) 82:1569–76. doi: 10.1128/AEM.03473-15
59. Oliphant K, Allen-Vercoe E. Macronutrient metabolism by the human gut microbiome: major fermentation by-products and their impact on host health. *Microbiome.* (2019) 7:91. doi: 10.1186/s40168-019-0704-8
60. Brown CT, Davis-Richardson AG, Giongo A, Gano KA, Crabb DB, Mukherjee N, et al. Gut microbiome metagenomics analysis suggests a functional model for the development of autoimmunity for type 1 diabetes. *PLoS ONE.* (2011) 6:e25792. doi: 10.1371/journal.pone.0025792
61. Iida N, Dzutsev A, Stewart CA, Smith L, Bouladoux N, Weingarten RA, et al. Commensal bacteria control cancer response to therapy by modulating the tumor microenvironment. *Science*. (2013) 342:967–70. doi: 10.1126/science.1240527
62. Bindels LB, Porporato P, Dewulf EM, Verrax J, Neyrinck AM, Martin JC, et al. Gut microbiota-derived propionate reduces cancer cell proliferation in the liver. *Br J Cancer*. (2012) 107:1337–44. doi: 10.1038/bjc.2012.409
63. Oldgren J, Healey JS, Ezekowitz M, Commerford P, Avezum A, Pais P, et al. Variations in cause and management of atrial fibrillation in a prospective registry of 15,400 emergency department patients in 46 countries: the RE-LY Atrial Fibrillation Registry. *Circulation*. (2014) 129:1568–76. doi: 10.1161/CIRCULATIONAHA.113.005451

64. Kirchhof P, Benussi S, Kotecha D, Ahlsson A, Atar D, Casadei B, et al. 2016 ESC Guidelines for the management of atrial fibrillation developed in collaboration with EACTS. *Eur J Cardiothorac Surg*. (2016) 50:e1–e88. doi: 10.5603/KP.2016.0172
65. Rodriguez-Palacios A, Kodani T, Kaydo L, Pietropaoli D, Corridoni D, Howell S, et al. Stereomicroscopic 3d-pattern profiling of murine and human intestinal inflammation reveals unique structural phenotypes. *Nat Commun*. (2015) 6:7577.
66. Rodriguez-Palacios A, Conger M, Hopperton A, Ezeji JC, Erkkila HL, Fiocchi C, et al. Identification of pathogenic bacteria in severe Crohn's disease. *Inflamm Bowel Dis*. (2019) 25(Suppl 1):S70. doi: 10.1093/ibd/izy393.175
67. Yang F, Kumar A, Davenport KW, Kelliher JM, Ezeji JC, Good CE, et al. Complete genome sequence of a parabacteroides distasonis strain (CavFT hAR46) isolated from a gut wall-cavitating microlesion in a patient with severe Crohn's disease. *Microbiology Resource Announcements*. (2019) 8:e00585-19. doi: 10.1128/MRA.00585-19
68. Butera A, Di Paola M, Pavarini L, Strati F, Pindo M, Sanchez M, et al. Nod2 Deficiency in mice is associated with microbiota variation favouring the expansion of mucosal CD4+ LAP+ regulatory cells. *Sci Rep*. (2018) 8:14241. doi: 10.1038/s41598-018-32583-z
69. Shen L, Liu L, Ji HF. Regulative effects of curcumin spice administration on gut microbiota and its pharmacological implications. *Food Nutr Res*. (2017) 61:1361780. doi: 10.1080/16546628.2017.1361780
70. Frank DN, St Amand AL, Feldman RA, Boedeker EC, Harpaz N, Pace NR. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc Natl Acad Sci USA*. (2007) 104:13780–5. doi: 10.1073/pnas.0706625104
71. Rodriguez-Palacios AAN, Cominelli F. "Stereomicroscopy and 3d-Target myeloperoxidase intestinal phenotyping following a fecal flora homogenization protocol," In: *Protocol Exchange*. (2015). doi: 10.1038/protex.2015.065
72. Rodriguez-Palacios A, Harding A, Menghini P, Himmelman C, Retuerto M, Nickerson KP, et al. The artificial sweetener splenda promotes gut proteobacteria, dysbiosis, and myeloperoxidase reactivity in Crohn's disease-like ileitis. *Inflamm Bowel Dis*. (2018) 24:1005–20. doi: 10.1093/ibd/izy060
73. Goetz DH, Holmes MA, Borregaard N, Bluhm ME, Raymond KN, Strong RK. The neutrophil lipocalin NGAL is a bacteriostatic agent that interferes with siderophore-mediated iron acquisition. *Mol Cell*. (2002) 10:1033–43. doi: 10.1016/S1097-2765(02)00708-6
74. Jin Y, Dong H, Xia L, Yang Y, Zhu Y, Shen Y, et al. The diversity of gut microbiome is associated with favorable responses to anti-PD-1 immunotherapy in Chinese non-small cell lung cancer patients. *J Thorac Oncol*. (2019) 14:1378–89. doi: 10.1016/j.jtho.2019.04.007
75. Naseribafrouei A, Hestad K, Avershina E, Sekelja M, Linløkken A, Wilson R, et al. Correlation between the human fecal microbiota and depression. *Neurogastroenterol Motil*. (2014) 26:1155–62. doi: 10.1111/nmo.12378
76. MacFabe DF, Cain NE, Boon F, Ossenkopp KP, Cain DP. Effects of the enteric bacterial metabolic product propionic acid on object-directed behavior, social behavior, cognition, and neuroinflammation in adolescent rats: relevance to autism spectrum disorder. *Behav Brain Res*. (2011) 217:47–54. doi: 10.1016/j.bbr.2010.10.005
77. Merrill AH. Sphingolipid and glycosphingolipid metabolic pathways in the era of sphingolipidomics. *Chem Rev*. (2011) 111:6387–422. doi: 10.1021/cr2002917
78. Walker A, Pfitzner B, Harir M, Schaubeck M, Calasan J, Heinzmann SS, et al. Sulfonolipids as novel metabolite markers of *Alistipes* and *Odoribacter* affected by high-fat diets. *Sci Rep*. (2017) 7:11047. doi: 10.1038/s41598-017-10369-z
79. Thanikachalam K, Khan G. Colorectal cancer and nutrition. *Nutrients*. (2019) 11:164. doi: 10.3390/nu11010164
80. Windey K, De Preter V, Verbeke K. Relevance of protein fermentation to gut health. *Mol Nutr Food Res*. (2012) 56:184–96. doi: 10.1002/mnfr.201100542
81. Hughes R, Magee EA, Bingham S. Protein degradation in the large intestine: relevance to colorectal cancer. *Curr Issues Intest Microbiol*. (2000) 1:51–8.
82. Yao CK, Muir JG, Gibson PR. Review article: insights into colonic protein fermentation, its modulation and potential health implications. *Aliment Pharmacol Ther*. (2016) 43:181–96. doi: 10.1111/apt.13456
83. Fung KY, Ooi CC, Zucker MH, Lockett T, Williams DB, Cosgrove LJ, et al. Colorectal carcinogenesis: a cellular response to sustained risk environment. *Int J Mol Sci*. (2013) 14:13525–41. doi: 10.3390/ijms140713525
84. Lin HC, Visek WJ. Colon mucosal cell damage by ammonia in rats. *J Nutr*. (1991) 121:887–93. doi: 10.1093/jn/121.6.887

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

PERSPECTIVE
published: 16 June 2020
doi: 10.3389/fimmu.2020.01086

Role of Kupffer Cells in Driving Hepatic Inflammation and Fibrosis in HIV Infection

*Lumin Zhang and Meena B. Bansal**

Divison of Liver Diseases, Icahn School of Medicine at Mount Sinai, New York, NY, United States

While the interactions between HIV and various liver cell populations have been explored, the relevance of these interactions when patients are well-controlled on ART is less clear. Therefore, we focus this perspective on HIV-related alterations that may drive hepatic inflammation and fibrosis in aviremic patients, with a focus on Kupffer cells and Hepatic Stellate Cells. Persistent CD4+ T cell depletion in the gut resulting in increased gut permeability has been postulated to play a role in systemic immune activation in HIV patients. The liver, with its unique location, remains the gatekeeper between the gut and the systemic circulation. The resident liver macrophage, Kupffer cell, is responsible for clearing and responding to these products. We propose that changes in Kupffer cell biology, in the context of HIV infection, creates a mileu that drives hepatic inflammation and fibrosis in response to microbial translocation. Targeting these pathways may be helpful in improving liver-related outcomes in HIV patients.

Keywords: liver fibrosis, HIV - human immunodeficiency virus, hepatic stellate cell (HSCs), Kupffer cells, microbial translocation

OPEN ACCESS

Edited by:
M. Victoria Delpino, CONICET Institute of Immunology, Genetics and Metabolism (INIGEM), Argentina

Reviewed by:
Larisa Y. Poluektova, University of Nebraska Medical Center, United States
Natalia A. Osna, University of Nebraska Medical Center, United States

***Correspondence:**
Meena B. Bansal
meena.bansal@mssm.edu

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

Received: *10 February 2020*
Accepted: *05 May 2020*
Published: *16 June 2020*

Citation:
Zhang L and Bansal MB (2020) Role of Kupffer Cells in Driving Hepatic Inflammation and Fibrosis in HIV Infection. Front. Immunol. 11:1086. doi: 10.3389/fimmu.2020.01086

INTRODUCTION

End-stage liver disease is a major cause of non-AIDS related mortality in HIV+ patients even with effective anti-retroviral therapy, accounting for almost 15% of deaths (1–7). As a result of shared routes of transmission, HCV and HBV are the most common liver diseases in HIV-infected patients, although other chronic liver diseases are emerging (8, 9). Most data, therefore, regarding fibrosis progression rates is derived from those with coinfection. These patients have a higher relative risk (RR) of cirrhosis, increased development of decompensated cirrhosis and accelerated fibrosis progression rates compared with those who are only infected with HCV or HBV (10, 11). Furthermore, rapid fibrosis correlates with reduced CD4+ T cell counts and detectable plasma HIV levels. HIV patients are also more susceptible to other liver diseases, which synergize to accelerative liver fibrosis. Alcohol consumption is associated with increased relative risk of fibrosis progression in HIV mono-infected patients (12) while NASH is emerging as a major cause of liver disease, with half of mono-infected patients with unexplained liver enzyme elevations having NASH (13). While many may have an unrecognized chronic liver injury, a higher frequency of liver fibrosis was demonstrated in HIV-1–monoinfected patients (range 11–40.9%) compared with uninfected patients even without coinfection of hepatitis viruses and alcohol abuse, suggesting a correlation between HIV-1 infection and advanced liver fibrosis (14–19). Therefore, persistent HIV-1 infection and viral associated liver immune dysfunction may independently contribute to the progression of liver diseases (20). Lastly, in those on ART, drug-induced liver injury and increased rates of NASH due to both medications and metabolic derangements common in HIV

are being observed. While hepatic stellate cells are the downstream effector of liver fibrosis, this perspective focuses on the role Kupffer cells play in promoting a mileu conducive to fibrosis progression in patients with HIV infection, particularly in aviremic patients.

THE LIVER AS THE GATEKEEPER

Shortly after HIV infection, a severe CD4+ T cell depletion in the gut-associated lymphoid tissues leads to a disruption of the intestinal barrier, consequently promoting translocation of microbial products into the portal circulation. The liver, which derives the majority of its blood flow from the portal circulation, is uniquely positioned to protect the systemic circulation from gut-derived products. In particular, the resident hepatic macrophage, the Kupffer cell, located within the hepatic sinusoid is charged with clearing translocated bacterial products in an immunotolerant manner. However, when products provoke a pro-inflammatory response by Kuppffer cells, a cascade of intrahepatic inflammatory respones is initiated with numerous secreted cytokines, such as IL-1β, TNF-α, and IL-6, serving as major drivers in the progression of liver injury and fibrosis.

KUPFFER CELLS AT THE NEXUS OF LIVER INFLAMMATORY RESPONSES

Kupffer cells (KCs) are the largest population of resident tissue macrophages in the liver. They reside within the hepatic sinusoid in close proximity to hepatic stellate cells, liver sinusoidal endothelia cells, and intrahepatic lymphocytes. Both the low flow state of the portal circulation and the uniquely fenestrated endothelium create a conducive environment for interaction of KCs with neighboring cells and circulating cells of the immune system. Physiologically, KCs are the first line of defense to eliminate macromolecules, immune complexes, senescent cells, virally-infected cells, and translocated microbial products from the gut to avoid liver injury and systemic immune responses (21). Given the dynamic nature of cell surface receptor expression on macrophage populations and some controversy regarding their origins, CD163 or CD68, CD14 and CD16 are often used to identify human KCs. However, murine KCs display phenotypic patterns characterized by F4/80^{+}, MHCII, and CD11b^{Int} expression. A detailed discussion of markers for various macrophage subpopulations within the liver is beyond the scope of this perspective and discussed elsewhere (22). The focus of this perspective is on the role of CD68+human KCs in promoting liver inflammation and fibrosis in patients with HIV.

The importance of KCs in liver injury and inflammation have been established with depletion studies wherein $GdCl_3$ was associated with AST reduction and inflammation in an alcohol model of liver injury (23). Crosstalk between KCs and HSCs is also evidenced by KC depletion as mRNA levels of TGF-β, α-SMA and collagen I are significantly decreased (24). Although GdCl3 is not specific to KCs, and thus interpretation is complex, GdCl3 treatment dramatically decreased cytokines predominantly produced by KCs, TNF-α, IL-6, and IL-1β, in response to LPS stimulation in murine livers (25, 26). Similarly, liposome/clodronate can suppress pro-inflammatory responses through the depletion of KCs (27).

In homeostasis, KCs are central to intrahepatic immune tolerance through an antigen-mediated induction of functional arrest of CD4 cells and regulatory T cells. However, in an inflamed microenvironment this delicate equilibrium is disrupted resulting in immune dysfunction and tolerance break (28). Indeed, knockout of TREM-1 (Triggering receptor expressed on myeloid cells), which is highly expressed on KCs in liver fibrosis, reduced liver fibrosis through the inhibition of TNF-α and IL-6 responses in a number of chronic injury models (29). Similarly, knock down of Jun N-terminal kinase ½ (JNK-1/2) from KCs reversed liver fibrosis in a choline-deficient L-aminoacid-defined (CDAA) model, with a decline in inflammatory responses, including TNF-α, IL6, IL-1β, and TGF-β (30).

While KCs display M1-like features in acute liver injury, with protracted chronic inflammation, due to exhaustion of M1-like macrophages and immune cells, M2-like macrophages emerge and secrete protective cytokines upon chronic cytotoxic stimulation such as IL-4, IL-10, and TGF-β (31, 32). IL-10, an anti-inflammatory cytokine, down-regulates macrophage effector functions and differentiation of neighboring cells to maintain immune microenvironment homeostasis. For example, administration of IL-10 decreased TNF-α produced from LPS-treated KCs (33). While very complex, the manipulation of KC mediated immune responses or approaches to limit their stimulation may be exploited therapeutically.

MICROBIAL TRANSLOCATION AND KUPFFER CELLS

The impact of translocated microbial products on KCs is well-established. pretreatment with 2.5% dextran sulphate sodium (DSS) causes increased intestinal permeability and promotes translocation of microbial products into the portal blood in mice. The resulting amplified TLR4 mediated inflammatory responses in KCs resulted in significant livery injury (34). Using a liver slice model, LPS stimulation increased IL-1β and TNF-α production compared to the control (35). Consistently, in mouse models, LPS administration rapidly induces the release of inflammatory cytokines in the liver with a higher IL-6 production obtained from LPS stimulated KCs than splenic and alveolar macrophages (36).

THE ROLE OF TLR4 SIGNALING IN INFLAMMATORY RESPONSES OF KUPFFER CELLS

TLR4, as one member of Toll-like receptors, belongs to the pattern recognition receptor (PRR) family. After stimulation by TLR4 ligands, for example lipopolysaccharides, TLR4 is activated through conformational changes and interaction with TIR-domain-containing adapter proteins via hydrophilic interactions. Intracellular TLR4 signaling is mediated by two classical

pathways: the TIRAP–MyD88-NF-κB pathway and the TRIF–TRAM-interferon regulatory factor-3 (IRF3)-NF-κB pathway. TLR4 signaling participates in the initiation of pro-inflammatory response, especially TRIF mediated TNF-α and synthesis of chemokines and have been reveiwed in detail elswhere (37).

In addition to TNF-α, TLR4 signaling also contributes to the transmission of two priming signals for the IL-1β pathway through the NLRP3 inflammasome. IL-1β is a crucial proinflammatory cytokine in response to microbial infection. IL-1β from LPS-treated KCs can produce a deleterious effect on hepatocytes and promote the secretion of VLDL apo B and lipid (38). IL-1β was also found to inhibit IFN-α induced STAT1 activation in hepatocytes, attenuating the innate immune response to viral infection in hepatocytes (39). In general, NLRP3 mediated-cleavage of caspase 1 is the critical step to promote the maturation of IL-1β. The formation of the NLRP3 inflammasome is initiated by ATP or microbial stimulation (40). Blockage of NLRP3 activation in KCs decreased IL-1β response to Ischemia/Reperfusion induced liver injury and improved survival (41, 42). Administration of MCC950, a small molecule selective inhibitor of NLRP3, suppressed LPS primed IL-1β response in NPC cells, subsequently, decreasing liver injury (43). Given the important role of TLR4 signaling in KCs, the modulation of this pathway in the context of HIV infection and persistent microbial translocation is critical.

MODULATION OF INFLAMMATORY RESPONSES BY HIV-1 INFECTION IN KCS

In addition to CD4, both CCR5 and CXCR4, HIV-1 co-receptors, are detected on human KCs isolated from non-HIV-1 individuals, suggesting that KCs are permissive for HIV-1 infection. HIV-1 infection of KCs in viremic patients has been shown by *in situ* hybridization for HIV-1 RNA and PCR for proviral DNA on FACS-purifed KCs from livers of patients with Acquired Immunodeficiency Sydnrome (AIDs) (44–46). Moreover, retrieval of HIV-1 from KCs derived from patients either not on ART (47) or on ART for short durations has been shown and supported by studies in SIV_{DH12R}-infected macaques (48, 49). Recently it has been shown that KCs derived from patients on long term ART, while containing evidence of HIV-1 transcripts, do not secrete replication competent virus (50). While macrophages are known to be able to transmit infectious virus to susceptible CD4+ cells via cell-cell contact (51, 52), the ability of KCs in patients on long-term ART to do so has not yet been explored though warrants investigation.

We have shown that Kupffer cells are highly permissive for HIV-1 infection *in vitro* with robust and sustained viral replication (53). $HIV\text{-}1_{BaL}$, a laboratory adapted CCR5-tropic HIV, infection rendered KCs more sensitive to LPS treatment through an increase in CD14 and TLR4 expression on the cell surface, resulting in increased secretion of TNF-α and IL-6, which was blocked by a small molecule TLR4 inhibitor. Interestingly, despite AZT and ritonavir abrogated viral replication, KCs maintained their sensitivity to the pro-inflammatory response to LPS. These findings suggest that even in patients on ART, KC biology may be impacted and promote a mileu supporting hepatic inflammation and fibrosis in response to microbial translocation. While no change in IFNα or IFNβ expression in HIV-1 infected KCs was observed, IL-1β mRNA and both intracellular and secreted IL-1β was increased by $HIV\text{-}1_{BaL}$ infection. Similar to the IL-6 and TNF-α response, this HIV-related sensitization was found to be TLR4-dependent and further determined to be via the NLRP3-caspase 1 pathway. Immunostaining on liver tissue derived from aviremic HIV+ patients demonstrated an increased expression of IL-1β compared to normal liver with a high degree of colocalization in CD68+ macrophages (54). These studies show that TLR4 mediated NLRP3 activation is critical for the inflammatory responses to microbial products in KCs. Importantly, liver injury and resulting damage-associated molecular patterns (DAMPS) also activate TLR4 signals in KCs and thus may play a role in other forms of liver injury in HIV patients such as drug-induced liver injury. Interestingly, it has also been shown that CCR5 and TLR may co-cluster on monocyte-derived macrophages (MDMs) as secretion of CCL2 and CXCL8 in response to either R5 gp120, recombinant envelope protein from CCR5-tropic HIV-1, or LPS can be blocked by either a CCR5 inhibitor or TLR4 blocking. These results suggest another mechanism for synergistic effects of HIV and LPS on macrophage biology and should be specifically examined in human KCs (55).

INFLAMMATORY RESPONSES TO HIV-1 INFECTION IN OTHER LIVER IMMUNE CELLS

While beyond the scope of this perspective, HIV-1 infection impacts a number of other cells critical to the inflammatory response in the liver. In line with circulating CD4+ T cells, HIV infection leads to a depletion of CD4+ T cell in the liver with relative reversal of CD4/CD8 ratio typically seen. Viral infection also makes IL2+ CD4+ T cells dysfunctional and attenuates hepatic immune response to microbial infection (56–58). CD4+ T cells from HIV mono-infected patients exhibit a low regulatory effect on Natural killer (NK). Co-cultured with NK cells, CD4+ T cells from HIV-1+ individuals greatly reduced anti-fibrotic effect of NK cells on HSCs (59). Therefore, reduction in CD4+ T cells influences progression of liver fibrosis in HIV+ patients (10, 60) while increased relative CD8+T cells correlates with a higher fibrosis scores in HIV-1 infected patients (61).

NK cells, which account for up to 30–50% human liver lymphocytes, play an important role in clearing virally infected cells trough NK cell antibody dependent cell cytotoxicity (ADCC). The activation and NK cellular numbers are spontaneously increased early in response to HIV-1 infection but with chronic infection exhaustion results in NK dysfunction with persistent viremia (62).

While the role of DCs in HIV-1 infection and progression and ability to transmit infectious virus to CD4 cells by cell-cell contact has been shown, HIV interaction with DCs in the liver is less studied. TLR7 is constitutively expressed by human pDCs. The delivery of HIV-1 viral nucleic acids in early endosome of pDCs

can be blocked by TLR7 inhibitor (63), suggesting that TLR7 is involved in the antigen presentation by pDCs. In addition, mDC express TLR4 and the frequency of mDCs, especially CXCL16-producing mDCs has been shown to be associated with the level of microbial products in the liver of HIV+ patients (64).

INTERACTIONS BETWEEN HIV AND HEPATOCYTES AND IMPLICATIONS FOR HEPATIC INFLAMMATION AND FIBROSIS

In vitro studies have shown that the envelope protein, HIV gp 120, which binds either CXCR4 (X4) or CCR5 (R5) on its target cell can promote hepatocyte apoptosis (65) and along with the HCV glycoprotein E2 promote the secretion of the pro-inflammatory cytokine IL-8 (66, 67). It has been known that Kupffer cells play a primary role in the clearance of apoptotic hepatocytes/cellular debris within the liver and thus play a key role in sterile inflammation and repair (68). More recently, Ganesan et. al demonstrated that ethanol exposure promotes HIV accumulation within hepatocytes, ultimately leading to increased oxidative stress and apoptosis. These apoptotic hepatocytes then stimulate inflammasome activation in KCs and pro-fibrogenic genes in hepatic stellate cells (69). Moreover, hepatic stellate cells can also engulf apoptotic hepatocytes resulting in NAPDH oxidation, stellate cell activation, and fibrogenesis (70). Therefore, effects on HIV on hepatocytes can promote both KC and HSC activation, synergistically driving hepatic inflammation and fibrosis.

DIRECT INTERACTIONS BETWEEN HIV AND HUMAN STELLATE CELLS

HSCs express both HIV CCR5 and CXCR4 co-receptors. We have shown that HIV and its envelope protein gp120 promote HSC activation, collagen I production, and CCL2 secretion through interactions with CXCR4 (71) and others have shown that the envelope protein on HIV that preferentially uses CCR5 for cellular entry (R5 gp120) promotes HSC chemotaxis and CCL2 secretion (72). While HIV can infect HSCs *in vitro*, infection *in vivo* has not been established. Similar to what has recently been shown for KCs, *in vitro* infected HSCs do not secrete replication competent virus though, like DCs, may be able to transmit virus by cell-cell contact (71, 73, 74). Similar to what has been shown on MDMs, CCR5 and TLR4 seem to co-cluster on HSCs and result in increased CCL2 and CXCL8 in response to gp120 and LPS, with effects of ligands blocked by inhibiting either receptor alone (55). As CCL2 is an important chemokine for attracting circulating monocytes into the liver, this may be important for propagating hepatic inflammation. R5 gp120 also promotes IL-6 secretion from HSCs through Jun-NF-kB activation (75). These studies suggest that HIV promotes inflammation and fibrosis by interacting with CXCR4 and CCR5 via gp120 and synergizes with TLR4 signals. While the

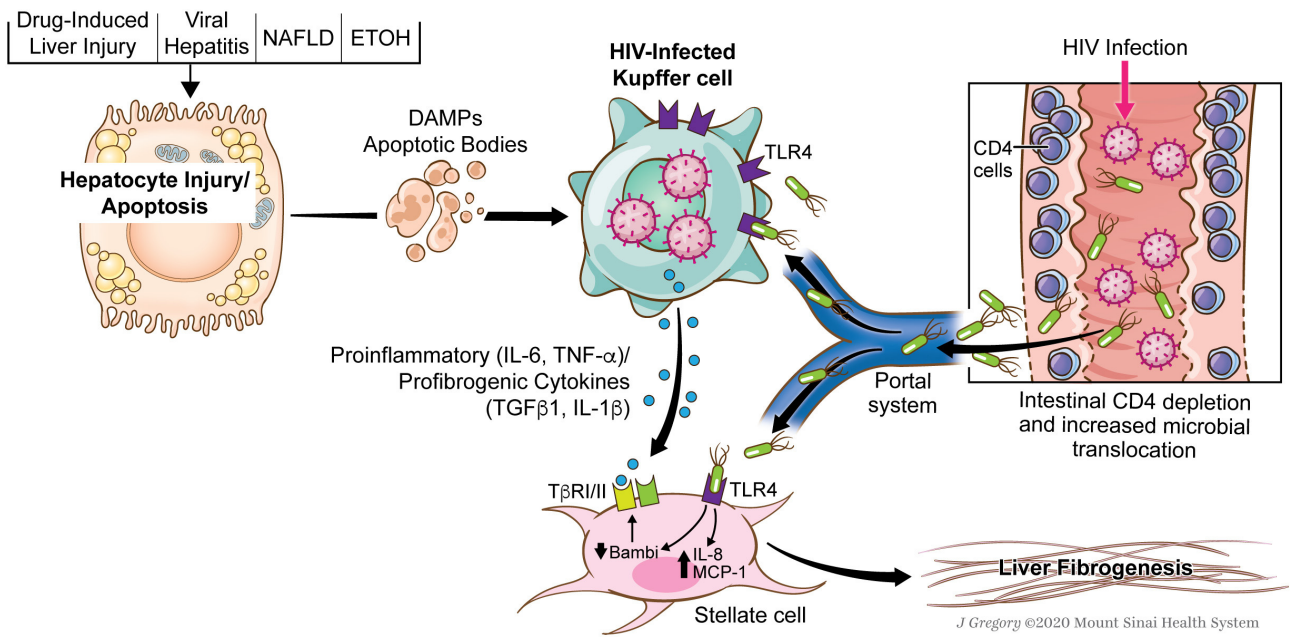


FIGURE 1 | Role of HIV and associated microbial translocation in driving hepatic inflammation and fibrosis through kupffer cell and stellate cell interactions. HIV infection causes early CD4 gut depletion which promotes increased microbial translocation and the delivery of pathogen-associated molecular patterns to the liver (PAMPs). At the same time, patients with HIV have multiple secondary chronic liver injuries (NAFLD, DILI, HCV, HBV, Alcohol) which promote direct injury to hepatocytes and give rise to damage-associated molecular patterns (DAMPs) or endogenous TLR ligands. HIV infected KCs are sensitized to effects of both the translocated microbial products as well as DAMPs. These signals converge on the HIV-infected KCs resulting in the secretion of a number of pro-inflammatory (IL-6, TNF-α) and pro-fibrogenic cytokines (IL-1β and TGFβ1). Additionally, TLR ligands have pro-inflammatory effects on HSCs and sensitize them to KC-derived TGFβ1. Used with permission from @Mount Sinai Health System.

latter is important in viremic patients, relevance for those on ART are not clear. For those on ART, the impact of HIV on microbial translocation and KC biology may be more important.

INTERPLAY BETWEEN KUPFFER CELLS AND HEPATIC STELLATE CELLS

While the ultimate effector cell in liver fibrosis is the hepatic stellate cell, the signals generated by KCs are critically important in promoting the activation of HSCs and then perpetuating the activated state. TLR4 activation on HSCs results in downregulation of the TGFβ1 pseudoreceptor, BAMBI, which sensitizes HSCs to the pro-fibrogenic effects of TGFβ1 (76), much of which is derived by KCs. Therefore, in the context of HIV-1 and associated microbial translocation, the effects on both KCs and HSCs are compounded and drive hepatic inflammation and fibrosis (**Figure 1**). Overall association between microbial translocation and liver fibrosis progression has been shown in a variety of liver diseases and thus HIV simply compounds this effect.

CONCLUSION

As patients with HIV live longer, liver disease will continue to emerge as a leading cause of morbidity and mortality. Understanding how HIV may set the stage for hepatic injury, inflammation and fibrosis may lead to novel therapeutic strategies. While treatment of underlying diseases, ranging from viral hepatitis to NASH or alcohol, remains the most imporant strategy, the alterations unique to this population need to be kept in mind. With this perspective, HIV related alterations in KC biology and microbial translocation may be at the nexus of creating a milue conducive to hepatic fibrosis. Targeting either the KC response to TLR4 ligands, PAMPs or DAMPS, decreasing the burden of microbial products from reaching the portal circulation, or blocking downstream pro-inflammatory or pro-fibrogenic effects on stellate cells are important to consider. Much will be learned from current treatments undergoing investigation for non-HIV related liver fibrosis that may be additionally leveraged for this special population.

AUTHOR CONTRIBUTIONS

LZ and MB wrote and reviewed this manuscript.

FUNDING

This work was supported by research grants NIH R01DK108364 to MB.

REFERENCES

1. Lifson AR, Group ICODW, Belloso WH, Carey C, Davey RT, Duprez D, et al. Determination of the underlying cause of death in three multicenter international HIV clinical trials. *HIV Clin Trials*. (2008) 9:177–85. doi: 10.1310/hct0903-177
2. Data Collection on Adverse Events of Anti-HIV drugs (D:A:D) Study Group, Smith C, Sabin CA, Lundgren JD, Thiebaut R, Weber R, et al. Factors associated with specific causes of death amongst HIV-positive individuals in the D:A:D Study. *AIDS*. (2010) 24:1537–48. doi: 10.1097/QAD.0b013e32833a0918
3. Price JC, Thio CL. Liver disease in the HIV-infected individual. *Clin Gastroenterol Hepatol*. (2010) 8:1002–12. doi: 10.1016/j.cgh.2010.08.024
4. Leone S, Gregis G, Quinzan G, Velenti D, Cologni G, Soavi L, et al. Causes of death and risk factors among HIV-infected persons in the HAART era: analysis of a large urban cohort. *Infection*. (2011) 39:13–20. doi: 10.1007/s15010-010-0079-z
5. Puoti M, Moioli MC, Travi G, Rossotti R. The burden of liver disease in human immunodeficiency virus-infected patients. *Semin Liver Dis*. (2012) 32:103–13. doi: 10.1055/s-0032-1316473
6. Weber R, Ruppik M, Rickenbach M, Spoerri A, Furrer H, Battegay M, et al. Decreasing mortality and changing patterns of causes of death in the Swiss HIV Cohort Study. *HIV Med*. (2013) 14:195–207. doi: 10.1111/j.1468-1293.2012.01051.x
7. Salmon-Ceron D, Rosenthal E, Lewden C, Bouteloup V, May T, Burty C, et al. Emerging role of hepatocellular carcinoma among liver-related causes of deaths in HIV-infected patients: the French national Mortalite 2005 study. *J Hepatol*. (2009) 50:736–45. doi: 10.1016/j.jhep.2008.11.018
8. Vallet-Pichard A, Mallet V, Pol S. Nonalcoholic fatty liver disease and HIV infection. *Semin Liver Dis*. (2012). 32:158–66. doi: 10.1055/s-0032-1316471
9. Stabinski L, Reynolds SJ, Ocama P, Laeyendecker O, Ndyanabo A, Kiggundu V. et al. High prevalence of liver fibrosis associated with HIV infection: a study in rural Rakai, Uganda. *Antivir Ther*. (2011) 16:405–11. doi: 10.3851/IMP1783
10. Benhamou Y, Bochet M, Di Martino V, Charlotte F, Azria F, Coutellier A, et al. Liver fibrosis progression in human immunodeficiency virus and hepatitis C virus coinfected patients. *The Multivirc Group Hepatol*. (1999) 30:1054–8. doi: 10.1002/hep.510300409
11. Macías J, Berenguer J, Japón MA, Girón JA, Rivero A, López-Cortés LF, et al., Fast fibrosis progression between repeated liver biopsies in patients coinfected with human immunodeficiency virus/hepatitis C virus. *Hepatology*. (2009) 50:1056–63. doi: 10.1002/hep.23136
12. Chaudhry AA, Sulkowski MS, Chander G, Moore RD. Hazardous drinking is associated with an elevated aspartate aminotransferase to platelet ratio index in an urban HIV-infected clinical cohort. *HIV Med*. (2009) 10:133–42. doi: 10.1111/j.1468-1293.2008.00662.x
13. Joshi D, O'grady J, Dieterich D, Gazzard B, Agarwal K. Increasing burden of liver disease in patients with HIV infection. *Lancet*. (2011) 377:1198–209. doi: 10.1016/S0140-6736(10)62001-6
14. Dallapiazza M, Amorosa VK, Localio R, Kostman JR, Lo Re V III. Prevalence and risk factors for significant liver fibrosis among HIV-monoinfected patients. *BMC Infect Dis*. (2010). 10:116. doi: 10.1186/1471-2334-10-116
15. Merchante N, Perez-Camacho I, Mira JA, Rivero A, Macias J, Camacho A, et al. Prevalence and risk factors for abnormal liver stiffness in HIV-infected patients without viral hepatitis coinfection: role of didanosine. *Antivir Ther*. (2010) 15:753–63. doi: 10.3851/IMP1612
16. Han SH, Kim SU, Kim CO, Jeong SJ, Park JY, Choi JY, et al. Abnormal liver stiffness assessed using transient elastography (Fibroscan(R)) in HIV-infected patients without HBV/HCV coinfection receiving combined antiretroviral treatment. *PLoS ONE*. (2013) 8:e52720. doi: 10.1371/journal.pone.0052720
17. Tahiri M, Sodqi M, Lahdami FE, Marih L, Lamdini H, Hliwa W, et al. Risk factors for liver fibrosis among human immunodeficiency virus monoinfected patients using the FIB4 index in Morocco. *World J Hepatol*. (2013) 5:584–8. doi: 10.4254/wjh.v5.i10.584
18. Rockstroh JK, Mohr R, Behrens G, Spengler U. Liver fibrosis in HIV: which role does HIV itself, long-term drug toxicities and metabolic changes play? *Curr Opin HIV AIDS*. (2014) 9:365–70. doi: 10.1097/COH.0000000000000064
19. Anadol E, Lust K, Boesecke C, Schwarze-Zander C, Mohr R, Wasmuth JC, et al. Exposure to previous cART is associated with significant liver fibrosis and cirrhosis in human immunodeficiency virus-infected patients. *PLoS ONE*. (2018) 13:e0191118. doi: 10.1371/journal.pone.0191118

20. Lamers SL, Rose R, Maidji E, Agsalda-Garcia M, Nolan DJ, Fogel GB, et al. HIV DNA is frequently present within pathologic tissues evaluated at autopsy from combined antiretroviral therapy-treated patients with undetectable viral loads. *J Virol*. (2016) 90:8968–83. doi: 10.1128/JVI.00674-16
21. Shi J, Fujieda H, Kokubo Y, Wake K. Apoptosis of neutrophils and their elimination by Kupffer cells in rat liver. *Hepatology*. (1996) 24:1256–63. doi: 10.1002/hep.510240545
22. Guillot A, Tacke F. Liver macrophages: old dogmas and new insights. *Hepatol Commun*. (2019) 3:730–43. doi: 10.1002/hep4.1356
23. Adachi Y, Bradford BU, Gao W, Bojes HK, Thurman RG. Inactivation of Kupffer cells prevents early alcohol-induced liver injury. *Hepatology*. (1994) 20:453–60. doi: 10.1002/hep.1840200227
24. Canbay A, Feldstein AE, Higuchi H, Werneburg N, Grambihler A, Bronk SF, et al. Kupffer cell engulfment of apoptotic bodies stimulates death ligand and cytokine expression. *Hepatology*. (2003) 38:1188–98. doi: 10.1053/jhep.2003.50472
25. Rizzardini M, Zappone M, Villa P, Gnocchi P, Sironi M, Diomede L, et al. Kupffer cell depletion partially prevents hepatic heme oxygenase 1 messenger RNA accumulation in systemic inflammation in mice: role of interleukin 1beta. *Hepatology*. (1998) 27:703–10. doi: 10.1002/hep.510270311
26. Tomiyama K, Ikeda A, Ueki S, Nakao A, Stolz DB, Koike Y, et al. Inhibition of Kupffer cell-mediated early proinflammatory response with carbon monoxide in transplant-induced hepatic ischemia/reperfusion injury in rats. *Hepatology*. (2008) 48:1608–20. doi: 10.1002/hep.22482
27. Ju C, Reilly TP, Bourdi M, Radonovich MF, Brady JN, George JW, et al. Protective role of Kupffer cells in acetaminophen-induced hepatic injury in mice. *Chem Res Toxicol*. (2002) 15:1504–13. doi: 10.1021/tx0255976
28. Heymann F, Peusquens J, Ludwig-Portugall I, Kohlhepp M, Ergen C, Niemietz P, et al. Liver inflammation abrogates immunological tolerance induced by Kupffer cells. *Hepatology*. (2015) 62:279–91. doi: 10.1002/hep.27793
29. Nguyen-Lefebvre AT, Ajith A, Portik-Dobos V, Horuzsko DD, Arbab AS, Dzutsev A, et al. The innate immune receptor TREM-1 promotes liver injury and fibrosis. *J Clin Invest*. (2018) 128:4870–83. doi: 10.1172/JCI98156
30. Kodama Y, Kisseleva T, Iwaisako K, Miura K, Taura K, De Minicis S, et al. c-Jun N-terminal kinase-1 from hematopoietic cells mediates progression from hepatic steatosis to steatohepatitis and fibrosis in mice. *Gastroenterology*. (2009) 137:1467–77 e1465. doi: 10.1053/j.gastro.2009.06.045
31. Mantovani A, Sica A. Macrophages, innate immunity and cancer: balance, tolerance, and diversity. *Curr Opin Immunol*. (2010) 22:231–7. doi: 10.1016/j.coi.2010.01.009
32. Coussens LM, Zitvogel L, Palucka AK. Neutralizing tumor-promoting chronic inflammation: a magic bullet? *Science*. (2013) 339:286–91. doi: 10.1126/science.1232227
33. Wan J, Benkdane M, Teixeira-Clerc F, Bonnafous S, Louvet A, Lafdil F, et al. M2 Kupffer cells promote M1 Kupffer cell apoptosis: a protective mechanism against alcoholic and nonalcoholic fatty liver disease. *Hepatology*. (2014) 59:130–42. doi: 10.1002/hep.26607
34. El Kasmi KC, Anderson AL, Devereaux MW, Fillon SA, Harris JK, Lovell MA, et al. Toll-like receptor 4-dependent Kupffer cell activation and liver injury in a novel mouse model of parenteral nutrition and intestinal injury. *Hepatology*. (2012) 55:1518–28. doi: 10.1002/hep.25500
35. Olinga P, Merema MT, De Jager MH, Derks F, Melgert BN, Moshage H, et al. Rat liver slices as a tool to study LPS-induced inflammatory response in the liver. *J Hepatol*. (2001) 35:187–94. doi: 10.1016/S0168-8278(01)00103-9
36. Ogle CK, Wu JZ, Mao X, Szczur K, Alexander JW, Ogle JD. Heterogeneity of Kupffer cells and splenic, alveolar, and peritoneal macrophages for the production of TNF, IL-1, and IL-6. *Inflammation*. (1994) 18:511–23. doi: 10.1007/BF01560698
37. Vaure C, Liu Y. A comparative review of toll-like receptor 4 expression and functionality in different animal species. *Front Immunol*. (2014) 5:316. doi: 10.3389/fimmu.2014.00316
38. Bartolome N, Arteta B, Martinez MJ, Chico Y, Ochoa B. Kupffer cell products and interleukin 1beta directly promote VLDL secretion and apoB mRNA up-regulation in rodent hepatocytes. *Innate Immun*. (2008) 14:255–66. doi: 10.1177/1753425908094718
39. Tian Z, Shen X, Feng H, Gao B. IL-1 beta attenuates IFN-alpha beta-induced antiviral activity and STAT1 activation in the liver: involvement of proteasome-dependent pathway. *J Immunol*. (2000) 165:3959–65. doi: 10.4049/jimmunol.165.7.3959
40. He Y, Hara H, Nunez G. Mechanism and regulation of NLRP3 inflammasome activation. *Trends Biochem Sci*. (2016) 41:1012–21. doi: 10.1016/j.tibs.2016.09.002
41. Huang H, Chen HW, Evankovich J, Yan W, Rosborough BR, Nace GW, et al. Histones activate the NLRP3 inflammasome in Kupffer cells during sterile inflammatory liver injury. *J Immunol*. (2013) 191:2665–79. doi: 10.4049/jimmunol.1202733
42. Xu Y, Yao J, Zou C, Zhang H, Zhang S, Liu J, et al. Asiatic acid protects against hepatic ischemia/reperfusion injury by inactivation of Kupffer cells via PPARgamma/NLRP3 inflammasome signaling pathway. *Oncotarget*. (2017) 8:86339–55. doi: 10.18632/oncotarget.21151
43. Mridha AR, Wree A, Robertson A, a,B, Yeh MM, Johnson CD, et al. NLRP3 inflammasome blockade reduces liver inflammation and fibrosis in experimental NASH in mice. *J Hepatol*. (2017) 66:1037–46. doi: 10.1016/j.jhep.2017.01.022
44. Housset C, Lamas E, Brechot C. Detection of HIV1 RNA and p24 antigen in HIV1-infected human liver. *Res Virol*. (1990) 141:153–9. doi: 10.1016/0923-2516(90)90017-D
45. Hoda SA, White JE, Gerber MA. Immunohistochemical studies of human immunodeficiency virus-1 in liver tissues of patients with AIDS. *Mod Pathol*. (1991) 4:578–81.
46. Schmitt MP, Steffan AM, Gendrault JL, Jaeck D, Royer C, Schweitzer C, et al. Multiplication of human immunodeficiency virus in primary cultures of human Kupffer cells–possible role of liver macrophage infection in the physiopathology of AIDS. *Res Virol*. (1990) 141:143–52. doi: 10.1016/0923-2516(90)90016-C
47. Schweitzer C, Keller F, Schmitt MP, Jaeck D, Adloff M, Schmitt C, et al. Morphine stimulates HIV replication in primary cultures of human Kupffer cells. *Res Virol*. (1991) 142:189–95. doi: 10.1016/0923-2516(91)90056-9
48. Persidsky Y, Steffan AM, Gendrault JL, Hurtrel B, Berger S, Royer C, et al. Permissiveness of Kupffer cells for simian immunodeficiency virus (SIV) and morphological changes in the liver of rhesus monkeys at different periods of SIV infection. *Hepatology*. (1995) 21:1215–25. doi: 10.1002/hep.1840210502
49. Igarashi T, Brown CR, Endo Y, Buckler-White A, Plishka R, Bischofberger N, et al. Macrophage are the principal reservoir and sustain high virus loads in rhesus macaques after the depletion of CD4+ T cells by a highly pathogenic simian immunodeficiency virus/HIV type 1 chimera (SHIV): implications for HIV-1 infections of humans. *Proc Natl Acad Sci USA*. (2001) 98:658–63. doi: 10.1073/pnas.98.2.658
50. Kandathil AJ, Sugawara S, Goyal A, Durand CM, Quinn J, Sachithanandham J, et al. No recovery of replication-competent HIV-1 from human liver macrophages. *J Clin Invest*. (2018) 128:4501–9. doi: 10.1172/JCI121678
51. Poli G. Cell-to-cell vs. cell-free HIV-1 transmission from macrophages to CD4+ T lymphocytes: lessons from the virology textbook. *AIDS*. (2013) 27:2307–8. doi: 10.1097/QAD.0b013e328363619a
52. Bracq L, Xie M, Benichou S, Bouchet J. Mechanisms for Cell-to-Cell Transmission of HIV-1. *Front Immunol*. (2018) 9:260. doi: 10.3389/fimmu.2018.00260
53. Mosoian A, Zhang L, Hong F, Cunyat F, Rahman A, Bhalla R, et al. Frontline Science: HIV infection of Kupffer cells results in an amplified proinflammatory response to LPS. *J Leukoc Biol*. (2017) 101:1083–90. doi: 10.1189/jlb.3HI0516-242R
54. Zhang L, Mosoian A, Schwartz ME, Florman SS, Gunasekaran G, Schiano T, et al. HIV infection modulates IL-1beta response to LPS stimulation through a TLR4-NLRP3 pathway in human liver macrophages. *J Leukoc Biol*. (2019) 105:783–95. doi: 10.1002/JLB.4A1018-381R
55. Del Corno M, Cappon A, Donninelli G, Varano B, Marra F, Gessani S. HIV-1 gp120 signaling through TLR4 modulates innate immune activation in human macrophages and the biology of hepatic stellate cells. *J Leukoc Biol*. (2016) 100:599–606. doi: 10.1189/jlb.4A1215-534R
56. Ostrowski SR, Gerstoft J, Pedersen BK, Ullum H. Impaired production of cytokines is an independent predictor of mortality in HIV-1-infected patients. *AIDS*. (2003) 17:521–30. doi: 10.1097/00002030-200303070-00007
57. Sun Z, Denton PW, Estes JD, Othieno FA, Wei BL, Wege AK, et al. Intrarectal transmission, systemic infection, and CD4+ T cell depletion

in humanized mice infected with HIV-1. *J Exp Med*. (2007) 204:705–14. doi: 10.1084/jem.20062411
58. Erikstrup C, Kronborg G, Lohse N, Ostrowski SR, Gerstoft J, Ullum H. T-cell dysfunction in HIV-1-infected patients with impaired recovery of CD4 cells despite suppression of viral replication. *J Acquir Immune Defic Syndr*. (2010) 53:303–10. doi: 10.1097/QAI.0b013e3181ca3f7c
59. Glassner A, Eisenhardt M, Kokordelis P, Kramer B, Wolter F, Nischalke HD, et al. Impaired CD4(+) T cell stimulation of NK cell anti-fibrotic activity may contribute to accelerated liver fibrosis progression in HIV/HCV patients. *J Hepatol*. (2013) 59:427–33. doi: 10.1016/j.jhep.2013.04.029
60. Brau N, Salvatore M, Rios-Bedoya CF, Fernandez-Carbia A, Paronetto F, Rodriguez-Orengo JF, et al. Slower fibrosis progression in HIV/HCV-coinfected patients with successful HIV suppression using antiretroviral therapy. *J Hepatol*. (2006) 44:47–55. doi: 10.1016/j.jhep.2005.07.006
61. Kooij KW, Wit FW, Van Zoest RA, Schouten J, Kootstra NA, Van Vugt M, et al. Liver fibrosis in HIV-infected individuals on long-term antiretroviral therapy: associated with immune activation, immunodeficiency and prior use of didanosine. *AIDS*. (2016) 30:1771–80. doi: 10.1097/QAD.0000000000001119
62. Lucar O, Reeves RK, Jost S. A Natural Impact: NK Cells at the Intersection of Cancer and HIV Disease. *Front Immunol*. (2019) 10:1850. doi: 10.3389/fimmu.2019.01850
63. Beignon AS, Mckenna K, Skoberne M, Manches O, Dasilva I, Kavanagh DG, et al. Endocytosis of HIV-1 activates plasmacytoid dendritic cells via Toll-like receptor-viral RNA interactions. *J Clin Invest*. (2005) 115:3265–75. doi: 10.1172/JCI26032
64. Evans TI, Li H, Schafer JL, Klatt NR, Hao XP, Traslavina RP, et al. SIV-induced translocation of bacterial products in the liver mobilizes myeloid dendritic and natural killer cells associated with liver damage. *J Infect Dis*. (2016) 213:361–9. doi: 10.1093/infdis/jiv404
65. Vlahakis S, Villasis-Keever A, Gomez T, Al E. Human immunodeficiency virus-induced apoptosis of human hepatocytes via CXCR4. *J Infect Dis*. (2003) 188:1455–60. doi: 10.1086/379738
66. Balasubramanian S, Ganju R, Groopman J. Hepatitis C virus and HIV envelope proteins collaboratively mediate interleukin-8 secretion through activation of p38 MAP kinase and SHP2 in hepatocytes. *J Biol Chem*. (2003) 278:35755–66. doi: 10.1074/jbc.M302889200
67. Munshi N, Balasbramanian A, Koziel M, Ganju R, Groopman J. Hepatitis C and human immunodeficiency virus envelope proteins cooperatively induce hepatocytic apoptosis via an innocent bystander mechanism. *J Infect Dis*. (2003) 188:1192–204. doi: 10.1086/378643
68. Yang L, Seki E. Toll-like receptors in liver fibrosis: cellular crosstalk and mechanisms. *Front Physiol*. (2012) 3:138. doi: 10.3389/fphys.2012.00138
69. Ganesan M, New-Aaron M, Dagur RS, Makarov E, Wang W, Kharbanda KK, et al. Alcohol metabolism potentiates HIV-induced hepatotoxicity: contribution to end-stage liver disease. *Biomolecules*. (2019) 9:20851. doi: 10.3390/biom9120851
70. Zhan SS, Jiang JX, Wu J, Halsted C, Friedman SL, Zern MA, et al. Phagocytosis of apoptotic bodies by hepatic stellate cells induces NADPH oxidase and is associated with liver fibrosis *in vivo*. *Hepatology*. (2006) 43:435–43. doi: 10.1002/hep.21093
71. Hong F, Saiman Y, Si C, Mosoian A, Bansal MB. X4 Human immunodeficiency virus type 1 gp120 promotes human hepatic stellate cell activation and collagen I expression through interactions with CXCR4. *PLoS ONE*. (2012) 7:e33659. doi: 10.1371/journal.pone.0033659
72. Bruno R, Galastri S, Sacchi P, Cima S, Caligiuri A, Defranco R, et al. gp120 modulates the biology of human hepatic stellate cells: a link between HIV infection and liver fibrogenesis. *Gut*. (2010) 59:513–20. doi: 10.1136/gut.2008.163287
73. Tuyama AC, Hong F, Saiman Y, Wang C, Ozkok D, Mosoian A, et al. Human immunodeficiency virus (HIV)-1 infects human hepatic stellate cells and promotes collagen I and monocyte chemoattractant protein-1 expression: implications for the pathogenesis of HIV/hepatitis C virus-induced liver fibrosis. *Hepatology*. (2010) 52:612–22. doi: 10.1002/hep.23679
74. Salloum S, Holmes JA, Jindal R, Bale SS, Brisac C, Alatrakchi N, et al. Exposure to human immunodeficiency virus/hepatitis C virus in hepatic and stellate cell lines reveals cooperative profibrotic transcriptional activation between viruses and cell types. *Hepatology*. (2016) 64:1951–68. doi: 10.1002/hep.28766
75. Gupta D, Rani M, Khan N, Jameel S. HIV-1 infected peripheral blood mononuclear cells modulate the fibrogenic activity of hepatic stellate cells through secreted TGF-beta and JNK signaling. *PLoS ONE*. (2014) 9:e91569. doi: 10.1371/journal.pone.0091569
76. Seki E, De Minicis S, Osterreicher CH, Kluwe J, Osawa Y, Brenner DA, et al. TLR4 enhances TGF-beta signaling and hepatic fibrosis. *Nat Med*. (2007) 13:1324–32. doi: 10.1038/nm1663

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