CELLULAR STRESS AND INFLAMMATION: HOW THE IMMUNE SYSTEM DRIVES TISSUE HOMEOSTASIS

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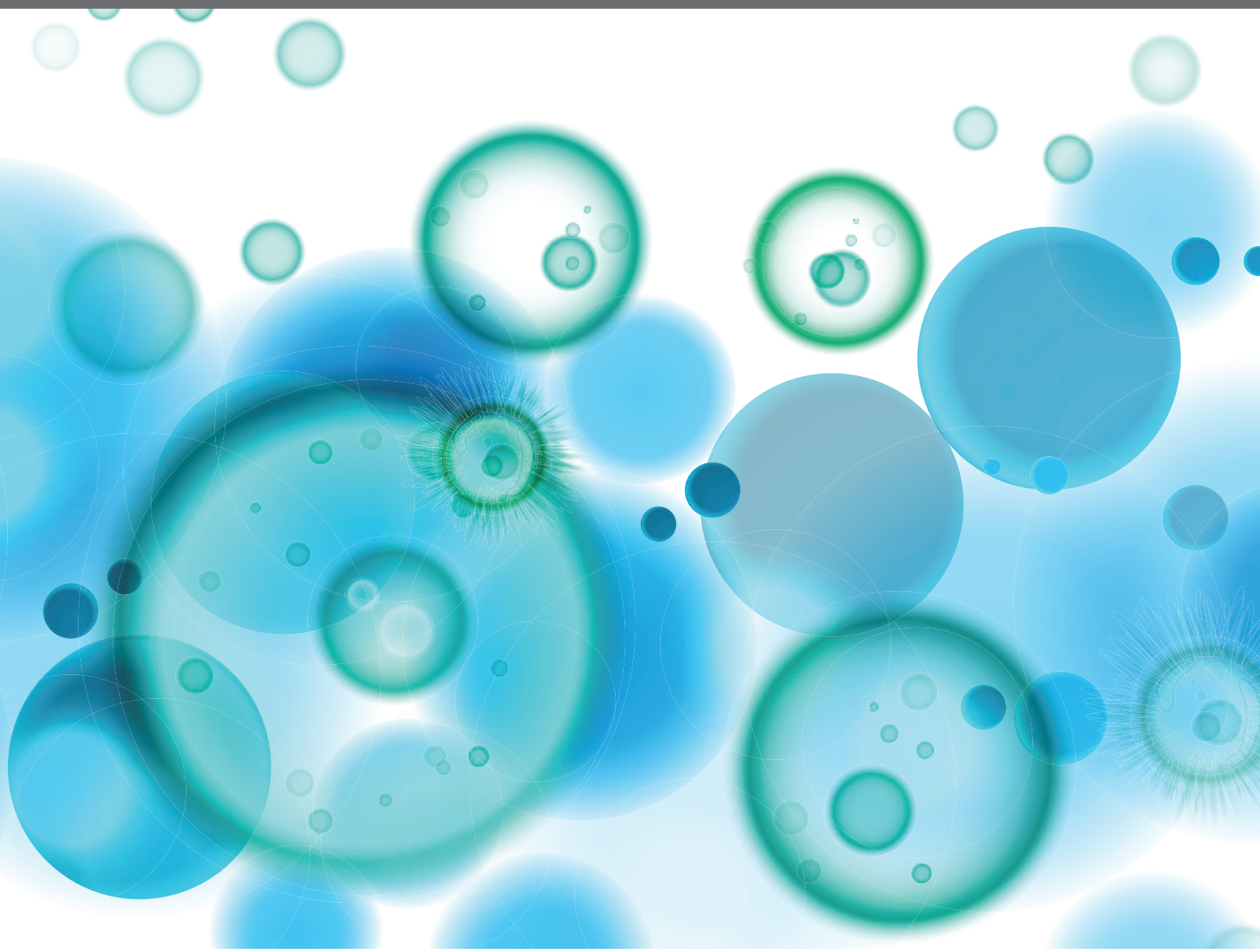

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CELLULAR STRESS AND INFLAMMATION: HOW THE IMMUNE SYSTEM DRIVES TISSUE HOMEOSTASIS

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EDITORIAL
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Editorial: Cellular Stress and Inflammation: How the Immune System Drives Tissue Homeostasis

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Keywords: inflammation, immune system, tissue homeostasis, metabolism, stress response, fibrosis, PD-L1, granzymes

Editorial on the Research Topic

Cellular Stress and Inflammation: How the Immune System Drives Tissue Homeostasis

For a long time, the immune system (IS) has been referred to only as a defense mechanism against pathogens and external threats. Nowadays, its pivotal role in orchestrating tissue remodeling of multicellular organisms is undoubtful. Cells facing cellular stress, by expressing stress-induced molecules or through the release of a plethora of soluble factors, undertake a dialog with the IS. In this way, cells link intracellular stress to systemic homeostasis, a process that has likely evolved during the establishment of the colonial way of life.

In this *Frontiers in Immunology* Research Topic, the interaction of parenchyma cells with the IS during peculiar inflammatory processes has been addressed by leading investigators, providing different point of views on the contribution of the IS to tissue homeostasis. Fibrosis is a prototypical response of damaged tissues largely guided by the IS, here well described by Trionfetti et al. in the peritoneum. The authors highlight the complex network among stromal cells, resident and recirculating leukocytes, which sustain peritoneal fibrosis. Mesothelial cell plasticity is at the base of the fibrotic process and is mostly affected by the cytokine milieu produced by locally activated or recruited immune cells. In particular, the crosstalk between mesothelial cells and macrophages is characterized by a bidirectional mode of action.

Antonangeli et al., considering the evidence that the key inflammatory mediator NF-κB is emerging as a positive regulator of PD-L1 expression in cancer, suggest that PD-L1 upregulation takes part in cancer biology as a maladaptive consequence of its physiological role during inflammation, namely by mirroring a process aimed at restoring tissue integrity during epithelial stress response, alike the epithelial to mesenchymal transition (EMT). It is conceivable that tissue morphogenesis, inflammation and immune response have a common evolutionary root, with NF-κB representing the link and the immune checkpoint PD-L1 a consequence.

The contribution by Mandatori et al. is focused on regulatory T cells (Tregs) during atherogenesis, a pathological process of the blood vessels that largely involves the IS. The authors show that autophagy sustains Treg maturation and they hypothesize that an impairment of the

autophagic processes, by affecting Tregs, contributes to the inflammation of the atherosclerotic plaques. The article sheds light to the intricated relationship between metabolism and the IS.

In this collection, two other peculiar aspects of the interplay between cellular metabolism and the IS are highlighted. Bilotta et al. summarize several studies showing how cholesterol and cholesterol metabolism influence immunity and inflammation. Indeed, the cholesterol receptors LXRs are able, depending on the cellular context, to act as activators or repressors of genes regulating different cellular responses including apoptosis, cytokine production, and cell cycle inhibition. These effects are particularly relevant to autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis, and atherosclerosis. Moreover, the cholesterol metabolism can also play a role in the regulation of tumor growth, as demonstrated in ovarian, colon, and prostatic cancers.

In the review article by Lauro and Limatola the metabolism-IS interconnection is analyzed in the context of neuroinflammation. Microglia, the sentries of the central nervous system, undergo a metabolic switch depending on the activation status. It is emerging that homeostatic microglia are characterized by an oxidative metabolism, while reactive microglia (similarly to activated macrophages) switch towards a glycolytic and pentose phosphate metabolism to support cell proliferation. This finding is mostly important because reactive microglia release cytokines and free radicals contributing to brain neurodegeneration and inflammation, while homeostatic microglia promote brain repair and has anti-inflammatory properties. Thus, targeting microglia metabolism represents an intriguing opportunity for the treatment of neurodegenerative diseases.

Macrophages are crucial in tissue homeostasis by catching apoptotic cells, removing cellular debris, and secreting cytokines. Two studies in this Research Topic pay attention to alveolar macrophages (AMs). AMs from pulmonary surfactant secretoglobin family 1A member 1 (SCGB1A1) deficient mice show alterations in different inflammatory pathways as evaluated by high-throughput RNA-sequencing and gene expression analysis. Xu et al. suggest that SCGB1A1 is necessary for AMs to stimulate an appropriate adaptive immune response and that SCGB1A1 supplementation could reduce cytokine surge during airway infections.

AMs control neutrophil homeostasis in pulmonary alveoli by expressing neutrophil chemotactic factors and by clearing apoptotic neutrophils (a process called efferocytosis). Alpha 1 antitrypsin deficiency (AATD) is a genetic disorder characterized by neutrophil accumulation in the lungs. Lee et al. propose a mechanism by which the mutated allelic variant of AAT, Z-AAT, is retained intracellularly and induces the expression of CXCL8 and TNF-α by AMs. CXCL8 is responsible for the increased neutrophil recruitment while TNF-α, by affecting CD14, CD36, and RARα, reduces AM efferocytosis ability, thus contributing to the pulmonary disease in AATD individuals.

Innovative considerations come from three articles included in the present collection that are all focused on the multifunctional pro-inflammatory role of granzymes (Gzms), a family of serine proteases expressed by immune, non-immune, and tumor cells. In particular, the involvement of Gzms in the pathogenesis of serious inflammatory diseases highlights the potential therapeutic effect of targeting Gzms to reduce organ damage. Garzòn-Tituaña et al. present evidence suggesting the relevant role of Gzms in sepsis pathophysiology. The authors show the role of GzmA and GzmM in regulating the inflammatory cytokine network, contributing to the cytokine storm, as well as in the regulation of the coagulation cascade, platelet function and endothelial barrier permeability, during the hyper-inflammatory initial-stage sepsis. Furthermore, a possible role of GzmB in the immunosuppressive late-stage sepsis is discussed.

In a research article, Cimini et al. nicely describe the expression of GzmB in both visceral adipose tissue (VAT) and serum of obese patients and report its association with VAT inflammation and glucose homeostasis dysregulation, suggesting that GzmB pro-apoptotic and extracellular proteolytic activities contribute to obesity-related VAT inflammatory remodeling and fibrosis, leading to adipose tissue dysfunction in metabolic diseases.

Finally, Velotti et al. provide an overview on recent data concerning the multifunctional pro-inflammatory activity of GzmB, consisting of perforin-dependent and perforin-independent (anoikis) apoptosis, IL-1 cytokine family activation, extracellular matrix remodeling, induction of EMT and fibrosis, that might underlie inflammaging and the pathogenicity and/or severity of acute and chronic inflammatory diseases, such as cardiovascular, pulmonary, metabolic diseases, and cancer.

Altogether, the articles included in the present Research Topic provide a fascinating view of the IS as a key player of tissue homeostasis, hopefully inspiring new developments in this challenging field. The articles well describe the plasticity of the IS in both physiological and pathological conditions. As a result, the idea of exploiting the plasticity of the IS, by altering cell metabolism and cell polarization or by targeting specific component, is emerging, with the aim of counteracting pathologies characterized by tissue homeostasis dysregulation such as cancer, neurodegenerative, and metabolic diseases.

AUTHOR CONTRIBUTIONS

FA, OG, MNR and FV edited the topic and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Altered Tregs Differentiation and Impaired Autophagy Correlate to Atherosclerotic Disease

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Atherosclerosis is a progressive vascular disease representing the primary cause of morbidity and mortality in developed countries. Formerly, atherosclerosis was considered as a mere passive accumulation of lipids in blood vessels. However, it is now clear that atherosclerosis is a complex and multifactorial disease, in which the involvement of immune cells and inflammation play a key role. A variety of studies have shown that autophagy—a cellular catalytic mechanism able to remove injured cytoplasmic components in response to cellular stress—may be proatherogenic. So far, in this context, its role has been investigated in smooth muscle cells, macrophages, and endothelial cells, while the function of this catabolic protective process in lymphocyte functionality has been overlooked. The few studies carried out so far, however, suggested that autophagy modulation in lymphocyte subsets may be functionally related to plaque formation and development. Therefore, in this research, we aimed at better clarifying the role of lymphocyte subsets, mainly regulatory T cells (Tregs), in human atherosclerotic plaques and in animal models of atherosclerosis investigating the contribution of autophagy on immune cell homeostasis. Here, we investigate basal autophagy in a mouse model of atherosclerosis, apolipoprotein E (ApoE)-knockout (KO) mice, and we analyze the role of autophagy in driving Tregs polarization. We observed defective maturation of Tregs from ApoE-KO mice in response to tumor growth factor-β (TGFβ). TGFβ is a well-known autophagy inducer, and Tregs maturation defects in ApoE-KO mice seem to be related to autophagy impairment. In this work, we propose that autophagy underlies Tregs maturation, advocating that the study of this process in atherosclerosis may open new therapeutic strategies.

Keywords: atherosclerosis, autophagy, ApoE-KO, regulatory T cells, plaques

INTRODUCTION

Atherosclerosis is a chronic inflammatory and multifactorial disease affecting the cardiovascular system and one of the leading causes of mortality in industrialized countries. Smoking, unhealthy diet, aging population, lack of physical activity, arterial hypertension, and diabetes promote atherosclerosis and possibly cardiovascular diseases such as myocardial infarction or stroke (1, 2). The early steps of atherosclerosis are characterized by the adhesion of blood leukocytes to the endothelial monolayer, the migration of the bound leukocytes into the intima, the maturation of monocytes into macrophages, and the subsequent enhanced uptake of lipids leading to their transformation into foam cells. Lesion progression involves the migration of smooth muscle cells (SMCs) from tunica media to intima. In advancing lesions, it has been reported that plaque macrophages and SMCs undergo cell death and contribute to the progression of the plaque (3–5). Extracellular lipids accumulate in the central region of the plaque, forming the lipid or necrotic core, thus promoting the progression of the lesion and, eventually, the plaque rupture, thrombus formation, and the subsequent myocardial infarction or stroke (6, 7).

Although a strong involvement of immune system response has been reported in the pathogenesis of atherosclerosis, the cellular and molecular mechanisms involved and the crosslink between immune cells during atherogenesis remain unclear (8, 9).

It is commonly known that preserving tolerance, while preventing autoimmunity and chronic inflammation, is decisive for slowing down the onset of atherosclerosis (10, 11). Regulatory T cells (Tregs) are the main CD4 T-cell subpopulation responsible for the maintenance of immunologic tolerance by suppressing T-cell responses, preventing autoimmunity, and suppressing inflammatory and proatherogenic immune response (12–14). T-cell differentiation into Tregs is driven by the lineage-determining transcription factor forkhead box P3 (FOXP3) (15). The tumor growth factor-β (TGFβ), a cytokine secreted by many cell types, including macrophages, promotes FOXP3 expression in naïve peripheral CD4+ T cells and converts them into FOXP3-expressing Tregs (16, 17). Recently, it has been reported that Tregs deletion of autophagy-related gene 7 (Atg7) or autophagy-related gene 5 (Atg5) alters immune homeostasis, establishing autophagy as a central and intrinsic regulator of Tregs (18). Autophagy, a self-protecting cellular catabolic process appointed to the recycling of biomolecules, is interestingly one of the molecular processes involved in the pathology of atherosclerosis (19, 20). So far, three forms of autophagy have been described: macroautophagy, microautophagy, and chaperone-mediated autophagy; hereafter, we refer to macroautophagy as autophagy (21, 22). In early atherosclerotic plaques, autophagy preserves normal cellular function to protect cells against oxidative injury, metabolic stress, and inflammation (23–25). Conversely, autophagy defects in macrophages, SMCs and endothelial cells, lead to cell senescence and apoptosis and promote atherosclerosis onset and progression (26).

Based on this evidence showing that autophagy is a relevant factor in the function and homeostasis of cells in the immune system and that chronic inflammation leads to the progression of atherosclerotic plaque, we hypothesized that an altered autophagic flux during Tregs differentiation might contribute to altering the inflammatory state of the lesions, promoting atherosclerotic plaque progression. Apolipoprotein E (ApoE)-knockout (KO) mice are the elective animal model to study atherosclerosis (27). ApoE is a component of lipoproteins (LPs), a biochemical assembly whose primary purpose is to transport lipids, fat-soluble vitamins and cholesterol into the lymph system and then into the blood. Mice lacking ApoE show a significant increase in total plasma cholesterol compared to wild-type animals and display atherosclerotic plaque development (28). In ApoE-KO mice, the progression of atherosclerotic disease is accelerated by treatment with aldosterone, a hormone which regulates blood pressure and promotes vascular function (29, 30). In this research, we characterize lymphocyte populations in human and murine atherosclerotic plaque, and we investigate the role of autophagy in Tregs differentiation.

RESULTS

Characterization of Human Atherosclerotic Plaques: Tregs Accumulation in AP and APR

As a starting point, we characterized lymphocyte populations in human atherosclerotic plaques obtained by endarterectomy, a surgical procedure to remove the atheromatous plaque from carotids (31). Donor's characteristics are presented in **Table 1** (section Materials and Methods). As shown in **Figure 1A**, in order to better characterize Tregs diversity, each carotid artery has been divided into an adjacent atherosclerotic plaque region (APR) and atherosclerotic plaque (AP), using peripheral blood mononuclear cells (PBMC) as a control of circulating immune cells. We performed a multiparameter flow cytometry analysis as described in **Supplementary Table 1**.

We estimated the frequency (%) of leukocyte populations (CD45+), CD4+ T cells (CD4+CD8–), CD8+ T cells (CD8+CD4–), conventional T (Tconv) cells (CD127+/–FOXP3–/CD4+ T cells), and Tregs ($CD127^{low}$FOXP3+/CD4+ T cells) by flow cytometry using the gating strategy shown in **Supplementary Figure 1**. We observed a significant decrease of CD45+ cells in APR compared to PBMC and a significant decrease of CD8+ T cells in AP compared to APR, while CD4+ cells were comparable among the experimental groups. Interestingly, the frequency of Tregs was significantly increased in APR and in AP, when compared to PBMC, suggesting a higher frequency of Tregs in the atherosclerotic plaque microenvironment (**Figure 1B**). We did not observe any differences in Tregs frequency between AP and APR, indicating that infiltration of Tregs takes place also in the adjoining plaque region.

Recent observation highlighted the pivotal role of the expression of OX40, a member of the TNFR/TNF superfamily, on activated CD4+ and CD8+ T cells (32), in supporting Tregs fitness in mouse models of homeostatic proliferation and colitis and in promoting the expansion of stable and

TABLE 1 | Characteristics of all carotid endarterectomy (CEA) patients included in the study ($n = 9$).

		Stenosis		Diseases							
Pt	**Sex**	**Surgery side**	**Opposite surgery side**	**Hypertension**	**Diabetes**	**Mi**	**POA**	**Dyslipidemia**	**COPD**	**Smoke**	**Statins**
1	F	Left	80%	Yes	No	No	No	Yes	No	No	Yes
2	M	Left	90%	Yes	Yes	No	No	Yes	No	Yes	Yes
3	M	Right	80%	Yes	No	No	No	Yes	No	No	Yes
4	M	Right	90%	Yes	No	No	Yes	No	No	Yes	No
5	F	Right	70%	Yes	Yes	Yes	No	Yes	No	Yes	Yes
6	F	Right	80%	Yes	No	No	No	Yes	Yes	Yes	Yes
7	F	Right	70%	Yes	No	No	No	Yes	Yes	Yes	Yes
8	M	Right	70%	Yes	No	No	No	Yes	Yes	Yes	No
9	M	Left	80%	Yes	No	No	No	Yes	Yes	Yes	Yes

The surgery side represents carotid occlusion (%); the opposite surgery side represents the condition of the opposite carotid artery. MI, Myocardial infarction; POA, Peripheral obliterant arteriopathy; COPD, Chronic obstructive pulmonary disease.

suppressive Tregs in human cancers (33–35). Based on these findings, we investigated whether Tregs and Tconv in AP were characterized by higher levels of OX40 expression (**Figure 1C**). The geometric mean fluorescence intensity (gMFI) of Tconv-OX40+ was significantly increased in AP if compared to PBMC. Furthermore, we found a significant increment of the frequency and the gMFI of Tregs-OX40+ in AP and APR when compared to PBMC (**Figure 1C**). These results suggest that Tregs in the atherosclerotic plaque microenvironment expressed higher levels of OX40, possibly contributing to Tregs expansion.

It has been recently demonstrated that, besides inducing a huge frequency of Tregs, OX40 expression was associated with fatty acid accumulation and with the promotion of selective proliferation of lipid-laden Tregs (36). Based on this evidence, we stained the cells with BODIPY, a cell-membrane-permeable fluorophore specific for neutral lipid stores; however, we did not find any significant difference in the intracellular lipid content of CD8+ T cells, Tconv, and Tregs derived from both APR and AP when compared to PBMC (**Figure 1D**).

Overall, these results show that a significant fraction of Tregs are found in APR and even more in the AP site when compared to PBMC, suggesting Tregs-mediated modulation of inflammation at the atherosclerotic plaque and its associated region.

Characterization of Atherosclerotic Plaques in ApoE-KO Mice Treated With Aldosterone

To confirm our results *in vivo*, we took advantage of the ApoE-KO mouse, one of the most widely used models to study atherosclerosis (37). ApoE is a glycosylated protein, mainly bound to plasma lipoproteins but also expressed in hematopoietic stem and progenitor cells (HSPC) (37–39). In order to better resemble the human cardiovascular disease and to speed up plaque progression, we treated ApoE-KO mice with aldosterone at a dose of 6 μg/kg/day. Such concentration is able to raise serum aldosterone levels to a range which promotes atherosclerosis, without inducing hypertension (27, 30, 40). Aldosterone promotes rapid lipid accumulation in the aortic arch section of ApoE-KO mice, as shown in **Figure 2A**. In order to estimate the frequency of leukocytes (CD45+), CD4 T cells (CD4+CD3+CD45+), Th1 (CXCR3+CCR6–CD4+), Th17 (CCR6+CXCR3–CD4+), and Tregs (FOXP3+CD4+), we performed multiparameter flow cytometry (**Supplementary Table 2**) on splenocytes and aortic arches obtained from 8-week-old ApoE-KO mice treated with aldosterone or vehicle for 4 weeks (the gating strategy is shown in **Supplementary Figure 2A**).

We first observed a significant increase of CD45+ cells and a significant decrease of CD4+ T cells in splenocytes of ApoE-KO mice treated with aldosterone (**Figure 2B**). Gated on CD4+ T cells, we checked the frequency of Th1 cells (CD4+CXCR3+), Th17 cells (CD4+CCR6+), and Tregs (CD4+FOXP3+), and we found a significant decrease of Th17 and a significant increase of Tregs in ApoE-KO mouse spleens treated with aldosterone (**Figure 2B**). However, we did not observe significant differences of Th17 and Tregs percentages in aortic arches. The calculation of the absolute cell numbers (%Cells of interest × CD4 × 100) of different T-cell populations revealed a statistically significant decrease in Th1 cells and Th17 and a significant increase of Tregs in spleens of ApoE-KO mice treated with aldosterone (**Figure 2C**). In agreement with previous results, the absolute cell number in aortic arches does not show any significant difference. Overall, these data show that Tregs accumulate in the spleens of ApoE-KO mice under the atherosclerotic development condition (with aldosterone treatment), whereas no changes in the presence of Tregs were observed in ApoE-KO mouse atherosclerotic plaque.

In order to investigate the possible role played by autophagy in lymphocyte homeostasis and in T-cell polarization, we analyzed the levels of microtubule-associated proteins 1A/1B light chain 3B (LC3), as a marker of autophagy in splenocytes and aortic arches obtained from 8-week-old ApoE-KO mice in the presence or absence of aldosterone. In particular, we detected the phosphatidylethanolamine-conjugated form of LC3, named LC3-II, which is closely correlated with the number of autophagosomes and serves as a good indicator of autophagosome formation (41). We then calculated gMFI

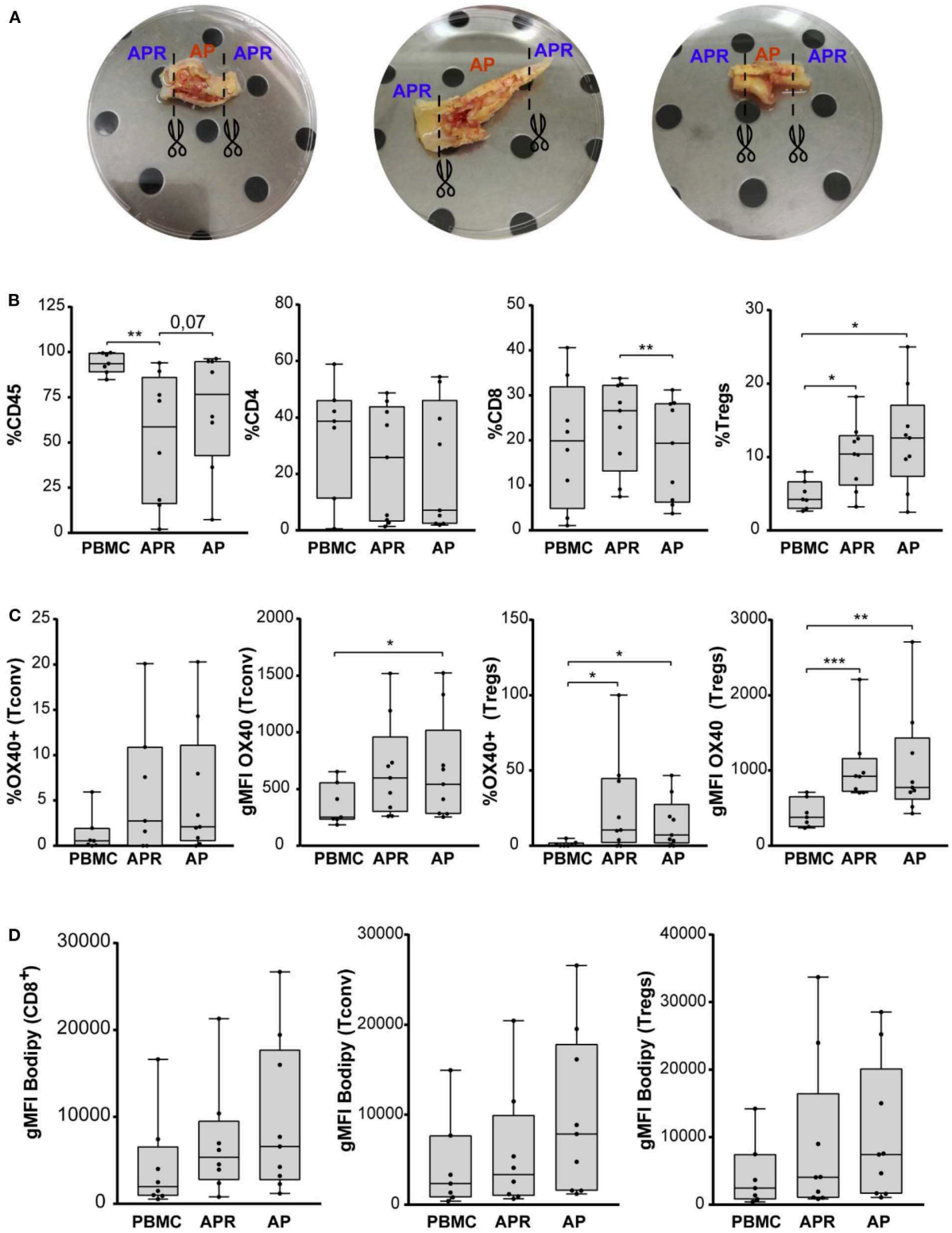

FIGURE 1 | (A) Three representative carotid endarterectomy samples. The image shows sample division strategy: APR (adjacent atherosclerotic plaque region), AP (atherosclerotic plaque). **(B)** Frequency of CD45, CD4, CD8, and Tregs (in gated CD4+ T cells) in PBMC, APR, and AP from CEA patients ($n = 9$). **(C)** Percentage of OX40 in Tconv and Tregs and gMFI of OX40 gated on Tconv and Tregs. **(D)** gMFI of BODIPY in CD8, Tconv, and Tregs. In all figures, Tregs have been identified as FOXP3+ CD127^{low} within the CD14– CD8– viability dye– CD4+ CD25+ gate. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, by paired t-test, two-tailed.

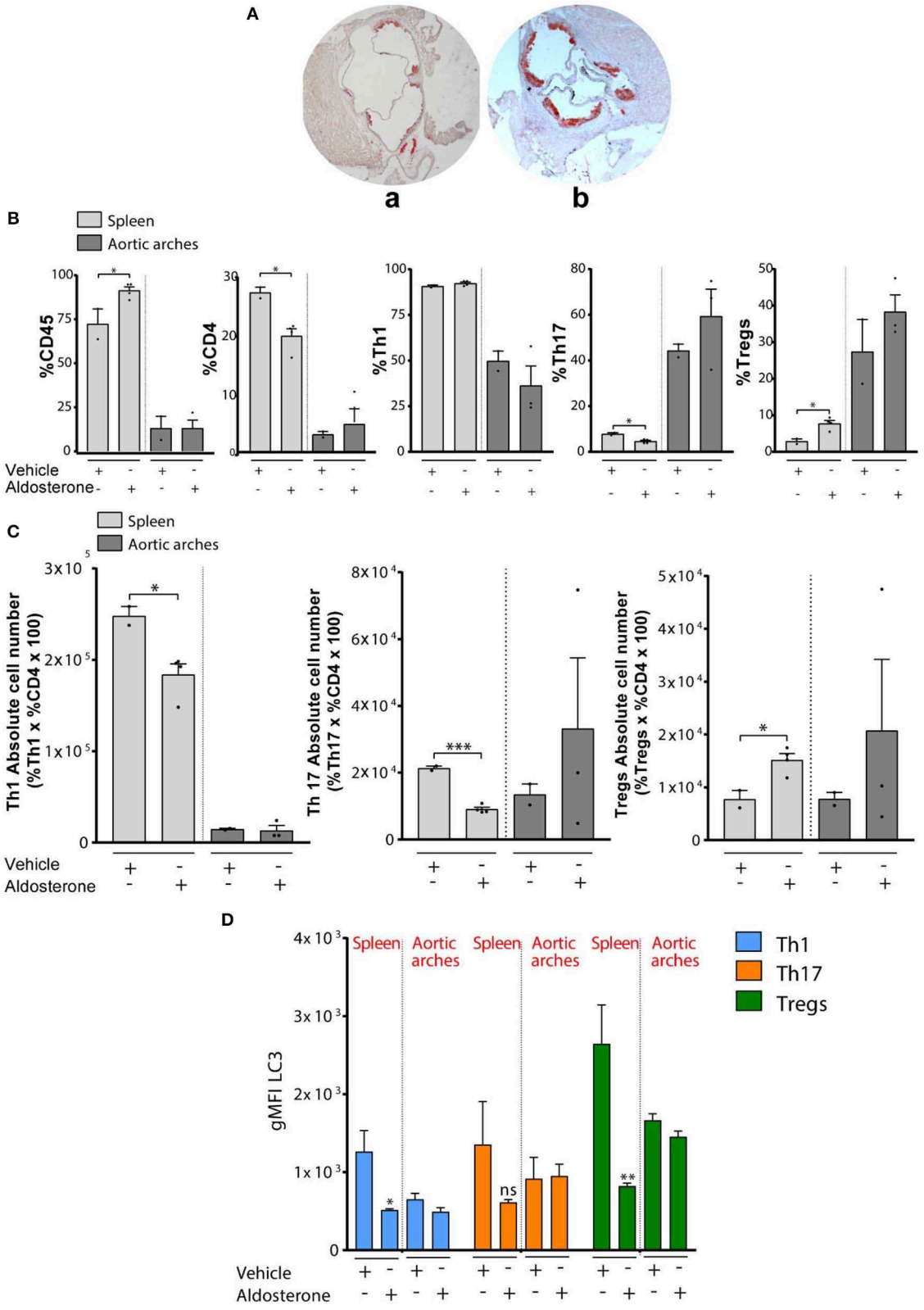

FIGURE 2 | (A) Quantification of atherosclerotic plaque composition in vehicle- or aldosterone-treated ApoE-KO: Representative Red Oil O stained cross sections of aortic root in 8- to 10-week-old ApoE-KO mice treated with vehicle (a) or aldosterone (6 μg/mouse/day) (b) and fed an atherogenic diet for 4 weeks. **(B)** Percentage

(Continued)

FIGURE 2 | of CD45, CD4, CD8 Th1, Th17, and Tregs (in gated CD4+ T cells) in spleens and aortic arches from ApoE-KO mice treated or not with aldosterone (n = 8, four mice each group). **(C)** Absolute number of Th1, Th17, and Tregs calculated on the percentage in **(B)**. **(D)** gMFI of LC3 in Th1, Th17, and Tregs. In all figures, Th1, Th17, and Tregs have been identified as CXCR3+CCR6–CD4+, CCR6+CXCR3–CD4+, and FOXP3+CD4+, respectively, within the CD8– CD4+ gate. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, by unpaired t-test, two-tailed.

LC3-II by flow cytometric analysis, and we observed a significant decrease of LC3-II in Th1 and Tregs in ApoE-KO mouse spleens treated with aldosterone (**Figure 2D**); however, similar to what was observed before, we did not find any difference in aortic arches of ApoE-KO mice in the presence or absence of aldosterone. A decrease in LC3-II levels could be an indication of (i) impaired autophagy, (ii) enhanced autophagic flux, or (iii) decreased LC3 transcription. We performed additional experiments on isolated lymphocytes to investigate this aspect.

Impaired Tregs Polarization in ApoE-KO Mice After TGFβ Stimulus

Based on our results and previous studies about Tregs differentiation during atherosclerotic plaque progression, we estimated the frequency of Tregs (FOXP3+CD4+ T cells) by flow cytometry in splenocytes derived from 8-week-old C57BL/6 wild-type (*wt*) and ApoE-KO mice treated or not with aldosterone. In line with previous literature (42), we observed a reduction of the percentage of Tregs in ApoE-KO mice compared with *wt* mice (**Figure 3A**). Surprisingly, we found that the frequency of Tregs (CD4+FOXP3+ gated on CD4+ T cells) in splenocytes was significantly increased in ApoE-KO mice when treated with aldosterone (**Figure 3A**). To clarify if the differences observed derive from aldosterone treatment itself or from the atherogenic-prone background, naïve CD4 T cells from *wt* mice were cultured in the presence of aldosterone for 96 h and with TGFβ to stimulate Tregs differentiation. However, as shown in **Supplementary Figures 2B–D**, no significant differences have been observed upon aldosterone treatment either in %CD4 T cells or in %Tregs, suggesting that the genetic background of ApoE-KO mice, rather than aldosterone treatment, is the determinant of the differential percentage of circulating Tregs. Although this is an interesting result, for subsequent *in vitro* experiments, we isolated cells from ApoE-KO mice that were not treated with aldosterone, in order to focus only on the genetic background. Our data, indeed, suggest that the aldosterone effect is presumably not directly interfering with immune cells but rather with the hormonal response in the animal that we exploited solely to speed up plaque formation (43). Hereby, we focused on the differences in Tregs frequencies between *wt* and ApoE-KO genetic background.

Tregs differentiation and expansion can occur extrathymically from naïve CD4+ T-cell precursors upon antigenic stimulation (44). Given the differences between *wt* and ApoE-KO mice in the frequency of Tregs, we first investigated the differences either in expanding Tregs starting from total CD4+ T cells or in the differentiating Tregs starting from naïve CD4+ T cells obtained from *wt* and ApoE-KO mice. Total CD4+ T cells (>5% FOXP3+), taken as a heterogeneous population derived from *wt* and ApoE-KO mouse spleens, were cultured for different times (24, 48, and 96 h) with interleukin-2 (IL2) in the presence or absence of TGFβ. Since CD4+ T cells include naïve CD4+ T cells, the precursors of Tregs, we could not determine the difference in proliferating Tregs or in induced Tregs. However, we observed a significant Tregs expansion in *wt* mice, already detected after 24 h of TGFβ treatment, reaching a higher extent after 96 h in culture (**Figures 3B**,**C**). In contrast, the treatment with TGFβ of ApoE-KO CD4+ T cells did not show any Tregs expansion. In line with this observation, the frequency of Tregs induced by TGFβ in *wt* was significantly much higher with respect to ApoE-KO cells. Calculation of Tregs absolute cell number (%Tregs × %CD4 × 100) shows how Tregs derived from *wt* animals were responsive to TGFβ stimulus differently from ApoE-KO cells (**Supplementary Figure 3A**). In order to quantify flow cytometry data, we also evaluated the geometric mean of FOXP3 which showed a significant reduction of intensity in ApoE-KO mice at 48 and 96 h, in agreement with the previous results (**Supplementary Figure 4**).

Considering that naïve CD4+ T cells are the precursors of both effector and memory T-cell subsets and that Tregs can be generated in the thymus or can differentiate from peripheral naïve CD4+ T cells (45), we stimulated Tregs polarization by culturing naïve CD4+ T cells obtained from *wt* and ApoE-KO mouse spleens (46). In particular, we cultured naïve CD4+ T cells in the presence of IL2 for 24 and 96 h, adding or not TGFβ and maintaining the same conditions used for CD4+ T cells. We found that TGFβ treatment did not induce ApoE-KO CD4+ Tregs differentiation, while *wt* cells respond to the treatment at both 24 and 96 h (**Figures 3D**,**E**). Thus, the frequency of Tregs induced by TGFβ in *wt* was significantly much higher with respect to ApoE-KO cells. To better appreciate the differences between *wt* and ApoE-KO, we calculated the Tregs absolute cell number. In accordance with the data previously shown, Tregs differentiation from *wt* mice cells was detectable after 24 h of TGFβ treatment, reaching the highest levels after 96 h. Although the absolute cell number of Tregs differentiation in ApoE-KO mice increased upon TGFβ treatment at both 24 and 96 h, the extent was much lower with respect to *wt* counterparts, thus suggesting a deficiency in TGFβ-induced maturation of ApoE-KO Tregs (**Supplementary Figure 3B**). In summary, our results show that Tregs differentiation in ApoE-KO is significantly impaired compared to *wt* counterparts.

TGFβ Modulates Autophagy in Tregs

Since autophagy is an important mechanism protecting Tregs lineage and survival integrity, we tested the hypothesis that autophagy might affect Tregs differentiation in ApoE-KO mice (18). In order to determine if autophagy was related to Tregs differentiation and to analyze its contribution in the defective Tregs differentiation observed in ApoE-KO mice, we first

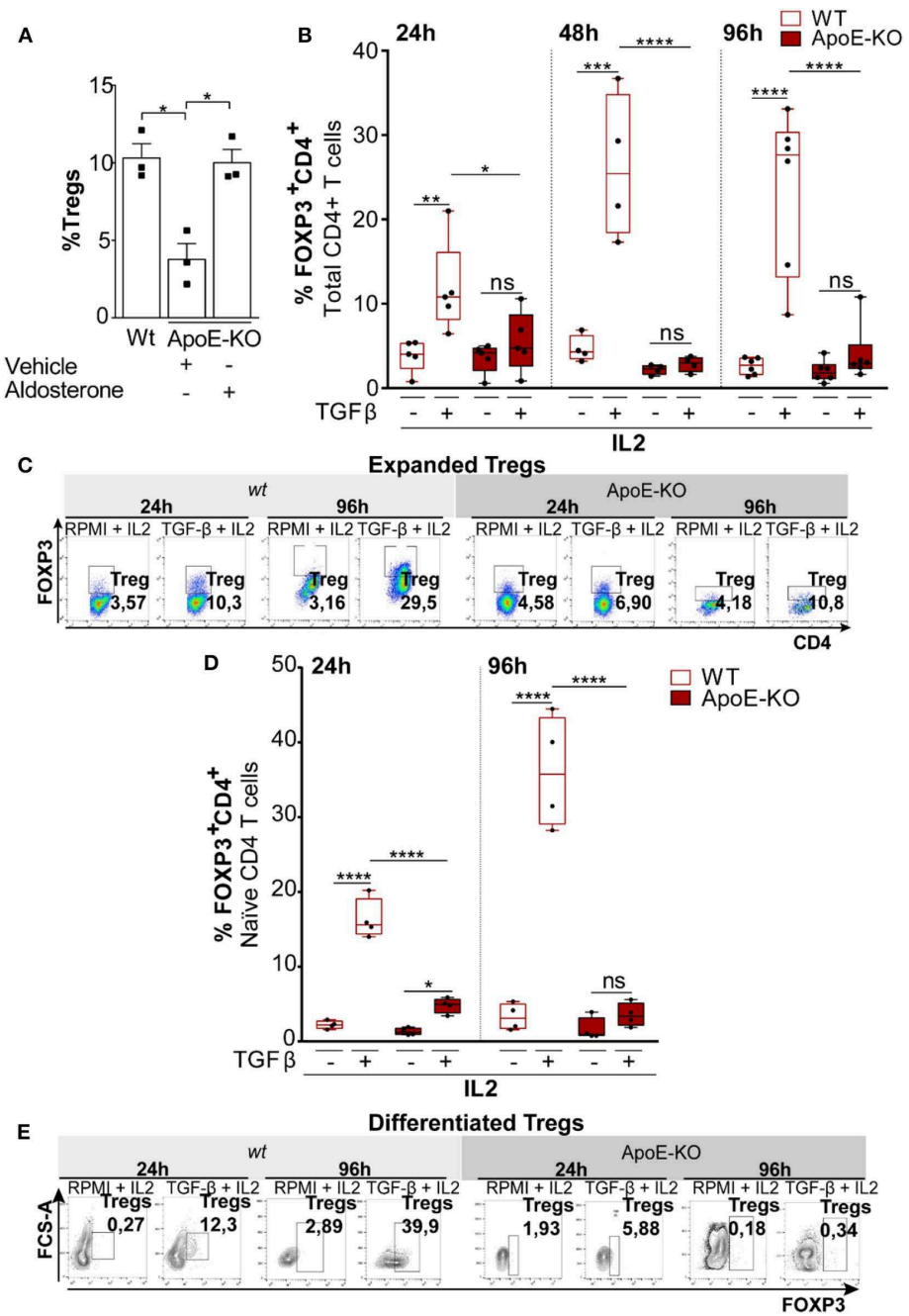

FIGURE 3 | (A) Percentage of Tregs (FOXP3+CD4+) in splenocytes obtained from *wt* ($n = 3$), ApoE-KO ($n = 3$), and ApoE-KO + aldosterone ($n = 3$). **(B)** Expanded Tregs treated with TGFβ (2 ng/ml) and IL2 (100 U/ml) in *wt* ($n = 5$) and ApoE-KO mice ($n = 5$) started from CD4+ T cell. The histograms represent % of Tregs (CD4+FOXP3+) analyzed by flow cytometry. The experiments were conducted at different times, 24, 48, and 96 h, by culturing CD4+ T cells isolated from spleens of wild type (*wt*) mice and ApoE-KO mice, in the presence of IL2, treated or not with TGFβ and stimulated with plate-bound anti-CD3 and anti-CD28. **(C)** Representative dot plots of expanded Tregs from five independent experiments in *wt* and ApoE-KO mice. **(D)** Differentiated Tregs with TGFβ (2 ng/ml) and IL2 (100 U/ml) in *wt* ($n = 4$) and ApoE-KO mice ($n = 4$). The bar graph indicates % of Tregs (CD4+FOXP3+) at two different times, 24 and 96 h, by culturing naïve CD4+ T cells isolated from spleens of *wt* mice and ApoE-KO mice, in the presence of IL2, treated or not with TGFβ and stimulated with plate-bound anti-CD3 and anti-CD28. **(E)** Representative dot plots of differentiated Tregs from four independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, by two-way analysis of variance (ANOVA).

investigated the kinetics of autophagy in TGFβ-induced Tregs derived from naïve CD4+ T cells. Cells were cultured in Tregs-polarizing conditions (IL2 treatment with or without TGFβ) and analyzed at two time points: 24 and 96 h. The autophagy flux has been analyzed by flow cytometry comparing the LC3-II amount in the presence and absence of the lysosomal inhibitor

bafilomycin (Baf), added 4 h before the end of the experiment (47). In order to evaluate the autophagic flux of CD4+ T cells, we calculated the LC3-II ratio between Baf-treated and untreated cells. On expanded Tregs gated on CD4, we did not observe any significant difference in the LC3-II ratio analyzed at two different time points: 24 and 96 h (data not shown). We therefore repeated the analysis during Tregs differentiation induced from naïve CD4+ T cells isolated from *wt* and ApoE-KO mice. We observed that the LC3-II ratio significantly increased in *wt* Tregs upon TGFβ treatment at 24 h, while no difference was observed in ApoE-KO Tregs (**Figure 4A**). These results indicate that TGFβ induces autophagy in *wt* but not in ApoE-KO Tregs. In particular, by blocking the autophagosome–lysosome fusion by Baf, 4 h before the end of the experiment, ApoE-KO mice did not show any significant increase in LC3-II ratio when challenged with TGFβ, suggesting a block of autophagic machinery during Tregs differentiation in ApoE-KO mice (**Figure 4A**).

We previously showed a reduction in LC3-II signal in ApoE-KO mouse spleen treated with aldosterone (**Figure 2A**). In order to exclude any commitment of aldosterone in LC3 lipidation in Tregs, we analyzed LC3-II-positive cells during Tregs differentiation by treating cells in the presence or absence of aldosterone at the same dose used for the *in vivo* experiment. After 96 h of Tregs differentiation, aldosterone did not interfere with LC3 lipidation (**Supplementary Figure 5**), suggesting that all the differences that we observed in the ApoE-KO mouse spleen, derived from the genetic background and not from the aldosterone treatment.

Due to the fact that Tregs derived from ApoE-KO mice seem to be autophagy impaired, we pharmacologically modulated autophagy in sorted *wt* naïve CD4+ T cells (CD4+CD62L+CD25–) under a TGFβ stimulus for the subsequent experiments. TGFβ has been reported to induce autophagy through the SMAD 2/3 pathway in different cell lines and by the increase in the mRNA levels of autophagic genes such as *BECLIN1*, *ATG5*, and *ATG7* (48, 49). Based on this knowledge, we performed RTqPCR at either 24 or 96 h by culturing naïve CD4+ T cells in IL2 in the presence or absence of TGFβ, in order to evaluate the mRNA levels of *Foxp3* and autophagic genes such as *p62*, *LC3A*, and *LC3B*. The obtained results showed an increase of mRNA levels of *Foxp3* after TGFβ and IL2 treatment at both 24 and 96 h (**Figure 4B**). As expected, the inhibition of autophagy by Baf or chloroquine, a lysosome acidification inhibitor, does not interfere with the transcriptional activation of *Foxp3* induced by TGFβ. We then investigated the mRNA levels of autophagic genes after 24 h of TGFβ and IL2 treatment; we did not observe any significant increase of *p62*, *LC3A*, and *LC3B*. Interestingly, at 24 h, the addition of Baf and chloroquine led to a significant increase of *LC3A* mRNA (**Figure 4B**, top), probably due to feedback mechanism of cells trying to overcome autophagy inhibition. The mRNA expression of *Foxp3*, already boosted by TGFβ at early time points, was remarkably increased at 96 h, as well as the mRNA expression of autophagy-related genes (*p62* and *LC3A*), confirming the transcriptional activation of autophagy by TGFβ (**Figure 4B**, bottom).

FOXP3 Accumulates Upon Autophagy Inhibition

Autophagy is a crucial process controlling the turnover of proteins and organelles. In our experiments, we noticed that the percentage of Tregs (CD4+FOXP3+) was significantly increased after Baf treatment when added during the last 4 h of differentiation (**Figures 5A**,**B**) even if the same treatment did not affect the transcription of *Foxp3* gene. Therefore, we investigated whether FOXP3 levels were affected by autophagy. The main pathway through which FOXP3 is degraded relies on the ubiquitination on lysine-48 and its subsequent proteasome degradation. However, several evidence reported that the natural p300 inhibitor, garcinol, can induce FOXP3 degradation via a lysosome-dependent pathway (50, 51). In order to investigate more in depth the potential autophagy-mediated FOXP3 degradation, we cultured sorted *wt* naïve CD4+ T cells (CD4+CD62L+CD25–) obtained from mouse spleens in the presence or absence of TGFβ and IL2 for 96 h, and we treated them with Baf or chloroquine for the last 4 h, in order to block the last steps of the autophagic flux. By flow cytometry analysis, we observed that the treatment with autophagy inhibitors led to an increase of FOXP3 in unstimulated cells after 96 h in culture upon 4-h treatment with Baf or chloroquine (**Figure 5C**), while the differences were abolished after TGFβ treatment, presumably because, after 96 h of stimulation, the differentiation was complete, autophagy shut down, and *Foxp3* gene upregulated hundred-folds by the effect of the cytokine (as shown in **Figure 4B**). Although further experiments are needed to confirm the autophagy-mediated FOXP3 turnover, these data suggest that this transcription factor can be an autophagy target.

We additionally investigated this aspect in ApoE-KO mice, particularly by culturing mechanically sorted naïve CD4+ T cells in the presence or absence of Baf and TGFβ. However, we did not observe any FOXP3 accumulation with only Baf treatment (data not shown) probably due to both lack of Tregs differentiation upon TGFβ treatment and, as previously shown in **Figure 4A**, autophagy being blocked in ApoE-KO mice at both 24 and 96 h.

Autophagy Affects Tregs Differentiation

Finally, we modulated autophagy on sorted *wt* naïve CD4+ T cells (CD4+CD62L+CD25–) under a TGFβ stimulus in order to ascertain the involvement of autophagy in Tregs differentiation. We used for 24 h Baf or 3-methyladenine (3MA) (a PI3 kinase inhibitor) to inhibit autophagy or, alternatively, rapamycin (RAPA) [the inhibitor of the main physiological autophagy suppressor mammalian target of RAPA (mTOR)] to boost autophagic flux (52, 53). As shown in **Figures 6A**,**B**, both Baf and 3MA were able to inhibit differentiation of Tregs induced by TGFβ, while autophagy activation by RAPA has no effects on Tregs differentiation, presumably because autophagy was already activated by TGFβ. We obtained the same results by quantifying the percentage of FOXP3+ cells (**Figure 6A**) or the absolute Tregs cell number (**Figure 6B**). Finally, to better appreciate the results, we normalized the percentage of FOXP3 in TGFβ-treated or untreated samples, and we

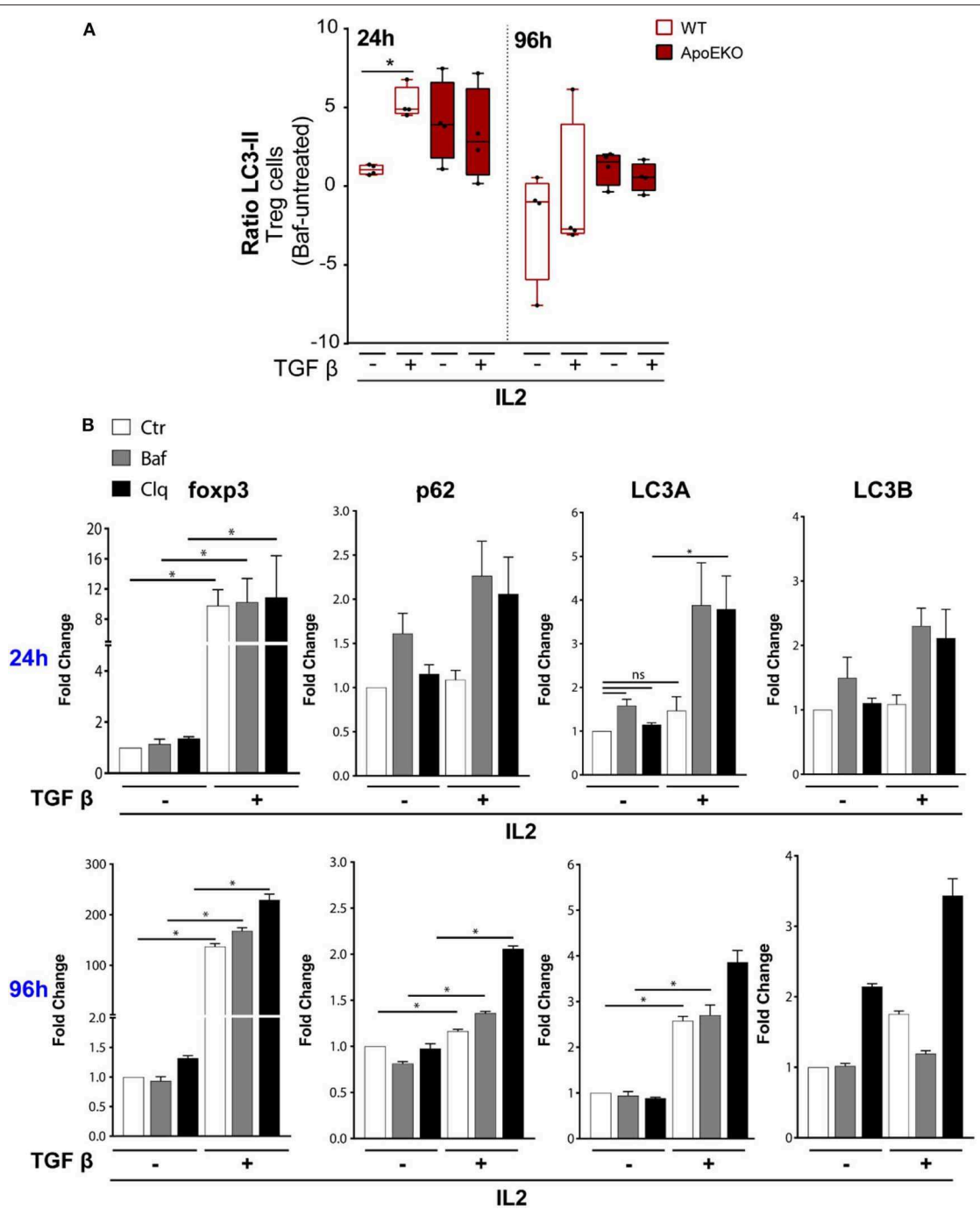

FIGURE 4 | (A) Autophagic flux during Tregs differentiation in *wt* ($n = 4$) and ApoE-KO mice ($n = 4$). Tregs were induced by culturing naïve CD4+ T cells isolated from splenocytes, in the presence of IL2 (100 U/ml) treated or not with TGFβ (2 ng/ml) and stimulated with plate-bound anti-CD3 and anti-CD28. The histograms show flow cytometry analysis for 24 and 96 h with and without bafilomycin (represented as LC3-II ratio: Baf-untreated) during the last 4 h of stimulation. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, by two-way analysis of variance (ANOVA). **(B)** Quantitative RTqPCR analysis of TGFβ-induced Tregs from naïve CD4+ T cells isolated from *wt* mice, in the presence of IL2 and stimulated with plate-bound anti-CD3 and anti-CD28 for 24 h ($n = 3$, 2 mice per experiment) and 96 h ($n = 2$, 8 mice per experiment). All the experiments were performed with and without bafilomycin (100 nM) or chloroquine (40 μM) during the last 4 h of differentiation. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, by unpaired *t*-test, two-tailed.

observed that autophagy inhibition by 3MA led to a significant decrease of Tregs differentiation (**Figure 6C**). Altogether, these results suggest that the modulation of autophagy impacts Tregs differentiation.

DISCUSSION

For many years, the therapy for the treatment of atherosclerosis has focused on lipid reduction methods. However, increasing

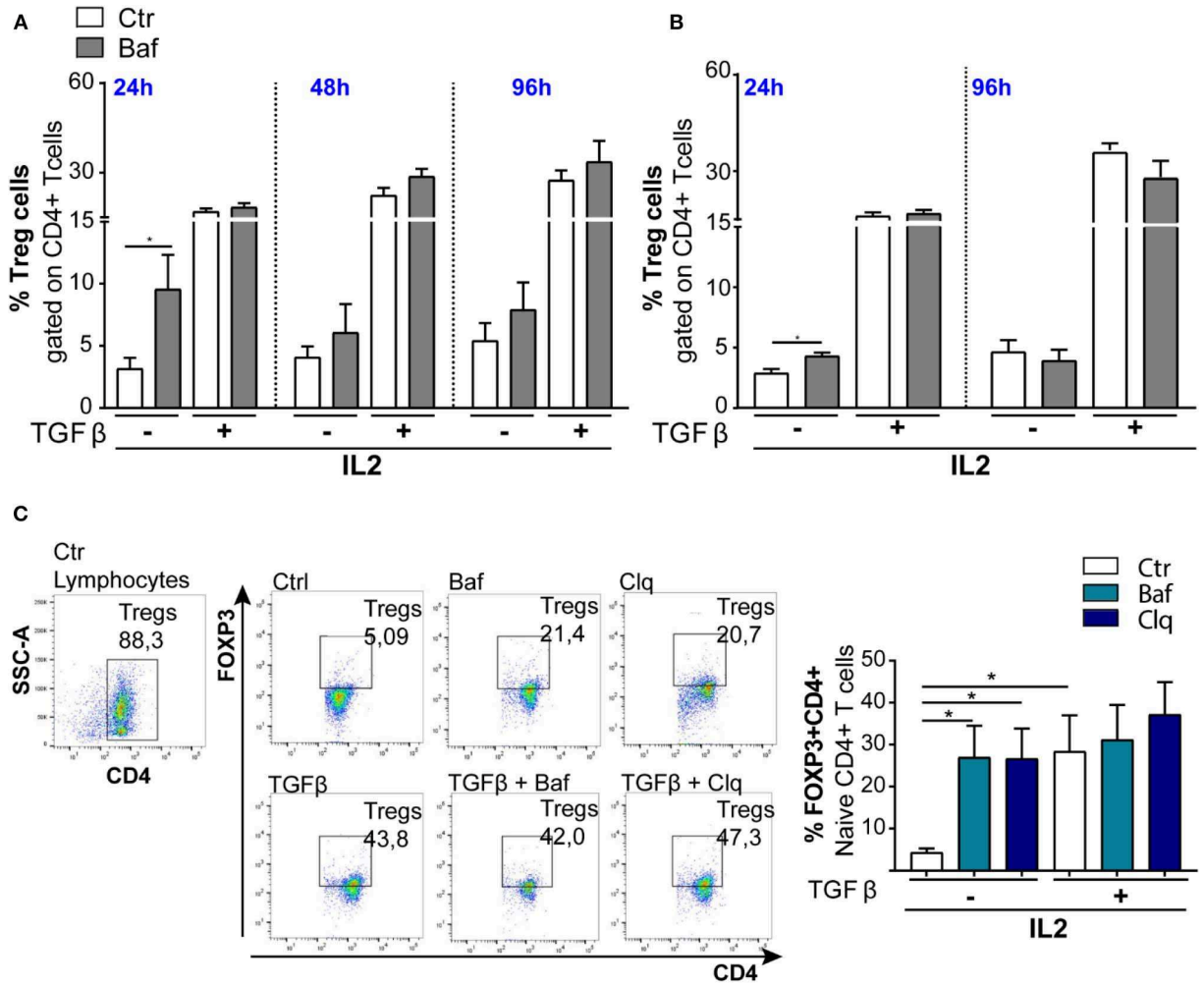

FIGURE 5 | Induction of Tregs with TGFβ (2 ng/ml) in *wt* mice ($n = 3$): The bar graphs represent % of Tregs (CD4+FOXP3+) analyzed by flow cytometry. The experiments were conducted at different times, 24, 48, and 96 h, by culturing CD4+ T cells **(A)** and naïve CD4+ T cells **(B)** in the presence of IL2, treated or not with TGFβ and stimulated with plate-bound anti-CD3 and anti-CD28. **(C)** Representative dot plots of FOXP3+ induction from three animals. *$P < 0.05$, by unpaired *t*-test, two-tailed.

evidence shows a strong involvement of the immune system in its pathogenesis and the idea of atherosclerosis as an autoimmune disease arose, paving the way toward new therapeutic approaches (54). A correlation between atherosclerosis and inflammation has been recognized in many studies, implying that immune cells and inflammatory signals are essential contributors to cardiovascular diseases (10, 55). In this scenario, it has been reported that Tregs exert atheroprotective properties by secretion of anti-inflammatory cytokines and suppression of T-cell proliferation (56). Coherently, atherosclerosis-prone mice harbor systemically less Tregs and a general imbalance of immune cells, potentially leading to atherogenesis (56). To add a further layer of complexity, it has been revealed that autophagy, the catabolic process responsible for the recycling of cellular components, when activated in immune cells within the atherosclerotic plaque preserves normal cellular function and protects plaque cells against oxidative injury, metabolic stress, and inflammation (26). Although the role of immune cells in regulating atherosclerosis onset and progression has been confirmed by many scientists, the contribution of autophagy in the maintenance of cellular homeostasis within atherosclerotic plaques is still neglected. With this research, we aimed at characterizing lymphocyte populations in human and murine plaques and addressing the effects of autophagy on Tregs differentiation in the context of atherosclerosis.

We started our study by characterizing human and mouse atherosclerotic plaques from an immunological point of view. The relative content of different lymphocyte subpopulations in the plaques, especially the frequency of the immunosuppressive Tregs, is crucial to understanding how the atherosclerotic disease progresses. Moreover, the establishment of immuno-profiles is of major importance in clinical practice as they could be used as predicting factor for cardiovascular atherosclerosis risk and therapy efficiency (57). Our initial characterization indicates that

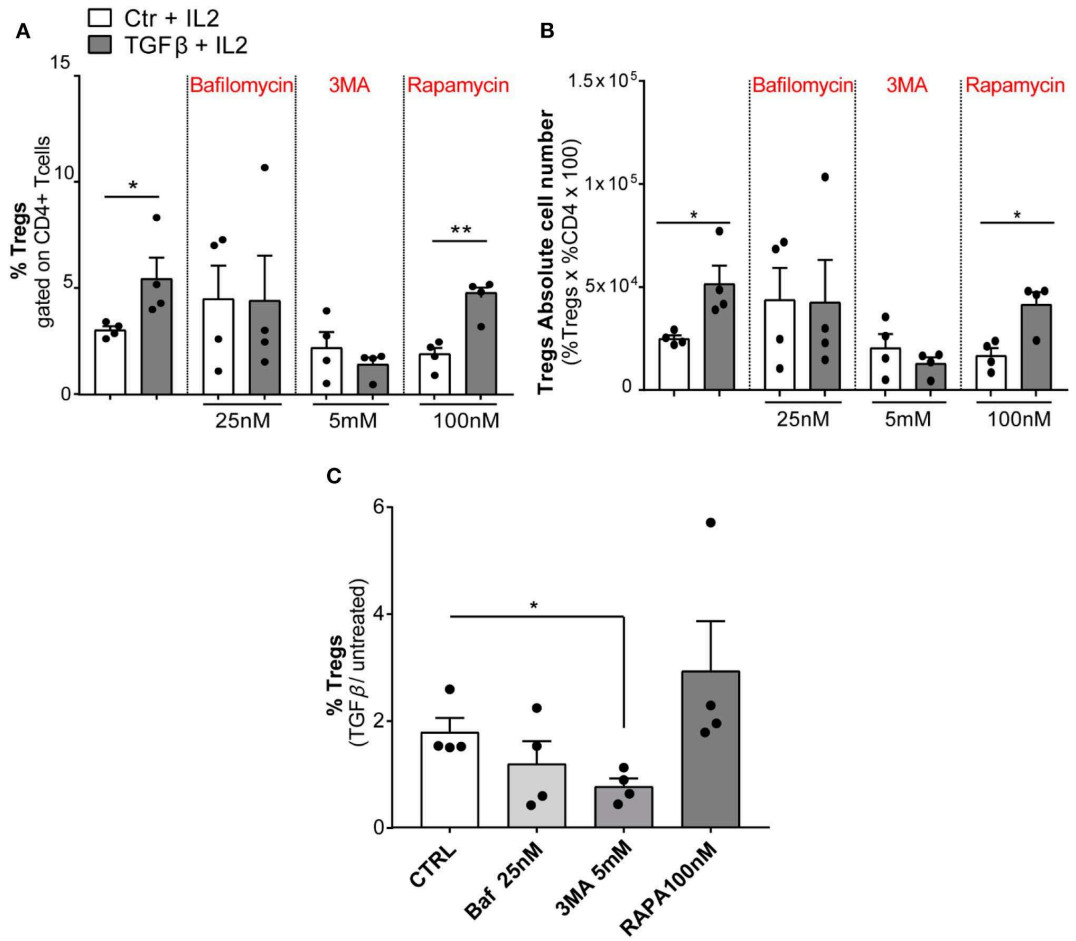

FIGURE 6 | Modulation of autophagic flux during regulatory T-cell (Treg) differentiation. Tregs were induced by culturing naïve CD4+ T cells isolated from *wt* mice ($n = 4$, two mice per experiment) in the presence of IL2 (100 U/ml), treated or not with TGFβ (2 ng/ml) and stimulated with plate-bound anti-CD3 and anti-CD28 for 24 h. The histograms show the frequency of Tregs **(A)**, absolute number of Tregs **(B)**, and the result represented as normalization TGFβ/untreated **(C)**. The experiment was performed with autophagy modulators: bafilomycin (25 nM), 3-methyladenine (3MA, 5 mM), and rapamycin (100 nM) for 24 h. $^{*}P < 0.05$, $^{**}P < 0.01$, by unpaired *t*-test, two-tailed.

plaques from both human and animal models display more infiltrating Tregs. Notably, since the structural integrity of the artery wall is compromised also in the surrounding area of atherosclerotic plaques, we analyzed the regions adjacent to the lesion, and also in this case, we observed high frequencies of Tregs. In order to obtain fresh murine atherosclerotic plaques for our studies, we took advantage of ApoE-KO mice treated with aldosterone—so far, the elective mouse model for this disease (58). Our aim, using this experimental model of atherosclerosis, was merely to obtain samples to analyze from an immunologic point of view, and therefore, we did not compare our results with *wt* non-atherosclerosis vessels, from which we could not extract any infiltrating cells to relate. Remarkably, *wt* mice do not develop atherosclerosis either during aging or under aldosterone treatment. Our research did not aim to dissect the etiological mechanism giving rise to the plaque or the pathologies (e.g., hypertension) leading to atherosclerosis.

In line with other studies, our results corroborate evidence that the frequency of Tregs is decreased in splenocytes of ApoE-KO mice, suggesting that the development of atherosclerotic plaques in the mouse could be related to depletion of a peripheral Tregs pool (42). Surprisingly, we also revealed that ApoE-KO mice treated with aldosterone display instead an increase of peripheral Tregs, presumably due to their recruitment at the plaque region to suppress the inflammation promoted by aldosterone. It is well-known, indeed, that this hormone is associated with vascular infiltration of immune cells, reactive oxidative stress, and pro-inflammatory cytokine production (59). Here, we also demonstrate that, differently from *wt*, ApoE-KO mouse-derived CD4+ T cells are unable to differentiate in Tregs when stimulated with IL2 and TGFβ. This result clearly indicates that ApoE-KO mice show a defect in Tregs maturation that could, potentially, contribute to atherosclerosis progression.

In order to characterize mechanisms responsible for the defects of Tregs maturation in atherosclerotic plaque, we directed

our studies toward the investigation of autophagy, due to its crucial role in sustaining the survival of immune cells at the level of atherosclerotic lesions. Although it is well-established that autophagy is essential for CD4+ T-cell proliferation, survival, cytokine production, and homeostasis in response to T-cell receptor activation (18, 60), the role of this catabolic process in Tregs maturation has been poorly dissected. Although far from being exhaustive, our experiments point toward a pivotal role of autophagy in Tregs differentiation that could inspire other researchers in this direction. We here show that TGFβ treatment in the presence of IL2, while inducing Tregs differentiation through FOXP3 transcription, triggers autophagy by inducing LC3 lipidation. At a later time point of stimulation, TGFβ is also responsible for the induction of the transcription of autophagic genes such as *p62* and *LC3A*. In order to functionally correlate autophagy induction with Tregs differentiation, we incubated naïve CD4+ T cells with autophagy pharmacological inhibitors (Baf or 3MA) for 24 h together with the differentiation mixture (IL2+TGFβ), and we observed an inhibition of the TGFβ-induced differentiation though autophagy induction, *per se*, with RAPA was not able to induce Tregs maturation. Overall, these data indicate that autophagy contributes to TGFβ-induced differentiation, but it is not the only driving force. In our experiments, we also noticed that short treatments with autophagic inhibitors lead to an accumulation of FOXP3, the main transcription factor driving Tregs differentiation, indicating it as a possible autophagy target and linking Tregs differentiation to autophagy.

Finally, and even more interestingly, we found that ApoE-KO mouse-derived CD4+ T cells that are unable to differentiate are also unable to activate autophagy upon TGFβ treatment. In this regard, we specify that lymphocytes derived from *wt* and ApoE-KO mice are phenotypically the same (ApoE expression in Tregs is virtually absent); however, they respond differently probably because of defects accumulated during the development in the mouse which persist even after we grow the cells in culture under controlled conditions. Dyslipidemia could, for instance, account for these lymphocyte defects, but this aspect was not investigated here. Moreover, the mechanism(s) leading to autophagy impairment in ApoE-KO mice is still unknown. However, with the data here provided, we can speculate that autophagy impairment (similarly to what is seen in other lymphocyte populations), by inhibiting Tregs maturation in ApoE-KO mice, enhances the inflammatory process at the level of atherosclerotic plaques.

In addition, seeking to analyze the autophagic flux in Tregs of human plaques, we checked the levels of phospho-S6 ribosomal protein kinase (p-S6)—a marker of mTOR pathway activation and autophagy inhibition—in Tregs cells derived from human plaques (61, 62). Although we observed an increase of p-S6 in a limited number of samples (data not shown), our knowledge regarding autophagy in Tregs of human plaques is still, unfortunately, not exhaustive.

Taken together, our results indicate that autophagy is a necessary process for Tregs differentiation and that the defective Tregs maturation in ApoE-KO mice could be connected to autophagy blockade. The present work underlines the complexity of the autophagy–Tregs–atherosclerosis axis and enlightens some important connections between immune cells and this catabolic process that could be exploited during the development of new prognostic and therapeutic strategies, which could be useful in rehabilitation programs for atherosclerotic patients. In conclusion, we suggest that, by enforcing the functional integrity of Tregs, the manipulation of autophagy may be exploited as a useful tool for the development of a novel therapeutic approach aimed at increasing functional Tregs in atherosclerosis therapy (18).

MATERIALS AND METHODS

Patients

Peripheral blood (PB), adjacent APR, and AP samples were obtained from nine patients undergoing carotid endarterectomy (CEA) (63) affected by different pathologies representing atherosclerosis risk factors. Patient's characteristics are shown in **Table 1**, displaying the percentage of occlusion (surgery-side) and associated pathologies.

PBMC were isolated from the PB of CEA patients by density gradient centrifugation with Lympholyte (Cedarlane Laboratories USA, cat. #CL5020) and collected in complete RPMI Dutch-modified medium containing 10% FBS (Gibco), 2 mM L-glutamine (Sigma-Aldrich), penicillin/streptomycin (Gibco), non-essential amino acids, sodium pyruvate (EuroClone), and 50 μM 2-mercaptoethanol (Sigma-Aldrich).

Mononuclear cells were extracted from APR and AP. Fragments of APR and AP were digested with Collagenase IV (C5138 Sigma) (2 mg/ml) + DNase I (0.1 mg/ml) in RPMI for 1–2 h at 37°C. Single-cell suspensions were obtained by disrupting the fragments with a syringe plunger over a cell strainer and pelleted through a 40% isotonic Percoll solution. Human studies were performed in accordance with the General Data Protection and Regulation (GDPR) and ethical guidelines of the 1975 Declaration of Helsinki and were approved by the Institutional Ethical Committee of the University of Rome "La Sapienza" (authorization: RIF. 3720_2015/23.07.2015 Prot. 2372/2015).

Mice

ApoE-KO mice were kindly provided by the Laboratory of Cardiovascular Endocrinology, IRCCS San Raffaele (Rome, Italy), maintained at Animal Facility Castel Romano (Rome) under protocols approved by the Italian Ministry of Health (493/2016-PR).

In 9-week-old male mice deficient for ApoE (ApoE-KO), osmotic minipump (Alzet model 1004) containing vehicle (ethanol/saline) or aldosterone (6 μg/mouse/day) was subcutaneously placed, and mice were fed with a proatherogenic HF diet (Harlan Teklad TD.88137) for 4 weeks (43). This dose of aldosterone was chosen on the basis of published studies demonstrating resultant serum aldosterone levels in physiologically relevant range, no change in blood pressure, and reproducible increases in atherosclerosis (30). At the time of sacrifice, animals were perfused with phosphate-buffered saline (PBS), and aortic valves were embedded in an optimal cutting compound (OCT) and collected at −80°C.

Cryosections of embedded aortic roots at the site where all three aortic valve leaflets could be visualized were taken at 10-μm intervals. Sequential sections were stained with Oil-Red O (ORO) in the aortic root at the level of the aortic valve.

To study the autophagy process, 8- to 12-weeks-old male C57BL/6J mice (Charles River Laboratories) housed at the animal facility of the Department of Anatomy, Histology, Forensic Medicine and Orthopaedics (DAHFMO), Unit of Histology and Medical Embryology (Sapienza University of Rome), were used. C57BL/6J mice were killed by CO_2 euthanasia.

Eight- to ten-weeks-old male C57BL/6N mice (Taconic), housed at the animal facility at the Danish Cancer Society Research Center (Copenhagen, Denmark), were used to study autophagy modulation (e.g., Baf, 3MA, and RAPA treatments) and FOXP3 accumulation (RTqPCR and cytofluorometry analyses).

Splenocyte Isolation and Aortic Arch Dissection From Murine Spleens

Characterization of ApoE-KO mouse spleens was performed in collaboration with the Laboratory of Cardiovascular Endocrinology, IRCCS San Raffaele (Rome, Italy). Splenocytes were obtained by spleen mechanical rupture with a syringe plunger followed by incubation with ACK (Lonza, Alpharetta, GA, USA, cat. no. 10-548E) for 5 min at room temperature (RT), and after washing, cells were filtered on a 70-μm cell strainer. The aortic arches were separated from the apical portion of the heart and were digested with collagenase IV (C5138 Sigma) (2 mg/ml) + DNase I (0.1 mg/ml) in RPMI 1640 for 1 h at 37°C.

In vitro Analysis of Tregs Differentiation

Splenocytes were obtained by spleen mechanical rupture with a syringe plunger followed by incubation with sterile ddH_2O for 10 s, blocking and washing with PBS 10×. CD4+ T cells were purified from splenocytes by using a CD4+ T-cell isolation kit (130-104-454 Miltenyi Biotec) and naïve CD4 T cells purified from splenocytes by using a naïve CD4+ T-cell isolation kit (130-104-453 Miltenyi Biotec) according to the manufacturer's instructions. Naïve CD4+ T cells and CD4+ T cells purified were cultured on 3 μg/ml of anti-CD3 (145-2C11) precoated wells (96-u bottom multiwell) at 37°C, at different time points (24, 48, and 96 h). Cells were plated in RPMI 1640 (R0883 Sigma) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, 50 μM 2-mercaptoethanol, 1 mM pyruvate, and 15 μg/ml gentamicin, in the presence of 2 μg/ml soluble anti-mouse CD28 (37.51 monoclonal antibody) and of human recombinant IL2 (354043 BD Bioscience) at 100 U/ml in the presence or absence of 2 ng/ml mouse recombinant TGFβ (14-8342 Affymetrix eBioscience). In some sets of experiments, cell cultures were treated with modulators of autophagy: Baf A1 (100 nM) (B1793 Sigma), chloroquine (40 μM) (C6626 Sigma), 3-methyladenine (5 mM) (M9281 Sigma), RAPA (100 nM) (R0395 Sigma). Aldosterone (10^{-8} M) treatment was used, maintaining the same conditions of the *in vitro* experiment (64).

Flow Cytometry

Cells obtained from human atherosclerotic plaques and ApoE-KO mice were analyzed following the complete panel of antibodies (**Tables S1**, **S2**). Surface staining was performed by incubating the cells with selected antibodies at 4°C for 30 min in PBS. Intracellular staining of cytokines and transcription factors was performed with Foxp3 Transcription Factor Staining Buffer (eBioscience) in accordance with the manufacturer's instructions. Dead cells were excluded using Fixable Viability Dye eFluor® 780 (eBioscience). Human samples were acquired on the BD LSRFortessa cell analyzer (BD Biosciences), while mouse samples were run on the BD LSRFortessa X20 (BD Bioscience).

Regarding *in vitro* experiments, intracellular FOXP3 and LC3-II staining was performed using PFA 4% and 90–100% Met-OH for fixation and permeabilization, according to Cell Signaling protocol. Dead cells were excluded by staining with NIR (Live Dead Fixable Near-IR Dead Cell stain kit; Invitrogen) according to the manufacturer's instructions. Samples were run on a Cyan cytometer (Beckman Coulter).

Autophagic flux was measured by FACS analysis using antibody against LC3-II-PE (Cell Signaling clone D-11), in the presence or absence of the modulators of autophagy as described above.

Samples were analyzed with FlowJo software, version 10.5.3.

Cell Sorting and RTqPCR

Naïve CD4+ T cells were stained with the antibodies CD4 eFluor-450, CD25 APC, and CD62L PECy7 and were sorted by using FACS Melody (BD).

Total RNA was extracted using the NucleoSpin RNA kit (740955.250 Macherey-Nagel). RNA concentration and purity were measured by NanoDrop (NanoDrop™ 2000/2000c spectrophotometers). Quantitative PCR was performed with 2 μl of cDNA, 0.4 μl of each primer (10 μmol/μl), and 10 μl of PowerUp SYBR Green qPCR Master Mix (Thermo Fisher Scientific) and analyzed with the QuantStudio software (Applied Biosystems).

The cycle threshold values were used to calculate the normalized expression of FOXP3, LC3A, LC3B, and p62 against β-actin.

The sequences of primer pairs are listed below:
mFOXP3f: AAT AGT TCC TTC CCA GAG TTC
mFOXP3r: GGT AGA TTT CAT TGA GTG TCC
mLC3Af: TTG GTC AAG ATC ATC CGG C
mLC3Ar: GCT CAC CAT GCT GTG CTG G
mLC3Bf: CCC ACC AAG ATC CCA GTG AT
mLC3Br: CCA GGA ACT TGG TCT TGT CCA
mSqstm1f: AAT GTG ATC TGT GAT GGT TG
mSqstm1r: GAG AGA AGC TAT CAG AGA GG
mActinf: CAC ACC CGC CAC CAG TTC GC
mActinr: TTG CAC ATG CCG GAG CCG TT.

Statistical Analysis

Statistical analysis was performed using the version 7.0 Prism software (GraphPad). A two-tailed paired Student's *t*-test was used to analyze human data to compare PBMC, APR, and AP in the same sample. The two-tailed unpaired Student's *t*-test was

applied to compare data from Th1, Th17, and Tregs in ApoE-KO vs. aldosterone. Two-way analysis of variance (ANOVA) was performed for *in vitro* analysis in which we compared *wt* vs. ApoE-KO mice upon TGFβ treatment. The two-tailed unpaired Student's *t*-test was applied to compare treatment with autophagy modulators when only one group was analyzed (*wt*). One-way ANOVA was performed for the *in vitro* experiment to analyze autophagic flux upon aldosterone treatment during Tregs differentiation. Every *in vitro* assay was performed in duplicate or triplicate, when possible. The number of repetitions is indicated in the figure legend for all experiments. In all graphs, bars show means ± SEM. In all tests, $P < 0.05$ was considered statistically significant.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Azienda Ospedaliera S. Andrea, Sapienza University of Rome—Rif. 3720_2015/23.07.2015. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by IRCCS San Raffaele (Rome, Italy), maintained at Animal Facility Castel Romano (Rome) under protocols approved by the Italian Ministry of Health (493/2016-PR).

AUTHOR CONTRIBUTIONS

AF, SP, FC, CG, and DS conceived the study. SM designed and performed the majority of the experiments and analyzed the data. FP contributed to the design of the multiparameter flow cytometry analysis. IP contributed to the experiments on human plaques. SM wrote the manuscript supported by SP and CG, and supervised by AF, MC, DD, and FC. FC and DD have hosted SM at the Danish Cancer Society Research Center (Denmark, DK) and contributed to the design of the experiments on autophagy. DD has supported SM to investigate autophagy and supervised the experiments regarding autophagy investigation. LV contributed to the design of the multiparameter flow cytometry in the murine model. MC, VM, CM, and AA provided the ApoE-KO mouse models and contributed to the experimental plan on the murine model. CS and MT provided the human atherosclerotic plaque and clinical characteristics of the patients. The manuscript was reviewed, revised, and edited by all authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00350/full#supplementary-material

Supplementary Figure 1 | Gating strategy for Tregs identification in PBMC, APR, AP by flow cytometry. Flow cytometry analysis was performed on lymphocytes extracted from **(A)** human peripheral blood mononuclear cells (PBMC), **(B)** adjacent Atherosclerotic Plaque Region (APR), **(C)** Atherosclerotic Plaque (AP). In gated CD4+ T cells, Tregs and Tconv were selected as FOXP3+ $CD127^{low}$ within the CD14– CD8– viability dye- CD4+ CD25+ gate.

Supplementary Figure 2 | (A) Gating strategy for Tregs identification in spleens and aortic arches obtained from ApoE-KO ± aldosterone (6 μg/mouse/day). In gated CD4+ T cells, Tregs, Th1 and Th17 were selected as CXCR3+CCR6-CD4+, CCR6+CXCR3–CD4+, FOXP3+CD4+, respectively, within the CD8– CD4+ gate. **(B)** Gating strategy for Tregs differentiation by culturing naïve CD4+ T cells isolated from *wt* mice ($n = 4$) and stimulated with plate-bound anti-CD3 and anti-CD28 for 96 h in presence of IL2 (100 U/ml), treated or not with TGFβ (2 ng/ml). In some set of experiment aldosterone [10^{-9} M] was added for 96 h. The histograms show the frequency of CD4+ T cells **(C)**, the frequency of Tregs represented as FOXP3+CD4+ cells (**D** left) and as TGFβ/Ctr fold change (**D** right).

Supplementary Figure 3 | Absolute cell number of Tregs, calculated on percentage of expanded Tregs at different times (24, 48, and 96 h) **(A)** and differentiated Tregs at two timepoints (24 and 96 h) **(B)**, starting from CD4+ T cells of *wt* and ApoE-KO mice, in presence of IL2 (100 U/ml), treated or not with TGFβ (2 ng/ml) and stimulated with plate-bound anti-CD3 and anti-CD28.

Supplementary Figure 4 | The bar graphs represent gMFI FOXP3 in Tregs analyzed by flow cytometry. The experiments were conducted at different times: 24, 48, and 96 h, by culturing CD4+ T cells in presence of IL2 (100 U/ml), treated or not with TGFβ (2 ng/ml) and stimulated with plate-bound anti-CD3 and anti-CD28.

Supplementary Figure 5 | Autophagic flux during Tregs differentiation under aldosterone treatment in *wt* ($n = 4$) mice for 96 h. Tregs were induced by culturing naïve CD4+ T cells isolated from splenocytes, in presence of IL2 (100 U/ml) treated or not with TGFβ (2 ng/ml) and stimulated with plate-bound anti-CD3 and anti-CD28. In some set of experiments aldosterone was added for 96 h. The histogram shows the frequency of Tregs-LC3-II+ (FOXP3+LC3-II+) cells gated on CD4+ T cells during 96 h of aldosterone [10^{-9} M] treatment. To analyze autophagic flux in some sets of experiments bafilomycin was added for during the last 4 h of stimulation. One-way Analysis of Variance (ANOVA) was performed without showing any significant differences.

Supplementary Table 1 | The multiparameter flow cytometry analysis was used to characterize human atherosclerotic plaque.

Supplementary Table 2 | The multiparameter flow cytometry analysis was used to characterize mice spleens and aortic arches.

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MINI REVIEW
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Metabolic Reprograming of Microglia in the Regulation of the Innate Inflammatory Response

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Microglia sustain normal brain functions continuously monitoring cerebral parenchyma to detect neuronal activities and alteration of homeostatic processes. The metabolic pathways involved in microglia activity adapt at and contribute to cell phenotypes. While the mitochondrial oxidative phosphorylation is highly efficient in ATP production, glycolysis enables microglia with a faster rate of ATP production, with the generation of intermediates for cell growth and cytokine production. In macrophages, pro-inflammatory stimuli induce a metabolic switch from oxidative phosphorylation to glycolysis, a phenomenon similar to the Warburg effect well characterized in tumor cells. Modification of metabolic functions allows macrophages to properly respond to a changing environment and many evidence suggest that, similarly to macrophages, microglial cells are capable of a plastic use of energy substrates. Neuroinflammation is a common condition in many neurodegenerative diseases and the metabolic reprograming of microglia has been reported in neurodegeneration. Here we review the existing data on microglia metabolism and the connections with neuroinflammatory diseases, highlighting how metabolic changes contribute to module the homeostatic functions of microglia.

Keywords: microglia, metabolism, neuroinflammation, neurodegeneration, homeostasis

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INTRODUCTION

Microglia Phenotypes and Metabolic States

Microglia are the resident immune cells of the central nervous system (CNS) and, depending on the brain region, they can represent from 5 to 12% of total cell population (1). Microglial cells continuously monitor the surrounding parenchyma to sense alteration of brain functions (2, 3) and are involved in controlling neuronal excitability, synaptic activity, neurogenesis, and clearance of apoptotic cells in the healthy adult brain (4). Microglia interact with the cerebral microenvironment through different molecules such as chemokines, cytokines, and trophic factors which, in turn, modulate microglia activities converting the homeostatic microglia into reactive microglia and *viceversa* (5). Alterations of functional phenotype are accompanied by dynamic changes of shape of cell body and processes, although no unique correlation among microglial cell morphology and functional phenotype has been identified (6). However, in early stages of brain development, and upon *in vitro* activation with pro-inflammatory stimuli, such as bacterial lipopolysaccharide (LPS), microglial cells display an ameboid profile, with large and round cell bodies, short and thick branches; this morphology is often accompanied by an increased phagocytic activity, production of specific molecules and gene expression signatures. At more mature stages

of development, microglia have usually a highly ramified morphology, dynamically reacting to brain parenchymal alterations and injuries (3) and changing phenotype from *surveillant* to pro- or anti-inflammatory in response to pathological conditions (7, 8). Under pathological conditions, it was shown that microglia comprise cells with diverse phenotypes (9). In fact, microglial-activated cells can be roughly divided into classically activated M1 cells, with cytotoxic and pro-inflammatory properties and alternatively activated M2 cells, with phagocytic activities. The M2 condition can be further divided into three classes: M2a, involved in repair and regeneration; M2b, an immune-regulatory phenotype; M2c, an acquired-deactivating phenotype (10, 11). Indeed, more recent transcriptomic analysis of microglia in different brain area and different disease conditions, reveal a much higher complexity, with several overlapping genes and few signature genes specifically expressed by microglia subgroups (12, 13). Upon aging, microglia phenotype changes further, and it was recently demonstrated an age-related senescent microglial phenotype in humans, possibly involved in pathological processes associated with brain aging (14). Like other cells, in order to perform their functions, microglia require a large amount of energy and it has been recently shown that different microglia phenotypes are associated with distinct metabolic pathways (15–18). Under normal oxygen supply, cells produce energy in the mitochondria, in the glycolytic pathway, through the oxidative phosphorylation (19); in hypoxic conditions, the anaerobic glycolysis converts pyruvate into lactate in the cytoplasm (20, 21). The bioinformatics analysis of a transcriptome database of mouse brain cells (22) showed that microglia express all the genes required for the glycolytic and the oxidative energy metabolism (16). It has been proposed that glucose metabolism exerts transcriptional control over microglial activation, and that the homeostatic phenotype of (cultured) microglia preferentially utilize oxidative metabolism (23–26). An essential fuel for microglia is glucose, which enters the cell through different transporters (GLUTs) (27). Microglia predominantly express GLUT3 (28) and the fructose transporter GLUT5 (29, 30), but under inflammatory conditions, GLUT1 expression is upregulated to increase glucose uptake and promote glycolysis (31). In the absence of glucose, microglia are able to use free fatty acids as alternative energy source, as also suggested by the accumulation of lipid droplets in glucose-deprived microglial cells (32). Microglia also express the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase NOX2 and the superoxide is used to kill pathogens (33, 34). Glucose metabolism controls NOX activation by the NADH-dependent transcriptional co-repressor C-terminal binding protein (CtBP) that affects nuclear factor kappa-light-chain-enhancer of activated B cell (NF-κB) signaling and the expression of inducible nitric oxide synthase (iNOS) (35, 36). Interestingly, microglia also express the monocarboxylic transporter (MCT) 1 and 2 and absorb lactate and ketons (37) and it has been demonstrated that a ketogenic diet is correlated with a suppression of microglia activation (38–40) likely due to the inhibition of histone deacetylases (HDACs) by ketonic bodies, which decreases NK-kB signaling (41–43). Moreover, silencing HDAC activity affects microglia during development and in adulthood, as a function of the activation state, suggesting that epigenetic changes affect cellular metabolism in activated microglia, modulating microglia function (44). Microglial activity, together with glucose availability and glycolytic rate, influences pro-inflammatory gene and protein expression (45). The oxidative phosphorylation occurs within the mitochondria and produces more ATP molecules; on the other hand, glycolysis permits a faster ATP production in activated microglia (46) allowing a rapid metabolism for cell growth, and the production of cytokines and reactive oxygen species (47). These pathways of energy production are both of primary importance for microglia to maintain their homeostatic functions and are critical for the progression and repair mechanisms upon CNS injury and neurodegeneration.

The "Warburg Effect" in Microglia

It is well-established that peripheral immune cells, such as macrophages and dendritic cells (DCs), switch from the oxidative phosphorylation to the aerobic glycolytic pathway when activated (48–50), similarly to what described in tumor cells (Warburg effect) (51–53), to foster cell proliferation. Even if microglia originate from a distinct embryological lineage, they share many characteristics with macrophages (54), as concern cell plasticity and the adaptable use of energy substrates. Several reports recently marked the metabolic similarity of microglia with DCs and macrophages: microglia exposed to inflammatory stimuli exhibit a transient upregulation of specific metabolic pathway's genes (45), indicating that energy metabolism is modulated during brain inflammation. Many studies have been performed with microglia cell lines: in particular, it was observed that upon activation, microglia alter the mitochondrial metabolism in a nitric oxide (NO)-dependent manner (24, 25). Another study demonstrated that lysophosphatidic acid (LPA) stimulates alteration in glycolysis, morphology and motility of C13NJ microglia cells (23). Furthermore, lipopolysaccharide (LPS) stimulation of the murine microglial cell line BV-2 increased lactate production, reduced the mitochondrial oxygen consumption and ATP production, with the resulting increase of glycolysis and decrease of oxidative phosphorylation (15), ultimately increasing nucleic acid production for gene transcription (55). It has also been observed that treatment of primary microglia with Deoxy-D-glucose (2-DG), a blocker of glycolytic pathway, reduced tumor necrosis factor α (TNFα) and interleukin-6 (IL-6) production through NF-kB inhibition, leading to microglia death (56, 57). On the other hand, primary rat microglia cultured with increasing glucose concentration (from 10 to 50 mM) boosted TNFα secretion (58, 59). More recently, Rubio-Araiz et al. showed that primary microglia exposure to LPS and amyloid-β (Aβ) induced an inflammatory state associated with the increase of the glycolytic enzyme 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3), with a boost in extracellular acidification rate (ECAR) (60). IFNγ and Aβ also increased microglia glycolysis together with an increase in PFKFB3, hexokinase II and Pyruvate kinase isozymes M2 (PKM2) (61), suggesting that inflammation affects microglia metabolism,

driving the glycolysis pathway through increased PFKFB3 activation. Consistently, classic anti-inflammatory stimuli, such as interleukin-4 (IL-4), decreased glucose consumption and lactate production (55) in BV2, and this was confirmed in primary microglia, where IL-4 increased oxygen consumption rate (OCR), basal respiration and ATP production (62); in addition, IL-4/IL-13 stimulation maintained an oxidative metabolic state (16), suggesting that this metabolic shift was associated with a reduced need for anabolic reactions. Pro-inflammatory activation of microglia leads to changes in mitochondrial dynamics and in particular to the metabolic switch from oxidative phosphorylation to glycolysis. It has been recently demonstrated that in inflammatory conditions, microglia upregulate GLUT1 to facilitate glucose uptake and promote glycolysis and that the blockade of GLUT1 reprogrammed back microglia from glycolysis to mitochondrial oxidative phosphorylation, thus altering microglial activation and reducing retinal neurodegeneration in a mouse model (31). These changes represent an adaptive mechanism, since the conversion of microglia from surveying to reactive is accompanied by increased energy consumption. In line with this view, Nair et al. showed that LPS-treated primary microglia increased mitochondrial fragmentation together with a reduction in oxidative phosphorylation and an increase in both oxygen consumption rate, glycolysis and cytokine production (63). In fact, fragmented mitochondria represent the preferred morphofunctional state when the respiratory activity is low (64). Moreover, when mitochondrial fragmentation increases, due to overmuch fission, it can increase the inflammatory response of microglia modulating DRP1 de-phosphorylation and ROS elimination, as already demonstrated for macrophages (65, 66). The same authors also demonstrated that normalizing mitochondrial membrane potential and ROS production with a putative mitochondrial division inhibitor (Mdivi-1) abolished the release of pro- and anti- inflammatory cytokines and chemokines (63). It fact, it has been shown that LPS induces an increase in proton leak and in membrane potential of primary microglia, partially mediated by the uncoupling proteins (UCPs) present in the mitochondrial inner membrane (67).

Microglia Dysfunction and Neurodegenerative Diseases

When exerting homeostatic activities, microglia rely on several membrane proteins: the Pattern Recognition Receptors (PRRs) and immune receptors such as the triggering receptor expressed on myeloid cells-2 (TREM2), the signal regulatory protein 1A (SIRP1A), the fractalkine receptor (CX3CR1), the cell surface transmembrane glycoprotein receptor CD200 (CD200R) and the colony stimulating factor 1 receptor (CSF-1R) (68–70) that recognize Damage-associated molecular patterns (DAMPs) or Neurodegeneration-associated molecular patterns (NAMPs) (71). Upon stimulation by potentially dangerous molecules, microglia assume a neurodegenerative phenotype (MGnD) or disease-associated microglia (DAM), also recently identified as "dark microglia" (72) in several neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), and Alzheimer's disease (AD) (71, 73, 74). It was shown that an aberrant microglia activation may result in a loss or alteration of their physiological functions with possible implications on the emergence or maintenance of pathological conditions; moreover, neuro-inflammation caused by microglia hyperactivity has been associated with several neurodegenerative diseases (12, 75–77) and many evidence support a metabolic reprograming of microglia in neurodegeneration (17). A possible mechanism explaining this microglial metabolic reprogramming has been described in a mouse model of AD, where Aβ directly triggers microglial inflammation together with a metabolic reprogramming from oxidative phosphorylation to glycolysis, in mTOR-HIF-1α pathway-dependent manner (78). Upon activation, microglia enter in a tolerant state with defects in cellular metabolism and reduced responses to inflammatory stimuli, including cytokine secretion and phagocytosis, suggesting that Aβ-induced microglial tolerance might represent a critical cue for AD progression (78). Nonetheless, when microglial glycolytic metabolism was reactivated by interferon-γ (IFN-γ) treatment, which is a known regulator of the mTOR (79) and glycolysis pathway (80), the phagocytic activity of microglia was restored, Aβ plaques and neuronal losses were reduced and cognitive impairment was rescued (78) indicating a (close) relation between the cellular metabolic pathways and functional phenotypes of microglia. The involvement of mTOR pathway in modulating microglial metabolism in AD was also previously suggested by Ulland et al., that identified TREM2 and the downstream mTOR signaling as mediators in maintaining microglial metabolic homeostasis (17). In particular they found that in AD patients carrying a TREM risk variant (81, 82) and in TREM2-deficient mice with AD-like pathology, microglia have an anomalous autophagy activity due to defective mTOR signaling. They demonstrated that upon AD development, TREM2 deficiency affects the mTOR pathway and the energetic metabolism in microglia: TREM2 deficiency was associated with decreased expression of genes for glucose transporters, glycolytic enzymes, and the transcription factor HIF1α, all involved in glycolysis (17). The role of TREM2 in microglial metabolic function was also confirmed in microglia produced by patient-derived iPSC expressing loss of function variants of TREM2: TREM2 variants could not perform the immune-metabolic switch toward glycolysis due to altered PPARg-p38MAPK-PFKFB3 signaling (83). Of note, in AD as well as in other diseases such as traumatic brain injury and ischemia, microglia phenotype changes from anti- to pro-inflammatory upon disease progression (84–86). In particular, in brain ischemia, a phenotypic change is well-documented (87, 88): few minutes after the ischemic attack, resident microglial cells, mainly in the peri-infarct region, acquire an anti-inflammatory phenotype in order to restrain brain damage. Few days after the ischemic insult, pro-inflammatory microglia predominate in the region adjacent the infarct zone (89, 90) and release ROS and pro-inflammatory cytokines that induce the activation of cerebrovascular endothelial cells and sustain the adhesion and transmigration of leukocytes into the injured tissue, contributing to further brain damage (91–94). It was recently demonstrated that upon permanent middle cerebral artery

occlusion (pMCAO), the expression profiles of anti- and pro-inflammatory genes in microglia correlates with the expression of genes related to the oxidative and glycolytic pathway, respectively (18), suggesting that a targeted modulation of microglia could be used to reduce the extent of tissue damage in brain ischemia. All these data indicate that a metabolic reprogramming is crucial for microglial function in several neuropathologies and the identification of tools to modulate microglial bioenergetics pathways might be a promising therapeutic strategy.

Microglia Metabolic Remodeling as Therapeutic Approach

Considering the heterogeneity of microglia phenotypes present in specific time windows in different CNS regions in pathophysiological conditions (87, 88, 95), studies based on general microglial depletion cannot be considered effective therapeutic strategies to eliminate potentially dangerous microglia phenotypes. Accordingly, since a given microglia subpopulation can plastically modify its phenotype and function in response to signals from the microenvironment (10), the targeting of specific microglial phenotypes in a proper time window could represent a more selective and efficacious approach and represent the current challenge of this field of research. One recent experimental approach proposes to induce a ketogenic state in microglia, suppressing glucose utilization to reduce inflammation, tissue loss and functional impairment after brain injury (41–43). The activation of the G-protein-coupled receptor 109A (GPR109A) with b-hydroxybutyrate (41, 43, 96) on microglial cells attenuates the NF-kB signaling and the production of pro-inflammatory cytokines, promoting a microglial neuroprotective phenotype in a mouse model of PD (42). Also, a metabolic switch toward oxidative metabolism might contribute to promote a protective microglia in some pathophysiological conditions, resulting in the production of metabolites beneficial for neurons (18, 97). Starting from the observation that in animal models of cerebral ischemia the increased anti-inflammatory polarization of microglia is associated with a smaller infarct area and the resolution of inflammation (98) it could be useful to identify a number of factors able to induce a metabolic switch in favor of an anti-inflammatory state of microglia. Among the possible candidates

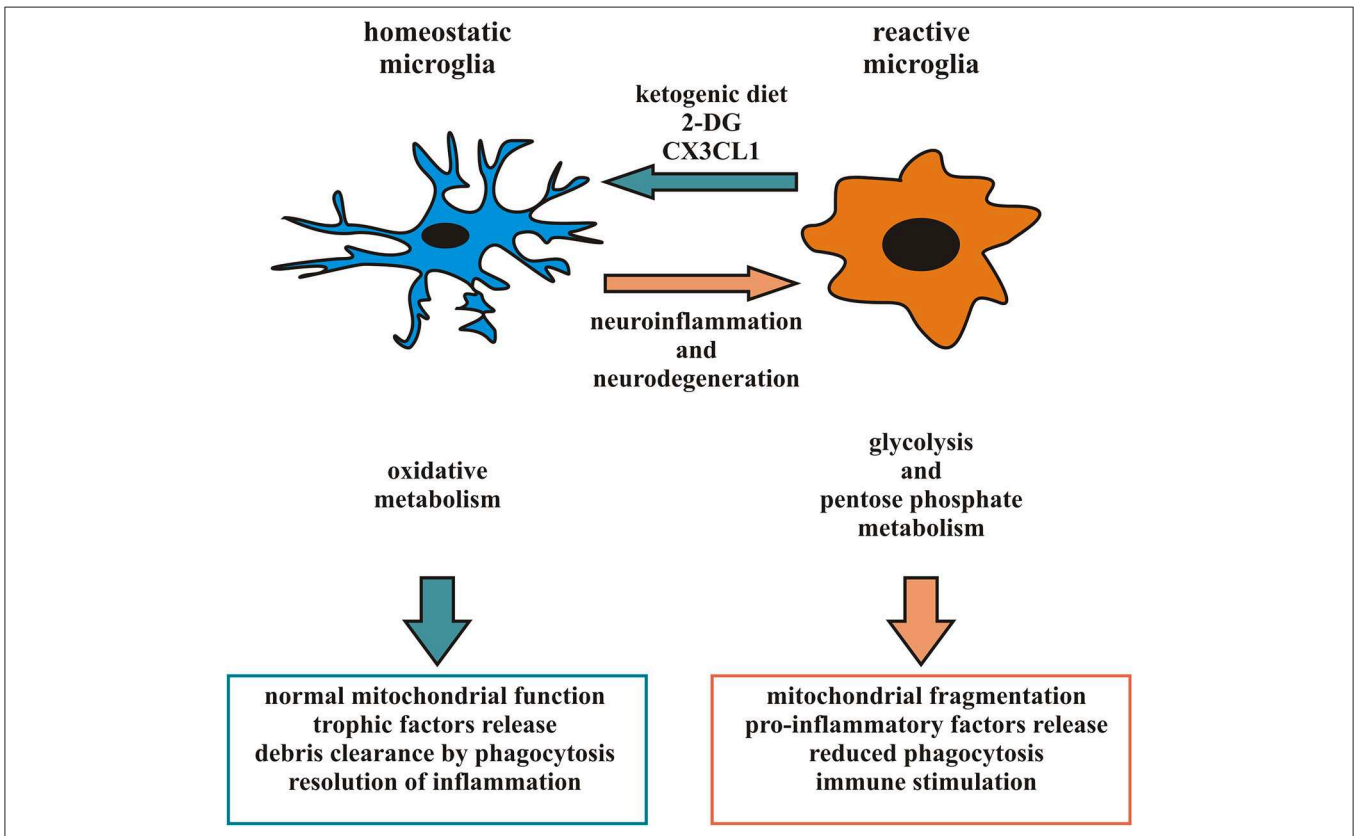

FIGURE 1 | Microglia phenotype and metabolic state: in response to appropriate signals, reactive microglia can switch from a pro-inflammatory to an anti-inflammatory phenotype and vice versa, reorganizing their structure and functions. In particular, pro-inflammatory microglia release cytokines and free radicals that impair brain repair and regeneration while anti-inflammatory microglia resolve cerebral inflammation and promote brain repair increasing phagocytosis and release of trophic factors. Different phenotypes of microglia are associated to distinct metabolic pathways, in order to perform their different functions and their activation leads to changes in mitochondrial dynamics and switch among oxidative phosphorylation and glycolytic metabolism. Several neurodegenerative diseases have been associated with neuro-inflammation related to microglia hyperactivity or mitochondrial dysfunction. Factors able to promote an anti-inflammatory microglia, such as a ketogenic diet, 2-DG and CX3CL1, may represent an intriguing approach to counteract some aspect of neurodegenerative diseases.

is CX3CL1, a chemokine released from neurons in response to ischemic insult that has neuroprotective properties in permanent focal cerebral ischemia (99), able to modulate the activation state of microglia and its metabolism, down-modulating the expression of several pro-inflammatory and glycolytic pathway-related genes and inducing an increase in the expression of several anti-inflammatory and oxidative pathway-related genes after the ischemic insult (18). CX3CL1 thus acts potentiating the anti-inflammatory function of microglia, prolonging this phenotype to limit neuro-inflammation and gaining time used by parenchymal cells to organize a neuroprotective response. Another possibility could be to regulate the dynamic of microglial mitochondria to prevent neurological disorders caused by aberrant microglial activation: as discussed above, microglia mitochondrial functions correlate with neuronal survival, as a function of microglial ROS production, but also indirectly affecting the activation state and cytokine production (63, 65). Therefore, targeting cytokines that promote the anti-inflammatory phenotype of microglia may result in protecting mitochondrial homeostasis and, on the other hand, direct approaches to enhance microglial mitochondrial function may promote the activation of the microglia anti-inflammatory state (**Figure 1**).

CONCLUSION

Many brain disorders are accompanied by changes in energy metabolism (100–105). While the mechanisms connecting inflammation to cell energy metabolism have been addressed (106), few information are available on how energy metabolism affects the inflammatory responses. Since microglia represent the sentries of the CNS, consistently, they respond to changes in brain metabolism; however, very little is known about their own metabolism, especially because most of the metabolic studies in microglia were conducted in dissociated populations of primary cultures, which do not mirror the complexity and diversity of multiple cell types which interact with other cells and external cues to adapt to bioenergetics changes. For this reason, it is essential to identify experimental approaches to study microglia metabolism in *in vivo* systems, in pathophysiological conditions. Moreover, most of our knowledge on microglia biology derives from rodents and, even if some *in vitro* studies suggest that polarization of human microglia might resemble that observed in rodents cells (107), there are several important differences between rodent microglia and their human counterparts (108) and additional studies using human biological systems, such as induced pluripotent stem cells, will be useful in the effort to translate the studies on microglia phenotype into preclinical biomedical research. However, despite these limitations, microglia represent an intriguing target for the treatment of neurodegenerative diseases and targeting their metabolism in order to change their immunological phenotype could represent a promising future therapeutic approach.

AUTHOR CONTRIBUTIONS

CLa made substantial contributions to conception and design of the review. CLi contributed to the manuscript revision, read, and approved the submitted version.

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REVIEW
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The Multifaceted Function of Granzymes in Sepsis: Some Facts and a Lot to Discover

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Sepsis is a serious global health problem. In addition to a high incidence, this syndrome has a high mortality and is responsible for huge health expenditure. The pathophysiology of sepsis is very complex and it is not well-understood yet. However, it is widely accepted that the initial phase of sepsis is characterized by a hyperinflammatory response while the late phase is characterized by immunosuppression and immune anergy, increasing the risk of secondary infections. Granzymes (Gzms) are a family of serine proteases classified according to their cleavage specificity. Traditionally, it was assumed that all Gzms acted as cytotoxic proteases. However, recent evidence suggests that GzmB is the one with the greatest cytotoxic capacity, while the cytotoxicity of others such as GzmA and GzmK is not clear. Recent studies have found that GzmA, GzmB, GzmK, and GzmM act as pro-inflammatory mediators. Specially, solid evidences show that GzmA and GzmK function as extracellular proteases that regulate the inflammatory response irrespectively of its ability to induce cell death. Indeed, studies in animal models indicate that GzmA is involved in the cytokine release syndrome characteristic of sepsis. Moreover, the GZM family also could regulate other biological processes involved in sepsis pathophysiology like the coagulation cascade, platelet function, endothelial barrier permeability, and, in addition, could be involved in the immunosuppressive stage of sepsis. In this review, we provide a comprehensive overview on the contribution of these novel functions of Gzms to sepsis and the new therapeutic opportunities emerging from targeting these proteases for the treatment of this serious health problem.

Keywords: sepsis, inflammatory cytokine, granzymes, coagulopathy, endothelial (dys)function, immunosuppression

INTRODUCTION

The immune system is the most important line of defense against pathogens. The immune response to infection is initiated after the recognition of pathogen derived molecules known as Pathogen Associated Molecular Patterns (PAMPs) by specific receptors known as PAMP receptors. This process triggers the production of inflammatory, angiogenic, and chemotactic factors involved in the activation of innate and adaptive immune mechanisms leading to pathogen clearance, tissue repair, and resolution of the inflammatory response. However, if not properly regulated, inflammation may act as a double-edged sword that can trigger serious complications like sepsis. This syndrome is an important health problem, defined as "a life-threatening organ dysfunction caused by a dysregulated host response to infection" (1). Recent epidemiological studies estimate an annual incidence of 48.9 million sepsis cases with 11 million sepsis-related deaths representing 20% of all global deaths (2).

The pathophysiology of sepsis is very complex and it is not well-understood yet. Simultaneous and interrelated inflammatory and anti-inflammatory responses are developed (3, 4), although it is widely accepted that the initial phase of sepsis is characterized by a hyperinflammatory response while the late phase is characterized by immunosuppression and immune anergy that increases the risk of other opportunistic infections, specially bacterial and fungal. These opposite reactions hamper the development of effective treatments to reduce the inflammatory damage, and, at the same time, do not increase the risk or even help to prevent secondary infections. Indeed, most experimental trials to reduce the inflammatory response with general or specific anti-inflammatory molecules targeting PAMPs like endotoxin, PAMPRs like TLR4, complement, cytokines, or coagulation factors have not positively impacted on patient survival (5).

The reasons for the low efficacy of anti-inflammatory therapy are not clear since reduction of tissue damage and coagulation syndrome together with an effective antimicrobial therapy should be beneficial to improve disease outcome. One reason could be that anti-inflammatory therapy might reduce host pathogen clearance due to inhibition of inflammatory pathways important for the protective immune response against the infection responsible of sepsis (5). In addition, it is not clear yet whether the anti-inflammatory therapies tested up to now are able to prevent immunosuppression and anergy during sepsis. Thus, although molecules involved in exacerbated inflammation and coagulation like inflammatory cytokines (i.e., IL6, IL1, TNFα) have shown a good prognostic value for sepsis progression and severity, targets to regulate inflammation without compromising host immune response against the sepsis-inducing pathogen or against secondary infections have not been found yet. Recently, some members of a family of serine-proteases named granzymes (Gzms), that are expressed by immune and non-immune cells (6), have been found in extracellular human fluids of different inflammatory disorders like sepsis. In addition, they have been found to regulate the inflammatory response *in vitro* and *in vivo* during different inflammatory disorders related to infections like sepsis.

The granule exocytosis pathway is a specialized form of intracellular protein delivery by which lymphocytes release perforin and Gzms. Perforin exerts its action allowing the passage of granzymes into the cytosol of target cells to carry out their effector functions, including cytotoxic and non-cytotoxic functions (7). On the other hand, Gzms can be released into the extracellular milieu where they will exert some extracellular functions including regulation of inflammation, pathogen inactivation, or extracellular matrix remodeling (6, 8). Gzms are a family of serine proteases classified according to their cleavage specificity. Five Gzms in humans (A, B, H, K, and M) and 10 in mice (A, B, C, D, E, F, G, K, M, and N) have been described. GzmA and GzmB are the most abundant and best characterized (6). Traditionally, it was assumed that all Gzms acted as cytotoxic proteases. However, recent evidence suggests that intracellulary-delivered GzmB is the one with the greatest cytotoxic capacity, while the cytotoxicity of others such as GzmA and GzmK is in controversy (7, 9–13). A recent study has shown that GzmA might mediate pyroptotic cell death in human and mouse tumor cells (14), although the relevance of this finding needs to be confirmed in different experimental models, since it has been previously reported that the cytotoxic potential of intracellulary-delivered GzmA might differ between mouse and human (15). Regarding human GzmH, a few studies have shown that it can induce cell death and inactivate viral proteins (7). However, at present there is not any study correlating GzmH with the regulation of the inflammatory response or sepsis, and, thus, GzmH will not be further discussed in this review.

Recent studies have found that GzmA, GzmB, GzmK, and GzmM act as pro-inflammatory mediators and could be involved in the pathophysiology of sepsis (7, 10–12) (**Table 1** and **Figure 1**). Thus, a detailed study of Gzms in different sepsis models as well as in patients undergoing different types of sepsis might provide new prognostic factors and therapeutic targets to overcome some of the limitations observed for other inflammation-related targets. This hypothesis is supported by different previous experimental findings indicating that deficiency in any single Gzm does not significantly reduce pathogen clearance, and, thus, their inhibition during sepsis would reduce inflammation without compromising host anti-pathogen immunity (6).

In this review, we will present the available evidence in animal models and humans that suggests that Gzms might have a relevant role in sepsis pathophysiology. In addition, we will discuss the potential advantages of using Gzms as therapeutic targets to reduce organ damage without compromising pathogen clearance and to prevent immunosuppression-associated secondary infections. Since novel functions of Gzms, non-related with its ability to induce cell death, have emerged in the last years, we will focus on the potential impact of these non-cytotoxic functions in the pathophysiology of sepsis. Some of them are originated in *in vitro* studies in non-sepsis models. However, since several of these non-cytotoxic functions (explained in more detail in the following sections) form part of the pathophysiology of sepsis, we think that it will be useful to discuss its potential impact on sepsis, indicating when appropriate the needed to specifically confirm their relevance in sepsis.

GRANZYMES ARE ELEVATED IN PATIENTS SUFFERING FROM SEPSIS

Some studies have analyzed the presence of soluble Gzms in fluids from patients suffering from sepsis. These data show an increase in cellular and serum levels of some Gzms like GzmA, GzmB, and GzmK. Increased levels of GzmA and GzmB have been found in patients with severe gram negative bacterial infections as well as in healthy volunteers with an experimental endotoxemia with lipopolysaccharide (LPS) (31). GzmM and GzmK were also found in serum from volunteers with experimental endotoxemia (32) and GzmM was also elevated in serum from meningococcal

TABLE 1 | Extracellular activity of granzymes on different cell types.

Enzyme substrate specificity	Species	Cell type	Effect	Receptor involved	References
GzmA Tryptase Lys, Arg	H	Monocytes	Induce expression of IL-6, IL-8, and TNF-α	–	(16)
		Monocytes	Induce expression of IL-1β, IL-6, and TNF-α	Inflammasomes/caspase-1	(17)
		Monocytes	Induce expression of IL-8 and MCP-1	Inflammasomes/caspase-1 TLR4	(18)
		Monocytes	Inactive gzmA potentiates the effect of LPS	TLR	(19)
		Lung Fibroblast	Induce expression of IL-6 and IL-8	–	(20)
		Intestinal Fibroblast	Induce expression of IL-6 and IL-8	–	
		Skin fibroblast	Induce expression of IL-8	–	
		Intestinal epithelial cells	Induce expression of IL-6 and IL-8	–	
	m	Neurons	Neurite retraction	PAR-1	(21)
		Macrophages	Induce expression of IL-1β	–	(17)
		Dendritic cells	Induce IFN-α and cell maturation	TLR-9	(22)
GzmB Aspartase Asp	H	Neurons	Neurotoxicity	PAR-1	(23)
		Smooth muscle cells	Cell death		(24)
	m	Endothelial cells	Cell death	–	(25)
		Endothelial cells	Disruptions of endothelial cell layer integrity by degrading proteins involved in tight junctions (Zonulin-1, PECAM, JAM, or cadherins)	–	(26)
GzmK Lys, Arg	H	Lung fibroblast	Induce the expression of IL-6, IL-8, and MCP-1	PAR-1	(27)
		Endothelial cells	Induce the expression of IL-6 and MCP-1	PAR-1	(28)
	m	Macrophages	Induce expression of IL-1β	–	(29)
GzmM[b] Metase Met	H	Endothelial cells	Cleaves vWF and avoid plasma FVIII activation	–	(30)
GzmC-G Chymase Phe	m	–	–	–	
GzmH Chymase Phe	h	–	–	–	

m, mouse; h, human; PAR, protease activated receptor; TLR, Toll like receptor. [b] *Extracellular GzmM cleaves vWF, releasing it from endothelial cell membrane and regulating its procoagulatory activity.*

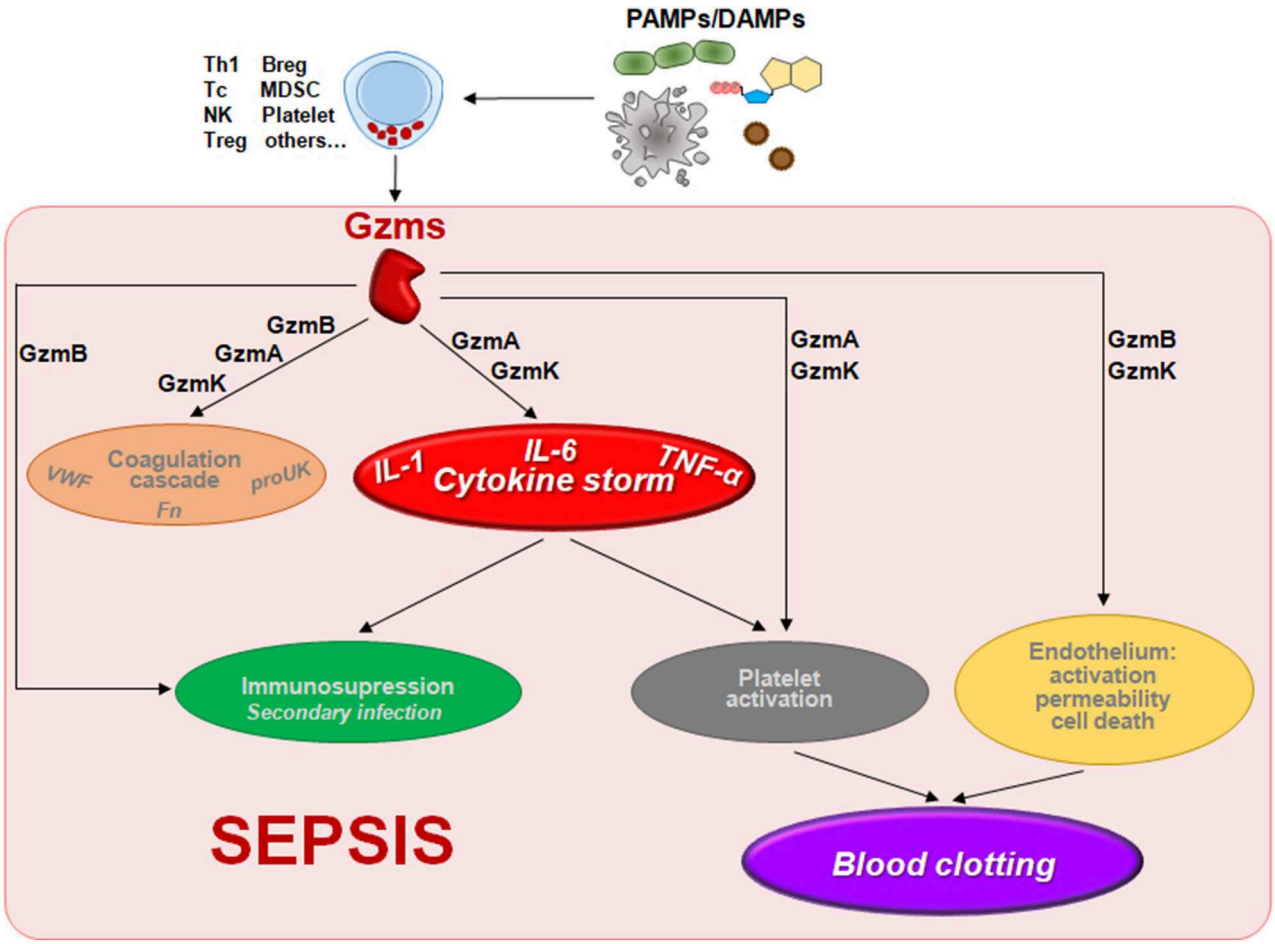

FIGURE 1 | The potential contribution of the non-cytotoxic functions of granzymes to the pathophysiology of sepsis. Gzms can be released to the extracelular millieu by different cell types including NK cells, T cells, mast cells, platelets, or other cells stimulated by molecules presented in pathogens (fungal, bacterial, or viral PAMPs) or released by damaged cells (DAMPs) during sepsis. There different Gzms could contribute to different alterations found during sepsis like the inflammatory cytokine storm, blood clotting, cardiovascular disorders (coagulation and endotelial permeability), or immunosuppresion as described in the text. Abbreviations: Breg, regulatory B cell; MDSC, myeloid-derived suppressor cell; NK, natural killer; Tc, cytotoxic T cell; Th1, type 1 T helper cell; Treg, regulatory T cell; VWF, Von Willebrand factor; proUK, prourokinase; Fn, fibrin.

sepsis (30). In contrast, GzmA levels were reduced in plasma during sepsis in burned patients (33). This result suggests that the level of GzmA in septic patients might depend on the underlying cause of sepsis. Supporting this hypothesis, Wensink et al. also showed that the release of GzmK and GzmM from human peripheral blood lymphocytes depended on the bacteria used for the stimulation (32). GzmK has also been found in the plasma of patients with sepsis as well as in bronchioalveolar fluid of patients with viral pneumonia (34, 35). However, the clinical significance of all these observations remains unknown.

A key question to understand the potential significance of soluble Gzms in sepsis is the regulation of its activity at the extracellular milieu. Like other proteases intracellular and extracellular Gzms might be tightly regulated to avoid unregulated proteolysis and cell/tissue damage. The main natural inhibitors that control Gzm activity are members of the Serpin (Serineprotease inhibitor) family. Different serpins have been found to inhibit extracellular Gzms like Inter-alpha-trypsin inhibitors (IaIs) (GzmA and GzmK), antithrombin III (GzmA), or bikunin (GzmK) (36). Thus, if extracellular Gzms are expected to play any role during sepsis it should be expected that they are not counteract by those inhibitors. Confirming this hypothesis and supporting a role for extracellular Gzms in sepsis it has been described that the levels of these inhibitors in plasma are reduced during sepsis. Indeed its concentration inversely correlated with mortality (37, 38).

Regarding the clinical utility of Gzms in sepsis prognosis, it was found that increased levels of GzmA and GzmB in $CD8^+$T cells were associated with a worse prognosis in severe sepsis patients (39). Pending of validating the clinical significance of the presence of soluble Gzms in septic patients, studies in animal models *in vivo* indicate a key role for some Gzms like GzmA and GzmM in inflammation and sepsis. GzmA deficiency has been shown to protect against LPS-induced endotoxicosis (17, 40) as

well as against sepsis induced by different bacterial pathogens including gram negative *Brucella microti* (41) or gram positive *Streptococcus pneumoniae* (42). These results suggest that the involvement of GzmA in sepsis is not related with Gram bacterial characteristics. Similarly, GzmM deficiency protects against LPS-induced endotoxicosis (40). In contrast, GzmB deficiency did not affect LPS-induced endotoxicosis (17, 40) or bacterial-induced sepsis (41). Importantly, increased survival in GzmA or GzmM deficient mice correlated with a reduction of the cytokine storm as measured by the levels of inflammatory cytokines in serum, organ damage, and coagulation activity confirming amelioration of the main sepsis pathological consequences. The course of sepsis in GzmK deficient mice remains to be elucidated.

The activity of Gzms during infectious diseases and sepsis seems to differ between anatomical site as well as the type of pathogen involved. For instance, a study showed that there was low level of GzmA in peritoneal lavage fluid of healthy mice while this level increased overtime during sepsis induced by *E. coli* (43). On the other hand, in a pneumonia-induced sepsis using a gram positive bacteria (*Streptococcus pneumoniae*) model the opposite was found (42). Here a small remark between the differences found in the pathophysiology of sepsis induced by gram negative and gram positive bacteria should be included. The onset and severity of sepsis may be defined by the molecular pathway involved in the activation of the immune system and the cytokines that can be induced in each case, both of which depend on the PAMPs expressed by the pathogen. Traditionally, the main responsible pathogens of sepsis have been considered gram negative bacteria. Nevertheless, it has been reported that gram positive bacteria are responsible for an ever-increasing number of septic events and have become the most frequent cause of sepsis, matching or even exceeding the sepsis caused by gram negative bacteria (44). It is known that the main receptors involved in the recognition of gram positive and negative bacteria are TLRs 2 and 4, respectively. This may be helpful for future studies and developments of sepsis treatments (44) and might help to explain the differences found regarding GzmA. For instance, a higher expression of IL-1β and IL-18 may indicate that the infection is due to a gram + bacteria while IL-6 and TNF would have a higher expression in gram negative infections (44). Indeed, GzmA has been found to produce very high levels of IL6 and TNF (Santiago et al. Cell Reports, Under review).

The inflammatory effect of GzmA does not seem to regulate bacterial control as GzmA deficient mice efficiently control infections like *Mycobacterium tuberculosis*, *Listeria monocytogenes, Brucella microti, Klebsiella pneumoniae, Streptococcus pneumoniae,* or *Escherichia coli* (6). Based on these experimental evidences it could be hypothesized that by targeting GzmA, bacteria-associated sepsis could be ameliorated without compromising the ability of the immune system to control infection. Thus, these findings open the opportunity to treat sepsis without causing immunosuppression and provide a new opportunity to overcome some of the limitations of other inflammatory targets unsuccessfully tested in clinical trials up to now.

WHICH COULD BE THE MECHANISMS BEHIND THE INFLAMMATORY ACTION OF Gzms IN SEPSIS?

Several biological functions of Gzms that could contribute to the different alterations found in sepsis have been described. Mainly those related with the regulation of inflammatory responses, the coagulation cascade, and changes in vascular permeability (summarized in **Figure 1**). In this section we will describe the mechanisms activated by Gzms that could potentially contribute to the pathophysiology of sepsis with a special focus on the hyperinflammatory stage.

Regulation of Inflammatory Cytokine Networks and Cytokine Storm

The hyperinflammatory response in sepsis is characterized by high levels of pro- and anti-inflammatory cytokines like IL-1β, IL-1α, IL-18, IL-17, TNFα, IL-6, MIF, HMGB1, IL-10, IL-4, and IL-13 (3, 45). This stage is also characterized by coagulation disorders, interstitial edema, hypotension, reduced perfusion, tissue hypoxia, mitochondrial dysfunction, and cell death (3, 4). It has been shown that extracellular GzmA is able to induce the production of different inflammatory cytokines such as IL-1β, TNF-α IL-6, or IL-8 in both mouse and human cells like monocytes, macrophages, fibroblasts, endothelial and epithelial cells (17, 20) (**Table 1**) which might contribute to the cytokine storm observed during sepsis. Indeed, most cytokines are reduced in GzmA deficient mice suffering from sepsis as indicated above. One of the first described substrates for GzmA is pro-interleukin 1β (pro IL-1 β), which after removal of the N-terminal part, generates the active inflammatory form IL-1β (46). However, direct cleavage of proIL1β and activation of IL1β has not been subsequently confirmed by different authors. Alternatively it has been reported that GzmA regulate the generation of inflammatory cytokines in human monocytes by caspase-1 and TLR4 dependent pathways (17, 18). Some other studies have reported that mouse GzmA is able to activate PAR1, a member of the Protease Activated Receptor (PAR) family, in mouse neurons (21). Since PARs are involved in platelet activation and thrombosis it could be suggested that PARs are involved in GzmA-mediated inflammation and coagulation alterations. However, as discussed below, the roles of PAR1 and 2 in sepsis are not clear yet and thus, *in vivo* extrapolation of all these results generated *in vitro* should be done with caution (47).

Alternatively, GzmA has been proposed to potentiate the effects of LPS on human monocytes by a mechanism dependent on TLR4 (19). Here authors showed that enzyme activity was not required for this effect, albeit the underlying mechanism was not clarified. This result contradicts previous findings that showed that human inactive GzmA was not able to induce the production of inflammatory cytokines in human monocytes (17). In addition, it has been shown that the inflammatory activity of mouse GzmA *in vivo* is significantly affected when GzmA inactivated with an inhibitor is used (48). The reasons for this discrepancy are not clear yet and will require further investigation.

GzmK displays a tryptase-like activity similar, but not identical (49, 50) to GzmA, and it might also induce the production of proinflammatory cytokines (**Table 1**). Nanomolar concentrations of GzmK induce the maturation and secretion of IL-1β suggesting that GzmK may augment GzmA-induced proinflammatory processes by differentially cleaving the same or different specific substrates (29). GzmK has also been found to activate PAR-1 in endothelial and fibroblast cells and to induce the generation of inflammatory cytokines (27, 28). However, as indicated above, its role in sepsis remains to be elucidated. Finally, as it has been previously discussed, GzmM is involved in endotoxicosis (40) although it is not known if GzmM is able to directly modulate inflammation on target cells (51).

The Coagulation Cascade

Systemic inflammation activates coagulation and inhibits anticoagulant and fibrinolytic mechanisms which leads to a dysregulated procoagulant state (52). The tissue factor (TF), expressed in monocytes and microparticles from living or apoptotic cells, is the responsible of the coagulation cascade activating factor VII forming the FT/VIIa complex which activates complexes IX and X. All of these play an important role in platelet activation (53, 54). TF is the main initiator of *in vivo* coagulation and it is not expressed in circulation under healthy conditions. Nevertheless, under inflammatory conditions, TF is expressed in endothelial and fibroblast cells and released to the circulatory system due to the action of proinflammatory cytokines, C reactive protein, and final products of advanced glycation (55–57). GzmA and GzmK could contribute to enhanced activation of the coagulation cascade in sepsis by generating cytokines involved in endothelial cell activation and coagulation like TNF-α or IL6 (58–60). GzmB is able to enhance the proinflammatory activity of IL-1α by proteolytic cleavage (61), although as indicated above, GzmB deficiency does not protect from sepsis.

Gzms can also modulate the coagulation cascade irrespectively of its ability to induce inflammatory cytokines. It has been reported that GzmB and GzmM can degrade coagulation-related substrates such as Von Willebrand factor (VWF) (30) or fibrinogen (only GzmB) (62). VWF is a homeostatic plasma protein that promotes platelet adhesion. Furthermore, VWF stabilizes coagulation factor VIII (FVIII) in plasma protecting it from proteolytic degradation and prolonging FVIII half-life. In septic patients, increased levels of VWF have been reported, so this protein could be a link between inflammation and thrombosis (63). VWF is regulated by the metalloprotease ADAMTS13, which specifically cleaves VWF in its A2 domain (64). It has been reported that GzmB and GzmM could mimic the action of ADAMTS13 by cleaving VWF regulating its adhesive function and preventing its binding to FVIII although did not affect platelet aggregation (30, 62). If these processes are relevant *in vivo* during sepsis, GzmB and GzmM would be expected to attenuate coagulation, preventing systemic coagulation and organ failure. This would explain why GzmB deficiency does not protect from sepsis, but would not explain the increased resistance of GzmM deficient mice to endotoxicosis and the reduced coagulation activity observed in these mice. It should be expected that VWF lost procoagulatory activity after cleavage and, thus, GzmM would protect from systemic coagulation disorders. A similar paradox has been found for GzmA. GzmA has been found to activate pro-urokinase, an enzyme that presents anticoagulant activity (65). However, the detrimental role of GzmA in sepsis has been confirmed by several independent groups, suggesting that the *in vitro* function of Gzms in regulating proteins involved in coagulation might not be relevant during sepsis *in vivo*. However, this hypothesis should be clarified by analyzing specifically the role of Gzms in regulating the coagulation system *in vivo*.

Regulation of Platelet Function

Platelets also play an important role in sepsis pathophysiology since the presence of thrombocytopenia is frequent in these patients. The role of platelets in sepsis is very complex as they can contribute to sepsis pathophysiology at different levels beyond regulation of haemostasis (66). Platelets can be directly activated by endotoxins, proinflammatory cytokines, or proteases such as thrombin (52, 67, 68). They contribute to the generation of inflammatory responses and, among other mediators, it has been shown that platelets can express GzmA and GzmB (18, 69, 70). Platelets can form clots on the endothelial damaged surface which produces elevated levels of VWF which is used as a marker for endothelial damage. It has been reported that the presence of proinflammatory cytokines such as IL-6, IL-8, and TNF-α (all of them induced by GzmA) are capable of induce the liberation of large VWF multimers that is a potent platelet aggregator. In septic patients, it has been observed elevated levels of VWF antigen which is related to a poor sickness prognosis (71). Here, as indicated above, since GzmB and GzmM have been found to cleave VWF and interfere with its procoagulant activity, it is tempting to speculate that these Gzms might have regulatory activities to prevent excessive VWF activity and clotting.

Curiously, GzmA was found to interact with the thrombin receptor (PAR1) in platelets and block thrombin-mediated responses (72). Although this interaction was not sufficient to induce *per se* platelet activation and aggregation, it might be possible that it can potentiate the effect of other ligands like endotoxin, a suggestion that has not been experimentally validated.

More recently it has been shown that during aging human platelets acquire GzmA expression which contributes to the generation of proinflammatory cytokines in monocytes (18). In light of these recent findings it will be interesting to analyse if Gzm expression is increased in older patients suffering from sepsis and its correlation with disease severity. Finally, as it will be discussed in the next section, it has been found that GzmB is expressed in platelets during sepsis using a mouse polymicrobial peritoneal sepsis model and might contribute to some of the alterations in vascular biology observed during sepsis (70).

Endothelial Barrier Permeability: Any Role for Protease Activating Receptors (PARs)?

The fluid redistribution to the extravascular space observed during sepsis is a consequence of an increase in the endothelial permeability or of the barrier function loss. Previous studies

suggest that the change in the membrane permeability is a consequence of the enzymatic cleavage of intercellular junction proteins, which results in a structural damage of endothelial cells. *In vivo* and *in vitro* studies have demonstrated that TNF-α is one of the responsible cytokines of this disorder and also that thrombin could potentiate them (73). Since some Gzms like GzmA and GzmM seem to be inflammatory mediators responsible of the detrimental effects of LPS in sepsis, including TNF-α production, they also could contribute to altered endothelial permeability.

The link between coagulation and inflammation has shown that the family of PARs play an important role because various proteases activated in the coagulation cascade will induce inflammation through PAR receptors. Activation of these receptors in endothelial cells critically contributes to the regulation of endothelial permeability. This family consists of four members, PAR-1,-2,-3,-4, that are expressed in cells present in the vasculature such as endothelial cells, monocytes, macrophages, platelets, fibroblasts and smooth muscle cells. PAR-1 is activated by thrombin, FXa (Activated factor X), trypsin and APC (Activated Protein C). It has also been reported that GzmA, B, and K are able to activate PAR1 in different cell types (21, 23, 27, 28). In contrast, PAR-2,-3 or−4 have not been found to be activated by any Gzm, albeit PAR2 participation in colitis has been indirectly proposed (74). Here it should be noted that neither activation of PAR2 by GzmA nor GzmA contribution to colitis were analyzed in the later study. Thus, it is not clear yet if PAR2 is activated by GzmA and the role of PAR2 in GzmA-mediated cytokine induction. A recent study found that a PAR-2 inhibitor (I-343) inhibited foot swelling induced by *in vivo* administration of GzmA (48). This result suggests that PAR-2 could be directly or indirectly involved in some of the biological functions of GzmA, but its specific role in the regulation of GzmA-mediated inflammatory cytokine production will require further experimental validation.

Despite the role of PAR-1 and 2 in the regulation of vascular biology and endothelial cell activation, its contribution to the pathophysiology of sepsis is complex and not completely clarified (47). Some data suggest that multiple activation of PAR receptors by coagulation proteases may contribute to inflammation in endotoxemia and in sepsis (75). However, it was found that PAR1 and/or PAR2 deficiency neither reduce the inflammatory response nor increase survival in a mouse model of endotoxemia, indicating that PAR1 and PAR2 are dispensable for LPS-induced sepsis (76, 77). In a later study, using PAR antagonists in wild type mice, it was shown that the role of PAR1 and PAR2 in sepsis is reversal, being detrimental in the early phase of sepsis and beneficial in later stages and thus, the net balance of PAR deficiency during sepsis was indistinguishable between wild type and PAR deficient mice (78). In contrast to PARs deficient mice, GzmA deficient mice are protected from endotoxemia and sepsis and, thus, PAR1 and 2 should not play an important role in GzmA-induced inflammation during sepsis.

PARs are expressed by a variety of cells and the function in each cell type might differ, which would explain the differences between the *in vitro* and the *in vivo* observations regarding PAR-1, GzmA, and sepsis. GzmA has been shown to activate PAR1 in neurons (21), but not in platelets (72) or monocytes (16). We have found that albeit a PAR-1 inhibitor, Vorapaxar, reduced IL6 production in mouse macrophages stimulated with GzmA, the protease was able to induce the same level of IL6 in macrophages from wt or PAR1 deficient mice (Santiago et al. Cell Reports, Accepted), indicating that PAR1 is dispensable for GzmA-induced inflammation in macrophages. This apparent contradictory result might be explained by the potential unspecific effects of Vorapaxar including toxicity (79). Whatever it is, it supports the previous findings suggesting that PAR1 is not activated by GzmA in platelets or monocytes (16, 72). In addition, it should be taken into account that even PAR1 could be activated in some cell types, its relative contribution to GzmA-mediated inflammation should be validated by using adequate inhibitors or genetic deficient cell models (KO or siRNA).

Regarding PAR-3 and−4, although they can be activated by thrombin and other proteases its role during sepsis has not been confirmed yet (80) and in addition there is not any evidence relating Gzms and them.

It seems that in light of the different results pointing to a complex regulation of PARs during sepsis, the involvement of PARs in the detrimental effects of GzmA on vascular permeability during sepsis will require further clarification and specific experimental validation.

Endothelial Barrier Permeability: Extracellular Matrix Degradation, Matrikines and More

In addition to inflammatory cytokines and direct endothelial cell activation, other factors involved in the maintenance of vascular permeability could contribute to the loss of endothelial barrier function during sepsis. Some of these biological processes have been shown to be activated by Gzms *in vitro* like extracellular matrix (ECM) degradation, generation of products from ECM degradation with biological activity (matrikines) or killing of endothelial and smooth muscle cells (**Table 1**). Although the involvement of these processes in the mechanisms activated by Gzms during sepsis has not been directly analyzed, we would like to speculate on the potential implications of some of them.

Although GzmB deficiency does not increase survival during endotoxemia (40) or bacterial sepsis (41, 81), some of the biological functions of this protease might contribute to some of the vascular alterations observed in sepsis. For example, it has been shown that extracellular GzmB is able to directly kill cells involved in the maintenance of vascular architecture like endothelial and smooth muscle cells (25).

Gzm B Induces Smooth Muscle Cell Apoptosis in the Absence of Perforin. This process is based in its ability to induce ECM degradation and cell detachment (82, 83), which might activate cell death by anoikis (26, 82). Alternatively GzmB could disrupt endothelial cell barrier integrity by degrading proteins involved in tight junctions like Zonulin-1, PECAM, JAM, or Cadherins as previously shown (26). Later on it was found a role for GzmB in VE-cadherin cleavage and endothelial permeability *in vitro* and *in vivo* (84). In addition, ECM degradation by GzmB has been shown to release vascular endothelial growth factor

which could affect vascular permeability (85). GzmB has also been found to affect wound healing, which was related to the ability of this protease to degrade fibronectin (86). Last but not least, it has been reported that GzmB may act on some of components of the EC involved in fibrillogenesis such as fibrillin-1 or decorin, increasing vascular permeability (87, 88), one of the most important pathological events that occur in sepsis.

Among other cell sources, platelets (70) and mast cells (26) have been found to express GzmB, but not perforin or GzmA, and thus, these cells could be the source of extracellular GzmB leading to its detrimental functions related with ECM degradation. In addition, it was found that GzmB of $CD8^+$T and NK cells could modulate endothelial cell permeability and immune cells transmigration as a mechanism involved in host protection against viral infections (89). NK cells have been involved in sepsis (90, 91) and thus unregulated NK cells responses leading to a high release of GzmB due to PAMP-induced activation of NK cell receptors could transform a physiological protective mechanism into a pathological insult affecting endothelial cell permeability.

We would like to reiterate that the roles of GzmB in coagulation or in vascular permeability have not been studied yet in the context of a septic response and thus, all these hypotheses will require experimental validation.

Regarding GzmA, this protease can also degrade some proteins of the ECM like fibronectin or collagen IV (92, 93) and also could be released by NK cells during sepsis (41), although the biological functions of these processes remain unexplored.

GRANZYMES AT THE IMMUNOSUPPRESSIVE EDGE

With the improvement of intensive care services, the majority of patients with sepsis are able to survive the hyperinflammatory phase but enter a prolonged immunosuppression stage that has been called "immunoparalysis" in which they will be susceptible to secondary infections (94).

In the late stages of sepsis, murine studies reveal a relatively constant balance in proinflammatory and anti-inflammatory cytokines, although with a smaller magnitude when compared to the acute phase. One of the key features of immunosuppression in sepsis is the state of cellular anergy that can begin to occur even in the early stages of the disease (3, 95, 96). This immunosuppression stage is characterized by a decrease in antigen presentation, alteration in the expression of costimulatory molecules, and changes in lymphocytes populations. Here some experimental evidences acquired in different disease models suggest that some Gzms might also contribute to the different alterations leading to immunosuppression.

One of the causes of anergy is the decrease in antigen presentation and dendritic cell maturation leading to impaired protective cellular responses (97, 98). The function (expression for HLA-II and co-stimulatory molecules) and number of several antigen presenting cells has been found to be compromised in sepsis correlating with reduced survival like dendritic cells or monocytes (99–103). These alterations contribute to changes in the cytokine profiles and alterations in lymphocyte populations.

Although a direct impact of Gzms on antigen cell presentation has not been reported, GzmB might contribute to T cell immunosuppression by different means. All the hypotheses proposed below will require experimental validation in order to show if Gzms can contribute to the immunosuppressive stage in sepsis.

GzmB has been found to be expressed by human and mouse Treg cells (104, 105) and contribute to immunosuppression and cancer immune-evasion by inhibiting Tc and NK cell responses in tumor models (106) or to control viral-induced lung inflammation (107). Thus, it could be speculated that GzmB of Treg cell could contribute to the cellular immunosuppression observed in sepsis patients. T regulatory cells are known to contribute to immune homeostasis preventing reactions against self-healthy tissue and commensal microbiota. They inhibit the immune response at different levels like B, CD4 Th1, CD8 T cell, and NK cell activation or dendritic cell maturation and it has been found that sepsis patients present reduced numbers of CD4 Th1, CD8 T cell, and NK cell (108) and increased Treg cell number and activity (109–111), which contributes to secondary infection.

Alternatively, GzmB is able to cleave the zeta chain of the T cell antigen receptor (TCR) which renders un-functional T cells (112), being another potential mechanism by which GzmB could contribute to immunosuppression in septic patients.

More direct evidence on the role of Gzms in immunosuppression was recently provided by Freishtat et al., who reported that acute sepsis-induced alterations in the megakaryocyte-platelet transcriptional axis result in strongly cytotoxic platelets expressing GzmB. These platelets used GzmB to kill $CD4^+$T cells contributing to lymphodepletion (69). Later on the same group reported that the mechanism of platelet-mediated GzmB-dependent lymphotoxicity required cell to cell contact and was perforin-independent (70).

CONCLUSIONS AND FUTURE PERSPECTIVES

Sepsis is a global serious health problem for which early specific diagnosis and optimized treatment is crucial in order to reduce organ damage and improve survival. It is well-known the importance and influence of inflammation in the development and pathogenesis of sepsis (3, 5). However, the molecular bases to understand the dysregulated inflammatory response during sepsis are complex and often not well-understood. Indeed, most experimental trials to reduce inflammation during sepsis have not been successful and a positive impact on patient survival has not been reported. Among other reasons the impact of anti-inflammatory therapy on pathogen control by host immune response and the risk of secondary infections could contribute to the low efficacy observed during the trials with these therapies. During the last years, cumulative experimental evidences indicate that some members of the Gzm family, especially GzmA, have a key role in modulating inflammation and contribute to sepsis. Indeed, elevated levels of Gzms have

been found in patients suffering from sepsis albeit the clinical significance remains unclear. In addition, *in vivo* mouse models show that GzmA deficiency protects from bacterial sepsis and endotoxemia indicating that GzmA have the potential to be used as biomarker and/or therapeutic target in sepsis (17, 40, 41). A current limitation of these studies is that the therapeutic potential of GzmA should be validated using a GzmA inhibitor in animal models expressing the protease. In addition, all *in vivo* studies have been performed using purified endotoxin or a single bacterial agent, and sepsis is often induced by more than a single microbial agent like peritoneal sepsis, one of the most common causes of sepsis. Moreover, the model of endotoxemia induced by LPS, albeit useful to study septic shock, does not provide information on the impact of regulation of inflammation on microbial infection.

Most studies in this field has been focused on the ability of some Gzms like GzmA, GzmK, and GzmM to induce the production of inflammatory cytokines in different cell types *in vitro* including monocytes, macrophages, endothelial cells, or fibroblasts. However, the role of GzmK *in vivo* has not been analyzed yet and GzmM has only been validated in the LPS-induced endotoxemia *in vivo* model. Apart from the ability to regulate the generation of the cytokine storm associated to the septic process and the consequences of this response to coagulation disorders and organ damage, *in vitro* evidences suggest that Gzms might also directly regulate other alterations found in sepsis like coagulation cascades, platelets function, or vascular permeability. Further experimental studies will be required to find out the relevance of these results in animal models *in vivo,* although it can be anticipated that it will not be easy to separate the effects due to Gzm-induced inflammatory cytokines from those that could be directly regulated by these proteases.

Apart from the disorders related with the inflammatory response leading to disseminated coagulation and organ damage, as discussed above, Gzms might also contribute to the immunosuppressive stage commonly observed in septic patients, responsible for a high morbidity/mortality due to secondary infections. Indirect evidences from other experimental models suggest that some Gzms might contribute to immunosuppression and, thus, it might be worth to dedicate some efforts to find out the role of Gzms in promoting immunosuppression and/or anergy during sepsis.

Despite the limitations mentioned above and all the hypotheses and speculations pending of experimental validation, some Gzms like GzmA or GzmK might present an important advantage in comparison with other inflammatory molecules that have been proposed and tested as therapeutic targets. GzmA or GzmK deficiency does not predispose to infection and these animals are able to efficiently clear most experimental infections (6, 113). Thus, targeting Gzms might reduce sepsis pathology without compromising the host immune response against the offending pathogen/s and, thus, enhancing the chances of efficient antimicrobial treatment to re-establish immune homeostasis and reduce immune-associated damage. Before these therapies can be developed and tested in potential clinical trials, it will be required to better understand the biology of granzymes during sepsis and the mechanisms involved.

AUTHOR CONTRIBUTIONS

MG-T, MA, and JP-P designed and wrote the first draft. LM-L prepared **Figure 1**. All authors wrote and revised the manuscript.

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Lung Secretoglobin Scgb1a1 Influences Alveolar Macrophage-Mediated Inflammation and Immunity

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Alveolar macrophage (AM) is a mononuclear phagocyte key to the defense against respiratory infections. To understand AM's role in airway disease development, we examined the influence of Secretoglobin family 1a member 1 (SCGB1A1), a pulmonary surfactant protein, on AM development and function. In a murine model, high-throughput RNA-sequencing and gene expression analyses were performed on purified AMs isolated from mice lacking in *Scgb1a1* gene and were compared with that from mice expressing the wild type *Scgb1a1* at weaning (4 week), puberty (8 week), early adult (12 week), and middle age (40 week). AMs from early adult mice under *Scgb1a1* sufficiency demonstrated a total of 37 up-regulated biological pathways compared to that at weaning, from which 30 were directly involved with antigen presentation, anti-viral immunity and inflammation. Importantly, these pathways under *Scgb1a1* deficiency were significantly down-regulated compared to that in the age-matched *Scgb1a1*-sufficient counterparts. Furthermore, AMs from *Scgb1a1*-deficient mice showed an early activation of inflammatory pathways compared with that from *Scgb1a1*-sufficient mice. Our *in vitro* experiments with AM culture established that exogenous supplementation of SCGB1a1 protein significantly reduced AM responses to microbial stimuli where SCGB1a1 was effective in blunting the release of cytokines and chemokines (including IL-1b, IL-6, IL-8, MIP-1a, TNF-a, and MCP-1). Taken together, these findings suggest an important role for *Scgb1a1* in shaping the AM-mediated inflammation and immune responses, and in mitigating cytokine surges in the lungs.

Keywords: club cell, alveolar macrophage, lung surfactant, SCGB1A1, Clara cell secretory protein, cytokine storm

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INTRODUCTION

The ongoing pandemic of Coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has already affected over 30 million people across the globe resulting in more than one million deaths (1, 2). It produces a significant amount of severe illnesses that overwhelm health care infrastructure. Early studies have shown that the prognosis of COVID-19 may be variable among different populations and age groups. Particularly,

it disproportionately affects the elderly and patients with preexisting conditions including chronic obstructive pulmonary disease (COPD) and hypertension compared to any other conditions (3, 4). Mechanisms of SARS-CoV-2 persistence and pathogenicity remains largely unknown; however, its widespread infection and associated fatalities outnumber the past coronavirus outbreaks such as the Middle East respiratory syndrome-related coronavirus and severe acute respiratory syndrome-associated coronavirus. The rapid decline in lung function and some of the COVID-19 patients requiring oxygen support and/or mechanical ventilation has been attributed to cytokine storm (5). There is no targeted anti-viral therapy currently available for COVID-19 and efforts to develop vaccines are ongoing. In this context, examining the cellular, and/or molecular interactions within the lung microenvironment may provide useful insights into the pathogenesis and possible therapeutics of COVID-19 and other infectious respiratory diseases.

Alveolar macrophages (AMs) are stationary cells of embryonic origin that constitute greater than 95% of the phagocyte pool in the alveolar space (6, 7). Consequently, AMs perform crucial immune surveillance at the respiratory surface providing the first line defense against airborne pathogens, pollutants (8–12), and clearing of cellular debris (13). AMs function as professional antigen presenting cells in eliciting antigen specific T cells and antibodies targeting pathogens (14, 15) and autoantigens (16, 17). AMs also can respond to various microbial stimuli producing an array of cytokines and chemokines that may influence the landscape of inflammation and immunologic outcomes (16–18).

On the other hand, club cells are a significant contributor to the homeostatic and reparative processes in the lungs (19). These non-ciliated and non-mucous-producing cells in the bronchiolar epithelium can differentiate into pulmonary epithelial and endothelial cells following tissue injury (20–23), and via secreted secretoglobin family 1A member 1 (SCGB1A1) protein, a component of the pulmonary surfactant, can exert anti-inflammatory and anti-fibrotic functions (24). Specifically, SCGB1A1 binds and sequesters key inflammatory mediators of airway diseases including prostaglandins (25, 26), phospholipase (PL) A2 (27–29) and PLC (28), and inhibits activation and translocation of NF-κB (30, 31). Lung infection by viruses and bacteria are known to elicit greater inflammatory responses in the absence of SCGB1A1 (32–34), whereas respiratory distress decreases SCGB1A1 levels following acute lung injury (35); exposures to pollutants (36), cigarette smoke (37) and ozone (38); lung allograft rejection (39–41); respiratory infections (42, 43); and chronic lung diseases (44, 45). Overexpression of *Scgb1a1* in airways has shown to limit ventilator induced lung injury and inflammation (46), and supplementation of exogenous SCGB1A1 has mitigated the increased proinflammatory cytokines and inflammatory buildup caused by *Scgb1a1* germline-deficiency (24).

Because AMs are non-migratory cells that adhere to the alveolar epithelium (47), they are constantly immersed in pulmonary surfactant and are likely to be influenced by the surfactant components. Under steady state, AMs exhibit a non-inflammatory phenotype while SCGB1A1 occurs at its physiologic maximum. Club cells, on the other hand, succumb to respiratory distress resulting in decreased SCGB1A1 levels. As the crosstalk between club cells and AMs is largely unknown, for the first time in the present study, we examine the transcriptomic profiles of mouse AMs during development and compare the shift in gene expression under *Scgb1a1*-deficiency. Furthermore, we also analyze the effects of SCGB1A1 protein on purified AMs delineating therapeutic implications of SCGB1A1 in inflammatory and fibrotic lung diseases.

MATERIALS AND METHODS

Experimental Animals

Wild type (WT, *Scgb1a1*$^{+/+}$) C57BL/6 mice were procured from Charles River Laboratories and Uteroglobin gene knockout (KO) mice (*Scgb1a1*$^{-/-}$) (48) were obtained from National Institutes of Health. Mice were housed at the Washington University School of Medicine according to institutional guidelines and approved protocols. Mice from WT and KO groups were euthanized at 4, 8, 12, and 40 weeks of age [respectively, equivalent to 6 months, 12 years, 20 years, and 40 years in human age (49)] and relevant samples were collected for further study (n = 3/group).

Flow Cytometry Analysis

We performed multicolor flow cytometry to analyze expression of a panel of phenotypic and functional markers. Bronchoalveolar lavage (BAL) cells were isolated per our established protocol (50) and incubated with Fc block (BD Bioscience) to prevent non-specific antibody binding. They were incubated with fluorophore tagged antibodies for CD45, CD11c and Siglec-F, and were analyzed with a BD LSR Fortessa cell analyzer (BD Bioscience). The functional states of AM at 4, 8, 12, and 40 weeks were compared between the KO and WT groups. Single stain control and fluorescence minus one control were included in every study and data were analyzed by FlowJo v10.6.2 (BD Life Sciences).

AM Isolation and RNA Purification

Alveolar macrophages, defined as CD45^{+}, CD11c^{hi}, and Siglec-F^{hi} granular cells, were isolated from BAL fluid via flow cytometry cell sorting technique following our previous study (50), and age-matched samples were collected and processed on the same day to minimize batch-to-batch variation. AMs were isolated individually from three mice per group and were sorted directly into RNA Lysis buffer using a PureLink RNA kit (Thermo Fisher Scientific). Total RNA was treated by DNase I (Thermo Fisher Scientific), quantitated by Nanodrop (Thermo Fisher Scientific), and stored at -80°C.

RNA Sequencing and Bioinformatics Analysis

Three biologic replicates per group were included to ensure a strong statistical power for detecting the inter- and intra-group variations. Total RNA (up to 100 pg) was subjected to the picoRNA workflow (Cofactor Genomics). A poly-A library

was constructed and 6×10^7 single-end reads were performed. Following RIN score (>7) determination and enrichment for mRNA, RNA sequencing was performed (Cofactor Genomics) for 60 million single-end reads covering >75 base pairs/read. RNA-seq reads were then aligned to the 76 primary assemblies for *Mus musculus* with STAR version 2.5.1a. Gene counts were derived from the number of uniquely aligned unambiguous reads by Sub read: feature Count version 1.4.6-p5. All gene counts were then imported into the R/Bioconductor package EdgeR and TMM normalization size factors were calculated to adjust for samples for differences in library size. The TMM size factors and the matrix of counts were then imported into the R/Bioconductor package Limma, and analyzed for differential expression using Limma/voom and sequencing data was analyzed by Genomics Suite (Partek). The data set has been submitted to Gene Expression Omnibus (GEO) with accession no GSE148647. The data were examined for changes in transcription profile in comparison with their WT counterpart by three-dimensional PCA plot, hierarchical clustering, Venn diagram, volcano plot, and profile trellis and gene ontology enrichment. Generally applicable gene set enrichment (GAGE) method was applied for pathway analysis (51) through databases including gene ontology (GO, molecular function and biological process) and Kyoto encyclopedia of genes and genome (KEGG, metabolism and disease pathway). The Benjamini and Hochberg's False Discovery Rate (FDR) correction was performed to determine the significance of gene expression (51).

Influence of SCGB1a1 Protein on AM Response to Inflammatory Stimuli

The effect of SCGB1a1 protein on AM responsiveness to inflammatory stimuli were studied *in vitro*. Flow sorted AMs were plated (50) in 12-well plates at 1×10^5 cells/well in triplicates and were incubated with Toll-like receptor (TLR) agonists (Invivogen) 2- heat-killed *Listeria monocytogenes* (HKLM), TLR4-Lipopolysaccharide from *Escherichia coli* K12 (LPS) and TLR5- *Salmonella typhimurium* Flagellin (FLA) in presence or absence of recombinant SCGB1a1 protein at 5 μg/mL (Creative BioMart). Culture supernatant was collected and total RNA was isolated at 72 h post-stimulation. The release of

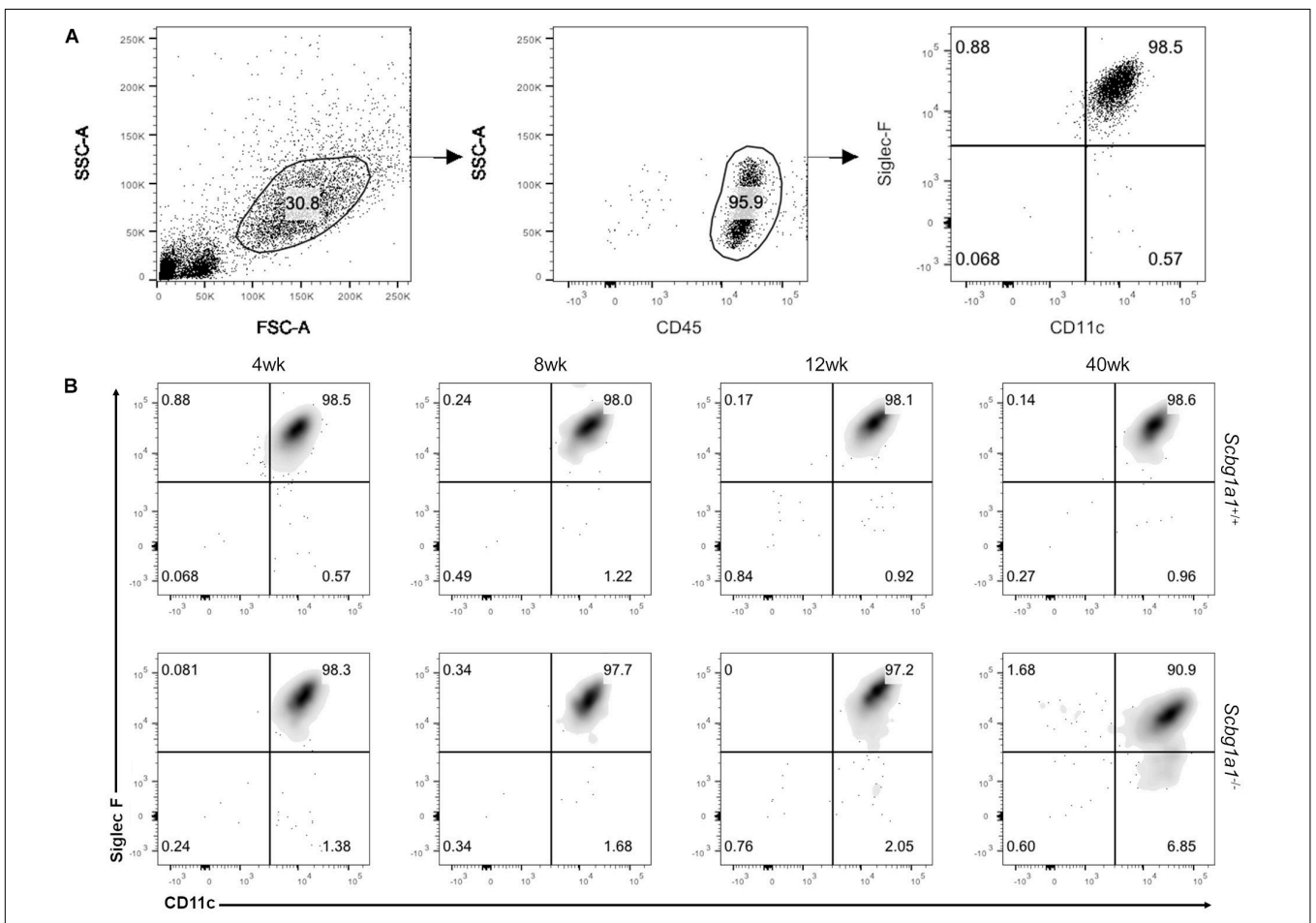

FIGURE 1 | Influence of *Secretoglobin family 1A member 1* (*Scgb1a1*) deficiency on alveolar macrophage (AM) phenotypes. **(A)** Flow cytometric analysis of mouse AMs ($CD45^+$, $CD11c^{hi}$, and Siglec-F^{hi} cells) from bronchoalveolar lavage fluid. **(B)** AMs from WT (*Scgb1a1*$^{+/+}$) and KO (*Scgb1a1*$^{-/-}$) mice were analyzed for phenotypic differences at 4, 8, 12, and 40 weeks of age.

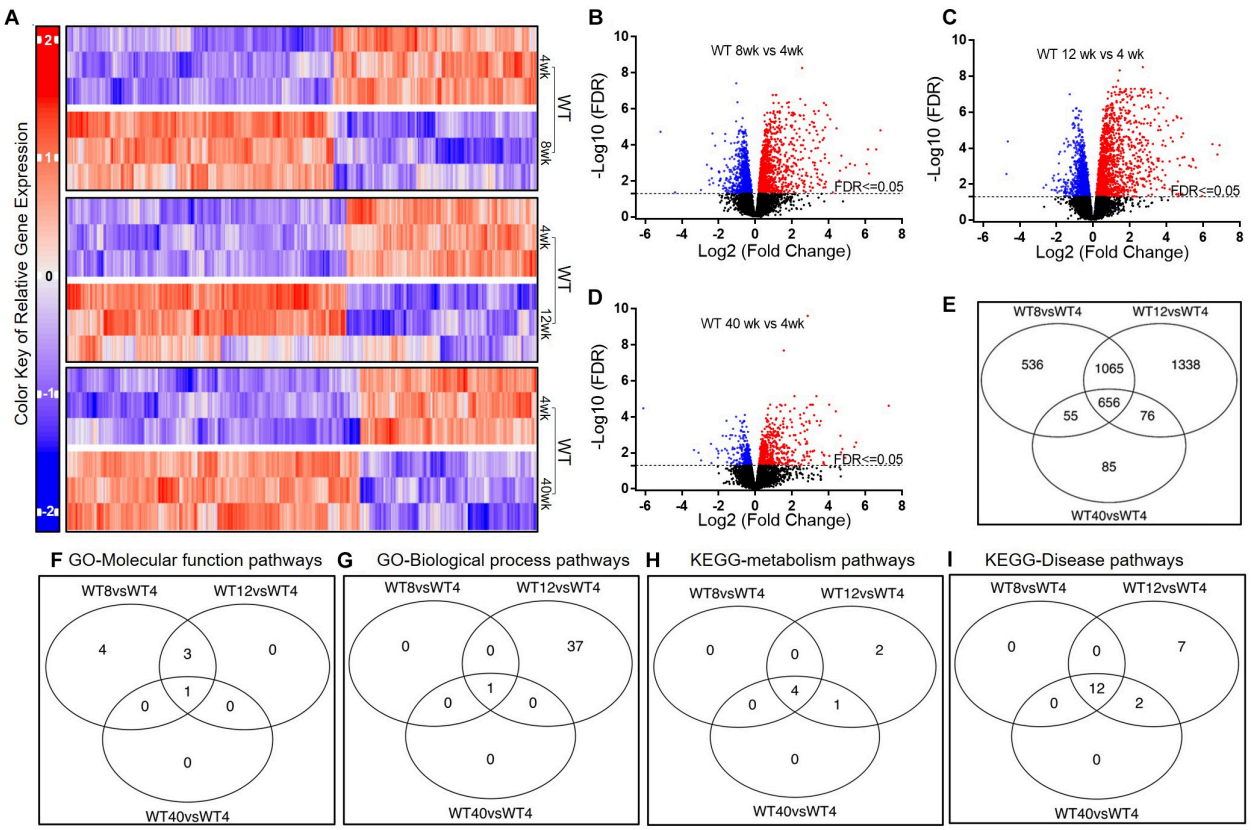

FIGURE 2 | Gene expression in AMs from wild type mice across age groups. **(A)** Up-regulated and down-regulated genes in AMs at 4, 8, 12, and 40 weeks; **(B–D)** Volcano plots of significantly up-regulated (red) and down-regulated (blue) genes (FDR < 0.05); **(E)** Venn diagrams of the significantly changed genes; Venn diagrams of the significantly changed gene sets in AMs of the WT mice at different ages including GO-Molecular function pathways **(F)**, GO-Biological process pathways **(G)**, KEGG-metabolism pathways **(H)**, and KEGG-Disease pathways **(I)**. FDR < 0.05 was considered significant; FC, fold change.

cytokines and chemokines in culture supernatant was analyzed by a Bio-Plex 200 system (Bio-Rad) using multiplex immunoassays.

RESULTS

Influence of Scgb1a1 Deficiency on AM Phenotype

We evaluated the prevalence and phenotype of AMs in age, gender and strain matched *Scgb1a1*$^{-/-}$ and *Scgb1a1*$^{+/+}$ mice. AMs, identified as BAL cell leukocytes with expression of CD11c^{hi} and Siglec-F^{hi}, were nearly identical between the KO and WT groups at earlier time points of 4 and 8 weeks (**Figure 1**). Interestingly, as early as 12-week, a unique population, *albeit* minor, with CD11c^{hi} and Siglec-F^{low} began to appear in the KO only. By 40-week, this population in KO had grown to represent ∼7% cells compared to ∼1% of that in the age-matched WT. Developmental origin and physiologic significance of the CD11c^{hi} and Siglec-F^{low} cells in *Scgb1a1*$^{-/-}$ is currently unknown; however, some unrelated studies have suggested a monocytic precursor for the Siglec-F^{low} pulmonary macrophages (52, 53). This phenotypic difference in AMs as early as 12 weeks of age suggests a variation in myelopoiesis and/or activation status associated with *Scgb1a1* deficiency.

Genes and Gene Sets Differentially Expressed in WT AMs

To study alteration in gene expression due to aging, we compared the AM expressed genes at 8, 12, and 40 weeks with that at 4 weeks in WT mice (**Figure 2A**). As shown in **Figures 2B–D**, there were 2312 genes at 8-week, 3135 genes at 12-week, and 872 genes at 40-week significantly up-regulated compared to that at the 4-week (FDR ≤ 0.05). The overlapping pattern of gene expression was categorized in a Venn diagram indicating that 656 genes were remarkably altered at all time-points beyond 4 weeks of age (**Figure 2E**). Additionally, ten most significantly up-regulated and down-regulated genes along with FDR values are presented in **Supplementary Table 1**.

To delineate the biological significance of the shift observed in gene expression, we analyzed the significantly changed gene sets using GAGE method and screened through GO and KEGG databases (**Figures 2F–I**). As shown in **Supplementary Table 2**, there were seven and three up-regulated GO molecular function pathways at 8 and 12 weeks, respectively, in comparison to

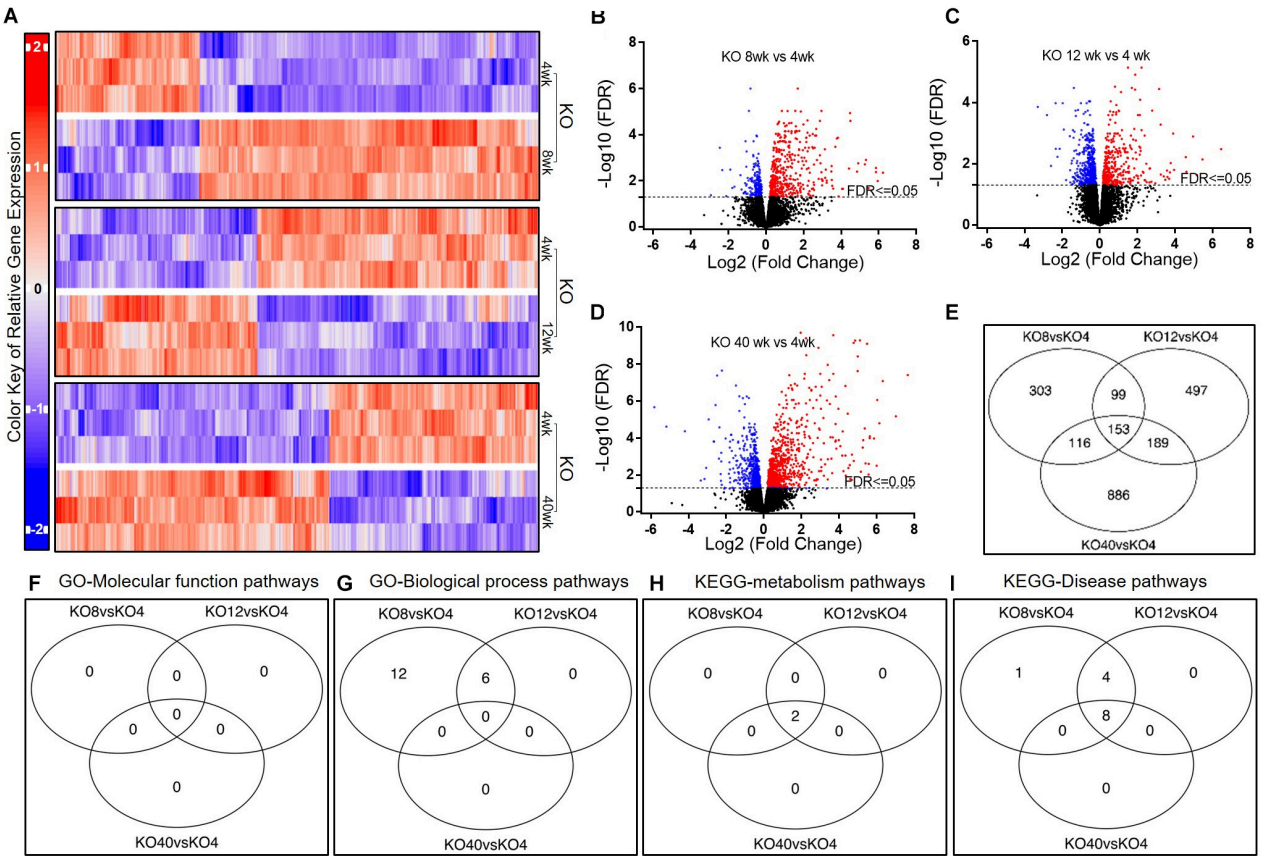

FIGURE 3 | Gene expression in AMs from *Scgb1a1* KO mice across age groups. **(A)** Up-regulated and down-regulated genes in AMs at 4, 8, 12, and 40 weeks; **(B–D)** Volcano plots of significantly up-regulated (red) and down-regulated (blue) genes (FDR < 0.05); **(E)** Venn diagrams of the significantly changed genes. Venn diagrams of the significantly changed gene sets in AMs from KO mice at different ages including GO-Molecular function pathways **(F)**, GO-Biological process pathways **(G)**, KEGG-metabolism pathways **(H)**, and KEGG-Disease pathways **(I)**. FDR < 0.05 was considered significant; FC, fold change.

that at 4 weeks. In contrast, the ribosome related pathway was down-regulated at 8, 12, and 40 weeks compared to that at 4 weeks. Importantly, most of the GO biological processes including the innate immune response pathway were up-regulated at 12-week compared with that at 4-week. There were 37 significantly up-regulated biological processes evident at 12-week, among which 30 biological pathways were directly involved with immune defenses and inflammatory process. No significant change in GO biological process was, however, found at 40-week compared to that at 4-week. In the KEGG metabolism pathway analysis, antigen processing and presentation, phagosome, and cell adhesion molecule pathways were significantly up-regulated at 8, 12, and 40 weeks than that at 4 weeks. In the KEGG disease pathway analysis, several infectious diseases and immune/metabolism related pathways including that of Influenza A, Epstein-Barr virus (EBV), Measles and *Staphylococcus aureus*, and non-alcoholic liver disease were significantly changed at 8, 12, and 40 weeks than that at 4 weeks.

In summary, most of the pathways for immunologic and inflammatory responses were activated by 12 weeks of age. This suggests that early adult WT mice may have a more pronounced immunological defense compared to any other age groups studied.

Significant Changes in Gene Expression in KO AMs

To assess the patterns of AM gene expression due to aging under *Scgb1a1* deficiency, we studied flow-sorted AMs isolated from KO mice (**Figure 3A**). Compared to gene expression at 4 weeks, there were 671 genes at 8-week, 938 genes at 12-week, and 1344 genes at 40-week that were significantly up-regulated (**Figures 3B–D**, FDR $\leq$ 0.05). The Venn diagram indicated that expression of 153 genes was remarkably altered at all time-points in comparison to that at 4-week (**Figure 3E**). The ten most significantly up-regulated and down-regulated genes along with FDR values are presented in **Supplementary Table 3**.

To explore the biomedical significance of KO expressed genes, we analyzed the pathways by using GAGE method and screening through GO and KEGG databases (**Figures 3F–I**). Interestingly, there was no significant change in GO molecular function pathway among the KO age groups (**Supplementary Table 4**). On the other hand, 18 and 6 GO biological processes

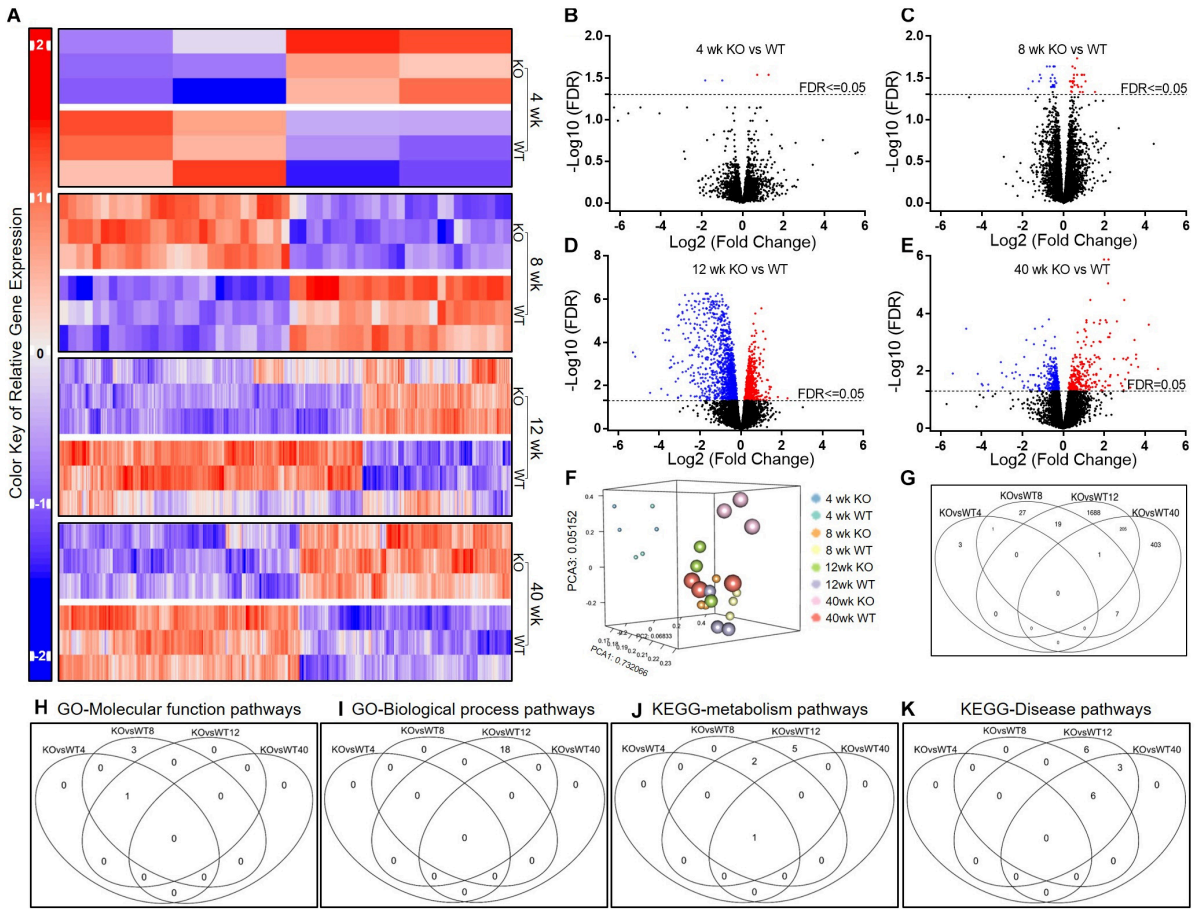

FIGURE 4 | Comparison of the AM gene expression patterns in *Scgb1a1* KO and WT mice. **(A)** Up-regulated and down-regulated genes in AMs at 4, 8, 12, and 40 weeks; **(B–E)**. Volcano plots of significantly up-regulated (red) and down-regulated (blue) genes (FDR < 0.05); **(F)** PCA plot of the variant feature of gene expression; **(G)** Venn diagrams of the significantly changed genes. Venn diagrams of the significantly changed gene sets in AMs from KO vs WT mice at different ages including GO-Molecular function pathways **(H)**, GO-Biological process pathways **(I)**, KEGG-metabolism pathways **(J)**, and KEGG-Disease pathways **(K)**. FDR < 0.05 was considered significant; FC, fold change.

were significantly up-regulated at 8 and 12 weeks of age, respectively, compared to that at 4 weeks. At 40-week, no significant change was, however, found in GO biological process. In the KEGG metabolism pathway analysis, antigen processing and presentation, and cell adhesion molecule pathways were significantly up-regulated at 8-week and 12-week than that at 4-week. In the KEGG disease pathway analysis, several infectious disease-related pathways including that of Influenza A, EBV, viral myocarditis, tuberculosis, and Herpes simplex virus (HSV) were found significantly up-regulated at 8, 12, and 40 weeks of age.

In brief, most of the pathways for immunologic and inflammatory responses were activated by 8 weeks and these pathways were less pronounced than that in the WT mice, suggesting an early onset of inflammation in KO mice.

Differential Expression of Genes and Gene Sets in KO and WT AMs

To evaluate the effect of *Scgb1a1* deficiency on AM transcriptome, we compared the gene expression patterns between KO and WT across the age groups (**Figure 4A**). As shown in **Figures 4B–E**, 4 genes at 4-week, 55 genes at 8-week, 1913 genes at 12-week, and 616 genes at 40-week were significantly altered in the KO AMs. The PCA plot showed gene expression patterns over time in the KO and WT mice (**Figure 4F**). The overlap between these genes was summarized in **Figure 4G** showing that the mice at 12-week had the most significant changes in gene expression. It was further presented in a Venn diagram indicating that 656 genes were remarkably altered at all time-points compared to that at 4-week (**Figure 4G**). The ten most significantly up-regulated and down-regulated genes are presented in **Supplementary Table 5**.

To decipher biologic relevance of gene expression patterns, we analyzed the significantly changed gene sets by using GAGE method and screened through GO and KEGG databases (**Figures 4H–K**). As shown in **Supplementary Table 6**, Ribosome was the only one GO molecular function pathway that was significantly down-regulated in 4-week old KO mice compared to that in WT. In addition to Ribosome pathway, there were three GO molecular function pathways that were down-regulated in the KO mice at 8 weeks of age. Surprisingly, 17 GO biological

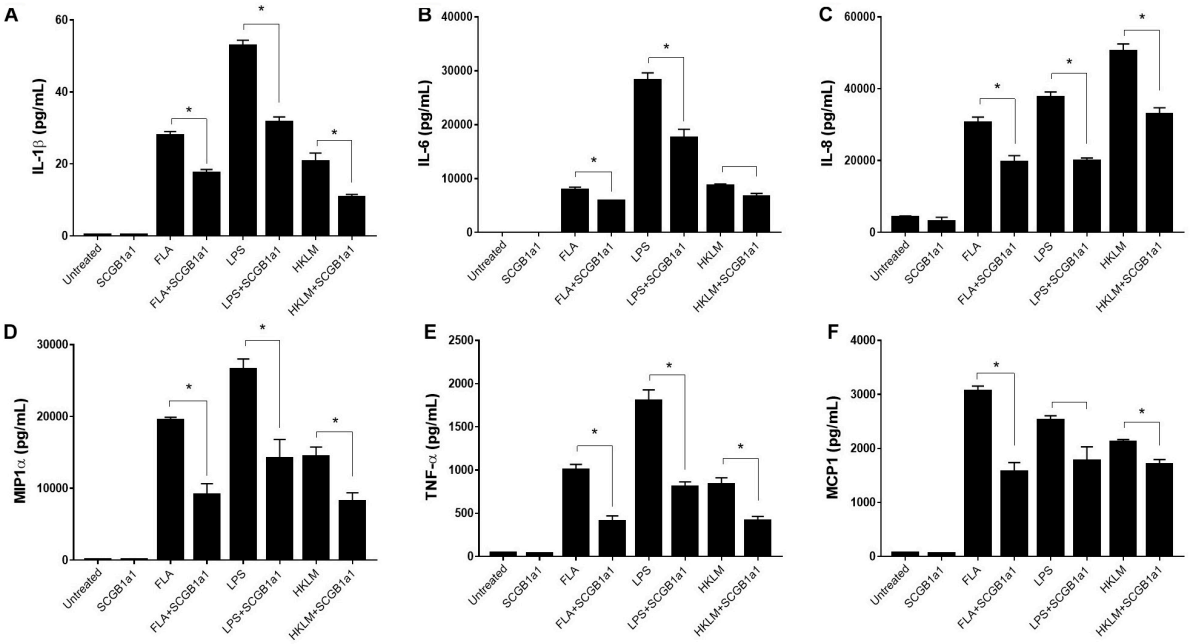

FIGURE 5 | SCGB1A1 attenuates AM-mediated inflammation. Cytokine and chemokine release from purified C57BL/6 AMs in response to toll-like receptor (TLR) agonists were measured: TLR2- heat-killed Listeria monocytogenes (HKLM), TLR4-Lipopolysaccharide from Escherichia coli K12 (LPS), and TLR5- Salmonella typhimurium Flagellin (FLA) in the presence or absence of recombinant SCGB1a1 protein. The panels depict IL-1b **(A)**, IL-6 **(B)**, IL-8 **(C)**, MIP1a **(D)**, TNF-α **(E)**, and MCP1 **(F)** responses to TLR stimuli. Data from three biological replicates are plotted as mean ± SEM, two-tailed unpaired *t*-tests were applied, and $p < 0.05$ was considered significant (*).

processes that are related to immune response or inflammation were significantly down-regulated in the KO at 12-week. From analysis of KEGG metabolism pathways, the antigen processing and presentation, proteasome, TNF signaling, and NOD-like receptor signaling were also significantly down-regulated in KO at 12-week. In KEGG disease pathway analysis, several infectious disease and immune related pathways including that of Influenza A, EBV, HCV, and HSV were significantly down-regulated whereas the non-alcoholic liver disease was significantly up-regulated at 12-week in the KO mice.

In sum, pathways for immunologic and inflammatory responses were less pronounced in KO AMs suggesting that *Scgb1a1* deficiency may undermine the repertoire of immune defenses available to this age group.

Attenuation of AM-Mediated Inflammation by SCGB1A1

We measured the cytokines and chemokines including interleukin (IL)-1β, IL-6, IL-8, MIP-1α, tumor necrosis factor (TNF)-α, and MCP-1 released by the flow-sorted AMs from C57BL/6 mice in response to TLR agonists: TLR2- heat-killed Listeria monocytogenes (HKLM), TLR4-Lipopolysaccharide from *Escherichia coli* K12 (LPS) and TLR5- *Salmonella typhimurium* Flagellin (FLA) in presence or absence of recombinant SCGB1a1 protein. As shown in **Figure 5A**, there was no significant change in the IL-1β level in the SCGB1a1 treated AMs (0.233 ± 0.033 pg/mL) than that in the untreated AMs (0.167 ± 0.120 pg/mL). However, the IL-1β release by AM was strikingly high when AMs were stimulated by LPS (52.660 ± 1.719 pg/mL, $p < 0.001$), HKLM (20.613 ± 2.426 pg/mL, $p = 0.001$), or FLA (27.707 ± 1.304 pg/mL, $p < 0.001$) than that in the untreated group. Moreover, the TLR stimulated cytokine/chemokine release was significantly reduced by supplementation of SCGB1a1. The IL-1β levels in LPS + SCGB1a1 (31.490 ± 1.588 pg/mL, $p < 0.001$), HKLM + SCGB1a1 (10.673 ± 0.876 pg/mL, $p = 0.001$), or FLA + SCGB1a1 (17.347 ± 1.130 pg/mL, $p = 0.001$) treated AMs were significantly lower than that the LPS, HKLM, or FLA only treated AMs. Furthermore, the administration of SCGB1a1 consistently lowered IL-6, IL-8, MIP-1α, TNF-α, and MCP-1 release from TLR induced AMs (**Figures 5B–F**). These data indicated that SCGB1a1 protein supplementation reduced the cytokine release induced by various microbial stimuli.

DISCUSSION

Alveolar macrophages are long-lived lung-resident phagocytes that occur in the alveoli up to 6×10^9 cells per healthy human adult (54) and are known to persist multitudes of infection, inflammation and autoimmune conditions. While contribution of AMs in eliciting pathogen and self-antigen specific immune responses (14–17) have been well recognized, their role in the inflammatory complications has not been fully understood.

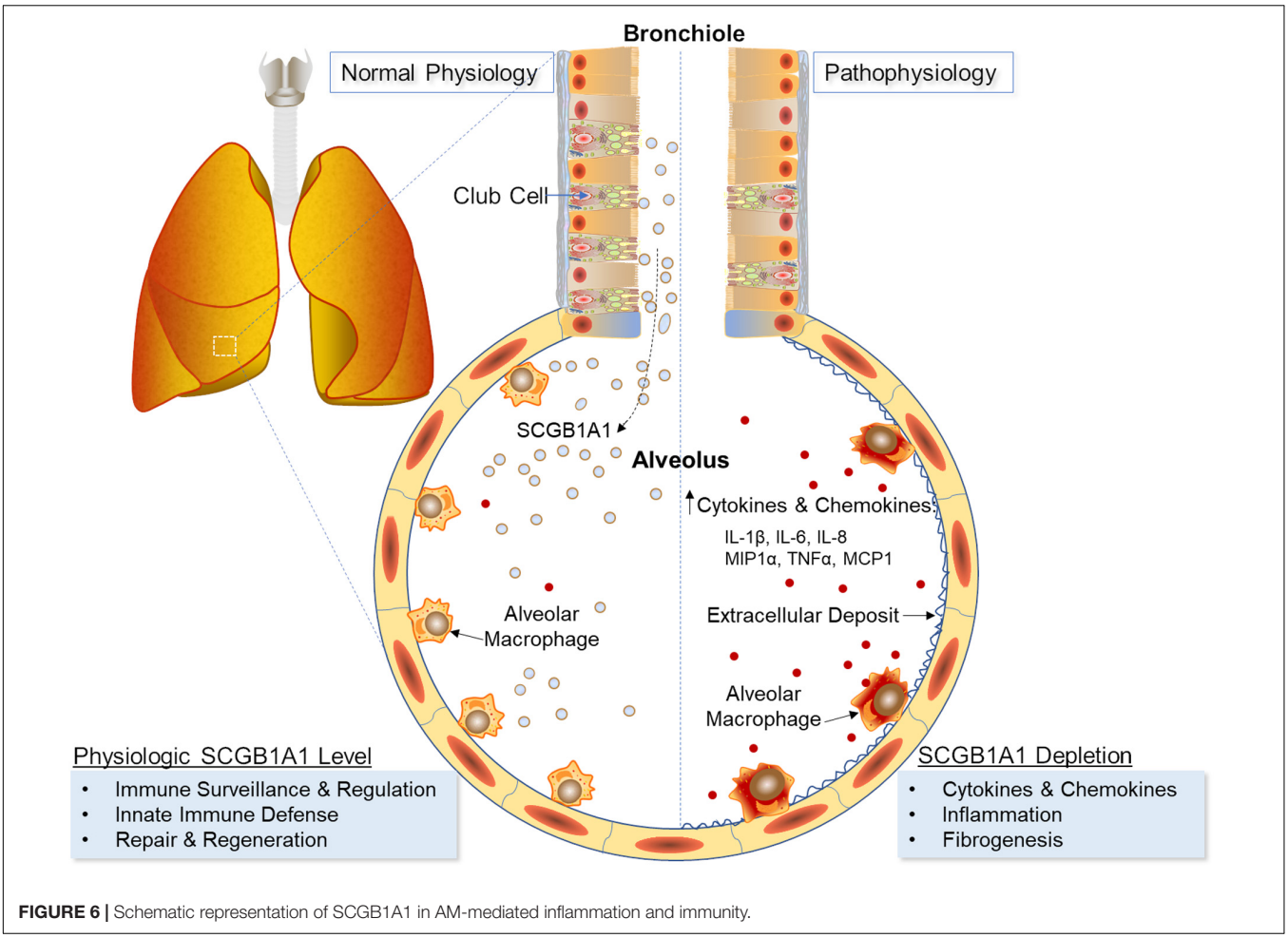

FIGURE 6 | Schematic representation of SCGB1A1 in AM-mediated inflammation and immunity.

To investigate potential anomalies in AM development and function in the absence of SCGB1A1 protein, we studied the phenotypes of AMs in age and gender matched *Scgb1a1*$^{-/-}$ and *Scgb1a1*$^{+/+}$ mice. While vast majority (>90%) of AMs were identified as CD11c^{hi} and Siglec-F^{hi} leukocytes in BAL cells, a minor population exhibited a phenotypic variation to be CD11c^{hi} and Siglec-F^{low} consisting of 6.85% in the KO compared to 0.96% that in the WT. Developmental origin and pathogenic significance of the CD11c^{hi} and Siglec-F^{low} cells in *Scgb1a1*$^{-/-}$ are currently unknown; however, some unrelated studies have suggested inflammatory monocytes as precursor for the Siglec-F^{low} pulmonary macrophages (52, 53). This phenotypic difference in AMs as early as 12 weeks of age (early adult) suggests a variation in the myelopoiesis and/or activation status associated with *Scgb1a1* deficiency. Furthermore, purified AMs from WT and *Scgb1a1*$^{-/-}$ KO mice were studied by RNA sequencing to capture their transcriptome profiles in a time dependent manner.

Our study reveals a progressive AM transcriptome from the *Scgb1a1* WT mice at weaning (4 week), puberty (8 week), early adult (12 week), and middle age (40 week) whereas that in *Scgb1a1* deficiency resembles the disorder of SCGB1A1 depletion induced by smoking, COPD and other types acute and chronic lung injuries in the humans. Specifically, in the WT mice, we found that the biological process pathways involving antigen presentation, innate immune and anti-viral defenses were significantly up-regulated at puberty, early adult, and middle age than that at weaning. Strikingly, WT mice at the early adult age had the most up-regulated immunological pathways suggesting that this age group can harness higher anti-viral immunity mediated by AMs. Although an increase in these processes was observed in the KO mice with most activated pathways found at puberty that further declined at early adult age and beyond, this indicated a premature and weakened immune system in the KO mice. These immunological changes between the KO and WT mice were also observed when analyzed by an age-paired comparison where the KO mice, at early adult age, had the most compromised immune system than that in the WT mice. These dynamic changes in anti-viral immunity during AM development may have a clinical relevance on the varied outcomes of COVID-19 among stratified age groups.

Both of the SARS-COV causing SARS (55) and SARS-COV-2 causing COVID-19 (56) are known to utilize angiotensin-converting enzyme 2 (ACE2) as a cell attachment receptor and entry site. Hence, the tissue distribution of ACE2 and its dynamic expression are crucial to understand the pathophysiology of coronavirus infection. It has been shown that ACE2 is expressed

in numerous tissues, including epithelial cells of the lung, intestine, kidney and blood vessels (56). Within the lung, the ACE2 is heavily expressed on alveolar type (AT) II cells (57–59) and it is also found on the SCGB1A1-producing club cells (60) that make these cell types vulnerable to the coronavirus infection. This is particularly important since COVID-19 is an airborne disease and SARS-COV-2 can travel through the inhaled air to the permissive sites of infection in respiratory bronchioles and alveoli. It is also concerning that smoking up-regulates ACE2 expression in lungs (61) and a recent study found current smokers to be 120% more likely to die from SARS-COV-2 infection than non-smokers (4). The human AMs are also susceptible to a strain of coronavirus infection (62). Together, these findings highlight the importance of AMs and club cells in the possible entry and transmission of coronaviruses.

Anti-inflammatory effector function of SCGB1A1 has been well studied and it has been shown that viral and bacterial infections of lungs deficient in *Scgb1a1* elicit greater inflammatory responses. The pro-inflammatory cytokine concentration and BAL cell count were significantly higher in S*cgb1a1*-deficient lungs following an acute Adenovirus infection (34). Similarly, infection by Respiratory syncytial virus led to increased T-helper 2 cytokines, neutrophil chemokines and viral replication following *Scgb1a1* deficiency whereas restoration of *Scgb1a1* expression in the airway abrogated the viral persistence and lung inflammation (33). It has been reported that club cells from mouse airways can modulate cytokine production by macrophages in the lung periphery (63). Another *in vitro* study also found that supplementation of exogenous SCGB1a1 can reverse cigarette smoke-induced IL-8 release and attenuate airway inflammation in biopsy specimens from patients with COPD (64). Currently, COVID-19 is treated with supportive care and respiratory failure from acute respiratory distress syndrome is the leading cause of death (65). Accumulating evidence suggests that a subgroup of patients with severe COVID-19 might have cytokine storm syndrome (5) indicating an urgent need for additional intervention and/or prophylaxis beyond supportive care. In the present study, we found exogenous supplementation of SCGB1a1 protein significantly blunted AM release of cytokine storm syndrome mediators including IL-6, IL-1β, TNF-α, IL-8, MIP-1α, and MCP-1. Moreover, AMs are critical in eliciting anti-coronavirus $CD4^+$ T cells that remain important in mounting a specific and lasting immune defense (66). Cytokine storm is believed to dampen AM's antigen presentation ability and cytokine neutralization results in a greater frequency of anti-viral T cells. Therefore, implementation of an early curb on AM inflammation and cytokine surge may produce better outcome measures in managing COVID-19 with lower mortality and higher virus specific immunity.

Although findings from this study are interesting and likely to have a high clinical relevance, there are several limitations in the study. For instance, the study did not assess bi-directional interactions between AMs and club cells, and their influence on lung tissue repair, remodeling, and regeneration. Such crosstalk may provide important insights into pulmonary health and serve as early indicators of AM activation and/or club cell damage under physiologic and pathologic conditions. Additionally, we did not evaluate the association of SCGB1A1 concentration with ACE2 expression in AT II cells. Due to current non-availability of humanized ACE2 transgenic mouse model, we were unable to perform *in vivo* infection studies with SARS-CoV-2 in the setting of *Scgb1a1* deficiency. In sum, SCGB1A1 influenced AM functionality where AMs developing under Scgb1a1 deficiency showed a diminished ability to stimulate adaptive immune responses. Given the high density of AMs in lung tissue, exogenous supplementation of SCGB1A1 may be helpful to restore AM SteadyState functions and prevent local cytokine surges in infectious and autoimmune diseases (**Figure 6**).

CONCLUSION

Although AMs and lung surfactants have been widely studied, their respective roles have been analyzed more in isolation than in tandem. To our knowledge, this is the first investigation that establishes a functional link between these two entities where Scgb1a1, a constituent of the lung surfactant, regulates AM development, gene transcription, and responsiveness to inflammation. In general, AMs developing under Scgb1a1-deficiency were skewed toward an inflammatory phenotype, whereas exogenous supplementation of recombinant SCGB1A1 protein exhibited anti-inflammatory effects on AM activation. While it is imperative to maintain physiologic levels of SCGB1A1 in the lung milieu for an optimal SteadyState respiration, we speculate that a lung locale overexpression of Scgb1a1 via gene therapy or an airway delivery of recombinant protein might be helpful in curbing lung inflammation and cytokine surge in the management of COVID-19 and other inflammatory lung diseases.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in the Gene Expression Omnibus (GEO) repository with accession number GSE148647 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE148647).

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee, Washington University in St. Louis School of Medicine, St. Louis, MO, United States.

AUTHOR CONTRIBUTIONS

MX performed data analysis and manuscript writing. WY performed sequence data analysis and interpretation. XW

reviewed the manuscript. DN designed the study, and performed data analysis and manuscript writing and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.584310/full#supplementary-material

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Liver X Receptors: Regulators of Cholesterol Metabolism, Inflammation, Autoimmunity, and Cancer

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The interplay between cellular stress and immune response can be variable and sometimes contradictory. The mechanisms by which stress-activated pathways regulate the inflammatory response to a pathogen, in autoimmunity or during cancer progression remain unclear in many aspects, despite our recent knowledge of the signalling and transcriptional pathways involved in these diseases. In this context, over the last decade many studies demonstrated that cholesterol metabolism is an important checkpoint for immune homeostasis and cancer progression. Indeed, cholesterol is actively metabolized and can regulate, through its mobilization and/or production of active derivatives, many aspects of immunity and inflammation. Moreover, accumulation of cholesterol has been described in cancer cells, indicating metabolic addiction. The nuclear receptors liver-X-receptors (LXRs) are important regulators of intracellular cholesterol and lipids homeostasis. They have also key regulatory roles in immune response, as they can regulate inflammation, innate and adaptive immunity. Moreover, activation of LXRs has been reported to affect the proliferation and survival of different cancer cell types that show altered metabolic pathways and accumulation of cholesterol. In this minireview we will give an overview of the recent understandings about the mechanisms through which LXRs regulate inflammation, autoimmunity, and cancer, and the therapeutic potential for future treatment of these diseases through modulation of cholesterol metabolism.

Keywords: liver-X-receptor, cholesterol, inflammation, autoimmunity, cancer metabolism, antitumor immune responses

INTRODUCTION

Cholesterol metabolism is deeply linked to different aspects of immunity and inflammation. It is generally thought as an exogenous player on immunity during disease, as in the case of pathologic cholesterol overloading of foam cells in atherosclerosis or more in general in hypercholesterolaemia. However, increasing evidences have recently changed this view by demonstrating that a number of

immune receptors and transcription factors such as Toll-like Receptors (TLRs), C-X-C motif chemokine receptor 2 (CXCR2), Stimulator of IFN genes (STING) and retinoic acid-related orphan receptor-γt (ROR-γt) are profoundly regulated by sterols (1–7). Moreover, regulation of intracellular cholesterol homeostasis controls lymphocyte proliferation and adaptive immune responses (8).

In this review we will discuss recent literature regarding aspects of lipid and cholesterol metabolism in tissues homeostasis, providing to the readers a synthetic overview of the main connections and regulatory interactions between cholesterol cellular metabolism and the activity of LXRs in the context of inflammation, autoimmunity and cancer. LXRs are transcription factors able to regulate specific gene networks implicated in cholesterol and lipid metabolism both in homeostatic and pathological conditions. Moreover, LXRs can mediate anti-inflammatory activities and modulate the immune response, promoting the expression of mediators which have a role in the control of inflammatory disorders and in the response to microbial infection. In a different scenario, accumulation of cholesterol has been also described in many types of cancer cells indicating metabolic addiction. This further expands the possible implications of its dysregulation in cancer progression (9, 10), configuring cholesterol as an important metabolic determinant. LXRs play relevant roles in cancer biology and in anti-tumor immune responses, opening new therapeutic possibilities (**Figure 1**) and (**Table 1**).

LXRS: A LINK BETWEEN LIPID METABOLISM AND IMMUNE RESPONSE

LXRs are transcription factors belonging to the nuclear receptors (NRs) superfamily. They are master regulators of cholesterol and lipid intracellular homeostasis (47). There are two isoforms of LXRs, LXRα (NR1H3), and LXRβ (NR1H2) (48, 49) that share extensive sequence homology [(77% identity in both the DNA binding domain (DBD) and ligand binding domain (LBD)]. Despite this similarity, they have rather different expression patterns (50); indeed, the expression of these NRs depends on the cell type and tissues analyzed, with LXRα more expressed in liver, intestine, adipose tissue and cells of the myelomonocytic lineage, while LXRβ is expressed more ubiquitously (51). Thus, their transcriptional role seems to be determined by their relative expression levels in specific tissues or cells, although important differences have also been identified in vivo between the two isoforms (52).

Different studies *in vitro* and *in vivo* have characterized a number of cholesterol derivatives including oxysterols, oxidized forms of cholesterol and cholesterol precursors (e.g., desmosterol) as LXR activators, able to bind with different affinities to the LXR LBD (47, 53, 54). When these endogenous ligands, or synthetic pharmacological agonists, trigger activation of LXRs, they heterodimerize with retinoid X receptors (RXR) and bind to target gene promoters on LXR-responsive-elements (LXREs), canonical binding sites composed of a repeated 6-mer sequence (5'-AGGTCA-3') separated by four nucleotides (55).

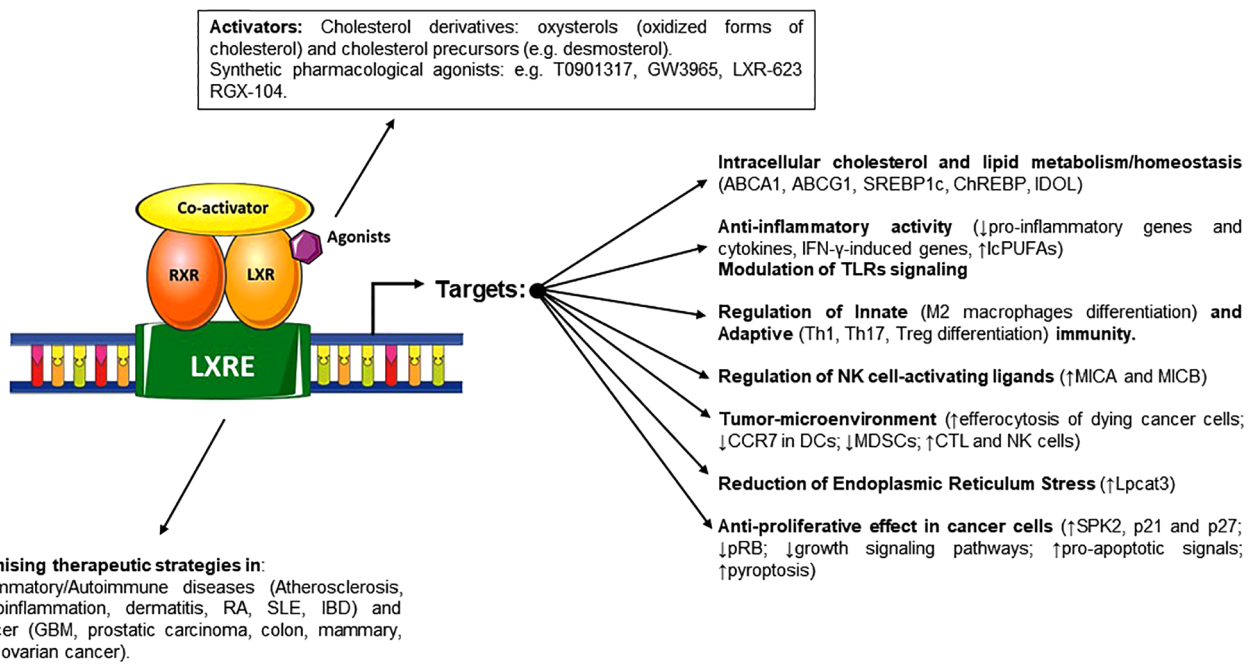

FIGURE 1 | Schematic representation of LXRs activators and the different effects on regulated genes and pathways involved in cholesterol and lipid homeostasis, regulation of immune system and cancer proliferation and progression. When these ligands trigger the activation of LXRs, they heterodimerize with RXR and bind to target gene promoters on LXR-responsive-elements, regulating the transcription and expression of specific target genes.

TABLE 1 | Activities of LXR in inflammation, autoimmunity, and cancer.

LXR, Inflammation, and Autoimmunity		
LXR/cholesterol-mediated responses	**Immune mechanisms**	**Experimental models**
Cholesterol enrichment in macrophage plasma membrane promotes the activity of TLRs (11, 12).	Cholesterol crystals uptake in macrophages activate NLRP3/inflammasome, and the pro-inflammatory cytokines IL-1β and IL-18 (13).	Atherosclerosis susceptibility (14). Atherosclerosis plaque (13).
Upregulation of ABCA1 and ABCG1 on engulfed apoptotic cells (15, 16). Differentiation of M2 macrophages (15, 16).	Prevention immune system anomalous activation (15, 16).	Efferocytosis (15, 16).
Transrepression: LXR binds to the NCoR-SMRT co-repressor preventing signal-dependent clearance from the promoter of pro-inflammatory genes (17, 18).	Transcriptional repression of NF-kB, AP-1, STAT1. Inhibition of primary cytokine production (17, 19, 20). Repression of pro-inflammatory cytokine maturation to their active form (e.g., IL-18) (21).	Inflammation and autoimmune diseases (atherosclerosis, dermatitis, neuroinflammation, lupus and arthritis) (22–23).
Indirect activity on inflammation: induction of lcPUFAs (e.g., omega 3 fatty acid) (24).	Decrease of transactivation mediated by NF-kB of inflammatory genes (24).	Inflammation control.
LXRα maintains BBB integrity and its activation modulates the pro-inflammatory response in astrocytes/microglia (25–27). Activation of LXR leads SREBP-1 to act on IL-17 promoter (28).	Reduced production of the pro-inflammatory cytokines IL-17 and IFN-γ and reduced expression of IL-23R (28, 29).	Demyelinating disease (30).
Activation of LXR by pharmacologic agonists or ligands present in synovial fluid.	Decreased pro-inflammatory cytokines production in CIA models (23, 31–32). Enhanced TLR-driven cytokines and chemokines secretion in RA synovitis (33–34).	Rheumatoid Arthritis (35). CIA models (23, 31–32).
LXR activation mediates anti-inflammatory effects in colon epithelial cells (36). Lack of LXR induces colitis in DSS and TNBS murine models.	LXR activation can suppress Th1 and Th17 polarization *in vitro* and promote the differentiation of gut associated Tregs (37).	Intestinal bowel disease (36, 37).

LXR and Cancer		
LXR-mediated cellular response	**Immune mechanisms**	**Cancer models and LXR activity**
Induction of cholesterol efflux and reduction of its uptake with consequent reduced tumor cell proliferation and survival (38–39). Reduced expression/activity of cell-cycle regulators (SPK2) (40), higher expression of cell-cycle inhibitors (p21, p27) and decreased phospho-RB protein levels (41, 42). Delayed progression of androgen-dependent tumors towards androgen independence (41, 42).	Decrease MDSCs through the induction of ApoE and potentiate activation of cytotoxic lymphocytes (43). Oxysterols impairs DC migration through the inhibition of CCR7 (44). Activation of LXRα in macrophages stimulates phagocytosis of dying cancer cells (45). LXR upregulates the expression of the NKG2D ligands MICA and MICB in MM and improved NK cell cytotoxicity (46).	Glioblastoma multiforme (38–39) Non-small-cell lung carcinoma (NSCLC) (9) Prostatic carcinoma (9) Ovarian cancer (9) Colon cancer (9) Mammary and Skin cancer (9) Multiple Myeloma (46)

The implications of direct LXR-mediated actions and regulation of cholesterol metabolism in the control of inflammatory diseases and cancer progression. This table summarizes the different experimental models and the roles of LXR in these pathologic conditions.

To activate target gene transcription, unliganded LXRs and co-repressors such as nuclear receptor corepressor 1 (NCoR1) and silencing mediator of retinoic acid and thyroid hormone receptor (SMRT), bound to LXREs, have to be displaced from chromatin to allow the binding of transcriptional co-activators [i.e., nuclear receptor co-activator 1 (NCOA1) and activating signal co-integrator 2 (ASC2)], leading to transcription (17).

Recent findings suggest that LXRs may be also recruited *de novo* to the promoter of target genes when triggered by ligands (18).

Once activated, they regulate the expression of genes involved in lipid and glucose metabolism (51, 56). In this context, LXRs are master regulators of cholesterol sensing; they counteract aberrant cellular sterol overload by upregulating the expression of sterol transporters such as the ATP binding cassette (ABC) family members ABCA1 and ABCG1, together with the transcription factors sterol regulatory element-binding protein 1c (SREBP1c) and carbohydrate-response element-binding protein (ChREBP) that regulate critical lipogenic pathways. Moreover, the activation of LXRs also induces the expression of inducible degrader of the LDL-receptor (IDOL), which is able to reduce the expression of low-density lipoprotein receptor (LDLR)s on the cell surface and the uptake of LDL/cholesterol particles (57).

Besides the regulation of cholesterol homeostasis, genetic and pharmacological studies have pointed out the role of LXRs as an important link between lipid metabolism, regulation of immune cell function and inflammation (58). Indeed, these NRs can both promote and repress the expression of specific immune regulatory gene networks (59). As discussed below, LXRs can induce anti-inflammatory activities in macrophages and Dendritic Cells (DCs) and represent a critical link between cholesterol metabolism, proliferation and migration of activated T and B lymphocytes (8, 15, 21, 28, 60–65), thus playing an important role in the control of inflammatory, autoimmune and infectious diseases.

LXRS, CHOLESTEROL, AND INFLAMMATION

Different pathways link inflammation to cholesterol metabolism and LXRs activity. Alteration of cellular cholesterol homeostasis

can both enhance or reduce innate receptor signalling and inflammasome activation. Cholesterol enrichment in macrophage plasma membrane promotes the activity of TLRs as in the case of the TLR4-MD2 and TLR4-CD14 complexes activated in response to lipopolysaccharide (LPS) (11, 12). On the other hand, the activation of the reverse cholesterol transport (RCT) mediated by ABCA1 and ABCG1 transporters limits the formation of cholesterol-enriched lipid rafts in the plasma membrane and/or in the endosomal system. This inhibits MyD88-dependent TLRs trafficking by selective reduction of free cholesterol content and suppresses macrophage inflammatory responses (66). This mechanism has been elegantly demonstrated in mouse models deficient for ABCA1 and ABCG1, shown to accumulate cholesterol in peritoneal macrophages and to exhibit enhanced inflammatory responses to TLR agonists (11). In line with these observations, in a model of atherosclerosis susceptibility, pathogens can interfere with macrophage cholesterol metabolism through inhibition of the LXRs. Here, the activation of TLR-3 and -4 by microbial ligands has been shown to repress the expression of selected target genes including ABCA1 in macrophages, as clearly shown in aortic tissue *in vivo*, with a mechanism connected to reduced cholesterol efflux from macrophages regulated by interferon regulatory factor-3 (IRF3)-mediated inhibition of LXRs on their target promoters (14). Activation of efferocytosis is also associated to the activity of LXRs, which results in the efflux of free cholesterol derived from engulfed apoptotic cells by upregulating ABCA1 and ABCG1 transporters. This mechanism, together with the LXR-mediated alternative (M2) macrophage differentiation, can prevent aberrant activation of the immune system (15, 16). Moreover, the removal of apoptotic cells helps avoiding autoimmunity, as shown in murine models of lupus-like autoimmunity where treatment with LXR agonists ameliorated disease progression (15, 67). In a different context, increased cellular content of cholesterol can trigger cholesterol crystal formation, as shown in atherosclerotic plaques. In this disease model, cholesterol crystals uptake or formation in macrophages has been shown to activate NLR family pyrin domain containing 3 (NLRP3)/inflammasome with the secretion of the pro-inflammatory cytokines interleukin-1β (IL-1β) and IL-18 and to promote the progression of atherogenesis (13).

As shown for other NRs, LXRs are anti-inflammatory; they can inhibit the transcriptional induction of pro-inflammatory genes mediated by critical transcription factors as NF-kB, AP-1 or STAT-1. In this regard, pharmacological activation of LXRs has been shown to ameliorate the severity of the inflammatory response in murine models of atherosclerosis (22), neuroinflammation (30, 68), dermatitis (22, 69), lupus (67) and arthritis (23), inhibiting primary cytokine production. Mechanistically, studies using LXR agonists in macrophages have shown that, depending on the LXR isoform, these NRs can repress the induction of pro-inflammatory genes through a molecular mechanism known as "transrepression". Here, after histone deacetylase-4 (HDAC-4)-dependent conjugation of LXR with small ubiquitin-related modifier (SUMO)-2/3 at specific lysine residues in the LBD, LXR becomes able to bind to the NCoR-SMRT co-repressor, thus preventing signal-dependent clearance from the promoters of pro-inflammatory genes (17, 19). With a different mechanism, LXRs can inhibit Interferon-γ-induced genes in astrocytes, where LXRα and LXRβ are SUMO-conjugated by HDAC4 or by protein inhibitor of activated STAT1 (PIAS1), respectively, and interact with phosphorylated signal transducer and activator of transcription-1 (STAT-1) preventing its binding to gene promoters (20). Furthermore, LXRs activation can repress pro-inflammatory cytokine maturation to their active form as demonstrated for IL-18 and can induce specific endogenous inhibitors (i.e., IL-18BP) (21).

In addition to direct transrepression activity on pro-inflammatory genes, LXRs can mediate other important integrated mechanisms contributing to the control of inflammation. LXRs can induce the synthesis of long-chain polyunsaturated fatty acids (lcPUFAs) such as omega 3 fatty acids. The presence of lcPUFAs can decrease transactivation mediated by NF-kB of inflammatory genes, modifying histone acetylation in their regulatory regions (24). Moreover, lcPUFAs have been shown to increase the production of eicosanoids and selected pro-resolving lipid mediators (70, 71). Interestingly, increased LXRs activity can also induce macrophage polarization toward a more pro-resolving phenotype, directly upregulating the expression of MER proto-oncogene Tyrosine Kinase (MERTK), a receptor that promotes the synthesis of mediators implicated in inflammation resolution (15, 72). Furthermore, as demonstrated in hepatic inflammation models, induction of the polyunsaturated phospholipids (PLs) remodeling enzyme lysophosphatidylcholine acyltransferase 3 (Lpcat3) by LXRs increases the formation of PLs and decreases membrane saturation, counteracting endoplasmic reticulum stress induced by fatty acids in hepatocytes, improving hepatic metabolic stress and inflammation by modulating aberrant c-Src activation (73). An additional consideration that can add a layer of complexity is that LXRs are highly expressed by haematopoietic stem cells (HSCs) and myeloid progenitor cells. In these cells, activation of LXRs can increase the ABCA1/ABCG1/apolipoprotein E (APOE)-mediated cholesterol efflux, which is able to reduce their proliferative responses to IL-3 and GM-CSF, thus indirectly modulating the production of inflammatory cells (74).

LXRS AND AUTOIMMUNITY

The activity of LXRs and cholesterol metabolites is implicated in the control and progression of several autoimmune diseases.

Altered lipid profiles have been associated with poor outcome of multiple sclerosis (MS) (75–80), an autoimmune disease characterized by inflammatory cell infiltrates and demyelination (81, 82). In this regard, obesity, among other environmental factors, has been described as a risk factor for MS in several epidemiological studies (83–86). In animal models of experimental autoimmune encephalomyelitis (EAE), the most common experimental model for human inflammatory demyelinating disease, selected agonists of LXRs (e.g., T0901317) have been shown to improve the severity of central nervous system inflammation (30). In line with this

evidence, the activity of LXRα is indispensable for maintaining blood-brain barrier (BBB) integrity and its immune quiescence. Indeed, in a model of EAE, the specific knockout of LXRα in brain endothelial cells has been shown to increase BBB permeability and endothelial inflammation (25). Morover, activation of LXRs using agonists *in vivo* has been shown to repress the production of the pro-inflammatory cytokine IL-17 (28), together with IFNγ and IL-23R expression (29). Noteworthy, Th17 cell differentiation is modulated by LXRs *via* induction of sterol regulatory element-binding protein 1c (SREBP-1c), which is able to bind to the E-box element on the IL-17 promoter and to physically interact with the aryl hydrocarbon receptor (AHR), inhibiting its transcriptional activity (28). Interestingly, the activity of LXRs mediated by oxysterols can also modulate pro-inflammatory responses in microglial and astrocytes (26, 27) possibly contributing to ameliorate inflammation.

LXRs have also been hypothesized as a possible therapeutic target for rheumatoid arthritis (RA), a chronic autoimmune disorder characterized by infiltration of inflammatory leukocytes in the synovial compartment, which causes cartilage and bone damage (87). Initial conflicting reports have described both protective and promoting actions of LXRs-mediated pathways in murine models of inflammatory arthritis. LXR agonists such as T0901317 or GW3965, attenuated the symptoms, decreasing the production of pro-inflammatory cytokines in different murine collagen-induced arthritis (CIA) models (23, 31, 32, 88) and suppressed inflammatory gene expressions in RA fibroblast-like synoviocytes (35). By contrast, other reports described increased inflammation and cartilage destruction mediated by ligand activated LXRs (TO901317 or GW3965) in CIA models and found that LXR pathways are significantly upregulated in RA synovial macrophages. Interestingly, in these models the activity of both LXR isoforms was required in control mice to induce the progression of inflammation, in respect to single LXRα$^{-/-}$ or LXRβ$^{-/-}$ mice (33), thus implying overlapping and exclusive effects in these models. Moreover, activation of LXRs by ligands present within synovial fluids enhanced TLR-driven cytokine and chemokine secretion, suggesting a novel mechanism that can promote RA synovitis (33, 34, 89).

In a different scenario, both LXR subtypes are expressed in human and murine colon and were described to mediate anti-inflammatory effects in colon epithelial cells (36). Furthermore, in a murine experimental model of intestinal bowel disease (IBD), it was reported that LXR-deficient mice were more susceptible to dextran sodium sulphate (DSS) and 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis. In this regard, the activation of LXRs can suppress Th1 and Th17 polarization *in vitro*, lowering the expression of their secreted pro-inflammatory cytokines and promoting differentiation of protective gut-associated regulatory T cells in mice, where systemic LXR activation was obtained by oral treatment with the LXR agonist GW3965 (37). These data confirmed a dual role of LXR in the control of inflammation by the suppression of pro-inflammatory T cells and the parallel induction of regulatory T cells.

LXRS AS REGULATORS OF LIPID METABOLISM, CANCER PROGRESSION, AND ANTITUMOR IMMUNITY

Genes involved in cholesterol homeostasis are often mutated or dysregulated in cancer cells (10, 90). A higher intracellular cholesterol level due to an enhanced uptake by LDLRs, a decreased efflux by ABC transporters and the upregulation of *de novo* synthesis can sustain the metabolic need for cancer cell proliferation (90–93), and accumulation of cholesterol has been described in many types of tumors (9, 49, 91–93).

Cells usually obtain cholesterol *via* different mechanisms including direct synthesis *via* the transcriptional activity of SREBPs, which promote the transcription of enzymes involved in cholesterol and fatty acid biosynthesis [i.e., 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA) reductase] (94, 95). In this regard, the recent use of HMG-CoA inhibitors (Statins) to block the mevalonate pathway and cholesterol *de novo* biosynthesis showed promising results (96). However, cancer cells often gain selective proliferative advantage by enhancing LDLR-mediated uptake of exogenous cholesterol (38), rendering these therapies often unsuccessful. Perhaps, one of the best characterized examples of cancer cholesterol addiction is glioblastoma multiforme (GBM). The treatment of these cancer cells with LXR agonists induced degradation of LDLR and increased apoptosis in glioblastoma cells expressing mutant epidermal growth factor receptor (EGFR), where tumor growth and survival is strongly dependent on SREBP-1-mediated lipogenesis (38). Moreover, triggering of LXRs increases cellular cholesterol efflux by ABCA1, lowering its levels and inducing severe GBM cell death. Accordingly, LXR agonists (e.g., LXR-623) prolonged survival of mice models bearing GBM, indicating that targeting cholesterol metabolism may be a promising strategy in the treatment of this cancer (39, 97).

Pharmacological studies on various types of cancer models such as prostatic carcinoma, colon, mammary and skin cancer have shown that the activation of LXRs generates anti-proliferative effects due to the destruction of growth signalling pathways and to the activation of pro-apoptotic signals (9). LXRs can reduce the expression/activity of cell-cycle regulators, as shown for S-phase Kinase associated protein (SPK2) in cancer cell lines (40) and, at the same time, are able to induce the expression of cell-cycle inhibitors as demonstrated for p21 and p27 (cyclin-dependent kinase inhibitors) in prostate and ovarian cancer cells, with a concomitant reduction in phospho-RB protein levels (41, 98). Moreover, in mouse models, activation of LXRs delayed the progression of androgen-dependent tumors towards androgen independence (41, 42).

In addition to these direct activities on cancer cell metabolism and survival, in the last few years experimental evidences have highlighted the importance of LXRs in anti-tumor immune responses. In this context, the role of LXR activation is still controversial. Several tumors can produce oxysterols that play an essential role in cholesterol homeostasis by activating LXRs (99, 100), and many of these metabolites can have antiproliferative activity in cancer cells (101). However, oxysterols can also inhibit

the expression of CCR7 on DCs, a chemokine receptor critical for the migration of DCs to tumor-draining lymph nodes (44). Circulating and tumor-derived oxysterols have been also described to recruit pro-tumor neutrophils and to increase neo-angiogenesis and immunosuppression in a CXCR2-dependent and LXR-independent manner (6, 102). This highlights the capability of selected oxysterols to regulate a broad range of pro-tumor activities—depending on the LXR isoform expressed by the tissue from which tumor cells originate and on the surrounding microenvironment. Moreover, in breast cancer, 27-hydroxycholesterol has been shown to act as an estrogen receptor agonist inducing tumor growth and metastasis (103). On the other hand, LXRs were reported to control cancer cell growth by inducing LXRβ-dependent pyroptosis of cancer cells and the activation of LXRα in macrophages, promoted the phagocytosis of dying cancer cells (45). More recently, in different mouse cancer models treated with specific LXRs agonists (i.e., RGX-104), has been observed a slower tumor growth which correlated with a decreased expansion of myeloid derived suppressor cells (MDSCs); these data were also validated in cancer patients, in a multicentre dose escalation phase 1 trial (43). Moreover, RGX-104 also partially abrogated the immunosuppressive effects of radiotherapy in a murine model of Non-Small-Cell Lung Carcinoma (NSCLC) (104). Mechanistically, the induction of ApoE, a transcriptional target of LXR, can induce MDSC depletion by triggering the low-density lipoprotein receptor-related 8 (LRP8) receptor on these cells, and potentiate activation of cytotoxic lymphocytes. In these settings, activation of LXRs together with PD-1 inhibition, improved the efficacy of cytotoxic T lymphocyte (CTL) and natural killer (NK) cells from cancer patients (43). In a different scenario, LXRs activation could upregulate MHC class I polypeptide-related sequence-A (MICA) and MICB expression in multiple myeloma cells, two ligands of the NK cell-activating receptor NK group 2 member D (NKG2D), by enhancing MICA promoter activity and inhibiting MICB degradation in lysosomes, thus improving NK cell-cytotoxicity (46).

CONCLUSION

The implication of cholesterol metabolism in the control of inflammatory diseases and cancer progression is the object of an interesting and controversial debate. Our increasing knowledge of the different roles mediated by LXRs in lipid homeostasis supports the idea that lipid metabolism and inflammation are closely connected and that their crosstalk is crucial for the evolution of different inflammatory diseases and, more in general, in the

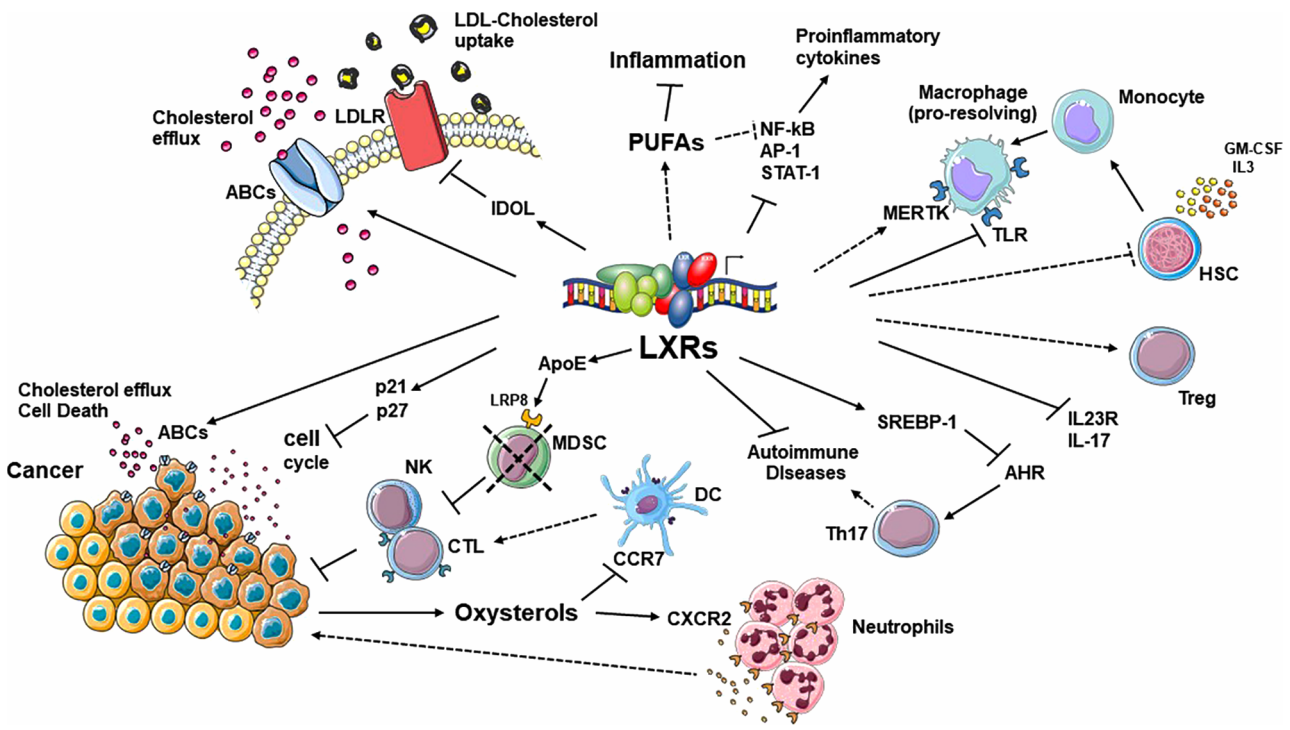

FIGURE 2 | Summary of the most relevant pathways regulated by LXRs in the context of cholesterol homeostasis, inflammation, autoimmunity and tumor progression. These NRs act in a cell-, tissue- and context-dependent manner. In addition, many LXR-dependent and -independent pleiotropic effects mediated by oxysterols, produced in inflamed or tumoral microenvironments, add an additional level of complexity to these regulatory pathways. ABC, ATP binding cassette; AHR, aryl hydrocarbon receptor; APOE, apolipoprotein E; CTL, cytotoxic T lymphocyte; DC, dendritic Cell; DSS, IDOL, inducible degrader of the LDL-receptor; IRF3, interferon regulatory factor 3; LBD, ligand binding domain; PUFA, long-chain polyunsaturated fatty acid; LDLR, low-density lipoprotein receptor; LRP8, low-density lipoprotein receptor-related 8; MDSC, myeloid derived suppressor cells; MERTK, MER proto-oncogene tyrosine kinase; NK, natural killer; SREBP-1, sterol regulatory element-binding protein 1; TLR, Toll-like receptor.

regulation of the immune response. The involvement of specific pathways regulated by LXRs during tumor progression and the possibility to pharmacologically modulate LXR activity, as an additional weapon for cancer therapy and for immunotherapy, have opened new therapeutic possibilities in this context. However, the activities of these NRs are often cell-, tissue-, and context-dependent, which makes it difficult to fully characterize their effects in disease conditions and to optimize specific therapeutic interventions in inflammatory disorders or in cancer therapy. In addition, many LXR-dependent and -independent pleiotropic effects of oxysterols produced in inflamed or tumoral microenvironments have been described in recent years, adding additional levels of complexity to these regulatory pathways (**Figure 2**). Another important issue is whether synthetic ligands that uncouple the anti-inflammatory and anti-cancer effects of LXRs from their role in cholesterol homeostasis can be developed. This is particularly important also in the context of different metabolic disorders with increased risk of developing diseases such as type 2 diabetes or cardiovascular disease, where beneficial effects of LXRs have been described (105). At the moment, different synthetic LXRs agonists have been optimized; however, their clinical application is limited by undesirable hyperlipidemic effects and other adverse side effects encountered in the central nervous system (106–111). The future development of isoform- and/or tissue-specific LXR modulators and the possibility to target LXR-interacting co-factors involved in LXR transcriptional activation will open new therapeutical possibilities for treating these diseases.

AUTHOR CONTRIBUTIONS

MB and SP made substantial contributions to conception and design of the review. MC organized the study, together with AS, contributed to revision, and approved the submitted version. All authors contributed to the article and approved the submitted version.

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Granzyme B in Inflammatory Diseases: Apoptosis, Inflammation, Extracellular Matrix Remodeling, Epithelial-to-Mesenchymal Transition and Fibrosis

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Inflammation is strictly interconnected to anti-inflammatory mechanisms to maintain tissue homeostasis. The disruption of immune homeostasis can lead to acute and chronic inflammatory diseases, as cardiovascular, pulmonary, metabolic diseases and cancer. The knowledge of the mechanisms involved in the development and progression of these pathological conditions is important to find effective therapies. Granzyme B (GrB) is a serine protease produced by a variety of immune, non-immune and tumor cells. Apoptotic intracellular and multiple extracellular functions of GrB have been recently identified. Its capability of cleaving extracellular matrix (ECM) components, cytokines, cell receptors and clotting proteins, revealed GrB as a potential multifunctional pro-inflammatory molecule with the capability of contributing to the pathogenesis of different inflammatory conditions, including inflammaging, acute and chronic inflammatory diseases and cancer. Here we give an overview of recent data concerning GrB activity on multiple targets, potentially allowing this enzyme to regulate a wide range of crucial biological processes that play a role in the development, progression and/or severity of inflammatory diseases. We focus our attention on the promotion by GrB of perforin-dependent and perforin-independent (anoikis) apoptosis, inflammation derived by the activation of some cytokines belonging to the IL-1 cytokine family, ECM remodeling, epithelial-to-mesenchymal transition (EMT) and fibrosis. A greater comprehension of the pathophysiological consequences of GrB-mediated multiple activities may favor the design of new therapies aim to inhibit different inflammatory pathological conditions such as inflammaging and age-related diseases, EMT and organ fibrosis.

Keywords: granzyme B, inflammatory cytokines, inflammaging, extracellular matrix remodeling, anoikis, apoptosis, epithelial-to-mesenchymal transition, fibrosis

INTRODUCTION

Inflammation is a physiological response to infections or tissue injury and is essential for survival, having beneficial effects towards the neutralization of dangerous or harmful agents. Inflammation is strictly interconnected with anti-inflammatory mechanisms, which control and resolve the inflammatory process to maintain immune homeostasis (1). Under some circumstances, this immune homeostasis is disrupted and inflammation becomes excessive and/or persistent, leading to the development of inflammatory diseases (1). In this context, aging can be characterized by an uncontrolled and unresolved chronic, low-grade inflammation, the so-called "inflamm-aging", which can lead to inflammatory age-related diseases, as cardiovascular, pulmonary, metabolic diseases (as type 2 diabetes, T2D) and cancer (2). Multiple factors underlie the pathogenesis of inflammatory diseases and can lead to tissue fibrosis and organ dysfunction, associated with high morbidity and mortality (3). Therefore, the knowledge of mechanisms involved in the development and/or progression of these pathological conditions is important to find specific and effective therapies.

Granzyme B (GrB) is a serine protease traditionally known for its perforin-dependent pro-apoptotic function underlying the capability of cytotoxic immune cells, as cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells, to kill tumor and virus-infected target cells (4–7). GrB expression has been recently demonstrated also in non-tumor or tumor immune and non-immune cells (8). Indeed, GrB is produced and secreted by immune cells, like T and B cell subpopulations, monocyte/macrophages, mast cells, and basophils (8–13), by non-immune cells, like vascular smooth muscle cells (V-SMCs), pneumocytes, keratinocytes, and chondrocytes (12, 14–16), as well as by tumor cells, like leukemia cells and breast, urothelial, prostate, pancreatic and colorectal cancer cells (17–21) (**Table 1**). GrB not only exerts a perforin-dependent intracellular activity, but also an extracellular perforin-independent function, consisting in the cleavage of multiple extracellular substrates, as extracellular matrix (ECM) components, cytokines, cell receptors, angiogenic and clotting proteins (28, 49, 50). Hence, the pathophysiological function of GrB has been redefined and a putative role for GrB in the pathogenesis of inflammatory and age-related diseases has emerged (8, 29) (**Table 1**).

In this review, we discuss data concerning GrB activity on multiple targets involved in inflammation, potentially allowing this enzyme to regulate a wide range of crucial processes that play a role in inflammatory disease development, progression and severity. We focus our attention on the possible impact of GrB on inflammatory events leading to tissue fibrosis in both acute and age-related inflammatory diseases.

GRANZYME B AS A MULTI-TARGETED PRO-INFLAMMATORY MOLECULE IN INFLAMMATORY DISEASES

The recent discovery of multiple intracellular and extracellular substrates for GrB has revealed this protease as a potential multifunctional pro-inflammatory molecule, contributing to the pathogenesis of multiple pathological inflammatory conditions.

Elevated extracellular GrB levels were found in biological fluids, as in plasma from patients with acute myocardial infarction (36), atherosclerosis (37), obesity and T2D (51, 52), in broncho-alveolar lavage (BAL) in chronic obstructive pulmonary disease (COPD), pneumonia, and asthma (8), and in the synovial fluid in rheumatoid arthritis (53).

Elevated GrB levels were also found in inflamed tissues, including V-SMCs and atherosclerotic plaque in cardiovascular diseases (14), CTLs, pneumocytes and alveolar macrophages in pulmonary diseases (12, 27), adipose tissue-T cells in obesity (33) and in skin diseases (49). Moreover, according to a putative contribution of GrB in inflammaging, increased GrB expression levels were found in the elderly affected by obesity, cardiovascular and skin diseases (29, 49, 54, 55).

GrB extracellular substrates include cytokines and ECM components (29, 31, 50). The potential pathophysiological consequences of their cleavage constitute the basis to envisage a crucial pro-inflammatory role for GrB in the pathogenesis of inflammatory diseases (29).

GrB has the ability to process and activate pro-inflammatory, pro-fibrotic and aging mediators belonging to the IL-1 cytokine

TABLE 1 | GrB in Inflammatory Diseases: GrB producing cells, GrB cellular and molecular targets, GrB-associated organ-specific diseases.

GrB producing cells	GrB targets	GrB-associated organ-specific diseases
◆ Cytotoxic lymphocytes (4, 7) (CTL, NK cells) ◆ Non-cytotoxic immune cells (8–13) (monocytes/macrophages, B, T, granulocytes, mast cells, dendritic cells) ◆Non-immune cells (12, 14–16) (V-SMC, pneumocytes, keratinocytes, chondrocytes) ◆Tumor cells (17–21) (breast, urothelial, pancreatic, colorectal, prostate, leukemia)	◆ Normal Cells: -smooth muscle cells (14) -endothelial cells (14) ◆ Extracellular Molecules: -ECM proteins (28, 29) (fibrinogen, fibronectin, laminin, smooth muscle cell matrix, VE-cadherin, vitronectin, ZO-1) -ECM proteoglycans (30) (decorin, biglycan, soluble β-glycan) -IL-1 family cytokines (29, 31) (IL-1α, IL-18)	◆ Lung (22–27): COPD, RSV infection, pneumonia, IPF ◆ Heart (32): cardiac fibrosis ◆ Adipose Tissue (33–35): adipose tissue fibrosis in metabolic diseases ◆ Blood vessels (8, 14, 36–48): atherosclerosis ◆ Skin (29, 49): skin fibrosis ◆ Breast, urothelial, pancreatic, colorectal carcinomas (18–21): invasion and EMT

CTL, cytotoxic T lymphocytes; NK, natural killer; V-SMC, vascular smooth muscle cells; ECM, extracellular matrix; VE, vascular endothelial; ZO-1, zonula occludens protein-1; IL, interleukin; COPD, chronic obstructive pulmonary disease; RSV, respiratory syncytial virus; IPF, idiopathic pulmonary fibrosis; EMT, epithelial-to-mesenchymal transition.

family (31, 56). Indeed, GrB processes IL-18 from its inactive to its active form and IL-1α into a significantly more potent pro-inflammatory fragment. IL-1α enhances persistent inflammation and stimulates fibroblasts to produce more interstitial collagenase and ECM remodeling, regulating normal and aberrant tissue repair (56, 57). IL-1α fragments, similar to those produced by GrB, were found in BAL in human airway inflammatory diseases, as COPD, cystic fibrosis and bronchiectasis (8), while GrB activity on IL-1α was demonstrated *in vivo* in GrB knockout mice (29), strongly suggesting that this activity also exists *in vivo*.

GrB has also the ability to degrade several ECM components, including proteins, as fibronectin, vitronectin, laminin, SMC matrix, VE-cadherin, and fibrillin-1, as well as proteoglycan, as biglycan and decorin, indicating GrB as a crucial player in ECM remodeling (28–30). Indeed, ECM undergoes remodeling, that is degradation by proteases and renewal and repair by fibroblasts, thus regulating tissue homeostasis and acting on tissue healing. ECM components assist cell attachment, ligate receptors and store growth factors, regulating cell survival, proliferation, differentiation, and migration. Therefore, abnormal ECM remodeling can result in cell detachment-dependent apoptosis and alterations in cell proliferation, differentiation and migration, as observed in several inflammatory conditions, such as cardiovascular, pulmonary and metabolic diseases, obesity, and cancer progression and metastasis (58, 59). Hence, GrB capability of targeting multiple ECM components, might allow this enzyme to regulate several fundamental biological processes involved in the development and/or progression of inflammatory diseases.

Thus, considering the extracellular and intracellular GrB function and the context in which GrB is produced, this molecule has the potential to contribute to the pathogenesis of non-neoplastic and neoplastic inflammatory diseases through a multitude of mechanisms ranging from the induction of perforin-dependent and/or –independent apoptosis and the promotion of epithelial-to-mesenchymal transition (EMT) and/or fibrosis, as illustrated below (**Figure 1**).

GRANZYME B AND PERFORIN-DEPENDENT AND/OR PERFORIN-INDEPENDENT APOPTOSIS IN INFLAMMATORY DISEASES

Apoptosis promotes tissue injury during inflammation and is involved in the pathogenesis of acute and chronic inflammatory

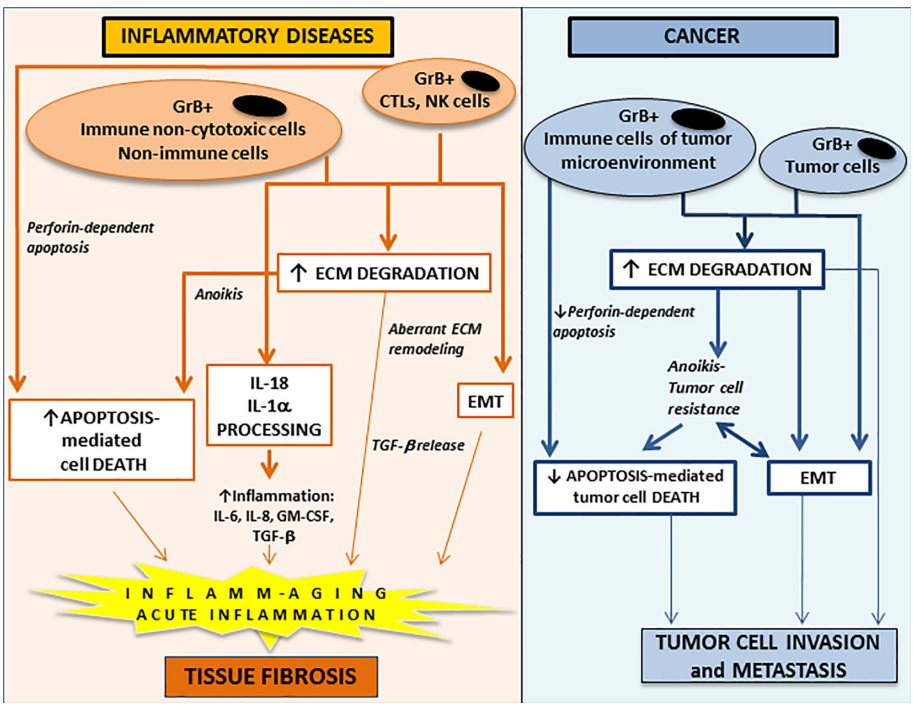

FIGURE 1 | The potential contribution of extracellular and intracellular GrB functions to the development and/or the progression of acute and chronic inflammatory diseases (*left panel*) and to cancer invasion and metastasis (*right panel*). GrB is a multifunctional pro-inflammatory molecule regulating a wide range of inflammatory events. GrB produced by perforin-expressing immune cells (CTL and NK cells) can induce perforin-dependent cell apoptosis, while GrB produced by perforin-deficient immune (e.g. non-cytotoxic T and B cell subpopulations, monocyte/macrophages/myeloid-derived suppressor cells, mast cells, basophils, neutrophils), non-immune (e.g. vascular smooth muscle cells, pneumocytes) and tumor (e.g. breast, urothelial, prostate, pancreatic, colorectal) cells can induce anoikis (anchorage-dependent cell death). Extracellular GrB can promote activation of pro-inflammatory cytokines (IL-18 and IL-1α), ECM degradation/remodeling, pathologic EMT and tissue fibrosis. GrB, granzyme B; CTL, cytotoxic T lymphocytes; NK, natural killer; ECM, extracellular matrix; EMT, epithelial-to-mesenchymal transition; IL, interleukin; TGF-β, transforming growth factor-β.

diseases, as cardiovascular and pulmonary diseases and metabolic syndrome (60–62). The capability of immune and non-immune cell-derived GrB of inducing apoptosis makes GrB a potential important player of apoptosis-mediated tissue damage in inflammation. GrB can induce two kinds of apoptotic cell death, the intracellular perforin-dependent apoptosis (7) and the extracellular perforin-independent apoptosis, named anoikis (63). Anoikis is due to the detachment of cells from ECM and from neighboring cells, playing a role in preventing inappropriate cell translocation and attachment, and assisting appropriate tissue renewal (62). In cancer, anoikis resistance characterizes cancer cell anchorage-independent growth and EMT, contributing to cancer cell invasion and metastasis (62, 64, 65). In inflammatory diseases, as cardiovascular (66), pulmonary (67) and skin (49) diseases, and diabetes-related cardiovascular complications and retinopathy (62), aberrant anoikis is involved in excessive cell death and tissue injury.

A role for GrB-mediated apoptosis -either perforin-dependent apoptosis or anoikis- has been reported in inflammatory pulmonary diseases, including age-related diseases, as COPD (23, 38), and acute severe lung inflammatory diseases, as respiratory syncytial virus (RSV) pulmonary infections (24, 25). In COPD patients, GrB was identified in type II pneumocytes, alveolar macrophages and in bronchial and alveolar wall-infiltrating CTLs, suggesting a role for GrB in bronchial and alveolar cell apoptosis (8, 12, 22, 38). Of note, in COPD, increased GrB- and perforin-expressing CTLs were found in BAL and blood, and GrB-expressing T cells in BAL positively correlated with bronchial epithelial cell apoptosis (22, 23, 38). In spite of these findings, the evidence of a causative role for GrB-mediated apoptosis in the pathogenesis of COPD is lacking. *In vivo* animal studies are made difficult, because of the lack of appropriate mouse COPD models. A role for GrB has been proposed also in acute pulmonary pathologies. There is evidence of high GrB expression by CD8+T, CD4+T, and NK cells in human RSV-induced acute severe lung injury (24), suggesting a role for GrB in amplifying pro-apoptotic and pro-inflammatory activities. Supporting this hypothesis, Bem et al. (25) showed GrB contribution to acute lung injury in pneumovirus-infected mice; GrB deficiency in pneumovirus-infected mice significantly delayed clinical response to fatal pneumovirus infection and this effect was associated with delayed neutrophil recruitment, decreased caspase-3 activation and reduced lung permeability, suggesting a role for GrB in acute disease progression due to alveolar injury.

In the last years, a putative role for GrB-mediated apoptosis in atherosclerosis is also emerged in both the elderly and insulin resistant young individuals (8, 26, 39–41). Elevated plasma GrB levels were found in patients with myocardial infarction (36, 42) and unstable carotid plaques associated with increased cerebrovascular events (37). GrB was absent in normal vessels and its expression appeared during atherosclerosis; studies on mild and advanced atherosclerotic human coronary arteries showed higher GrB expression in V-SMCs, CTLs and macrophages in advanced lesions (11, 14). GrB expression co-localized with V-SMCs and macrophages undergoing apoptosis, suggesting that GrB may mediate apoptosis in these cells (11, 14). Furthermore, peripheral blood mononuclear cells (PBMCs) from patients with unstable angina produced higher GrB levels than PBMCs from patients with stable angina, and PBMC-derived conditioned media induced apoptosis in cultured endothelial cells, supporting a possible role for GrB in atherosclerosis severity, possibly inducing vascular apoptosis in unstable angina (43). Finally, the proteinase inhibitor-9, the GrB endogenous inhibitor, was reduced in unstable atherosclerotic lesions compared to stable lesions (44), according to the hypothesis of a role for GrB in plaque instability and suggesting that GrB activity in atherosclerosis may be regulated by an imbalance between GrB and its inhibitor. Although these findings do not allow to definitively establish an *in vivo* role for GrB in the induction of apoptosis in atherosclerotic plaque instability and rupture, *in vitro* and animal studies support this hypothesis (14, 26, 45, 46). Indeed, GrB mediates anoikis of cultured human coronary artery SMCs and endothelial cells (14). Moreover, in angiotensin II-treated apolipoprotein E (ApoE), GrB deficiency was associated with decreased abdominal aortic aneurysms and increased survival (because of rare aneurism rupture) compared to perforin-deficient or control mice (26, 45). Finally, a role for NK and NKT cells in the promotion of atherosclerosis has also been proposed (47, 48). Increased atherosclerosis was observed when NK cells were transferred into ApoE(-/-)Rag2(-/-)IL2rγ(-/-) mice, whereas decreased atherosclerotic lesions were found in NK cell depleted ApoE(-/-) or when GrB/perforin-deficient NK cells were transferred (47). Transfer of CD4+NKT cells into T-, B- and NK-cell-deficient ApoE mice augmented aortic root atherosclerosis; this effect reversed when GrB/perforin-deficient NKT cells were transferred (48).

GRANZYME B: EPITHELIAL-TO-MESENCHYMAL TRANSITION AND FIBROSIS IN INFLAMMATORY DISEASES

Inflammation, characterized by excessive apoptosis and abnormal ECM remodeling, can lead to tissue fibrosis, which impairs the affected organ's function (3). Fibrosis is triggered by inflammatory cytokines and growth factors signaling abnormal ECM regulation; this leads to an imbalance between ECM degradation by proteases and excessive ECM deposition by different cells, mainly myofibroblast (3) derived by mesenchymal cells and by epithelial cells undergoing EMT (EMT-derived myofibroblasts) (68). Noteworthy, fibrosis and EMT share one of their major inducer that is transforming growth factor-β (TGF-β) (69).

Recent studies have proposed a role for GrB in heart, lung, adipose tissue and skin fibrosis (27, 29, 32, 49, 61, 69).

Elevated GrB expression was detected in human and murine fibrotic hearts (32). Moreover, a perforin-independent role for GrB in the pathogenesis of cardiac fibrosis was suggested *in vivo*, showing that GrB deficiency in mice protected against angiotensin II-induced

cardiac fibrosis, reducing microhemorrhage, inflammation, and fibroblast accumulation (32).

In COPD, GrB–expressing monocytes and granulocytes were identified, and CD8+T infiltrating cells and apoptosis increased in airway epithelial cells, while soluble GrB levels and GrB-expressing T cells increased in BAL, suggesting that GrB upregulation in CD8+ and CD8- cells may be involved in small airway wall remodeling (27).

In obesity, increased CD8+T cells and GrB expression were found in adipose tissue *in vivo*, suggesting a role for GrB in adipose tissue fibrosis (33, 61). Furthermore, CD8+T-cell-depletion in overfed mice improved obesity-induced insulin resistance and decreased adipose tissue pro-inflammatory macrophages; these effects were reversed when mice were reconstituted with CD8+T cells (34). These findings suggest that, in obesity, adipose tissue CD8+T cells induce the recruitment of macrophages and that both may induce adipose tissue dysfunction and insulin resistance.

A role for GrB has also been indicated in skin fibrosis, as extensively discuss elsewhere (29, 49).

The mechanisms by which GrB induces fibrosis have not been completely elucidated and multiple GrB-mediated activities have been proposed.

GrB can cleave the ECM proteoglycan decorin, a potent anti-fibrotic (30) and a pro-autophagic (35) molecule. Indeed, decorin, by attaching to cell surface receptor and ECM molecules, regulates signal transduction pathways controlling genes involved in ECM organization (30). In addition, by attaching to cell receptors, decorin promotes autophagy in endothelial cells leading to inhibition of angiogenesis (35). Therefore, decorin cleavage by GrB might underlie aberrant ECM and/or vascular remodeling, involved in the initiation and/or the progression of various fibroproliferative disorders. Note also that decreased autophagy is involved in the pathogenesis of inflammaging, fibrotic diseases and tumors. Studies have shown a reduction of decorin in different fibrotic organs, as in cardiac fibrosis following myocardial infarction and in acute exacerbation-idiopathic pulmonary fibrosis (IPF) (70–72). Animal experiments in decorin-null mice with myocardial infarction (70) or in hamster and mice models of lung fibrosis (73–75) showed both decorin requirement for proper fibrotic evolution of tissue injury and the potential therapeutic anti-fibrotic effect of decorin administration. Evidence also exists for a role of decorin in maintaining glucose tolerance in obesity (76).

Moreover, GrB, cleaving decorin and other ECM substrates as biglycan, beta-glycan and fibrillin-1 which act as reservoir of cytokines and growth factors as TGF-β, induces the release of active TGF-β, a key regulator of fibrosis (30, 69). Therefore, the aberrant release of sequestered TGF-β by GrB-mediated cleavage of ECM components represents another potential mechanism by which GrB may contribute to fibrosis.

Noteworthy, GrB (18, 21, 77), as some other granzymes (78–80), has been recently proposed as promoters of EMT, an important process linked the stimulation of the three following events: 1) tissue and organ formation during embryogenesis; 2) tissue and organ physiologic repair and pathologic fibrosis; 3) tumor cell invasion and metastasis (81). EMT is a process in which epithelial cells lose E-cadherin-mediated cell-cell adhesion and acquire some mesenchymal features, as N-cadherin expression and the capability of invasion, migration, and production of ECM. Inflammatory molecules, mainly TGF-β, trigger intracellular signaling cascades, activating EMT-transcription factors like Snail, ZEB, and TWIST (81). EMT is involved in multiple organ fibrosis, as those occurring in cardiovascular and pulmonary (COPD and IPF) diseases (81–88). Now interestingly, EMT-derived fibrosis has been also called to possibly account to pulmonary fibrosis in SARS-CoV-2 infection (89), suggesting a possible contribution of GrB in the severe pulmonary damage in COVID-19 (89, 90). A possible role for GrB in EMT promotion has emerged in human tumor models (18, 21, 77). Enzymatically active GrB was expressed, in absence of perforin, by tumor cells *in vitro* and in tissues (*ex vivo*) (17–21). Although GrB in cancer tissues is widely used as activation marker for cytotoxic lymphocytes, and lymphocyte-derived GrB-positive tumor immunostaining is associated with a favorable clinical outcome in a large spectrum of cancers, in some cases, GrB expression in tumors correlates to the severity of the disease, poor prognosis and therapy resistance (91–96). It has been documented GrB expression by urothelial carcinoma cells in primary urothelial cancer tissues and its expression was associated to EMT (analyzed by Snail-1, E- and N-cadherin expression) (18). Significantly, GrB expression was concentrated in urothelial neoplastic cells undergoing EMT at the cancer invasion front, suggesting that the expression of GrB and EMT molecules might be functionally related (18). A further support to the hypothesis of considering GrB as an EMT promoter, derives from the association that existed between GrB expression in tumor tissues and the pathological tumor spreading, in particular, the increasing invasiveness status of urothelial carcinomas (18). In addition, *in vitro* experiments of loss and gain of GrB function performed in CRC (including also CRC patient-derived Cancer Stem Cells), bladder and pancreatic carcinoma cells showed that GrB deficiency was associated to the loss of the EMT phenotype and the inhibition of invasion through matrigel, further supporting a role for GrB in tumor EMT promotion and cancer cell invasion (18, 21). Finally, GrB function in EMT was further supported by data indicating a contribution of GrB in the induction of TGF-β1-driven EMT in CRC cells (21). Indeed, TGF-β1 enhanced GrB expression while inducing EMT in CRC cells, whereas GrB depletion resulted in the inhibition of TGF-β1-driven EMT (21). However, research is needed to identify GrB targets involved in the mechanisms underlying EMT modulation by GrB. It should also be taken into account the possible regulation of GrB activity and function by the GrB-bound proteoglycan serglycin, considering that its intracellular activity consists in the promotion of secretory granule maturation and GrB storage, while its extracellular activity is implicated in the regulation of tumorigenesis, driving inflammation, EMT and tumor progression (97). Lastly, the examination of GrB expression in a large number of cancers in relation to the clinical outcome is needed, together with the evaluation of EMT in murine tumor and non-tumor models.

CONCLUSION

GrB is emerging as a multifunctional pro-inflammatory protease, acting with tissue and context dependence on multiple targets, thus representing a putative powerful regulator of a wide range of crucial processes involved in the pathogenicity and/or in the severity of inflammatory diseases, either acute or age-related. The major limitation of this assumption is the paucity of *in vivo* direct evidence for the multiple GrB pro-inflammatory activities. It should be considered that the *in vivo* function of human GrB is a challenging problem and difficult to deal with, in that, although few mechanistic animal studies connecting clinical observations with *in vitro* data exist, animal experiments might generate false interspecies functions of GrB, because of GrB interspecies structural and functional diversity (98–100). Therefore, further research is required to explore the multiple activities of GrB potentially occurring in the inflammatory events underlying acute and chronic inflammatory diseases.

A greater comprehension of GrB function may favor the design of new therapies aimed to inhibit and regulate GrB pro-inflammatory activities, counteracting excessive inflammation, fibrosis and abnormal EMT-derived processes. Current research is considering the development and the use of pharmacological GrB inhibitors as potential therapeutic options for the prevention and/or treatment of GrB-associated inflammatory pathological conditions (101–106). Progress in this field might be even more urgent if we consider the possibility to develop therapies that have an impact on inflammaging and chronic age-related diseases, as well as on excessive acute inflammatory reactions, as they occur in COVID-19, especially in aged individuals tending to excessive inflammatory responses resulting in lethal lung damage (89, 107, 108).

AUTHOR CONTRIBUTIONS

FV made substantial contributions to conception and design of the review. IB, FAC, and MGC contributed to the manuscript revision, read, and approved the submitted version. All authors contributed to the article and approved the submitted version.

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Granzyme B Expression in Visceral Adipose Tissue Associates With Local Inflammation and Glyco-Metabolic Alterations in Obesity

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Granzyme B (GrB) is a serine protease produced by immune and non-immune cells, able to promote multiple processes, like apoptosis, inflammation, extracellular matrix remodeling and fibrosis. GrB expression in visceral adipose tissue (VAT) was associated with tissue damage, local inflammation and insulin resistance in obesity murine model, but there is no data in humans. Aim of this study was to explore the expression of GrB in VAT from obese subjects in relation to adipose tissue injury, inflammation, metabolic alterations and GrB circulating levels. For this purpose, 85 obese individuals undergoing bariatric surgery and 35 healthy subjects (as control) were recruited at Sapienza University, Rome, Italy. Study participants underwent clinical work-up and routine biochemistry. mRNA expression of GrB in VAT and of a panel of VAT inflammatory markers was analyzed by real-time PCR. Serum GrB levels were measured by Elisa Affymetrix EBIO. We observed that 80% of obese patients expressed GrB mRNA in VAT, and GrB VAT expression was associated with the presence of local inflammation and glucose homeostasis alterations. Moreover, GrB serum levels, which were higher in obese subjects compared to non-obese healthy individuals, were associated with GrB expression in VAT and glyco-metabolic impairment. Our data show, for the first time in humans, that obese subjects with "sick" fat and altered glucose tolerance exhibit GrB expression in VAT, and suggest that GrB might contribute to obesity-related VAT inflammatory remodeling and glucose homeostasis dysregulation. Moreover, increased circulating GrB levels might represent a possible peripheral marker of VAT dysfunction in metabolic diseases.

Keywords: Granzyme B, visceral adipose tissue, inflammation, glyco-metabolic alterations, obesity

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INTRODUCTION

Obesity represents a global health problem and its prevalence is rapidly rising (1). The excessive accumulation of body fat and the consequent adipose tissue (AT) dysfunction is considered a crucial risk factor for the development of metabolic diseases (2), as type 2 diabetes (T2D) (3, 4).

Visceral AT (VAT) plays a major role in regulating systemic energy homeostasis, and in condition of obesity it expands and rearranges its structure. Essentially, in response to an excessive nutritional status and to the need for surplus lipid accumulation, the number and size of the adipocytes increases (5, 6) and angiogenesis cannot fulfill the oxygen requirement provoking hypoxia, an important metabolic stressor (5, 7, 8). Then, AT produces cytokine and chemokines, promoting tissue infiltration by immune cells, as cytotoxic lymphocytes (cytotoxic T lymphocytes –CTLs- and natural killer –NK- cells) and pro-inflammatory macrophages (9, 10). In this inflammatory context, adipocytes undergo apoptosis and extracellular matrix (ECM) endures degradation; thus, the instability of protein composition and the dynamics of ECM proteins lead to VAT remodeling and functional impairment (11, 12). Hence, this "sick" VAT loses its storage capacity releasing free fatty acids in the bloodstream and secretes several bioactive molecules that support local and systemic inflammation, leading to insulin resistance (13–16).

Granzyme B (GrB) is a serine protease expressed by several immune and non-immune cells, including CTLs, NK cells, B lymphocytes and macrophages (17–20). GrB exerts multiple activities, including apoptosis, ECM component cleavage and inflammation (21–23). Increased expression of GrB was observed in many human chronic inflammatory diseases (23), including atherosclerosis and cardiovascular diseases (CVD) (24–29). In addition, high GrB circulating levels were described in human obesity-related dysmetabolic conditions (30, 31), including T2D (32). In obese mice high levels of GrB were described in VAT, where they were associated with local inflammation and damage, as well as with alterations of insulin signaling (33). Despite these findings are suggestive of a potential role of GrB in the inflammatory and the reactive processes occurring in VAT during obesity, currently there is no data on GrB in human VAT.

Aims of this study were to evaluate the expression of GrB in VAT from obese subjects and to explore its relationship with local inflammation, metabolic alterations and GrB circulating levels.

MATERIALS AND METHODS

Study Population

We enrolled 85 consecutive obese subjects with or without T2D and/or metabolic syndrome (MS), referring to the Diabetes and Endocrinology outpatient clinics at Sapienza University of Rome, Italy, for pre-operative evaluations before undergoing bariatric surgery. T2D was diagnosed according to the American Diabetes Association 2009 criteria (34) and the presence of MS was defined according to the modified National Cholesterol Education Program Adult Treatment Panel III criteria (35). Inclusion criteria were as follows: (a) male and female aged between 25 to 65 years old; (b) Caucasian ethnicity; (c) clinical indication to sleeve gastrectomy; (d) full acceptance of informed consent to the study. Exclusion criteria were: (a) severe psychiatric illness; (b) heart failure ≥3 according to the New York Heart Association (NYHA) functional classification; (c) dialysis and/or end-stage renal disease; (d) absence of chronic terminal kidney disease or hepatic failure; (e) absence of active cancer of any type.

For the evaluation of circulating levels of GrB, we also recruited, as control group, 35 non-obese healthy subjects comparable for sex and age with the obese population.

This study was reviewed and approved by the Ethics Committee of Sapienza University of Rome and conducted in conformance with the Helsinki Declaration. A written informed consent was obtained from all subjects before participating to the study.

Clinical Work Up and Laboratory Determinations

The entire study population underwent medical history collection, physical examination and anthropometric measurements (**Table 1**). Weight and height were measured by wearing light clothes and shoes and the body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters (kg/m^2). Waist circumference (cm) was measured at the midpoint between the 12th rib and the iliac crest. Systemic blood pressure (systolic-SBP, diastolic-DBP; mmHg) was measured after 5 min of rest; three consecutive measurements were performed and the average of the second and third measurements was considered for statistical analysis.

The study population underwent fasting venous sampling for measuring serum levels of fasting blood glucose (FBG, mg/dl), fasting blood insulin (FBI, μU/L), total cholesterol (mg/dl), high-density lipoprotein (HDL, mg/dl), triglycerides (mg/dl), aspartate

TABLE 1 | Clinical and biochemical characteristics of the obese population in comparison with control group.

Parameters	Obese population n = 85	Control group n = 35	p-value
Age (years)	44 ± 9.8	45 ± 11	0.14
Sex (M/F)	17/68	12/23	0.03*
Body mass index (kg/m^2)	42.5 ± 4.8	23.2 ± 3.7	0.0001
Waist circumference (cm)	126.1 ± 12.8	90.1 ± 11.2	0.0001
Systolic blood pressure (mmHg)	129.6 ±14	122 ± 12.3	0.35
Diastolic blood pressure (mmHg)	84.3 ± 13.9	74.3 ± 10.1	0.04
Total cholesterol (mg/dl)	195.7 ± 33.5	178.7 ± 22.9	0.04
HDL- cholesterol (mg/dl)	47.4 ± 10.7	56 ± 14.2	0.01
LDL- cholesterol (mg/dl)	119.4 ± 31.3	90.1 ± 21.4	0.01
Triglycerides (mg/dl)	140.9 ± 66.5	88.9 ± 39.3	0.001
Fasting blood glucose (mg/dl)	100.3 ± 22.9	85.7 ± 10.2	0.006
Glycosylated hemoglobin (%)	5.5 ± 1.1	–	–
Fasting blood insulin(IU/ml)	13.2 ± 7.2	–	–
HOMA-IR	3.25 ± 1.87	–	–
HOMA-β%	162.1 ± 110.5	–	–
Aspartate aminotransferase (IU/l)	27.8 ± 14.3	20.1 ± 3.6	0.05
Alanine aminotransferase (IU/l)	36.1 ± 25.1	22.9 ± 10.4	0.08
Serum Granzyme B (pg/ml)	28.16 ± 18.5	8.3 ± 15.27	0.001
Type 2 Diabetes (%)	18%	0	0.0001*
Impaired fasting glucose (%)	9%	0	0.07*
Metabolic syndrome(%)	88%	0	0.02*

*Student's T test; *Chi-square test.*

aminotransferase (AST, IU/l), alanine aminotransferase (ALT, IU/l), and glycosylated hemoglobin (HbA1c, %, mmol/l) through standardized laboratory methods. Low-density lipoprotein (LDL, mg/dl) was obtained using Friedewald formula. The homeostasis model assessments of insulin resistance (HOMA-IR) and insulin secretion (HOMA-β%) were calculated as described by Matsuda (36).

Omental Biopsies and Gene Expression Analysis by Real-Time PCR

Omental biopsies (1 cm^3) from obese patients were collected during bariatric surgery. VAT fragments, fixed with 10% buffered formalin for 24 h and then paraffin-embedded (FFPE), were analyzed by real-time PCR for gene expression of a vast panel of molecules related to different processes underlying VAT impairment in obesity.

Total RNA from FFPE samples was extracted using RecoverAllTM Total Nucleic Acid Isolation Kit for FFPE (ThermoFisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. Purity and quantity of RNA were confirmed by NanoDrop ND-1000 Spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA). RNA was reverse transcribed into cDNA with High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA). PCR products of human GrB, IL6, TNFα, IL8, MIP1α, MIP2, TIMP1, Wisp-1, CASP3, CASP7, UNC5B, and HIFα were detected by using gene-specific primers and probes labeled with reporter dye FAM. GAPDH was used as an internal standard, which yielded a predicted amplicon of 171 bp. TaqMan real-time quantitative PCR was performed on an ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystem, Foster City, CA, USA). PCR reactions were carried out in triplicate on 96-well plates with 10 L per well using 1× TaqMan Master Mix and the results were evaluated using the ABI PRISM 7500 software (Applied Biosystem, Foster City, CA, USA). The cycle threshold (Ct) values were averaged for all subsequent calculations. The 2-ΔCt method was used to calculate relative changes in gene expression.

Serum GrB Measurement

Serum GrB levels were measured by Human Granzyme B Platinum–Kit Elisa-Affymetrix EBIO according to the manufacturer's instructions. Briefly, 50 µl of each sample with 50 µl of Dilution Buffer were incubated at room temperature for 1 h on a microplate shaker, then washed and incubated with 100 µl of Biotin-Conjugate. After washing, 100 µl of Streptavidin-HRP solution was added to all wells and incubated at room temperature for 30 min. TMB substrate solution was used to visualize HRP enzymatic reaction. The sensitivity of the assay is 0.2 pg/ml. The intra- and inter-assay coefficient of variation is 8.5% and 10.4%, respectively.

Statistical Analyses

The IBM statistical package for social sciences (SPSS) statistics (version 25.0; IBM, Armonk, NY) was used to perform all the analyses. Continuous variables were reported as median (25°–75°) or mean ± standard deviation (SD) and categorical variables were reported as percentages. Skewed variables underwent logarithmic transformation before the analyses. Student's T-test for continuous variables and $\chi 2$ test for categorical variables were used to compare mean values between two independent groups, as appropriate. Correlations between continuous variables were calculated by Pearson's coefficient, whereas Spearman's coefficient was used for dichotomic/ordinal parameters. In order to test the existence of an independent association between higher VAT GrB expression and the presence of altered glucose metabolism -as indicated by the diagnosis of IFG/T2D-, a multivariate regression analysis was built considering IFG/T2D (yes/no) as categorical dependent variable and entering variables significantly associated with IFG/T2D at the bivariate analysis, as potential confounding factors. Correlation coefficients were reported as r values in the text and tables. A p-value < 0.05 was considered statistically significant in all the analyses, with a 95% confidence interval.

RESULTS

GrB Is Expressed in VAT of Obese Subjects and Is Associated With Local Hypoxia, Apoptosis, and Inflammation

We analyzed a panel of VAT pro-inflammatory molecules, such as IL6, TNFα, IL8, MIP1α, MIP2, TIMP1, Wisp-1, CASP3, CASP7, UNC5B, and HIF (**Supplementary Materials, Table 1**), and we analyzed their expression in relation to the expression of GrB in VAT. We observed that GrB expression in VAT, considered as continuous variable, was associated with the local expression of the following markers: 1) hypoxia, as the hypoxia-inducible factor a (HIF1a; r= 0.21, p= 0.02); 2) leucocyte chemotaxis, as macrophage inflammatory proteins MIP1α/CCL3 (r= 0.6, p=0.000), MIP-2/CXCL2 (r= 0.39, p=0.015), IL-8 (r= 0.35, p=0.031), IL-6 (r=0.34, p=0.038) and TNFα (r=0.34, p=0.04); 3) apoptosis, as caspase 3 (r= 0.39, p=0.015) and caspase 7 (r= 0.28, p=0.018), and 4) adipocyte differentiation and function, as TIMP-1 (r= 0.37, p=0.019) and WISP-1 (r= 0.62, p=0.002) (**Table 2**).

VAT GrB Expression Is Associated With Glyco-Metabolic Alterations in Obese Subjects

We also investigated whether a relationship existed between GrB expression in VAT of obese patients and their clinical and biochemical parameters, such as BMI, waist circumference, SBP, DBP, FBG, FBI, total cholesterol, HDL, triglycerides, LDL, AST, ALT, HbA1c, HOMA-IR and HOMA-b. We found that VAT GrB expression was associated with the presence of glyco-metabolic alterations, in particular with higher FBG (r= 0.29, p=0.008), HbA1c (r= 0.23, p=0.01) and blood pressure (SBP, r= 0.26 p= 0.019; DBP, r=0.22, p=0.04) levels, as well as with the diagnosis of impaired fasting glucose (IFG; r=0.43, p=0.01) and T2D (r= 0.31, p=0.04) (**Table 3**).

In addition, when stratifying the obese cohort according to the glycemic state (normal glucose tolerance versus IFG/T2D), patients with IFG/T2D showed significantly higher VAT GrB expression than normo-glycemic individuals (1.27±1.13 vs 0.31±0.55 A.U., p = 0.02) (**Figure 1**). In our study population, the other parameters that

TABLE 2 | Correlation between Granzyme B (GrB) mRNA expression in visceral adipose tissue (VAT) and features of local inflammation in obese subjects (n= 85).

	Correlation coefficient	*p-value*
UNC5B	0.09	0.55
IL8	0.35	0.031
IL6	0.34	0.038
TNF α	0.34	0.04
MIP1α	0.60	0.0001
MIP2	0.39	0.015
TIMP1	0.37	0.019
WISP-1	0.62	0.002
CASP3	0.39	0.015
CASP7	0.28	0.018
HIF1a	0.21	0.02

Bivariate correlation analyses (Spearman's coefficient; GrB mRNA is considered as a continue variable)

significantly associated with the IFG/T2D diagnosis were sex (r= −0.26 p= 0.001), age (r= 0.36 p= 0.001), greater BMI (r= 0.37 p= 0.05) and waist circumference (r= 0.11 p= 0.05).

At the multivariate logistic regression analysis, greater GrB expression levels in VAT were significantly associated with the diagnosis of IFG/T2D, independently of confounding factors such as sex, age, BMI and waist circumference with an OR: 4.61 (95%CI: 1.6–13.5) (**Table 4**).

GrB Serum Levels Are Associated With GrB Expression in VAT of Obese Subjects

The measurement of GrB levels in the serum of the whole study population showed higher GrB levels in obese subjects compared to the control group (28.16 ± 18.5 pg/ml *vs* 8.3 ± 15.27 pg/ml, p= 0.001) (**Table 1**). Moreover, in the obese subjects circulating GrB positively correlated with BMI (r=0.58, p=0.001), waist circumference (r=0.37, p=0.05), triglycerides (r = 0.55, p = 0.02), FBG (r = 0.37, p = 0.05) and the presence of IFG (r=0.38, p=0.04) and MS (r = 0.35, p = 0.05) (**Table 5**). Remarkably, GrB levels in serum were associated with GrB expression in VAT (r = 0.31, p = 0.04) (**Table 5**), suggesting that GrB circulating levels predict the expression of GrB in VAT.

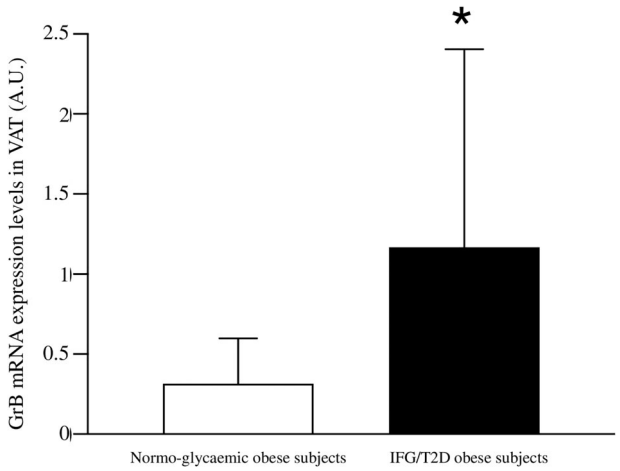

FIGURE 1 | Comparison between Granzyme B (GrB) expression in visceral adipose tissue (VAT) from normo-glycemic obese subjects (n= 62) and IFG/T2D obese subjects (n=23). GrB mRNA expression levels are shown as arbitrary units (A.U.). Data are shown as mean ± standard deviation. *p < 0.05.

TABLE 3 | Correlation between Granzyme B (GrB) mRNA expression in visceral adipose tissue (VAT) and clinical and biochemical parameters in obese subjects (n = 85).

	Correlation coefficient	*p-value*
Age (years)	0.12	0.26
Sex (M/F)	0.28	0.41
Body mass index (kg/m^2)	0.08	0.46
Waist circumference (cm)	0.04	0.74
Systolic blood pressure (mmHg)	0.26	0.019
Diastolic blood pressure (mmHg)	0.22	0.04
Total cholesterol (mg/dl)	0.18	0.12
HDL- cholesterol (mg/dl)	0.01	0.92
LDL- cholesterol (mg/dl)	0.15	0.20
Triglycerides (mg/dl)	0.10	0.37
Fasting blood glucose (mg/dl)	0.029	0.008
Glycosylated hemoglobin (%)	0.23	0.01
Fasting blood insulin(IU/ml)	0.11	0.59
HOMA-IR	0.16	0.64
HOMA-β%	0.13	0.61
Aspartate aminotransferase (IU/l)	0.04	0.71
Alanine aminotransferase (IU/l)	0.08	0.44
Serum GrB levels (pg/ml)	0.31	0.04
Type 2 diabetes (%)	0.31	0.04
Impaired fasting glucose (%)	0.43	0.01
Metabolic syndrome (%)	0.18	0.09

Bivariate correlation analyses (Spearman's coefficient; GrB mRNA is considered as a continue variable).

DISCUSSION

This study showed, for the first time in humans, that GrB is expressed in VAT of obese subjects and is associated with established mediators and markers of VAT dysfunction, as well as with glyco-metabolic alterations and GrB serum levels. Our findings prompt us to speculate, as depicted in **Figure 2**, on the possible association and function of GrB along the pathway that, from chronic caloric excess and VAT inflammation and dysfunction, leads to systemic low-grade inflammation up to glyco-metabolic impairment.

We observed that GrB expression in VAT was associated with HIF1a, a main marker of hypoxia, which is linked to the expansion of AT in obesity (7, 8). In the context of stressed AT, it takes place the production of cytokines and chemokines that stimulate VAT infiltration by inflammatory cells, including cytotoxic lymphocytes, B cells and macrophages. Indeed, GrB in VAT associated with chemiotactic molecules, such as IL8, MIP1α/CCL3, MIP2/CXCL2 and pro-inflammatory cytokines, such as IL6 and TNFα. In particular, IL8, is an adipokine known to be able to sustain VAT inflammation in obesity (15, 37, 38), and MIP1α and MIP1β are major factors produced by activated macrophages that, in turn, amplify VAT inflammation by potentiating the production of pro-inflammatory cytokines and the recruitment of immune cells,

TABLE 4 | Granzyme B (GrB) mRNA expression in visceral adipose tissue (VAT) is an independent predictor of glucose metabolism alteration.

				95% C.I.	
	Coefficient ß	**Standard Deviation Error**	**p-value**	**Lower**	**Upper**
Age	0.082	0.04	0.042	1.003	1.175
Sex	−1.087	0.783	0.165	0.073	1.566
Waist circumference	0.014	0.032	0.671	0.951	1.08
BMI	0.018	0.075	0.813	0.878	1.18
GrB expression in VAT	1.527	0.548	0.005	1.574	13.481
(Constant)	−7.624	4.444	0.086		

Multivariate logistic regression analysis. Glycemic alterations (yes/no) is the dependent variable. C.I., Confidential Interval.

TABLE 5 | Correlation between serum Granzyme B (GrB) levels and clinical and biochemical parameters in obese subjects (n = 85).

	Correlation coefficient	***p-value***
Age (years)	0.06	0.64
Sex (M/F)	0.18	0.58
Body mass index (kg/m^2)	0.58	0.001
Waist circumference (cm)	0.37	0.05
Systolic blood pressure (mmHg)	0.11	0.72
Diastolic blood pressure (mmHg)	0.27	0.11
Total cholesterol (mg/dl)	0.19	0.59
HDL- cholesterol (mg/dl)	0.31	0.44
LDL- cholesterol (mg/dl)	0.23	0.13
Triglycerides (mg/dl)	0.55	0.02
Fasting blood glucose (mg/dl)	0.37	0.05
Glycosylated hemoglobin (%)	0.11	0.13
Fasting blood insulin (IU/ml)	0.14	0.45
HOMA-IR	0.39	0.09
HOMA-β%	0.19	0.33
Aspartate aminotransferase (IU/l)	0.11	0.54
Alanine aminotransferase (IU/l)	0.06	0.73
Serum GrB levels (pg/ml)	0.31	0.04
Type 2 diabetes (%)	0.21	0.08
Impaired fasting glucose (%)	0.38	0.04
Metabolic syndrome (%)	0.35	0.05

Bivariate correlation analyses (Spearman's coefficient).

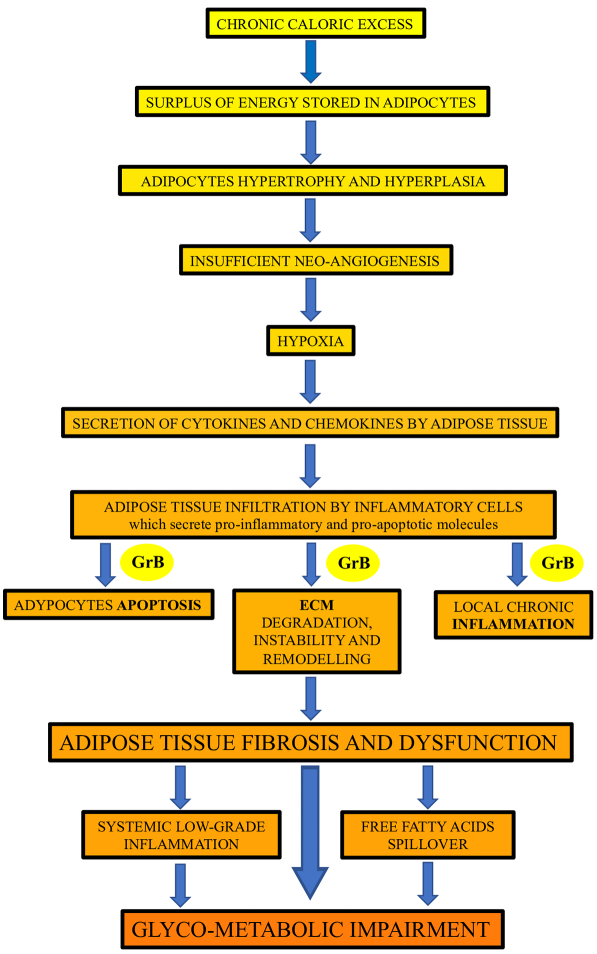

FIGURE 2 | The potential contribution of Granzyme B (GrB) to the inflammation and dysfunction of adipose tissue in obese subjects. GrB, produced by different adipose tissue infiltrating inflammatory cells, may contribute to the promotion of the apoptotic, inflammatory and extracellular matrix (ECM) remodeling processes occurring in adipose tissue in obesity, leading to adipose tissue fibrosis and dysfunction, and driving up to glyco-metabolic impairment.

including cytotoxic lymphocytes, B cells and monocytes (15, 39, 40). VAT infiltrating CTL, NK cells, B cells and activated macrophages produce GrB, which exerts its well-known intracellular pro-apoptotic function and multiple extracellular activities (21–23). We showed that GrB in VAT associated with markers of apoptosis, as caspase 3 and caspase 7, and, since inflamed VAT in obesity undergoes increased caspase-mediated apoptosis of adipocytes (41, 42), GrB in VAT might be indicative of a possible direct role of this serine protease in promoting adipocyte apoptosis. In fact, GrB can promote perforin-dependent apoptosis, when secreted by perforin-expressing cells as CTL and NK cells (17, 18), as well as perforin-independent apoptosis or anoikis (43), when secreted by cells lacking perforin as B cells and macrophages. Anoikis is a cell-detachment-induced apoptosis, derived by the loss of cell-ECM contact mediated by ECM proteins, including fibronectin, which represent an established substrate directly cleaved by GrB (22). Indeed, one of the main activities of extracellular GrB is its capability of ECM remodeling *via* cleavage of multiple ECM components (22, 23), and ECM degradation and VAT remodeling have been implicated in the regulation of obesity, inflammation and insulin resistance (11, 12). Another activity of extracellular GrB is its capability of cleaving and processing pro-inflammatory cytokines, as IL-1α, enhancing their

biological activity several fold (44), thus amplifying and supporting VAT inflammation. In addition, according to our previous study (32) showing a significant relationship between serum GrB levels and systemic markers of VAT inflammation such as WISP-1, here we demonstrated a strong correlation between VAT expression of GrB and WISP-1, further supporting a role for GrB in the induction of VAT dysfunction (15, 45, 46).

Our findings, suggesting that GrB expressed in VAT takes part in different steps involved in the development of VAT impairment (**Figure 2**), are in agreement with those obtained by Yang et al. (33), who showed that, in obese mice, T-cell derived GrB in VAT associated with adipocyte death, inflammatory insult and local damage. Other studies, conducted on animal models, suggested that GrB, produced by VAT infiltrating B cells, contribute to the phenotypic switch of adipocytes causing them to release adipokines, pro-inflammatory mediators and cell debris (19). Remarkably, according to the hypothesis that GrB in VAT plays a central role in VAT dysfunction, our data revealed that GrB VAT expression strongly associates with the presence of T2D and with early alterations of glucose homeostasis. The link between excess adiposity and impaired glucose metabolism is not explained simply by absolute fat mass, and accumulating evidence clearly indicates that the functional capacity of VAT is likely a major determinant of insulin resistance, glucose intolerance and T2D in obesity (4). This evidence further supports the possible crucial contribution of GrB in the development of the complex inflammatory process underlying obesity.

In summary, our data showed that GrB expression in VAT correlated with high levels of GrB in serum, which, in turn, associated with glyco-metabolic impairment. These findings, according to our previous study (32), provide additional evidence that high GrB circulating levels might be a marker of VAT dysfunction and alteration of glucose metabolism in metabolic diseases.

In conclusion, this study provides novel insights into the potential mechanisms underlying metabolic impairment and VAT inflammation, suggesting a possible involvement of GrB in the regulation of VAT homeostasis and inflammatory processes in the presence of obesity.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENTS

The studies involving human participants were reviewed and approved by Ethics Committee of Sapienza University of Rome, Italy. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

FV, FC, IB, and MC designed the study. FC and IB coordinated the study. LB, VC, AB, and FL oversaw patient recruitment and data collection, and finalized the dataset. GS performed bariatric surgery and VAT biopsies. FC, FS, VC, LB, CC, and CC performed laboratory experiments. IB and MB conducted the statistical analyses. FV, FC, IB, and MC drafted the paper, which was reviewed by all authors. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.589188/full#supplementary-material

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ORIGINAL RESEARCH
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Alpha 1 Antitrypsin-Deficient Macrophages Have Impaired Efferocytosis of Apoptotic Neutrophils

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Alpha 1 antitrypsin deficiency (AATD) is an autosomal co-dominant disorder characterized by a low level of circulating AAT, which significantly reduces protection for the lower airways against proteolytic burden caused by neutrophils. Neutrophils, which are terminally differentiated innate immune cells and play a critical role to clear pathogens, accumulate excessively in the lung of AATD individuals. The neutrophil burden in AATD individuals increases the risk for early-onset destructive lung diseases by producing neutrophil products such as reactive oxygen radicals and various proteases. The level of AAT in AATD individuals is not sufficient to inhibit the activity of neutrophil chemotactic factors such as CXCL-8 and LTB4, which could lead to alveolar neutrophil accumulation in AATD individuals. However, as neutrophils have a short lifespan, and apoptotic neutrophils are rapidly cleared by alveolar macrophages that outnumber the apoptotic neutrophils in the pulmonary alveolus, the increased chemotaxis activity does not fully explain the persistent neutrophil accumulation and the resulting chronic inflammation in AATD individuals. Here, we propose that the ability of alveolar macrophages to clear apoptotic neutrophils is impaired in AATD individuals and it could be the main driver to cause neutrophil accumulation in their lung. This study demonstrates that Z-AAT variant significantly increases the expression of pro-inflammatory cytokines including CXCL-8, CXCL1, LTB4, and TNFα in LPS-treated macrophages. These cytokines play a central role in neutrophil recruitment to the lung and in clearance of apoptotic neutrophils by macrophages. Our result shows that LPS treatment significantly reduces the efferocytosis ability of macrophages with the Z-AAT allele by inducing TNFα expression. We incubated monocyte-derived macrophages (MDMs) with apoptotic neutrophils and found that after 3 h of co-incubation, the expression level of CXCL-8 is reduced in M-MDMs but increased in Z-MDMs. This result shows that the expression of inflammatory cytokines could be increased by impaired efferocytosis. It indicates that the efferocytosis ability of

macrophages plays an important role in regulating cytokine expression and resolving inflammation. Findings from this study would help us better understand the multifaceted effect of AAT on regulating neutrophil balance in the lung and the underlying mechanisms.

Keywords: Alpha 1 antitrysin, AAT deficiency, neutrophil, macrophage, efferocytosis, cytokine

INTRODUCTION

Alpha-1-antitrypsin (AAT) is a protease inhibitor that regulates the proteolytic effects of neutrophil-derived serine proteases, including neutrophil elastase, cathepsin G, and thrombin (1, 2). It is produced mainly by hepatocytes but also by monocytes, macrophages, and bronchial cells (3). AAT is a classical acute phase response protein and its serum level is increased during states of acute inflammation (2). AATD results from mutations in the *SERPINA1* gene. Approximately 120 variant alleles of AAT have been reported to date. The most common allele is M, and Z is a mutated allele most commonly responsible for severe deficiency. It is characterized by a single amino acid substitution of lysine for glutamic acid at position 342, leading to conformational change to its latent form or polymerization, lowering the concentration of circulating AAT. Therefore, AAT does not reach lung tissues where it normally acts as the primary regulator against proteolytic activities in AATD individuals (4). AATD predisposes individuals to lung diseases, including chronic obstructive pulmonary disease (COPD) (5, 6). AATD is indeed responsible for 1%–2% of COPD, and AATD-associated lung disease shares major features of emphysema (7).

AATD individuals have a higher number of neutrophils in their lower respiratory tract than healthy normal individuals (8–10). Neutrophils could be considered a double-edged sword. They are important mediators of host defenses, typically being the first leukocytes recruited to an inflammatory site and eliminating pathogens to resolve the inflammation. However, uncontrolled neutrophilic activity and continued neutrophil recruitment to inflammatory sites can result in an excess of reactive oxygen radicals and various proteolytic enzymes that could cause damage to the surrounding healthy lung tissues and persistent inflammation, a feature of many human diseases including COPD and cystic fibrosis (11–13). Excessive numbers of neutrophils have been implicated in the pathogenesis of many acute and chronic lung diseases (14). Thus finding molecular mechanisms responsible for alveolar neutrophil accumulation in AATD individuals is important to understand risks of lung diseases in AATD individuals.

Alveolar macrophages reside at the interphase between air and lung tissue, serving as the front line of cellular defense against respiratory pathogens. They are the primary phagocytes of the innate immune system, clearing the lower respiratory tract of allergens and infectious or toxic particles (15). When faced with infectious particles or microbes, alveolar macrophages produce pro-inflammatory cytokines to initiate inflammatory responses and recruit neutrophils into the alveolar spaces (16). Neutrophils migrate out of the pulmonary capillaries into the air spaces to serve as the second-line defense. After the phagocytosis of infectious microbes, neutrophils undergo programmed cell death and are cleared by alveolar macrophages, orchestrating the resolution of inflammation and tissue repair (17). However, if apoptotic neutrophils are not efficiently cleared by alveolar macrophages, the apoptotic cells release potentially injurious cytoplasmic contents into the alveolus, causing further tissue injury and perpetuating inflammation (18, 19).

Neutrophil accumulation is considered to be the main source of proteolytic burden in the lung of AATD individuals, and airway neutrophilic inflammation is closely linked to tissue destruction and alveolar airspace enlargement, leading to disease progression (20, 21). Nonetheless, the role of AAT in maintaining alveolar neutrophil homeostasis has not been fully examined. In this study, we hypothesized that Z-AAT impairs neutrophil homeostasis in pulmonary alveoli by increasing the expression of neutrophil chemotactic factors and by decreasing the ability of macrophages to clear apoptotic cells. We compared the expression level of pro-inflammatory cytokines between lipopolysaccharide (LPS)-treated M- and Z-monocyte derived macrophages (MDMs). The cytokines examined in this study include potent neutrophil chemotactic factors chemokine (C-X-C motif) ligand 1 (CXCL-1) and ligand 8 (CXCL-8) (22, 23). Due to difficulty obtaining primary human alveolar macrophages, for the present study we used macrophages derived from monocytes (MDM). The macrophages were maturated in the presence of macrophage colony-stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) as previously described (24). MDMs from PiMM individuals are referred to as M-MDM while MDMs from PiZZ individuals are referred to as Z-MDM in this study. Efferocytosing macrophages promote resolution of inflammation by suppressing the expression of inflammatory cytokines (25). Therefore macrophage efferocytosis impaired by LPS, which is known to inhibit the efferocytosis of apoptotic neutrophils by macrophages, results in persistent expression of inflammatory cytokines (26). We investigated whether AAT regulates the inhibitory effect of LPS on macrophage efferocytosis. This study demonstrates that Z-AAT reinforces the inhibitory effect of LPS on macrophage efferocytosis by increasing TNFα expression and delays the suppression of CXCL-8 mediated by efferocytosis of apoptotic cells. Our results also show that TNFα could be responsible for the LPS-reduced macrophage efferocytosis by inhibiting the expression of efferocytosis-associated molecules, CD14, CD36, and RARα. Taken together, this study explains the function of Z-AAT to exacerbate neutrophil burden and consequent pulmonary inflammation. Therefore, the findings from this study may translate to identifying a targeted strategy to control alveolar neutrophil balance in AATD individuals.

MATERIALS AND METHODS

Monocyte Isolation and Macrophage Differentiation

Using ficoll-gradient centrifugation, peripheral blood mononuclear cells were isolated from the blood of outpatient volunteers (University of Florida Institutional Review Board protocol 2015-01051). Characteristics of the volunteers are shown in **Table 1**. Monocytes were purified using a monocyte enrichment kit (Stemcell Technology, Vancouver), following the manufacturer's instruction. Monocytes were plated in 12-well plates at 300,000 cells per well in macrophage differentiation media (RPMI 1640 containing 10% heat-inactivated FBS, 20 Units/ml penicillin, 20 µg/ml streptomycin, 250 ng/ml Amphotericin B, recombinant human GM-CSF (0.5 ng/ml) and recombinant human M-CSF (5 ng/ml)) and differentiated for 7 days. Both growth factors, GM- and M-CSF, exist in the lung, and especially GM-CSF is important to induce AAT expression (24). Supplemental medium (50% of the volume in each well) was added every 3 days after removal of half of the old media, and cells were used on day 7 for any treatment. To induce inflammatory signaling in MDMs, the cells were stimulated with 10 ng/ml LPS from Escherichia coli O111:B4 (Sigma-Aldrich, St. Louis) overnight. Non-treated and LPS-treated MDMs were harvested for RNA extraction using the Qiagen RNeasy kit (Qiagen, Hilden). To inhibit LPS-mediated TNFα signaling, MDMs were pretreated with LPS for 1 h and incubated with TNFα neutralizing antibody (MAB210, R&D Systems, Minneapolis) or isotype control (MAB002, R&D Systems, Minneapolis) for 18 h.

Immunofluorescence

To examine AAT distribution, MDMs were differentiated on glass slides and fixed in 4% paraformaldehyde for 20 min and permeabilized for 10 min in PBS containing 0.01% Triton X-100. The permeabilized cells were incubated with rabbit anti AAT polyclonal antibody (Abcam, Cambridge) at 1:400 dilution in PBS containing 0.1% Tween 20 for 1 h. After washing with PBS-Tween 20, cells were immunostained with Alexa Fluor488 goat anti-rabbit (Abcam, Cambridge) at 1:500 dilution at room temperature for 1 h. The immunostained cells were mounted on glass slides using VECTASHIELD mounting media with DAPI and examined using a fluorescence microscope (BZ-X700, Keyence, Osaka). For quantification of intracellular AAT, AAT fluorescent intensity and number of cells were measured with BZ software, and the fluorescent intensity was normalized to the cell number. To examine CD36 distribution, MDMs were incubated with mouse anti CD36 monoclonal antibody (ThermoFisher, Waltham) at 1:20 dilution in blocking solution (Invitrogen, Carlsbad) overnight at 4°C. After washing with PBS-Tween 20, cells were immunostained with Alexa Fluor647 goat anti-rabbit (Abcam, Cambridge) at 1:1000 dilution at room temperature for 1 h. For quantification of CD36, ~1000 MDMs were evaluated for each MDM group (n=4).

TABLE 1 | Characteristics of controls and AATD individuals.

Characteristic	PiMM (n=6)	PiZZ (n=6)	P-value
Age	48.5 (22–68)	51 (39–69)	0.82
Gender (M/F)	4/2	4/2	N/A
FEV1% predicted	86.6 (57–114)	60.8 (23.2–97)	0.14
FEV1/FVC	77.6 (59–91)	55.3 (31.6–71.8)	0.04
Current smoker	No	No	N/A

Definition of abbreviations: PiMM, individuals homozygous for normal PiM allele; PiZZ, individuals homozygous for mutant PiZ allele; N/A, not applicable; FEV1, forced expiratory volume in one second; FVC, forced vital capacity.

Western Blot Analysis

Total proteins were extracted from MDMs using RIPA lysis buffer (Cell Signaling, Danvers) plus protease and phosphatase inhibitors. The protein concentration of each sample was measured using a standard Bradford assay (BioRad, Hercules) and equal amounts of protein were loaded onto a 12% SDS polyacrylamide gel. After gel electrophoresis, the proteins were transferred onto a nitrocellulose membrane using a wet-transfer system, and the membrane was blocked in Tris-buffered saline with 0.1% Tween 20 (TBST) containing 5% nonfat dry milk. The membrane was immunoblotted overnight at 4°C with AAT rabbit polyclonal antibody (DAKO, Carpinteria) at a dilution of 1:5,000 in TBST. Horseradish peroxidase conjugated anti-rabbit antibody (BioRad, Hercules) was used for secondary labeling at 1:5,000 in TBST for 1 h at room temperature. The membrane was reprobed with GAPDH rabbit polyclonal antibody (Proteintech, Rosemont) at 1:5,000 in TBST. A horseradish peroxidase conjugated anti-rabbit (BioRad, Hercules) was used for secondary labeling. Protein bands were visualized by enhanced chemiluminescence (ECL, GE Healthcare, Chicago).

ELISA

AAT was measured in conditioned media of MDMs using a sandwich enzyme-linked immunosorbent assay (ELISA). Ninety-six well ELISA plates were coated with goat anti-human AAT antibody at 4°C overnight. The wells were blocked with PBS containing 0.05% Tween 20 and 0.5% BSA for 1 h at room temperature. Control and samples were added to the ELISA plate and incubated for 2 h at 37°C. After the ELISA plates were washed, bound AAT remained in each well. Rabbit anti-human AAT antibody (DAKO, Carpinteria) was added to the plate and incubated for 1 h at 37°C, followed by HRP-conjugated goat anti-mouse antibody (Bio-Rad, Hercules). After washing, QuantaBlu Fluorogenic Peroxidase Substrate (ThermoFisher, Waltham) was added to the wells and incubated for 5 min at room temperature. HRP activity was read at an excitation/emission maxima of 325/420 using a spectrophotometer (LS50B LuminSpectrometer, Perkin Elmer, Waltham). The concentrations of CXCL-8 (Abcam, Cambridge), LTB4 (Cayman Chemical, Ann Arbor), and TNFα (Abcam, Cambridge) were measured in conditioned media of MDMs by ELISA, following the manufacturer's instruction.

Neutrophil Isolation and Chemotaxis Assay

Primary neutrophils were isolated from PiMM volunteers using EasySep direct human neutrophil isolation kit (Stemcell

Technologies, Vancouver), following the manufacturer's instruction. The isolated cells were incubated with fluorescent CD16 and CD66b antibodies (BioLegend, San Diego). Percentage of CD16 and CD66b-positive cells was calculated using a Gallios flow cytometer with Kaluza software (Beckman Coulter, Brea, **Supplementary Figure 1**). Neutrophil chemotaxis was assayed in a Transwell system using polycarbonate membranes with 3-μm pore size (Corning, Corning). MDMs were incubated with or without LPS and, supernatant from the cell culture was transferred into the bottom layer of the chemotaxis chamber. Freshly isolated neutrophils (1 x 10^6) were added into the top layer of the chamber. The neutrophils migrated from the top to the bottom layer for 30 min. Migrated cells were counted using a hemocytometer and automated cell counter (Invitrogen, Carlsbad).

AAT Treatment

Lyophilized AAT (Prolastin-C) was reconstituted with deionized water, following the manufacturer's instruction and stored at -80°C. To examine whether AAT is able to inhibit LPS-mediated CXCL-8 expression, MDMs were incubated with LPS and different concentrations of AAT for 18 h. AAT-treated MDMs were lysed for RNA extraction using the Qiagen RNeasy kit (Qiagen, Hilden), and CXCL-8 expression was examined using qRT-PCR.

Neutrophil Apoptosis

To induce apoptosis in the cells, neutrophils were aged for 20 h and then incubated with 1 μM staurosporine for another 3 h. The apoptotic rate of the neutrophils was assessed by flow cytometry with Annexin V/propidium iodide staining (Invitrogen, Carlsbad). For efferocytosis assay, the apoptotic neutrophils were labeled with CellTracker Red CMTPX dye (Invitrogen, Carlsbad), following the manufacturer's instruction.

Efferocytosis Assay

Following 7 days of macrophage differentiation, MDMs were incubated with or without LPS for 18 h, and then the MDMs were incubated with CellTracker Red CMTPX dye-labeled apoptotic neutrophils suspended at 1 x 10^6/ml at 37°C for 30 min, providing a phagocyte to target ratio of 1:4. After the incubation, the non-ingested neutrophils were removed by repeated washing with PBS. Removal of the non-ingested neutrophils was confirmed with light microscope (Olympus 1X70), and phagocytosis of the neutrophils by MDMs was confirmed by fluorescence microscopy (BZ-X700, **Supplementary Figure 2**). MDMs were incubated with Accutase (Stemcell Technologies, Vancouver) at room temperature for 20 min, followed by 15 min on ice. After the incubation with Accutase, MDMs were collected by a gentle scraping with a plastic scraper and analyzed by Gallios flow cytometer with Kaluza software (Beckman Coulter, Brea). A minimum of 10,000 events was acquired per sample. For each MDM sample, the efferocytosis rate of control MDM was set to 100%.

Gene Expression Validation by qRT-PCR

Total RNA (1 μg), extracted from MDMs, was reverse-transcribed using SuperScript® VILO Master Mix (Invitrogen, Carlsbad), according to the manufacturer's instruction. Quantification of PCR products was performed with 7500 Fast Real-time PCR (Applied Biosystems, Foster City). SensiFAST Real-Time PCR Kit (Bioline, London) was used to produce fluorescence-labeled PCR products and to monitor increasing fluorescence during repetitive cycling of the amplification reaction. Taqman probes/primers specific for the CXCL-1, CXCL-8/IL-8, TNFα, CD14, CD36, RARα genes, and for the GNB2L1 gene, as a housekeeping gene, were used in the real-time PCR reaction. Expression levels of the genes were obtained using the classical 2^(-ΔΔCt) method.

CXCL-8 Suppression by the Efferocytosis of Apoptotic Cells

MDMs were treated with LPS (10 ng/ml) for 30 min and then they were incubated with apoptotic neutrophils or Jurkat cells for 3 h. To induce apoptosis in Jurkat cells, the cells were exposed to UV (200 mJ/cm^2) in PBS and the UV-treated cells were incubated in RPMI 1640 for 4 h before being incubated with MDMs. Non-ingested apoptotic cells were washed off with PBS five times and MDMs were lysed for RNA isolation. The expression level of CXCL-8 was examined in the RNA samples using qRT-PCR. The efferocytosis-mediated suppression of cytokine levels was compared between M- and Z-MDMs.

Statistical Analysis

Results are expressed as the mean of number of independent experiments using MDMs from different donors. The assessment was evaluated by two-tailed Student's t-test, one-way ANOVA test or two-way ANOVA test. Bonferroni test was used for multiple comparisons. P-values of 0.05 or less were considered to be statistically significant.

RESULTS

Z-AAT Retained in MDMs

AAT mRNA expression level was compared between M- and Z-MDMs. As shown in **Figure 1A**, the gene expression level of AAT was similar between the two groups (p-value=0.17). The concentration of AAT was also compared in conditioned media of M- and Z-MDMs, and the result shows that the AAT concentration is significantly higher in the media of M-MDMs than that of Z-MDMs (**Figure 1B**, p-value<0.0001), indicating a higher secretion rate of M-AAT than Z-AAT. MDMs were immunostained for AAT (**Figure 1C**), and the level of intracellular AAT was quantified in the cells. The level of intracellular AAT was significantly higher in Z-MDMs than M-MDMs (**Figure 1D**, p-value=0.0006).

Neutrophil Chemoattractant Production by LPS-Stimulated MDMs

The innate immune response usually begins with the activation of alveolar macrophages to produce various cytokines, including neutrophil chemoattractants in the lung, and chemotaxis by neutrophils plays a critical role in the innate immune response. CXCL-8 and CXCL-1 are potent neutrophil chemoattractants on

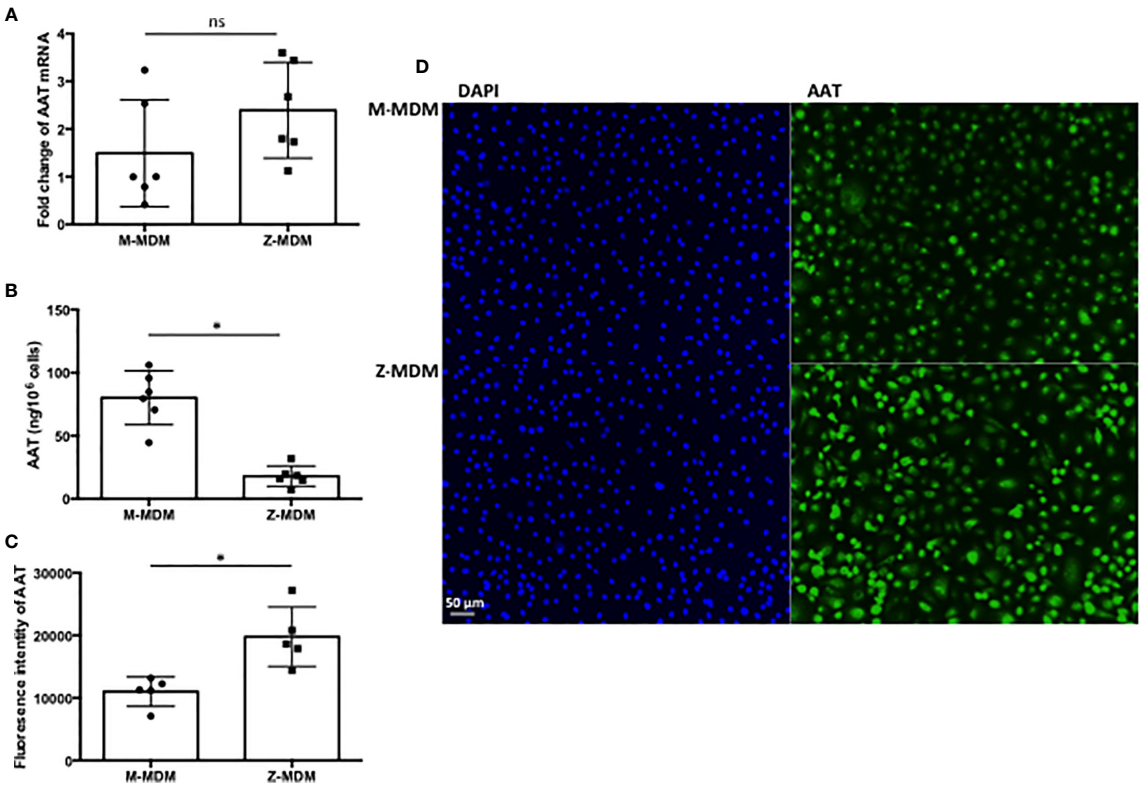

FIGURE 1 | AAT in M- and Z-MDMs. MDMs and their conditioned media were collected at day 7 of macrophage differentiation. AAT mRNA and protein levels are compared in M- and Z-MDMs (n=6). **(A)** The level of AAT mRNA was measured in M- and Z-MDMs using qRT-PCR. **(B)** The concentration of AAT was measured in conditioned media of M- and Z-MDMs using ELISA. **(C, D)** MDMs were immunostained for AAT (green) and the level of intracellular AAT was estimated based on the intensity of positive signal. 20x Images were taken using a fluorescence microscope; bar 50 µm. ~2,000 cells, originating from three separate experiments, were evaluated for each MDM group. *Denotes statistical significance (p-value < 0.05) according to two-tailed Student's t-test.

an equimolar basis (27–29). The concentration of AAT is about five-fold higher in conditioned media of M-MDM compared with Z-MDM (24). To examine the effect of AAT on the expression of CXCL-8 and CXCL-1, their expressions were compared between LPS-treated M- and Z-MDMs. LPS remarkably increased the expression of the two neutrophil chemoattractants in both M- and Z-MDMs (**Figures 2A, B**). The expression level of CXCL-8 was increased ~200-fold by LPS in Z-MDMs, and CXCL-1 expression was increased ~40-fold by LPS treatment in Z-MDMs. The expression level of CXCL-8 and CXCL-1 was significantly higher in LPS-treated Z-MDMs than M-MDMs (p-value < 0.05 for the comparison). It was previously reported that the level of CXCL-8 is significant higher in bronchoalveolar lavage fluid of AATD individuals than healthy controls (30). We measured the concentrations of CXCL-8 in conditioned media of LPS-treated MDMs using ELISA. The result shows that CXCL-8 concentration is significantly higher in conditioned media of LPS-treated Z-MDMs than M-MDMs (**Figures 3A**, p-value=0.0004), consistent with the previous finding. The higher expression level of CXCL-8 in Z-MDMs could result from lower concentration of extracellular AAT, higher level of accumulated intracellular AAT, or both. Exogenous AAT was added to M- and Z-MDM cultures to give a similar concentration of extracellular AAT between the two MDM cultures to eliminate the effect caused by different extracellular AAT concentrations on CXCL-8 expression between the cells. The result shows that even when the concentration of extracellular AAT is very similar between the two MDM cultures, the expression level of CXCL-8 is still significantly higher in Z-MDMs compared with M-MDMs (**Figure 3B**). It indicates that intracellular Z-AAT led to the higher expression of CXCL-8 in LPS-treated Z-MDMs. As AAT is an acute phase protein, its expression level is increased by LPS treatment, leading to a higher level of intracellular Z-AAT in the cells (**Figure 3C**). The level of intracellular AAT was quantified and compared between controls and LPS-treated MDMs. As shown in **Figure 3D**, the intracellular AAT level was not statistically different between M-MDM controls and LPS-treated M-MDMs, but it was significantly higher in LPS-treated Z-MDMs than their controls (p-value<0.0003). To examine whether AAT expression level is correlated with the expression of CXCL-8 in Z-MDMs, we calculated a Pearson's correlation coefficient and p-value between the expression levels of the two genes. The result shows that the expression of AAT is

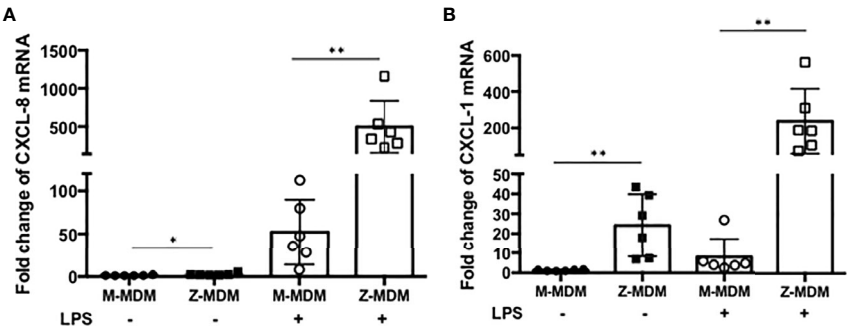

FIGURE 2 | LPS-induced neutrophil chemoattractant expression in M- and Z-MDMs. MDMs were incubated with LPS (10 ng/ml) overnight and the expression levels of **(A)** CXCL-8 and **(B)** CXCL-1 were compared between M- and Z-MDMs (n=6). The expression levels of the cytokines were normalized to GNB2L1, housekeeping gene.* and **Denote statistical significance (p-value < 0.05 and p-value < 0.01, respectively) according to two-tailed Student's t-test.

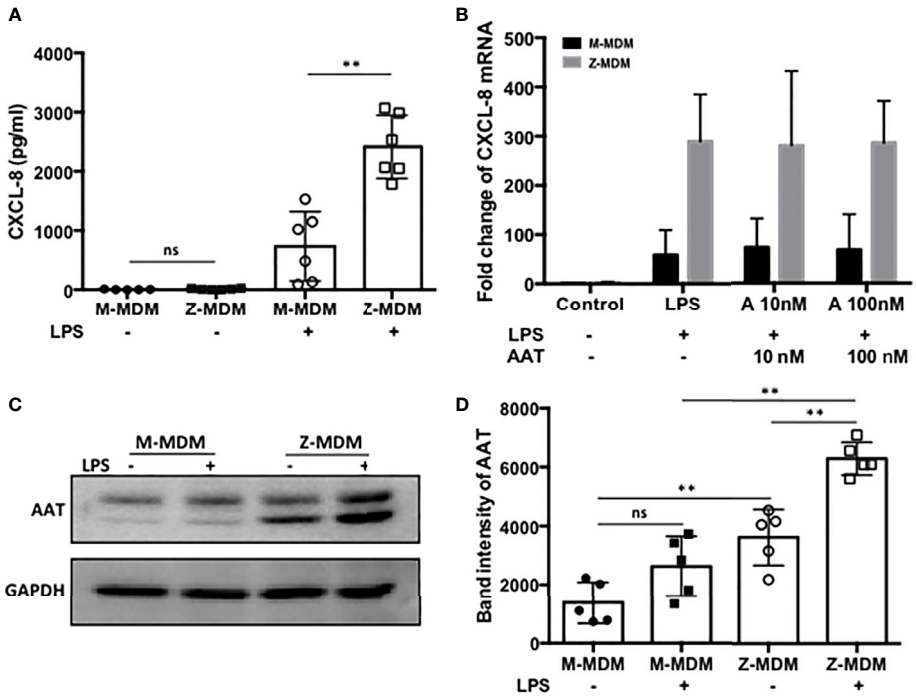

FIGURE 3 | A high level of CXCL-8 resulted from Z-AAT accumulation. MDMs were incubated with LPS (10 ng/ml) overnight. **(A)** CXCL-8 protein level was measured and compared between LPS-treated M- and Z-MDMs (n=6). **(B)** Two different concentrations of AAT were extracellularly added to M- and Z-MDM cultures and the cells were incubated with LPS and AAT overnight. Two-way ANOVA test was used to analyze the effect of genotype and treatment (AAT) on the expression of CXCL8 in the cells (n=5). P-values of interaction, genotype, and treatment were 0.96, 0.0001, and 0.996, respectively. *Denotes statistical significance (p-value < 0.05) according to two-tailed Student's t-test. **(C, D)** Total proteins were collected from control and LPS-treated MDMs and the increased intracellular AAT levels in LPS-treated cells were visualized and quantified using western blotting (n=5). One-way ANOVA was used to compare the level of intracellular among the samples, and p-value was less than 0.0001. **Denotes statistical significance (p-value < 0.01) according to one-way ANOVA multiple comparisons.

highly correlated with the expression of CXCL-8 in Z-MDMs (**Table 2**).

We also examined the level of leukotriene B4 (LTB4), a well-known neutrophil chemoattractant, in conditioned media of M- and Z-MDMs using ELISA. The concentration of LTB4 was below the lower limit of detection level in the media of M-MDM samples while it was detected in Z-MDM samples. The concentration of LTB4 was on average 93.6 ± 53.7 pg/ml in Z-MDM samples. The release of LTB4 is stimulated when alveolar macrophages are exposed to neutrophil elastases (10). It could explain the low level of LTB4 in conditioned media of the MDM cultures.

TABLE 2 | Correlation between the expression levels of AAT and CXCL-8.

Z-MDM culture condition	n	AAT	CXCL-8	Pearson r	P-value
Control	5	1.0	1.0		
LPS (10 ng/ml)	5	2.7	289.4		
LPS (10 ng/ml)+AAT (10 nM)	5	2.8	280.4		
LPS (10 ng/ml)+AAT (100 nM)	5	3.4	286.2		
LPS (10 ng/ml)+AAT (1 μM)	5	2.6	171.9		
LPS (10 ng/ml)+AAT (10 μM)	5	2.9	164.5	0.883	0.02

Neutrophil Chemotaxis Increased in Z-MDMs

AATD individuals have a higher number of alveolar neutrophils than non-AATD individuals (9, 31, 32). We suspected that the higher expression of the neutrophil chemoattractant factors in LPS-stimulated Z-MDMs could lead to higher neutrophil migration. We freshly isolated neutrophils from human blood and immediately used them for a neutrophil chemotaxis assay because their half-life is short, generally 6–8 h (33). We compared neutrophil migration rates between control and LPS-treated MDMs. Neutrophil migration rate was higher in the conditioned media of LPS-treated MDMs than that of non-treated controls, and the neutrophil migration rate of non-treated controls are shown in the **Figure 4A**. We calculated the neutrophil migration rate increased by LPS treatment in each MDM group. As shown in **Figure 4B**, LPS-mediated increase in the neutrophil migration rate was significantly higher in conditioned media of Z-MDMs than M-MDMs (p-value=0.039). This result correlates with our previous observation that the expression level of neutrophil chemoattractant factors was significantly higher in Z-MDMs than M-MDMs. To confirm that the neutrophils were migrated by chemotaxis other than chemokinesis, three different solutions of PBS, RPMI 1640, and macrophage-differentiated media were used as negative controls for the neutrophil migration assay. The result showed that random neutrophil migration rates in the three solutions were less than 3%, supporting that the observed neutrophil migration in conditioned media of MDMs was mediated by neutrophil chemoattractant factors produced by macrophages.

Efferocytosis of Apoptotic Neutrophils Reduced by LPS

Neutrophils recruited to the site of infection phagocytize bacteria and the process typically accelerates neutrophil apoptosis. Alveolar macrophages clear the apoptotic neutrophils, which ultimately promotes resolution of the bacterial infection (34). Thus the ability of alveolar macrophages to phagocytize apoptotic cells in a timely manner is critical to orchestrate the resolution of inflammation. To determine the ability of M- and Z-MDMs to efferocytose apoptotic neutrophils, we performed efferocytosis of apoptotic neutrophils by MDMs in the presence or absence of LPS. The efferocytosis rate was on average similar between the two MDM groups in the absence of LPS (**Table 3** and **Supplementary Figure 2D**). It was previously reported that LPS inhibits efferocytosis of apoptotic neutrophils by MDMs (26). We compared the reduced efferocytosis rate caused by LPS treatment between M- and Z-MDMs. The result showed that the inhibitory effect of LPS was significantly higher in Z-MDMs than M-MDMs (**Figures 5A–D** (p-value=0.01)). We compared the expression of macrophage efferocytosis-related genes in LPS-stimulated M- and Z-MDMs. The examined molecules were PPARα, PPARγ, CEBPβ, ADAM17, and TNFα. These genes have previously been reported to be involved in efferocytosis. However, unlike the expression of TNFα, the expression levels of all other genes were not statistically different between M- and Z-MDMs (data not shown). LPS stimulation increased the

TABLE 3 | The efferocytosis rate of M- and Z-MDMs.

	Efferocytosis rate (%)	P-value
M-MDM	28.7 ± 13.5	0.959
Z-MDM	28.3 ± 14.3	

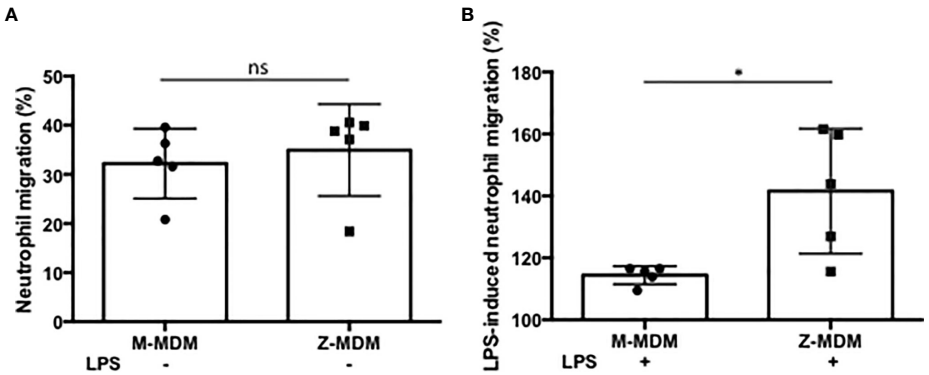

FIGURE 4 | The effect of AAT on LPS-induced neutrophil chemotaxis. MDMs were incubated with LPS (10 ng/ml) overnight. To examine the ability of AAT to regulate neutrophil transmigration, conditioned media of M- and Z-MDMs were collected and placed in the bottom chamber of transwell system. Freshly isolated neutrophils were placed in the top chamber of the transwell system. The number of neutrophils migrated to the bottom layer were counted after 30 min. **(A)** The number of neutrophils in the bottom layer was divided by the total number of neutrophils to calculate the migration rate of control MDMs. **(B)** To calculate the migration rate after LPS treatment, the migration rate of control MDM was set to 100%, and the migration rate of LPS-treated MDM was normalized to control for each individual; n=5. *Denotes statistical significance (p-value < 0.05) according to two-tailed Student's t-test.

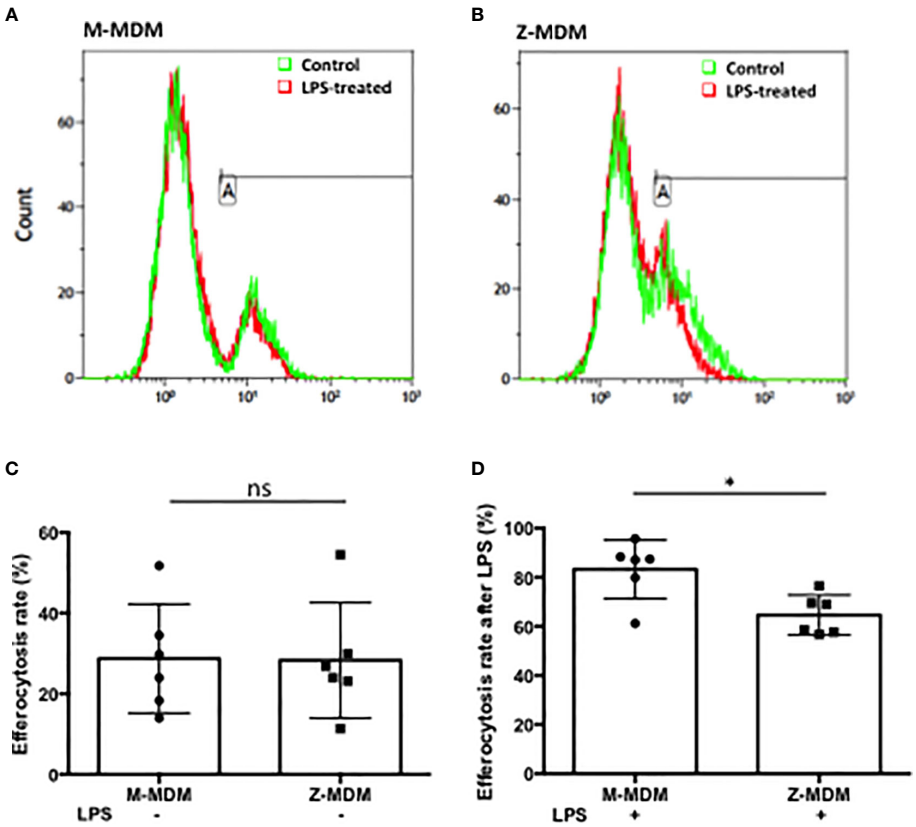

FIGURE 5 | Efferocytosis of apoptotic neutrophils by M- and Z-MDMs. MDMs were incubated with LPS (10 ng/ml) overnight and celltracker red-labeled apoptotic neutrophils were added to the MDM culture. Efferocytosis of the apoptotic cells by MDMs was assessed by flow cytometric analysis **(A)** M-MDM **(B)** Z-MDM. In each histogram plot, the green and red graphs indicate control and LPS-treated MDMs, respectively. The first peak of the each graph indicates non-phagocytosing MDM population and the second peak indicates phagocytosing MDM population. **(C)** The efferocytosis rates of MDM controls were assessed by flow cytometric analysis; n=6. **(D)** Efferocytosis rates of LPS-treated MDMs are expressed relative to those of MDM controls that are set to 100%; n=6. *Denotes statistical significance (p-value < 0.05) according to two-tailed Student's t-test.

expression of TNFα in both M-and Z-MDMs, but TNFα expression level was significantly higher in Z-MDMs (**Figure 6A**, p-value=0.027). The protein level of TNFα was also significantly higher in conditioned media of LPS-treated Z-MDMs than that of M-MDMs (**Figure 6B**, p-value=0.032). To investigate if extracellular AAT is able to inhibit LPS-induced TNFα expression in Z-MDMs, we incubated LPS-stimulated MDMs with two different concentrations, 1 and 10 μM, of M-AAT, and examined TNFα expression in the cells. The result shows that only a higher concentration of AAT, which is similar to the AAT concentration found in circulating blood of non-AATD individuals, could suppress TNFα expression in LPS-stimulated Z-MDMs, as shown in **Figure 6C** (p-value=0.018). It supports that AAT could inhibit LPS-mediated TNFα expression in macrophages. The ability of AAT to inhibit TNFα expression in human neutrophils was previously reported (35). We suspected that different TNFα expression levels between M- and Z-MDMs could be responsible for the different efferocytosis rates observed between the cells. In order for TNFα to exert its biological function, it has to bind to its specific receptors. To prevent TNFα from binding to its receptors, we incubated Z-MDMs with TNFα neutralizing antibodies. We then examined whether blocking TNFα signaling could abolish the inhibitory effect of LPS on the efferocytosis of apoptotic neutrophils by MDMs. The inhibitory effect of LPS on macrophage efferocytosis was significantly reduced when MDMs were incubated with the neutralizing antibody to TNFα (**Figure 7A**, p-value=0.011). LPS reduced efferocytosis rate by 53% in Z-MDMs but the efferocytosis rate was recovered to 94% by TNFα neutralizing antibody in the cells. The result proposes that LPS inhibits macrophage efferocytosis through TNFα in the cells. The efferocytosis rate of the MDMs that were incubated with a combination of LPS and non-specific isotype antibody was similar to that of the MDMs incubated with LPS alone.

The Expression of Neutrophil Chemoattractant Factors Regulated by TNFα

TNFα is known to self-regulate its own expression by activating NF-kB signaling (36–38). Regarding that NF-kB signaling regulates the expression of CXCL-8 and CXCL-1 (39, 40), we

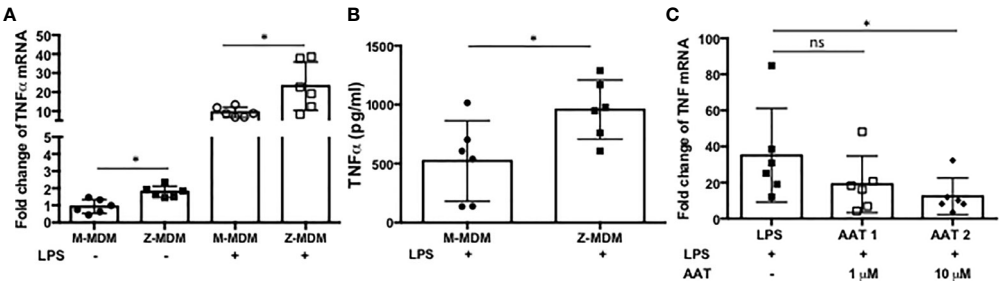

FIGURE 6 | LPS-induced TNFα expression in M- and Z-MDMs. MDMs were incubated with LPS (10 ng/ml) overnight and the gene expression level of **(A)** TNFα and **(B)** its protein level were compared between M-MDMs and Z-MDMs. **(C)** Z-MDMs were incubated with LPS (10 ng/ml) and two different concentrations of M-AAT overnight. The effect of M-AAT on the expression of TNFα was examined in the cells; n=6. *Denotes statistical significance (p-value < 0.05) according to two-tailed Student's t-test.

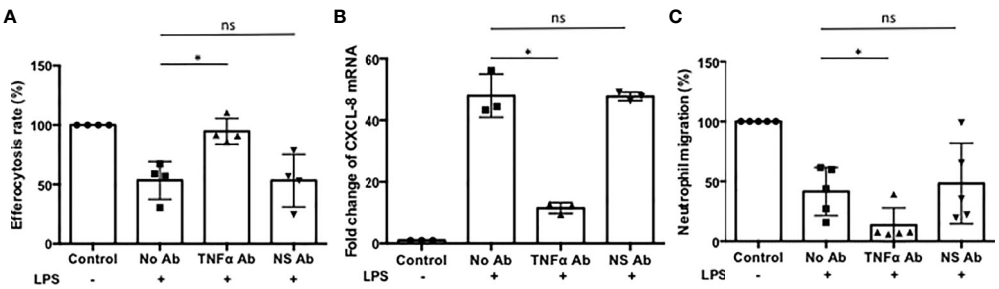

FIGURE 7 | The effect of TNFα on macrophage efferocytosis and neutrophil chemotaxis. Z-MDMs were incubated with LPS (10 ng/ml) and TNFα neutralizing antibody overnight. **(A)** The effect of TNFα neutralizing antibody on neutrophil efferocytosis by MDMs was examined (n=4). For each Z-MDM individual sample, the efferocytosis rate of control MDM was set to 100%, and the efferocytosis rates of the other three treatments were normalized to control MDM. **(B)** The relative expression levels of CXCL-8 in the LPS-treated cells were calculated in comparison to their non-treated controls (n=3). The CXCL-8 expression level of control MDM was set to 1, and the CXCL-8 expression levels of the other three treatments were normalized to control MDM. **(C)** Conditioned media of Z-MDM samples were collected for neutrophil chemotaxis assay (n=5). The number of migrated neutrophils was counted, and neutrophil migration rate was calculated based on the number. The migration rate of control MDM was set to 100%, and the migration rates of the other three treatments were normalized to control MDM. *Denotes statistical significance (p-value < 0.05) according to two-tailed Student's t-test.

suspected that TNFα might be responsible for the increased expression of CXCL-8 and CXCL-1 in LPS-stimulated Z-MDMs. We examined whether their expression is reduced when TNFα signaling is inhibited in Z-MDMs. The expression level of CXCL-8 was significantly reduced when MDMs were incubated with TNFα neutralizing antibody (**Figure 7B**, p-value=0.019). The expression level of CXCL-1 was also reduced in the cells but the degree of the reduction was not significant (data not shown). We then performed neutrophil migration in conditioned media of the Z-MDMs in which TNFα signaling was inhibited. The result confirmed that neutrophil migration rate is reduced when MDMs were incubated with TNFα neutralizing antibody in Z-MDMs (**Figure 7C**, p-value=0.024).

Suppression of CXCL-8 Through Macrophage Efferocytosis of Apoptotic Cells

Efferocytosis plays a critical role in the resolution of inflammation by preventing the secondary necrosis of dead cells and triggering several anti-inflammatory signalings. Emerging evidence suggests that the expression of inflammatory cytokines is suppressed in post-efferocytotic macrophages (41, 42). We induced inflammatory signaling in MDMs using LPS and examined whether the efferocytosis of apoptotic neutrophils is able to suppress LPS-induced CXCL-8 expression. As shown in **Figure 8A**, macrophage efferocytosis of apoptotic neutrophils suppressed the expression level of CXCL-8 by ~20% in M-MDMs but the cytokine suppression was not observed in Z-MDMs (p-value=0.039). We repeated the assay using apoptotic Jurkat cells (**Figure 8B**). The efferocytosis-mediated suppression in the expression level of CXCL-8 was observed in both M- and Z-MDMs, and the suppressed level was significantly higher in M-MDMs (p-value=0.032). The results imply that AAT plays an important role in macrophage efferocytosis-medicated cytokine suppression and probably in resolving inflammation.

The Effect of TNFα on the Expression of Efferocytosis-Related Genes

Our results showed that LPS-induced TNFα expression inhibits efferocytosis of apoptotic neutrophils by MDMs. It was

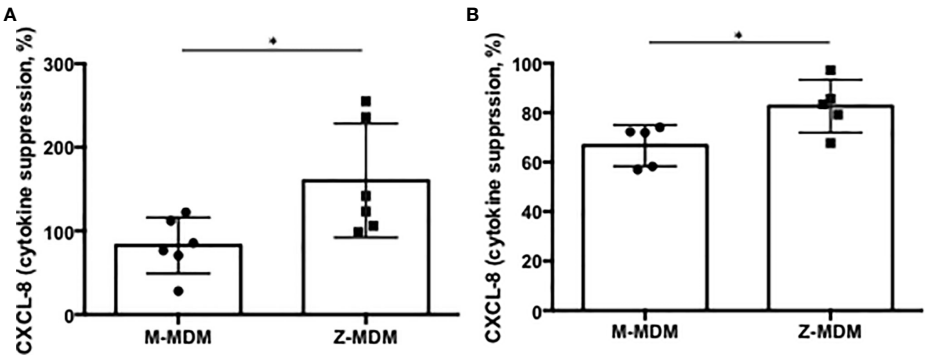

FIGURE 8 | Cytokine suppression in post-efferocytotic macrophages. LPS (10 ng/ml)-stimulated MDMs were incubated with apoptotic **(A)** neutrophils (n=6) or **(B)** Jurkat cells (n=5) for 3 h. Then, the expression level of CXCL-8 was compared between control and post-efferocytotic cells. Data are expressed as mRNA levels relative to control which are set to 100%. *Denotes statistical significance (p-value < 0.05) according to two-tailed Student's t-test.

previously reported that macrophages in a TNFα-rich inflammatory environment are less able to phagocytose apoptotic Jurkat cells (43). We wanted to identify the target molecules that TNFα modulates to inhibit macrophage efferocytosis. A number of genes are involved in the process of efferocytosis. We examined whether TNFα is able to reduce the expression of these genes. MDMs were incubated with TNFα for 1 or 18 h and the expression levels of the genes were examined in the TNFα-treated cells using qRT-PCR. We found that the expressions of CD14, CD36, and RARα are significantly down regulated by TNFα (**Figures 9A** p-value=0.005, **9B** p-value=0.0008, and **9C** p-value=0.0001). These three genes play an important role in macrophage efferocytosis of apoptotic cells; CD14 functions as a bridging molecule that tethers apoptotic cells to macrophages and associates with other molecules within the phagocytic synapse (44). CD36 is essential for macrophage recognition of phosphatidylserine on the surface of apoptotic cells (45). RARα increases apoptotic cell phagocytosis by inducing the expression of phagocytosis-related genes (46). Therefore, TNFα could mediate reduction in efferocytosis of apoptotic cells by inhibiting the expression of CD14, CD36 and RARα in macrophages. We compared the expression level of the genes between LPS-treated M-MDMs and Z-MDMs. LPS treatment reduced the expression of CD36 and RARα in both M- and Z-MDMs, but the reduced level was statistically significant only in Z-MDMs (**Figures 9D** p-value=0.0043 and **9E** p-value=0.0018). The cell surface distribution of CD36 was examined in MDMs. LPS reduced the cell surface level of CD36 in both M- and Z-MDMs, but the reduced level was statistically significant only in Z-MDMs (**Supplementary Figure 3**, p-value=0.014). The expression of CD14 was also reduced by LPS in both M- and Z-MDMs, and it was, on average, reduced more in Z-MDMs than M-MDMs. However, there was no statistical difference in CD14 expression between LPS treated M-MDMs and Z-MDMs (data not shown). We suspect that the combined effect of the reduced expression of CD14, CD36, and RARα lead to the statistically different efferocytosis rate of apoptotic neutrophils between M- and Z-MDMs. However, we could not rule out that there are other molecules, playing a role in reducing the efferocytosis rate of LPS-treated MDMs.

DISCUSSION

AATD is a genetic disorder leading to emphysema and chronic obstructive pulmonary disease mostly due to a significantly low level of AAT (47); the concentration of circulating AAT is 20–53 μM in normal individuals while it ranges from 3 to 7 μM in AATD individuals with homozygous Z genotype (5). A number of studies aimed to elucidate the pathogenesis of lung diseases associated with AATD and found that alveolar neutrophils are prevalent in AATD individuals (10). It suggests that AAT has an ability to limit excessive neutrophil accumulation to the pulmonary alveolus as well as its primary function of defense against the elastolytic burden in the lower airways posed by various proteases. It has been suggested that AAT has other capabilities that extend beyond its antiprotease role (20). In this study, we demonstrated that AAT has multifaceted abilities to maintain a neutrophil balance in the pulmonary alveolus and consequently a healthy lung. One previous study found that the chemotactic migration rate of PiZZ neutrophils is 2–8 times higher than PiMM neutrophils and extracellularly added AAT reduced neutrophil chemotaxis by binding to CXCL-8 (20). Another study showed that AAT is able to directly bind to LTB4, a potent neutrophil chemoattractant, and consequently inhibits LTB4–BLT1 interaction to reduce neutrophil chemotaxis (48). A common finding of the previous studies is that AAT inhibits neutrophil chemotaxis through its binding to those neutrophil chemotactic factors. Unlike the previous studies, we found that AAT is able to modify the expression of neutrophil chemotactic factors in LPS-stimulated macrophages. Our results showed that the expression level of neutrophil chemotactic factors is significantly higher in LPS-treated Z-MDMs than M-MDMs and that neutrophil migration rate is

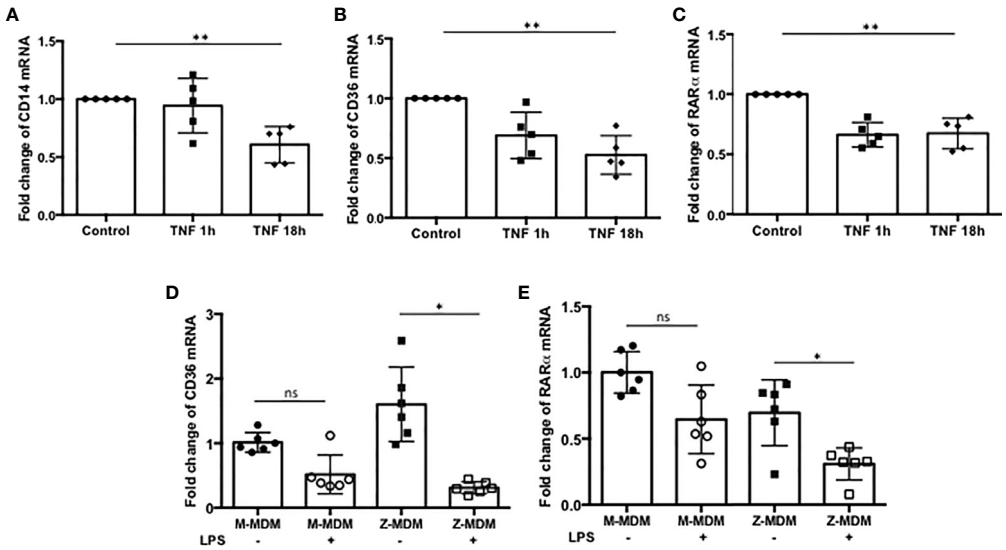

FIGURE 9 | The expression of efferocytosis-related genes regulated by TNFα. MDMs were incubated with TNFα (10 ng/ml) for 1 and 18 h, and the expressions of **(A)** CD14, **(B)** CD36, and **(C)** RARα were examined in the cells (n=5). **Denotes statistical significance (p-value < 0.01) according to one-way ANOVA test. MDMs were incubated with LPS (10 ng/ml) overnight and the relative expression levels of **(D)** CD36, and **(E)** RARα were calculated in comparison to non-treated controls for M-MDMs and Z-MDMs (n=6). *Denotes statistical significance (p-value < 0.05) according to two-tailed Student's t-test.

significantly higher in the conditioned media of Z-MDM culture than that of M-MDM culture. In addition, our data showed that the expression level of CXCL-8 is more significantly suppressed by the efferocytosis of apoptotic cells in M-MDMs than Z-MDMs. Taken together, the results support that AAT is able to inhibit the expression of the neutrophil chemotactic factors in macrophages and thus block excessive neutrophil infiltration to pulmonary alveoli during LPS-mediated inflammation while Z-AAT variant increases the number of alveolar neutrophils in AATD individuals by inducing the expression of the neutrophil chemotactic factors in alveolar macrophages.

Neutrophils are terminally differentiated cells and have a very short life span (49). Immediately after bacterial infection, a number of neutrophils migrate to the infection site. When the recruited neutrophils fulfill their role, neutrophils undergo programmed cell death. Lingering neutrophils exacerbate inflammation and cause tissue injury. Thus neutrophil apoptosis is essential to normal tissue homeostasis. However, inappropriate or premature apoptosis of neutrophils may compromise their function, impairing host defense (50–53). Our data showed that the expression level of TNFα is significantly higher in Z-MDMs than M-MDMs. It was previously reported that TNFα induces neutrophil apoptosis (54). Given that the apoptosis rate of PiZZ neutrophils is two-fold higher than PiMM neutrophils (53) and the expression level of TNFα promoting neutrophil apoptosis is higher in Z-MDMs, Z-AAT variant accelerates neutrophil apoptosis in the pulmonary alveoli of AATD individuals.

Recognition and efferocytosis of apoptotic cells by macrophages is a critical step to resolve inflammation by mediating secretion of anti-inflammatory cytokines TGF-β and IL-10 that inhibit inflammatory response (55, 56). It was previously demonstrated that efferocytosis of apoptotic cells by macrophages could accelerate resolution of LPS-induced lung inflammation in a TGF-β dependent manner (25, 57). Our result showed that LPS inhibited the efferocytosis of apoptotic neutrophils by macrophages and the inhibitory effect of LPS on the efferocytosis was significantly higher in Z-MDMs than M-MDMs, indicating that AAT combats LPS-reduced macrophage efferocytosis. Impaired macrophage efferocytosis could lead to a higher neutrophil infiltration rate to alveoli because prolonged presence of apoptotic neutrophils aggravates inflammation that further increases the expression of neutrophil chemotactic factors in alveolar macrophages. Previous studies on COPD, which is highly associated with Z-AAT variants, found that alveolar macrophages efferocytosis are impaired in COPD patients and suggest that the impaired efferocytosis or phagocytosis by alveolar macrophages could perpetuate an inflammatory response (18, 58–60). Our data showed that LPS-induced efferocytosis impairment was recovered in Z-MDMs when TNFα signaling was inhibited in the cells by TNFα neutralizing antibody. It suggests that TNFα signaling is responsible for the reduced macrophage efferocytosis in LPS-stimulated cells. When MDMs were stimulated with LPS, the expression level of TNFα was significantly higher in Z-MDMs than M-MDMs. It indicates that the clearance of apoptotic neutrophils by alveolar macrophages would be more impaired in AATD individuals by the increased TNFα level during LPS-mediated inflammation. Indeed, TNFα signaling has been proposed to drive immune cell dysfunction causing lung

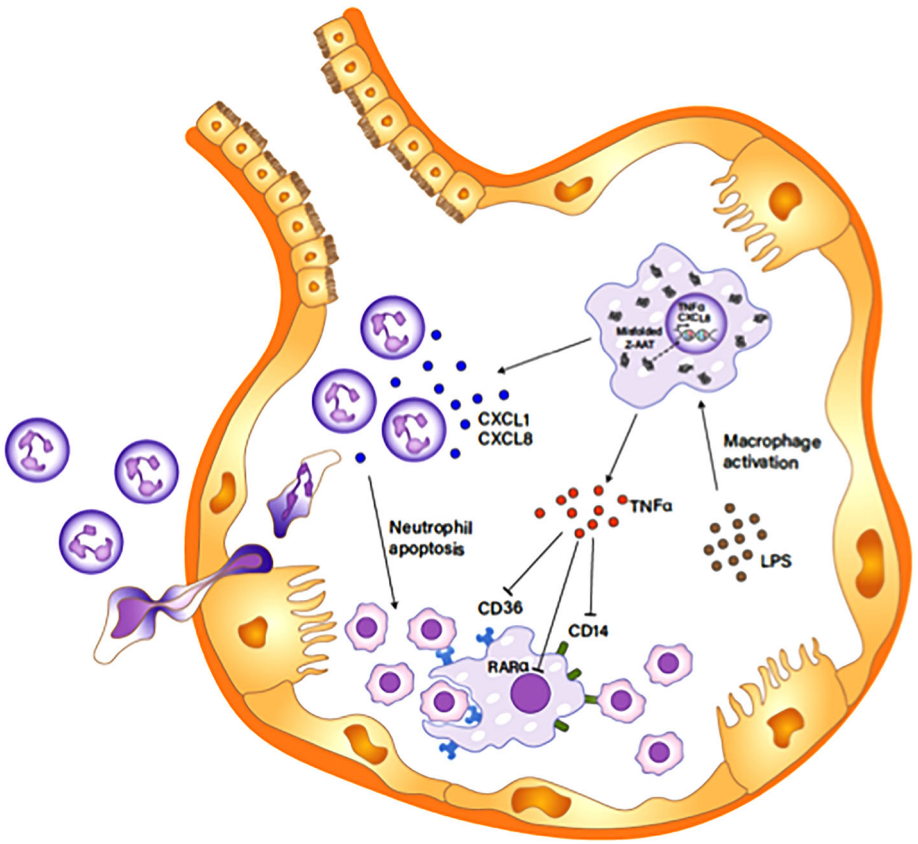

FIGURE 10 | Z-AAT causing excessive neutrophil accumulation in the pulmonary alveolus. Unfolded Z-AAT induces the expressions of CXCL-8 and TNFα in LPS-stimulated macrophages. The increased level of the chemotactic factor accelerates neutrophil infiltration to the pulmonary alveolus. LPS-induced TNFα expression inhibits the expression of CD14, CD36, and RARα that are important in macrophage efferocytosis of apoptotic cells, delaying the clearance of apoptotic neutrophils by macrophages. The impaired clearance of neutrophils aggravates and prolongs the neutrophil influx in the pulmonary alveolus.

diseases in AATD individuals (47). We attempted to explain how TNFα signaling reduces macrophage efferocytosis and found that the expression levels of CD14, CD36, and RARα were significantly reduced by TNFα in MDMs. This suggests that TNFα signaling inhibits the expression of the three genes to impair macrophage efferocytosis of apoptotic neutrophils. The expression level of the three genes was significantly lower in LPS-treated Z-MDMs and that, at least in part, explains the higher inhibitory effect of LPS on the efferocytosis by Z-MDMs.

The present study highlights the pivotal role of the AAT molecule in modulating the expression of pro-inflammatory cytokines in alveolar macrophages. Upon pro-inflammatory cytokine stimulation, alveolar macrophages produce cytokines and chemokines that attract and activate neutrophils. A high expression level of CXCL-8 has been found in pulmonary diseases, including acute respiratory distress syndrome and idiopathic pulmonary fibrosis (61, 62). TNFα has been suggested to be essential in the pathogenesis of lung diseases associated with AATD (47). The effect of extracellular AAT on regulating TNFα expression and CXCL-8 activity has been well studied. In this study, we focus on examining the effect of intracellular Z-AAT on the expression of CXCL-8 and TNFα in LPS-stimulated MDMs. We show that the level of intracellular Z-AAT is highly increased by LPS treatment, and the expressions of CXCL-8 and TNFα are significantly increased in LPS-treated Z-MDMs. We also show that inhibiting TNFα signaling reduces the expression of CXCL-8 and alleviates the inhibitory effect of LPS on macrophage efferocytosis of apoptotic neutrophils.

In conclusion, Z-AAT accumulation in alveolar macrophages is a main driver for excessive neutrophils in pulmonary alveoli of AATD individuals by inducing the expression of CXCL-8 and TNFα in the cells and, in addition to a low concentration of extracellular AAT, further exacerbates neutrophil burden in the individuals. We propose a mechanism to explain the role of Z-AAT in neutrophil accumulation in pulmonary alveoli of AATD individuals, as depicted in **Figure 10**. The knowledge gained from the present study helps us better understand the multifaceted effect of AAT on regulating neutrophil balance and the underlying mechanisms, which is critical to develop improved therapies to reinforce host defense and attenuate detrimental pulmonary diseases associated with AAT deficiency.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of Florida Institutional Review Board protocol 2015-01051. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JL and MB: designed the study, planned the experimental work, and analyzed the data. JL, YL, CM, and KH: performed experimental work and analyzed the data. RO and JW: collected biological samples. JL and MB: wrote the manuscript. YL, KH, CM, RO, and JW: critically reviewed the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.574410/full#supplementary-material

SUPPLEMENTARY FIGURE 1 | Neutrophil isolation and apoptosis. Primary neutrophils were isolated from PiMM volunteers for neutrophil chemotaxis assay. **(A)** The isolated neutrophils were incubated with fluorescent CD16 and CD66b antibodies, and percentage of CD16 and CD66b-positive cells were calculated using Gallios flow cytometer with Kaluza software. **(B)** The apoptotic rate of neutrophils was assessed by flow cytometry with Annexin V/propidium iodide staining.

SUPPLEMENTARY FIGURE 2 | Visualization of neutrophil efferocytosis by MDMs. **M**-MDMs were incubated with red color-labeled apoptotic neutrophils, and the images of phagocytizing MDMs were captured using fluorescent microscope. **(A–C)** indicate the phase contrast of MDMs, red color-labeled apoptotic neutrophils, and MDMs phagocytizing apoptotic neutrophils, respectively.

SUPPLEMENTARY FIGURE 3 | Calculation of neutrophil efferocytosis rate. Z-MDMs were incubated with apoptotic neutrophils in the absence or presence of LPS (10 ng/ml). **(A, C)** show control and LPS-treated cells that were gated on size and granularity, respectively. **(B, D)** show the percentage of control and LPS-treated MDMs phagocytozing apoptotic neutrophils, respectively. In the histogram plot, the first peak indicates non-phagocytosing MDM population and the second peak indicates phagocytosing MDM population.

SUPPLEMENTARY FIGURE 4 | Cell surface distribution of CD36 in MDMs. MDMs were incubated in the absence or presence of LPS (10 ng/ml) overnight. Blue and red fluorescence indicates the nucleus and CD36, respectively. 63x Images were taken using a fluorescence microscope.

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REVIEW
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Regulation of PD-L1 Expression by NF-κB in Cancer

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Immune checkpoints are inhibitory receptor/ligand pairs regulating immunity that are exploited as key targets of anti-cancer therapy. Although the PD-1/PD-L1 pair is one of the most studied immune checkpoints, several aspects of its biology remain to be clarified. It has been established that PD-1 is an inhibitory receptor up-regulated by activated T, B, and NK lymphocytes and that its ligand PD-L1 mediates a negative feedback of lymphocyte activation, contributing to the restoration of the steady state condition after acute immune responses. This loop might become detrimental in the presence of either a chronic infection or a growing tumor. PD-L1 expression in tumors is currently used as a biomarker to orient therapeutic decisions; nevertheless, our knowledge about the regulation of PD-L1 expression is limited. The present review discusses how NF-κB, a master transcription factor of inflammation and immunity, is emerging as a key positive regulator of PD-L1 expression in cancer. NF-κB directly induces *PD-L1* gene transcription by binding to its promoter, and it can also regulate PD-L1 post-transcriptionally through indirect pathways. These processes, which under conditions of cellular stress and acute inflammation drive tissue homeostasis and promote tissue healing, are largely dysregulated in tumors. Up-regulation of PD-L1 in cancer cells is controlled via NF-κB downstream of several signals, including oncogene- and stress-induced pathways, inflammatory cytokines, and chemotherapeutic drugs. Notably, a shared signaling pathway in epithelial cancers induces both PD-L1 expression and epithelial–mesenchymal transition, suggesting that PD-L1 is part of the tissue remodeling program. Furthermore, PD-L1 expression by tumor infiltrating myeloid cells can contribute to the immune suppressive features of the tumor environment. A better understanding of the interplay between NF-κB signaling and PD-L1 expression is highly relevant to cancer biology and therapy.

Keywords: tumor associated macrophages, T cells, immune checkpoint inhibitors, tumor immunity, non-small-cell-lung cancer, tissue homeostasis, epithelial-mesenchymal transition, inflammation

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INTRODUCTION

The immune system relies on a complex balance between activating and inhibitory mechanisms to counteract infections and other threats while avoiding excessive tissue damage. Among the inhibitory molecules, a distinct set of inhibitory receptors and their ligands, collectively called "immune checkpoints," has recently attracted a lot of attention for its relevance in cancer therapy, chronic infections, and autoimmune diseases (1). Programmed cell death protein-1 (PD-1) is a member of the CD28 family expressed by activated lymphocytes. PD-1 triggers immunosuppressive signals upon engagement by its ligands, i.e., PD-L1 (CD274 or B7-H1) and PD-L2 (CD273), which are members of the B7 family. While PD-L2 expression is largely restricted to antigen-presenting cells (APCs) and B1 lymphocytes, PD-L1 is expressed by APCs (mostly macrophages and dendritic cells), activated/exhausted T and B lymphocytes, and regulatory T cells (T_{reg}), among others (2, 3). PD-L1 is also expressed by the cardiac endothelium, placenta, and pancreatic islets, with a possible role in maintaining immunological tolerance in these districts (4). Cancer cells can express PD-L1 and exploit the PD-L1-driven inhibitory pathway to their benefit as a key mechanism of immune evasion (5).

Return to the steady state at the end of immune response is tightly regulated (6, 7), and it is widely recognized that the PD-1/PD-L1 axis plays a central role in physiological immune homeostasis, contributing to the prevention of lymphocyte over-activation and immunopathology (1, 8). A variety of mechanisms have been involved in PD-1-mediated suppression of activated T lymphocytes, including exhaustion, inflammatory cytokine secretion inhibition, anergy, and apoptosis (8). PD-1 expression by antigen-responding T and B cells is tightly regulated, which allows for a stringent control of lymphocyte response (9, 10). Accordingly, PD-1 deficiency is associated with the development of autoimmune diseases (11, 12). PD-1 can be expressed also by γδ T cells, natural killer (NK) cells, and innate lymphoid cells (ILCs), which are circulating and tissue-resident lymphocytes involved in tissue repair and early responses against pathogens and cellular stress (13–16).

Regulation of PD-L1 expression and function takes place at different levels, as extensively reviewed by Sun and colleagues (17). Several mediators of inflammation are PD-L1 inducers, including TNFα, IFN-γ, IL-10, IL-17, and C5a (18–21). The JAK/STAT, RAS/MAPK, and PTEN-PI3K/AKT pathways are involved in the control of *PD-L1* gene expression *via* different downstream transcription factors, such as STAT1, STAT3, IRF1, IRF3, HIF-1α, MYC, JUN, BRD4, and NF-κB (22). The corresponding DNA-binding elements, except for IRF3, have been described on the PD-L1 promoter (23–32). Additional mechanisms of regulation include microRNA-mediated post-transcriptional inhibition (e.g., miR-513, miR-34a, miR-200, and miR-570) and the presence of a soluble form of PD-L1 (sPD-L1) in the blood, which possibly competes with the membrane-bound PD-L1 for binding to PD-1 (17, 33). Though only partially investigated, reverse signaling of PD-L1 has been reported in tumor cells and macrophages, resulting in pro-survival and inhibitory signals, respectively (34, 35). In addition to the PD-1/PD-L1 pair, a further interaction between PD-L1 and B7.1 (CD80) has been implicated in the inhibition of T-cell proliferation and cytokine production (36).

IFN-γ is one of the most studied PD-L1 inducers in tumors but PD-L1 expression does not necessarily mirror the IFN-γ signature (37, 38). NF-κB, a central player of inflammation and immunity, is emerging as a key positive regulator of PD-L1 expression. Notably, two recent studies, by using a CRISPR-Cas9-based wide screening approach, have identified NF-κB as a major determinant of cancer cell resistance against immune attack (39, 40). Considering the pivotal role played by PD-L1 for tumor cell immune evasion, the disclosure of the relationship between NF-κB signaling and PD-L1 expression is of great relevance (41). The present review gives an overview of the experimental works linking NF-κB to the regulation of PD-L1 expression in tumors. Furthermore, the implications of NF-κB-mediated control of PD-L1 expression for tissue homeostasis, cancer biology, and immune-therapy are discussed.

NF-κB AMONG INFLAMMATION, IMMUNITY, AND CANCER

NF-κB [nuclear factor kappa-light-chain-enhancer of activated B cells, discovered by Sen and Baltimore in 1986 (42)] is a transcription factor supporting host responses to cellular stress and immune responses to pathogens and other challenges. NF-κB can be composed of different dimers of the NF-κB family, activated downstream of multiple signaling pathways [for a comprehensive review on NF-κB see (43)]. Briefly, five proteins belong to the NF-κB family: p50, p52, p65 (RelA), RelB, and c-Rel; they are encoded by *NFKB1*, *NFKB2*, *RELA*, *RELB*, and *REL* genes, respectively. *NFKB1* and *NFKB2* codify for the p105 and p100 precursors, which are then processed to the active forms p50 and p52, respectively. The canonical (or classical) pathway leads to the activation of the p50/p65 (RelA) or p50/c-Rel heterodimers, while the non-canonical (or alternative) pathway leads to the activation of the p52/RelB heterodimer. The different heterodimers play distinct biological roles, controlling lymphoid organ development, immune activation, and cell survival (44–46). In healthy cells, NF-κB complexes are retained in the cytoplasm by inhibitory proteins belonging to the IκB family. Activating signals of the canonical pathway, which include TNFα, IL-1, and Lypopolysaccharide (LPS), cause IκB phosphorylation *via* IκB kinase (IKK). IKKα and IKKβ are the catalytic subunits of the multimeric IKK, which also includes the scaffold protein IKKγ (also named NEMO). Upon phosphorylation, IκB is ubiquitinated and targeted to degradation by the proteasome. This allows NF-κB's translocation to the nucleus where it regulates gene transcription by binding to the promoters of its target genes. Direct phosphorylation of p65 further enhances NF-κB nuclear translocation. In the non-canonical pathway, the inactive precursor of p52/RelB heterodimer is matured into its active form by IKKα phosphorylation and proteasomal processing upon NF-κB-induced kinase (NIK) activation by signals such as CD40L and lymphotoxin (47). A third atypical IKK-independent pathway is mainly triggered by hypoxia and

TABLE 1 | Predicted binding sites for NF-κB on the *PD-L1* gene promoter.

Consensus sequence	**−5 −4 −3 −2 −1 0 +1 +2 +3 +4** **5′-G G G N N N N N C C-3′**	**N: any base**		
Position	**κB element**	**Validated by**	**Cell type**	**References**
From −387 to −378	GGGGG**A**CGCC	ChIP-PCR	TNBC	(67)
N/A	GGAAA**G**TCCA	Luciferase assay	Cervical cancer	(68)
N/A	GGAGC**G**TTCC	Luciferase assay		
From −1769 to −1760	GGCAA**A**TTCC		Macrophage	(69)
From −1293 to −1284	GGGAA**G**TCAC			
From −610 to −601	GGGAA**G**TTCT	ChIP-PCR		
From −75 to −66	GGAAA**G**TCCA			
From −606 to −597	N/A		Gastric cancer	(70)
From −238 to −229	N/A			
From −71 to −62	N/A	Luciferase assay		
From −49 to −40	N/A			

Position refers to the transcription starting site. TNBC, triple-negative breast cancer; N/A, not available.

UV radiation and leads to p50/p65 activation (48). NF-κB activation is regulated by several negative loops, including phosphorylation/de-phosphorylation and ubiquitination events. The NF-κB inhibitor IκBα is itself a transcriptional target of NF-κB. Different microRNAs (e.g., miR-146a, miR-302b) can contrast mRNA translation of proteins involved in the NF-κB cascade (49, 50).

Within the context of inflammation and immunity, NF-κB is activated downstream of the toll-like receptor (TLR)-MyD88 pathway that senses both pathogen-associated molecular patterns (PAMPs), such as LPS and other microbial products, and damage-associated molecular patterns (DAMPs), which are released by either stressed or dying host cells (51). NF-κB positively regulates the expression of inflammatory cytokines (TNFα, IL-1, IL-6), chemokines (CCL2, CCL5, CXCL8), adhesion molecules (VCAM1, ICAM1), angiogenic (VEGF), and anti-apoptotic factors (BCL-2, BCL-X_L, FLIP), enzymes required for prostaglandin and NO synthesis (COX2, iNOS) (52). Furthermore, NF-κB is activated downstream of both the T-cell receptor (TCR) and B-cell receptor (BCR), sustaining the adaptive immune response (e.g., by controlling IL-2 expression) (53). NF-κB is also involved in NK-cell activation regulating IFN-γ production (54).

Within the context of cancer, NF-κB activation can support the neoplastic process (55, 56). One of the earliest pieces of evidence dates back to the discovery of the retroviral oncogene *v-Rel* (57). Furthermore, NF-κB can induce the transcription of the mitogenic factors MYC and Cyclin-D (58, 59). Finally, the AKT-mediated NF-κB activation, which frequently occurs in tumors, promotes cell survival and contributes to chemotherapy resistance of cancer cells (60). Remarkably, NF-κB activation in cells of the tumor immune infiltrate can have both anti-tumoral and pro-tumoral consequences depending on the immune cell type. On one hand, T cells, NK cells, and NKT cells require NF-κB for their anti-tumoral effector activity (61–63), but on the other hand, NF-κB sustains T_{reg} and myeloid-derived suppressor cell (MDSC) activity, resulting in pro-tumoral effects (64–66).

NF-κB-MEDIATED EXPRESSION OF PD-L1 BY CANCER CELLS

PD-L1 expression is often observed in tumors and correlates with aggressive behavior and poor prognosis. Whether PD-L1 is expressed by cancers cells, especially at the later stages of the disease, as negative feedback of a chronic inflammation process intertwined with cancer progression or as a consequence of cell selection by the immune system is still unclear. Certainly, PD-L1 expression confers a selective advantage to cancer cells, e.g., by enabling them to avoid host immune response by activated CD8 T lymphocytes and NK cells.

Considering the key role played by NF-κB in inflammation, immunity, and cancer, perhaps it is not surprising that NF-κB regulates PD-L1 expression in tumors, either directly at the transcriptional level or via indirect mechanisms. Different binding sequences for NF-κB have been described on the promoter of the *PD-L1* gene (**Table 1**). The canonical consensus for NF-κB DNA binding, named κB, consists of a nearly palindromic sequence, 5′-GGGRNWYYCC-3′ (where R: purine, Y: pyrimidine, W: adenine or thymine, and N: any base), which recently has been broadened to 5′-GGGNNNNNCC-3′ (32, 71, 72).

PD-L1 expression by cancer cells can be related to endogenous oncogenic pathways or oncogenic virus infection. In addition, PD-L1 expression by either cancer cells or tumor infiltrating cells can be driven by different kinds of exogenous stimuli, including cellular stress, e.g. stress induced by UV exposure or chemotherapy, as well as pro-inflammatory cytokines (such as TNFα and IFN-γ) in the tumor bed (**Figure 1** and paragraphs below).

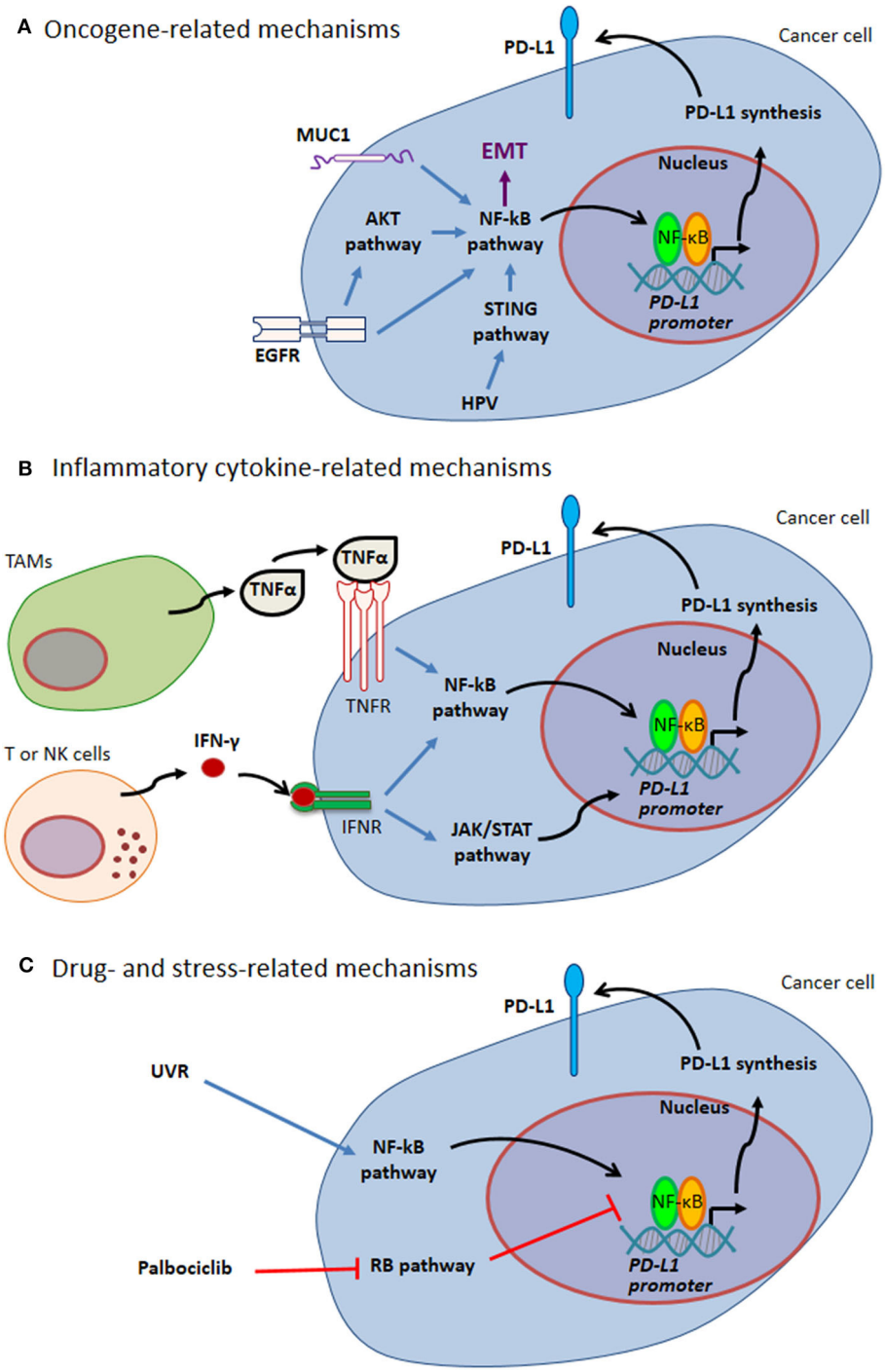

FIGURE 1 | Mechanisms of PD-L1 expression through NF-κB. **(A)** Oncogene-related mechanisms. MUC1 and EGFR up-regulate PD-L1 expression by activating NF-κB pathway. These pathways are intertwined with EMT. HPV modulates PD-L1 expression triggering STING that in turn activates NF-κB. **(B)** Inflammatory cytokine-related mechanisms. Tumor-infiltrating immune cells can produce several cytokines regulating PD-L1 expression. Two well-known cytokines acting via NF-κB pathway are TNFα produced by TAMs and IFN-γ produced by tumor infiltrating T and NK cells. **(C)** Drug- and stress-related mechanisms. Different drugs act on NF-κB transcriptional activity (e.g., Palbloclicib). Stress response to UVR activates NF-κB, thus mediating PD-L1 up-regulation. Blue arrows indicate activation of NF-κB pathway; black arrows indicate NF-κB-mediated PD-L1 up-regulation; red T-arrows indicate negative regulation. EGFR, Epidermal Growth Factor Receptor; EMT, Epithelial-Mesenchymal Transition; HPV, Human Papilloma Virus; IFN, Interferon; JAK, Janus Kinase; MUC1, Mucin 1; NK, Natural Killer; PD-L1, Programmed Cell Death Protein 1 Ligand; RB, Retinoblastoma; STAT, Signal Transducer and Activator of Transcription; STING, Stimulator of Interferon Genes; TAMs, Tumor Associated Macrophages; TNF, Tumor Necrosis Factor; UVR, ultraviolet radiation.

Oncogene-Related Mechanisms

A direct link between the epidermal growth factor receptor (EGFR) and PD-L1 expression *via* NF-κB has been described in non-small-cell lung cancer (NSCLC), the most common lung cancer (73, 74). EGFR mutations associated with constitutive tyrosine kinase-mediated phosphorylation lead to NF-κB activation along with PD-L1 overexpression. Accordingly, the tyrosine kinase inhibitor gefitinib, approved for NSCLC treatment, reduces PD-L1 levels by inhibiting the NF-κB pathway. Notably, even wild-type EGFR induces PD-L1 up-regulation after stimulation with EGF (73). Mechanistically, EGFR triggers ERK, AKT, and IκBα phosphorylation, resulting in HIF-1α and PD-L1 accumulation (74). Activation of AKT is reported to increase HIF-1α protein translation, and it is known that the PD-L1 promoter contains an HIF-1α response element (27, 75, 76). As a consequence, EGFR activation in NSCLC promotes PD-L1 expression directly by phosphorylating IκBα and indirectly *via* HIF-1α. Furthermore, HIF-1α can support NF-κB pathway activation by driving *IKKβ* gene transcription through a hypoxia response element present in the promoter and, at the same time, by directly inducing p65 (77, 78). Conversely, it has been reported that NF-κB can induce HIF-1α by binding directly to the HIF-1α promoter (79, 80). Thus, both HIF-1α and NF-κB pathways sustain PD-L1 expression and reinforce each other.

Overexpression of the oncoprotein Mucin 1 (MUC1) is correlated to NF-κB-mediated PD-L1 expression in both NSCLC and triple-negative breast cancer (TNBC) (81, 82). MUC1, through its MUC1-C subunit, activates the PI3K/AKT, MAPK, β-catenin/MYC, and NF-κB pathways (83). NF-κB activation by MUC1 occurs, at least, through three different mechanisms: (i) binding to p65, thus forming a p65/MUC1 transcriptionally active complex (84); (ii) binding to IKKβ-IKKγ complexes, which triggers IκBα phosphorylation (85); and (iii) associating with TGF-β-activated kinase-1 (TAK1), which phosphorylates IKKβ on Ser181 (86). Once activated, NF-κB enhances the expression of MUC1, thus creating a self-sustaining loop. At the same time, MUC1, *via* the YAP/β-catenin pathway, mediates the induction of the WNT target gene *MYC* (87). PD-L1 promoter contains both an E-box sequence (CAGCTT) for MYC binding at positions from −164 to −159 nt and a p65-binding site (GGGGGACGCC) at positions from −387 to −378 nt upstream of the transcription-starting site (67) (**Table 1**). Hence, MUC1 can induce the expression of PD-L1 triggering the occupancy of its promoter by both p65 and MYC.

It is remarkable that the signaling of PD-L1 induction shares important elements with that occurring during epithelial-mesenchymal transition (EMT), a tissue remodeling process typical of advanced epithelial cancers (88). PD-L1 expression is observed during EMT in NSCLC (89). Hypoxia and chronic inflammation are the main drivers of EMT, which is largely mediated by TGF-β1, TNFα, HGF, EGF, and PDGF (90, 91). Interestingly, PD-L1 expression is not regulated by the EMT-specific transcription factor SNAIL, but it involves a non-canonical NF-κB signaling. In particular, TNFα activates IKKε, which leads to p65 recruitment on the PD-L1 promoter. Concomitantly, TGF-β1 reduces DNA methyl transferase-1 (DNMT1) recruitment on the PD-L1 promoter, causing its de-methylation and thus enhancing its transcription. Notably, TNFα and TGF-β1 withdrawal reverts the EMT phenotype and, at the same time, abolishes PD-L1 expression (89). The interconnection between EMT and PD-L1 is evident in the MUC1 signaling with NF-κB as the matchmaker. NF-κB not only drives PD-L1 expression, but also the expression of ZEB1, a transcription factor that is able to mediate EMT (92). ZEB1, in turn, suppresses the transcription of miR-200, an inducer of epithelial differentiation, thus enhancing EMT and PD-L1 expression (93).

Taken together, these findings indicate that PD-L1 expression is a feature of EMT in cancer. Considering that (i) EMT occurs physiologically during embryonic development and (ii) PD-L1 expression can be enhanced by OCT4 and SOX2, two master "stemness" transcription factors driving the expression of genes necessary for the stem cell phenotype (94), it is tempting to speculate that NF-κB drives PD-L1 expression in the context of a general mechanism that has evolved to protect mesenchymal cells and stem cells from immune attack during physiological development.

It is now emerging that PD-L1 not only mediates negative feedback in the context of immune response, but it is also a component of a homeostatic response of epithelial cells to stress (95). In this case, PD-L1 expression can be linked to cellular conditions such as proliferation, adhesion, and migration (22). Supporting this consideration, MUC1, whose role in mammalians is to protect epithelial layers by forming a mucous barrier, senses cell stress and transduces signals that are able to activate the NF-κB pathway, which leads to PD-L1 expression (96). It is therefore convincing that NF-κB-mediated expression of PD-L1 takes part in cancer biology by echoing a process evolved to restore tissue integrity during the epithelial stress response.

Oncogenic viruses are also PD-L1 inducers. For example, infection by papilloma virus promotes PD-L1 expression in cervical cancer cells. Interferon-inducible 16 (IFI16) acts as viral DNA sensor and activates stimulator of IFN genes (STING), leading to TANK-binding kinase-1 (TBK1) activation that, in turn, initiates a cascade signaling that is able to recruit p-p65 on the PD-L1 promoter. The STING/TBK1 pathway activates both IRF3 and NF-κB, with NF-κB mediating a major contribution to *PD-L1* gene transcription (68). PD-L1 expression is also observed in NK/T cell lymphoma infected by Epstein-Barr virus. Both MAPK and NF-κB pathways are involved in PD-L1 induction in these cells (97).

Inflammatory Cytokine-Related Mechanisms

NF-κB regulates PD-L1 expression downstream of inflammatory cytokine-induced pathways in the tumor microenvironment, contributing to linking together two immune-related hallmarks of cancer, i.e., tumor-promoting chronic inflammation and immune escape (98). IFN-γ and TNFα will be considered here in more detail.

IFN-γ is one of the most studied PD-L1-inducer inflammatory cytokines, which is produced by highly activated T and NK cells infiltrating the tumor (38, 99). Although it is well-established that IFN-γ signals *via* JAK/STAT (100), there is evidence

that IFN-γ can also activate the NF-κB pathway, which in turn mediates PD-L1 up-regulation. For example, in melanoma cells, IFN-γ inducible expression of PD-L1 is linked to the activity of p50 and p65, and not to the activation of the interferon-related STAT factors (101). Moreover, inhibitors of the bromodomain and extra terminal (BET) proteins, a class of epigenetic regulators with immunomodulatory activity (102), reduce PD-L1 expression by inhibiting p50 transcription (103). Transcriptional up-regulation of *PD-L1* gene by NF-κB occurs also in clonal blasts from myelodysplastic diseases treated with IFN-γ and TNFα (104). Notably, also type I IFN, which can be produced by many types of cell stimulated by either PAMPs or DAMPs, is a PD-L1 inducer (105).

TNFα, which can be released by activated tumor associated macrophages (TAMs), is a major driver of inflammation and one of the main inducers of NF-κB in tumor microenvironment. It has been mentioned above that TNFα drives EMT and regulates PD-L1 expression (see previous paragraph). Apart from EMT, TNFα, IL-17, or a combination of both, can induce PD-L1 up-regulation *via* NF-κB (20). Moreover, Lim and colleagues have shown that TNFα-activated NF-κB can regulate PD-L1 post-transcriptionally through an indirect way (106). TNFα binds TNFR on cancer cells and induces, among other pathways, a signaling cascade that promotes p65 nuclear translocation *via* IKKβ. In the nucleus, p65 trans-activates the *COPS5* gene by binding to its promoter. *COPS5* codifies for COP9 signalosome 5 (CSN5), which is the catalytic subunit of a large multiprotein complex with deubiquitination activity (107). CSN5 is able to interact and deubiquitinate PD-L1 protein, increasing its stability and consequently its surface expression. The biological relevance of this regulatory mechanism is confirmed by the positive correlation observed in breast cancer specimens between p-p65, CSN5, and PD-L1 expression as well as the inverse correlation with granzyme B, a cytotoxic lymphocyte effector molecule (106). Notably, TNFα, through the activation of the NF-κB pathway, also up-regulates CSN2, which, by blocking the ubiquitination of the transcription factor SNAIL, promotes tumor invasiveness (108). Therefore, the inflammatory cytokine TNFα coordinates both EMT and tumor immune evasion by using NF-κB signaling (see also the previous paragraph). This pathway is negatively regulated by curcumin, a natural anti-inflammatory compound that is known to inhibit NF-κB signaling as well as CSN5 activity (109, 110). Accordingly, it has been shown that curcumin reduces TNFα-mediated PD-L1 stabilization (106).

Drug- and Stress-Related Mechanisms

Drugs currently used in the clinic or in pre-clinical studies can influence PD-L1 expression *via* NF-κB, also influencing epigenetic regulation. Epigenetic events, changing the chromatin structure *via* methylation or acetylation/deacetylation, modulate gene expression. For example, taxolo (named also paclitaxel) and gemcitabine induce transient expression of PD-L1 mRNA in ovarian cancer by up-regulating p65, even in the absence of IFN-γ signaling (111). NF-κB nuclear activity is regulated by reversible acetylation/deacetylation of p65 operated by HDAC3 (112). As a result, two histone deacetylase (HDAC) inhibitors, namely resminostat and entinostat, affect NF-κB mediated PD-L1 expression (113).

Palbociclib, a recently developed CDK4/6 inhibitor, promotes PD-L1 protein stabilization and increases *PD-L1* gene transcription (114, 115). The latest effect is due to an indirect mechanism involving the retinoblastoma protein (RB), which acts as a negative regulator of NF-κB functions, as follows. Hyper-phosphorylated RB (phosphorylation at S249 and T252) specifically interacts *via* its N-terminal portion with p65 in the nucleus and, in this manner, blocks NF-κB transcriptional activity, including the NF-κB-dependent transcription of PD-L1. Inhibition of the RB pathway by palbociclib induces the hypo-phosphorylated status of RB, thus enhancing NF-κB-mediated PD-L1 transcription (115).

Solar ultraviolet radiation (UVR) is a common environmental stress for the skin and is largely involved in the carcinogenesis of skin cancers. Besides the well-known mutagenic effects, UVR can establish an immunosuppressive environment by different mechanisms, including CTLA-4 and PD-L1 up-regulation (116). PD-L1 transcriptional up-regulation in melanocytes and melanoma cells has been linked to the transcriptional activity of either NRF2, a regulator of antioxidant proteins, or NF-κB (117, 118). Regarding NF-κB, UVR B exposure causes in keratinocytes and melanocytes the subcellular translocation from the nucleus and release outside the cell of the high mobility group box 1 protein (HMGB1), an early stress response DAMP. HMGB1 acts in an autocrine and/or paracrine fashion and binds the receptor for advanced glycation endproducts (RAGE) leading to downstream kinase TBK1 activation. TBK1, and not TAK1, is involved in starting the NF-κB cascade after UVR B exposure even though TAK1 has been shown to play a role in the DNA-damage induced NF-κB signaling (119). TBK1 phosphorylates its canonical target IRF3 and IKKβ, which, by phosphorylating IκBα, removes its inhibition on p65. PD-L1 promoter contains two putative IFN-stimulated response elements (ISRE) along with NF-κB binding sites. Once activated, p65 forms the canonical p50/p65 heterodimer but also interacts with IRF3 itself forming a complex that is recruited to PD-L1 promoter at the NF-κB binding sites, thus starting *PD-L1* gene transcription. In agreement with PD-L1 up-regulation, and possibly mediated also by additional mechanisms, melanoma cells show a reduced susceptibility to CTL-dependent cytotoxicity after UVR B treatment (118).

NF-κB-MEDIATED EXPRESSION OF PD-L1 BY TUMOR INFILTRATING MACROPHAGES

Chronic inflammation can pave the way to tumor onset and cancers are often embedded in an inflammatory microenvironment that enhance tumor progression (120). In this context, investigating the regulation of PD-L1 expression by tumor infiltrating cells can shed light on the link between chronic inflammation, tumor progression, and immune escape. TAMs are key cellular players of the tumor infiltrate that can regulate the inflammatory process and, at the same time, act as

antigen-presenting cells for CD4 T lymphocytes. Macrophages have heterogeneous phenotypes, ranging from classical M1 to alternative M2 cells, which represent extremes in a continuous spectrum of activation states, with M1 cells having tumoricidal activity and M2 cells favoring tumor progression (121, 122). In addition to the identification of PD-L1$^+$ TAMs (123), PD-L1 has been found highly expressed in MDSCs, a population of tumor-infiltrating myeloid cells implicated in inhibition of other cells of the immune system (124, 125), and a MDSC molecular program linked to NF-κB activation has been related to PD-L1 expression (126).

NF-κB can directly regulates PD-L1 expression in macrophages and other myeloid cells stimulated by inflammatory cytokines (IL-12, IFN-γ), PAMPs, and DAMPs (69, 107, 127, 128). LPS, a prototypical PAMP, can stimulate PD-L1 expression by macrophages via TLR signaling. Indeed, within 1 h after LPS sensing, p65 translocates to the nucleus where it binds to the PD-L1 promoter, inducing *PD-L1* gene transcription independently of AP-1 and IRF3, two canonical transcription factors activated by LPS-TLR4 signaling (69). The mechanism whereby LPS promotes *PD-L1* gene expression through the transcriptional activity of NF-κB links inflammation to its control, and it is not restricted to macrophages but also occurs in tumors, as demonstrated in gastric cancer cells (70). Furthermore, melanoma-derived extracellular vesicles carrying Heat Shock Protein (HSP)-86, a typical DAMP, can stimulate PD-L1 expression by myeloid cells *via* TLR4 signaling. Interestingly, a strong NF-κB activation is observed in the immortalized myeloid suppressor cell line MSC-2 stimulated with the extracellular vesicles, and PD-L1 up-regulation is reduced in a dose-dependent manner by the NF-κB inhibitor Bay11-8082 (128).

Despite a direct role for NF-κB in the expression of PD-L1 by macrophages has been demonstrated in response to inflammatory cytokines, NF-κB activation in TAMs is the result of the combined action of both microenvironmental signals and microphysiological conditions (i.e., hypoxia, glucose levels, and pH), and accumulating evidence indicates that different activation states of NF-κB regulate functions and phenotypic heterogeneity of TAMs (121). Moreover, along with tumor-promoting functions, TAMs and monocytic MDSCs share similar molecular traits, such as nuclear accumulation of p50 NF-κB inhibitory homodimer, which drives M2 macrophage polarization and suppressive activity (66, 129, 130). The nuclear accumulation of p50 homodimer hinders the expression of inflammatory cytokines (TNFα, IL-1, IL-6) while increasing anti-inflammatory cytokines (IL-10, TGFβ) and chemokines (CCL17, CCL2), being therefore essential for the resolution of the inflammatory response (131). It thus appears that tumors co-opt transcriptional mechanisms guiding the resolution of inflammation, to promote cancer development. Inhibition of classical NF-κB activation in TAMs has been also observed in response to the M2 polarizing signal TGFβ, through the induced expression of kinase IRAK-M, an inactive serine/threonine kinase that acts as a negative regulator of TLR signaling (132). Of relevance, a recent report has associated M2-like macrophage infiltration with PD-L1 expression in gastric adenocarcinoma (133). Hence, as a key transcriptional component setting the onset and the resolution phase of inflammation, the different forms of NF-κB activation appear as the main regulators of TAMs functional heterogeneity, including their suppressive activity mediated by PD-L1.

CONCLUDING REMARKS: TRANSLATIONAL IMPLICATIONS

NF-κB, being a master regulator of inflammation, represents a link between immune response and cell growth (134). According to this view, inhibiting NF-κB signaling might counteract inflammation, tumor growth, and possibly reduce PD-L1 expression. NF-κB pathway is the primary or secondary target of different currently used drugs for the treatment of multiple myeloma (135). IKK inhibitors are commercially available and have been tested in preclinical studies for the management of different tumoral and inflammatory pathologies [for comprehensive reviews on the therapeutic implications of IKK targeting see (136, 137)]. Nevertheless, several concerns exist regarding the administration of NF-κB inhibitors due to their pleiotropic effects. To address this issue, current approaches comprise intermittent administration and use as adjuvant therapy. Alternative approaches that can be considered to reduce unwanted effects include either targeting components of the NF-κB pathway other than IKK or modulating NF-κB-dependent downstream effectors. In brief, NF-κB inhibition could be especially relevant in the context of cancer immunotherapies aiming to prevent PD-L1 overexpression and to modulate TAM survival/polarization.

Conversely, immunotherapy based on immune checkpoint inhibition (ICI) has revolutionized cancer treatment and PD-1/PD-L1 axis is the target of different monoclonal antibodies approved for human use (Pembrolizumab and Nivolumab are approved anti-PD-1; Atezolizumab, Avelumab, Durvalumab are approved anti-PD-L1). It is still unclear if they have different activity and/or toxicity. The majority of these compounds are engineered in order to prevent antibody-dependent cell cytotoxicity (ADCC), but Avelumab, an anti-PD-L1 IgG1 isotype, is able to perform both immune checkpoint inhibition and ADCC so that TAMs, MDSCs, and T_{reg}, which can express high levels of PD-L1 in the tumor infiltrate, can be targeted as well as cancer cells (35, 138, 139). Immune-related adverse effects (irAEs) that resemble autoimmune responses occur during ICI therapy. Indeed, breakdown of the homeostatic PD-1/PD-L1 axis can provoke colitis, hepatitis, endocrinopathies, kidney injury, and skin problems (4, 140).

PD-L1 expression, as evaluated by immunohistochemistry, is routinely used as a biomarker for patient eligibility to anti-PD-1/PD-L1 therapy, and it is the only reliable molecular biomarker nowadays. Nevertheless, responsiveness to therapy does not mirror PD-L1 expression, and unfortunately a few PD-L1$^+$ patients undergo hyper-progressive disease after ICI therapy (141–143). These discrepancies can be ascribed to several issues, including technical limitations of PD-L1 expression analysis, intra-tumoral heterogeneity, tumor mutational burden,

inefficient priming of anti-tumoral T cells, and inadequate T-cell responses due to either tumor-intrinsic (compensatory immune checkpoints) or tumor-extrinsic (immune suppressive milieu) factors (144). It remains to be determined whether there are differences among individual drugs targeting PD-1/PD-L1 axis that are relevant for their clinical use and whether some patients would better benefit of either anti-PD-1 or anti-PD-L1 treatment. Although blocking either PD-L1 or PD-1 should similarly inhibit their molecular interaction, it is conceivable that, at least in some anti-PD-L1-treated patients, unblocked PD-L2 activity on PD-1 receptor can still inhibit anti-tumor response. Furthermore, considering that anti-CTLA-4 therapeutic efficacy can heavily depend on depletion of intra-tumoral CTLA-4^+ T_{reg} by ADCC (145), it is possible that anti-PD-1/PD-L1 therapy efficacy could similarly depend on the depletion of either PD-1^+ and/or PD-$L1^+$ tumor-infiltrating T_{reg}, at least in those patients in which these types of inhibitory cells dominate suppression of anti-tumor response. Accordingly, patient-tailored anti-PD-1/PD-L1 therapy is a subject of intense investigation [for example, see (146)].

It is conceivable that it is not PD-L1 expression *per se*, but rather the tumor microenvironment that induces PD-L1, that accounts for therapy success. A more comprehensive characterization of the tumor environment in terms of cytokine milieu, type of lymphocyte infiltration, macrophage phenotype would lead to improved approaches, most likely involving combined therapies (147, 148).

In this regard, the NF-κB state of activation, rather than PD-L1 alone, could have a prognostic value, as recently suggested by a detailed investigation of different types of human cancers, which reports how the local immune landscape drives clinical outcome (38). In patients with cancer, a positive or negative prognostic value has been correlated to the activation state of STAT1 and NF-κB pathways, respectively, despite the signaling of either being enough to lead to PD-L1 expression (38). It is tempting to speculate that the STAT factors, evolved in response to viral infections as components of the IFN-activated pathways that limit viremia, have anti-cancer features mostly by mediating pro-apoptotic effects. In contrast, NF-κB, evolved to regulate inflammation and tissue healing, and thus supporting cell survival and proliferation, drives pro-survival functions in cancer settings.

PD-L1 expression controls the strength of the immune response and acts as a rheostat of inflammation. Unfortunately, this mechanism is exploited by cancer cells to perform immune evasion. PD-L1 regulation during tumor progression evokes its physiological modulation, as discussed in the present review. In this scenario, uncovering the NF-κB-mediated regulation of PD-L1 in tumors can pave the way towards tailored therapeutic approaches targeting the PD-1/PD-L1 axis.

AUTHOR CONTRIBUTIONS

FA and FD searched for literature articles, conceived, and wrote the manuscript. AN, MG, ASi, and ASa revised and critically contributed to the manuscript drafting. FA, AN, MG, ASi, ASa, and FD approved the final version of the manuscript.

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Mechanisms of Peritoneal Fibrosis: Focus on Immune Cells–Peritoneal Stroma Interactions

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Peritoneal fibrosis is characterized by abnormal production of extracellular matrix proteins leading to progressive thickening of the submesothelial compact zone of the peritoneal membrane. This process may be caused by a number of insults including pathological conditions linked to clinical practice, such as peritoneal dialysis, abdominal surgery, hemoperitoneum, and infectious peritonitis. All these events may cause acute/chronic inflammation and injury to the peritoneal membrane, which undergoes progressive fibrosis, angiogenesis, and vasculopathy. Among the cellular processes implicated in these peritoneal alterations is the generation of myofibroblasts from mesothelial cells and other cellular sources that are central in the induction of fibrosis and in the subsequent functional deterioration of the peritoneal membrane. Myofibroblast generation and activity is actually integrated in a complex network of extracellular signals generated by the various cellular types, including leukocytes, stably residing or recirculating along the peritoneal membrane. Here, the main extracellular factors and the cellular players are described with emphasis on the cross-talk between immune system and cells of the peritoneal stroma. The understanding of cellular and molecular mechanisms underlying fibrosis of the peritoneal membrane has both a basic and a translational relevance, since it may be useful for setup of therapies aimed at counteracting the deterioration as well as restoring the homeostasis of the peritoneal membrane.

Keywords: peritoneal fibrosis, mesothelial cells, peritonitis, innate immunity, T cell subpopulations, pro-inflammatory cytokines

INTRODUCTION

Peritoneum is a serosal membrane forming the lining of the abdominal cavity. Peritoneum is a first line of defense against microorganisms and tumor cells. Moreover, peritoneum constitutes a slippery non-adhesive surface allowing frictionless movements of the viscera in the abdominal cavity. Peritoneum is composed of a continuous monolayer of cells of mesodermal origin, the mesothelial cells (MCs). MCs cover a submesothelial region made of a thin layer of connective tissue

composed mainly of bundles of collagen fibers with few fibroblasts, macrophages (MØs), mast cells, and hematic and lymphatic vessels (1, 2).

Peritoneal fibrosis is the end point of a progressive alteration of the peritoneal membrane due to a wide array of inflammatory and infectious events, many of which are directly related to clinical practices (3). A main cause of peritoneal fibrosis is, in fact, **peritoneal dialysis** (PD). PD is a form of renal replacement alternative to the hemodialysis, where peritoneal membrane is used as a dialysis membrane in therapeutic procedures for the treatment of end-stage renal disease. Currently, peritoneal dialysis (PD) accounts for around 10% of all forms of renal replacement therapy worldwide (4). During PD practice, signs of fibrosis are found in 50 to 80% of patients within one or two years of PD (3, 5).

Peritoneal fibrosis represents an important cause of PD discontinuation, together with peritonitis and death due to cardiovascular complications. PD is also a risk factor for the onset of **encapsulating peritoneal sclerosis** (**EPS**), the most serious complication of PD, with potentially fatal manifestation (6). EPS is a syndrome characterized by loss of ultrafiltration function, anorexia, weight loss, diarrhea, intestinal obstruction, inflammation, peritoneal thickening, fibrin deposition, sclerosis, calcification and encapsulation (7). However, peritoneum during PD practice often presents only limited complications and many patients develop a **simple peritoneal sclerosis** (**SPS**), characterized by thickening of the peritoneum, calcification, presence of inflammatory elements, angiogenesis and dilatation of blood and lymphatic vessels in the absence of systemic disease, and whose alterations are at least in part reversible after discontinuation of PD.

Besides fibrosis during PD practice, peritoneum is directly implicated in the genesis of post-surgical intra-abdominal adhesions **(peritoneal adhesions, PAs)**, which are fibrous bands tethering organs to one another or to the parietal peritoneal wall, leading to a significant cause of post-surgical morbidity and posing a major public health challenge (8). Their primary sequelae include bowel obstruction, female infertility, ectopic gestation, chronic abdominal and pelvic pain, poor quality of life, and death. It is estimated that ~93% of patients undergoing abdominal surgery develop adhesions and about 20% require re-hospitalization for adhesion-related complications (9, 10).

Finally, the insurgence of peritoneal fibrosis has a clinical relevance also for **peritoneal metastases**. In this context, metastatic tumors (generally ovary or colon cancers) instruct a fibrotic response in the peritoneal membrane, generating areas where tumor spreading and dissemination are facilitated (11–13). Although fibrosis related to peritoneal tumors is the object of increasing interest, due to its intrinsic specificities, this review article will not deal with this topic.

The induction of peritoneal fibrosis is a complex pathological event where peritoneal cells sense the pro-fibrotic stimuli and secrete extracellular mediators leading to the recruitment of circulating leukocytes playing a role in induction and amplification of the inflammatory response. The generation of myofibroblasts, cells of heterogenous origin with the ability of producing and remodeling the extracellular matrix proteins (ECM) is central for fibrosis onset. At the same time, the nature of the stimuli imparts signals promoting the resolution of the inflammatory state, with phagocytosis of dead cells and removal of debris. In this context, an implication of adaptive immunity has been proven relevant in its cross talk with peritoneal stroma or innate immunity components.

Therefore, the onset of peritoneal fibrosis is the final result of a tight network of signals between stromal resident and immune recirculating leukocytes, whose understanding may lead to a better medical containment of this deleterious pathologic event.

There is now plenty of information on the role of the non-immune components of peritoneal membrane (MCs, fibroblasts, endothelium) and activities of innate and adaptive immunity have been described by relevant studies (14); the main underlying intracellular mechanisms have been reviewed elsewhere (3, 15). The aim of this review article is to create a comprehensive synthetic description of how different signals from both stromal cells and immune system components are integrated and how cellular components are mutually influenced during the induction of peritoneal fibrosis.

PERITONEAL FIBROSIS: MULTIPLE INGREDIENTS FOR ONE CAKE

Peritoneal fibrosis onset is the final result of complex interactions between external stimuli, intrinsic properties of the peritoneal membrane, and subsequent activities of the local innate-adaptive immune system. A flowchart describing the stimuli discussed in this chapter and the main peritoneal stromal responses is shown in **Figure 1**.

Infectious Peritonitis

Peritonitis is a main cause of fibrosis induction in peritoneum. Peritonitis onset is one of the most serious complication of PD: it induces angiogenesis and fibrosis and is a major cause of morbidity and mortality in PD patients (16). Repeated episodes of peritonitis are often a cause of discontinuation of PD and may precede induction of EPS (17).

Many microorganisms may infect peritoneum; the peritoneal membrane is contiguous to the intestine which harbor bacteria than can leak towards the peritoneal cavity. Moreover, medical actions such as catheter positioning and maintenance, practice of peritoneal dialysis and abdominal surgery may favor the entry of microorganisms in the peritoneum space.

The majority of peritonitis episodes in PD can be ascribed to Gram-positive bacteria of the skin and, to a minor degree, to Gram-negative bacteria presumably originating from the enteric flora (18).

Compared to bacteria, there are limited reports on the role of viruses. The suspect of virus infection occurs when cultures from peritonitis appear negative, an event occurring around 20% of the cases: however, virus infection is not diagnosed by standard tests (19).

Similarly to pericardium, coxackievirus B1 infection has been reported in peritoneum and it is characterized by the presence of

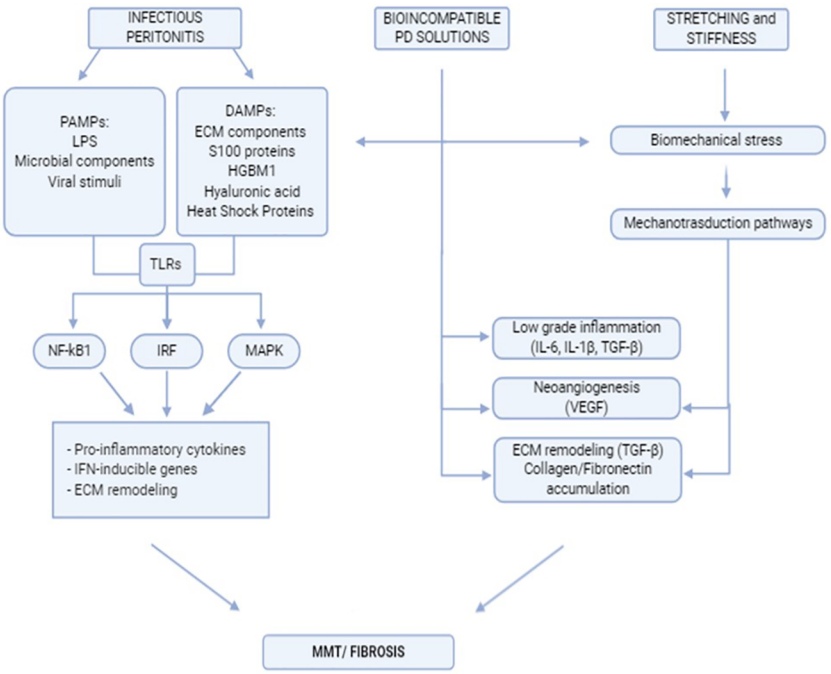

FIGURE 1 | Flowchart indicating the main extracellular stimuli promoting peritoneal fibrosis and subsequent mesothelial cell response.

monocytosis in PD effluent (20). Also, less studied are peritonitides caused by fungal infections. They constitute a serious complication of PD and account for between 1 and 15% of all PD-associated peritonitis episodes. The majority of these FP episodes are caused by Candida species such as *Candida albicans* (21, 22).

Mechanism of Inflammatory Response: Exogenous TLR Ligands

It is believed that the damage to the peritoneal membrane by infectious agents is mediated mainly by innate pattern recognition receptors (PRRs) on peritoneum, which include Toll-like receptors (TLRs), RIG-I-like receptors, NOD-like receptors, and C-type lectin receptors. The intracellular signaling cascades triggered by these PRRs lead to transcriptional expression of inflammatory mediators that coordinate the elimination of pathogens and infected cells (23).

Pathogens are recognized by PRRs through the interaction with molecules conserved among microbial species, which are called pathogen-associated molecular patterns (PAMPs). Besides PAMPs, PRRs also recognize endogenous molecules released from damaged cells, termed damage-associated molecular patterns (DAMPs) (24).

Among PRRs, TLRs play a critical role in innate immune responses by specifically recognizing molecular patterns from a wide range of microorganisms, including bacteria, fungi and viruses. TLRs are responsible for sensing invading pathogens outside of the cell and in intracellular endosomes and lysosomes (23). 10 different TLRs in humans and 12 in mice have been so far identified. Each of them recognizes different molecular patterns of microorganisms and self-components.

Human MCs respond to bacterial ligands through a specific subset of TLRs (*i.e.* TLR1, TLR2, TLR3, TLR5 and TLR6).

Gram positive bacteria are recognized by TLR2 and TLR5 (25), both singularly and cross-talking to better counteract microbial infections (26).

TLR2 recognizes an array of microbial molecules in part by hetero-dimerization with other TLRs (*e.g.* TLR1 and TLR6) or unrelated receptors (*e.g.* Dectin-1, CD36 and CD14). TLR activation triggers nuclear factor-kappa B (NF-κB), interferon regulatory factor (IRF) and mitogen-activated protein kinase (MAPK) signaling leading to altered gene expression, including pro-inflammatory cytokine and IFN-inducible genes (27).

TLR5 recognizes flagellin, a flagellum component in many motile bacteria (28). TLR5 expression on MCs may therefore be a critical signal of flagellated bacteria's invasion into the peritoneal cavity. Translocation of intestinal bacteria is a potential cause of infection in PD patients, along with access through the intraperitoneal catheter, and many flagellated bacteria are Gram-negative species, with a poor outcome in PD associated peritonitis (25).

Gram-negative bacteria induce responses through TLR4, initially identified as responsible for the recognition of lipopolysaccharide (LPS). Differently from murine MCs, human MCs do not directly respond to TLR4. However, TLR4 is present in MØ stably residing in the peritoneal membrane and their response may contribute to inflammation leading to fibrosis.

Recent studies have shown that the modulation of TLR2 and TLR4 activity through specific antibodies or soluble Toll-like receptor 2 (sTLR2), a TLR2 inhibitor, is able to cause a substantial reduction of inflammatory parameters to inhibit

fibrosis development in an experimental model of *S. epidermidis* infection (29).

A set of TLRs, comprising TLR3, TLR7, TLR8, and TLR9, act in the intracellular space in order to recognize nucleic acids derived from viruses and bacteria, as well as endogenous nucleic acids in pathogenic contexts (23). These TLRs respond by activating the production of type I IFNs and pro-inflammatory cytokines. Viral stimuli are recognized by the intracellular TLR3, which is functionally expressed in MCs (30). While for several exogenous TLRs the signaling pathway depends on MyD88, known as the inductor of the early phase response in MØs, TLR3, specifically, acts thought TRIF that plays an essential role in inducing a NF-κB mediated fibrosis and a late phase immune response activation (31, 32).

In human MCs, TLR3 is also involved in the regulation of the final common pathway of inflammation and fibrosis acting on matrix-remodeling proteins. In particular, TLR3 is correlated in time- and dose-dependent upregulation of MMP9 and TIMP1 (33).

Mechanisms of Inflammatory Response: Endogenous TLR Ligands

In addition to PAMPs, TLR mediated response can be stimulated by endogenous TLR molecules, inducing sterile inflammatory processes (34, 35). Many endogenous TLRs derive from ECM components, such as fibronectin or fibrinogen or ECM interacting proteins such as tenascin-C (36, 37).

Proteins with various functions may serve as endogenous TLRs such as cardiac myosin, S100 proteins, HGBM1 (38) (39–41). While the last protein may interact with several TLRs, the majority of these ligands are direct agonists of TLR2 and TLR4 (42, 43). Interestingly, exposure to PD fluids promotes the expression of Hsp60, Hsp70 and hyaluronic acid (HA), all TLR2 and TLR4 ligands, by leukocytes and MCs, thus driving an inflammatory response in the absence of infectious stimuli (see below) (44). Accordingly, treatment with soluble TLR2 (sTLR2) reduces pro-inflammatory and fibrotic response in mice exposed to PD fluids. These discoveries open to future clinical trials testing the clinical efficacy of these compound in patients undergoing long term PD (44).

Bioincompatibility of PD Solutions

The partial bioincompatibility of fluids used for the practice of PD may act as pro-fibrotic stimuli causing progressive morphological changes and leading to functional alterations that may cause ultrafiltration failure, discontinuation of PD and increased risk of developing EPS.

Traditional PD solutions, in fact, are hyperosmotic, hyperglycemic and acid. These solutions contain sodium, chloride, calcium, magnesium, lactate and a high concentration of glucose. Low pH in these solutions counteracts glucose oxidation that may release in the solution toxic glucose degradation products (GDPs) during the sterilization process. Moreover, glucose and reactive carbonyl compounds can form Advanced Glycation End-products (AGEs), binding to free amino groups on proteins or lipids (45, 46). The high osmolarity and the high glucose concentration favor ultrafiltration and toxin elimination by keeping the electrolyte balance (47).

All these factors may promote a low-grade inflammatory status in the peritoneal membrane, characterized by increase of inflammatory and profibrotic cytokine production such as IL-6, IL-1β, TGF-β1, VEGF, acceleration in TIMP release, causing a loss of balance in ECM remodeling and an accumulation of collagen and fibronectin. The same factors have a cytotoxic effect on MCs inducing mesothelial denudation of the peritoneal membrane and a decrease in the intercellular junctional proteins levels, causing hyperpermeability (48). *In vitro* evidence has demonstrated that the so called 'bioincompatible' PD fluid may induce apoptosis of MCs (49).

More recently, *in vitro* and *in vivo* studies have demonstrated that high glucose peritoneal dialysis solutions (HGPDS) may cause apoptosis and autophagy of MCs. However, further efforts will be necessary for the full understanding of the role of these mechanisms in the genesis of fibrosis (50).

This variety of stimuli also promotes a process known as mesothelial to mesenchymal transition (MMT) (see below) contributing to matrix deposition, increased stiffness and fibrosis (51). These cellular and molecular alterations parallel the induction of numerous morphological changes in the peritoneal membrane (PM) including increased thickness of the submesothelial space, vascular changes with subendothelial hyalinization, luminal narrowing or obliteration, increased density of blood vessels (52).

Clinically, these changes reflect an increase in small solute transport due to neoangiogenesis that extends the peritoneal surface area (a blood vessel density related parameter) and ultrafiltration reduction due to fibrosis and thickness of the submesothelial zone (53, 54). In certain cases, the simple peritoneal sclerosis common in peritoneum of PD patients can lead to EPS (7).

In order to mitigate the side effects of traditional PD solutions, a second generation of so called 'biocompatible' PD fluids has been designed that can be divided in two main groups: PD solutions with neutral pH, low GDPs and PD solutions where glucose is replaced with glucose polymers (icodextrin) or amino acids (55–57). The functionality of PD solutions is debated. It has been reported that these solutions better preserve the residual renal function and diuresis with a decrease in peritonitis frequency (3, 58). *In vitro* and *in vivo* effects of traditional *versus* biocompatible PD fluids are summarized in **Table 1**.

However, the effectiveness and the long-term benefits are currently being analyzed and there is not a definitive consensus on the benefits of this treatment, especially in the long term (66–69).

Icodextrin is a glucose polymer with a high molecular weight. PD solutions based on the use of icodextrin seem to increase peritoneal ultrafiltration, to reduce glucose absorption and to improve cardiac parameter (60). The use of a glucose polymer as an osmotic agent is particularly interesting as a glucose substitute in diabetic subjects. The reduced carbohydrate load also seems to provide a long-term metabolic advantage in terms of lipid control (61). However, icodextrin can interfere with blood glucose measurement by providing falsely elevated results. It can also cause hypersensitivity reactions, and it is more expensive than other PD solutions.

TABLE 1 | Table comparing the main characteristics of traditional *versus* biocompatible PD with emphasis on *in vitro/in vivo* mechanisms of toxicity.

PD solution	Traditional PD fluids	Biocompatible PD fluids		
PD solution type	**Traditional PD solutions**	**Neutral pH, Low GDPs**	**Icodextrin based**	**Amino acid based**
Osmotic agent	Glucose	Glucose	Icodextrin	Amino acids
pH	5.5	6.8-7.3	5.5	~6.7
Toxic Agents	GDPs, AGEs, ROS, acidic pH, lactate buffer	Significant reduction of toxic agents (GDPs, AGEs, ROS)	Acid pH, lactate buffer, ROS	High concentration of amino acids
Mechanisms of cytotoxicity	TGFβ and VEGF, acceleration of TIMP release, inflammatory cytokines (IL-6, IL-1β) production	↓ osmolality	Iron accumulation, ↑ maltose and maltotriose serum level	Protein accumulation
***in vitro* effects**	MMT induction, ECM deposition, increased stiffness, fibrosis, MC apoptosis	Improvement in cellular functions	pH-dependent apoptosis	Increase in nitrogenous waste metabolism
***in vivo* effects**	Peritonitis, vasculopathy, disruption of renal functions, anuria, infusion pain, diabetic glomerulosclerosis	Probable reduction in ultrafiltration; ↑ urine volume	Hypoglycemia, skin rush	Acidosis, uremia
Benefits	Ultrafiltration efficiency, Lower costs	Preservation of residual renal functions, reduction of peritonitis risk	Increased daily ultrafiltration, reduced glucose adsorption, increased glycemic control of diabetic PD patients, improvement of cardiac parameters	Improved surrogate markers of nutritional status of malnourished PD patients
References	45, 46, 48, 53, 54, 59	3, 55–58	3, 55, 56, 58, 60, 61	3, 55, 56, 58, 62–65

Solutions containing amino acids have been produced to improve the nutritional status of subjects on PD. PD causes a significant loss of protein in the dialysate, estimated to be 2–4 g of amino acids per day. Amino acid 1.1% solutions were found to be effective osmotic agents (62). In some studies, they have improved the nutritional status of malnourished PD patients (63). Common side effects include worsening of acidosis and an increase in blood urea linked to the increase in nitrogenous waste metabolism.

Glucose has been partially replaced by two osmometabolic agents, xylitol and L-carnitine. Treatment with this new formulation resulted a higher cell viability, better preservation of the integrity of the mesothelial layer, and reduced release of pro-inflammatory cytokines, as reported in a recent *in vitro* study (70).

Another field of investigation is the search of immunomodulators that may be added to mitigate the cellular effect of prolonged PD treatment. A recent discovery is the immunomodulatory effect of alanyl-glutamine (AlaGln) supplementation in PD solutions. This treatment seems to ameliorate peritoneal inflammation status and to improve healthy peritoneum biomarkers as well as tight junction organization and functionality (64, 65).

PM Damage by Biomechanical Cues: Stiffness and Stretching

Besides extracellular biochemical mediators, a vast body of evidence has demonstrated a role for biomechanical forces in mediating cell physiopathological responses.

Changes in biomechanical features of the extracellular matrix (ECM), such as ECM stiffness, can modify cell state and are major promoters of a fibrotic response (71). Beyond ECM stiffness, the sensing of mechanical stretching is characteristic of organs and tissues exposed to continuous variations of dynamic cues, such as respiratory and abdominal movements or the cyclic blood circulation pulse wave. In cells with epithelial features, the effects of cellular stretching have been analyzed especially on tissues composed of monocellular layers, such as lung epithelial cells and endothelium (72, 73). Biomechanical forces affect signal transduction (mechanotransduction) with a consequent impact on cellular behavior (74).

During exposure to PD fluid, the PM experiences continuous biomechanical cues. PD practice requires the injection of large PD solution volume (2 l). This causes mechanical stress by swelling the abdominal cavity, involving mechanical stretching of MCs. Other mechanical perturbations may arise from the trauma of the peritoneal membrane after abdominal laparotomies (75).

MCs upon exposure to cellular stretch *in vitro* increase the expression of VEGF and of TGF-β1 (76). It has been recently demonstrated that exposure of MCs to linear cyclic stretch *in vitro* leads to several cellular modifications corresponding to bona fide MMT induction. The experimental data are summarized in a model where a cross-talk between biomechanical and biochemical signals result in the induction of MMT (77).

Biomechanical forces are also involved in the formation of PAs, with a key contribution by MCs. It is believed that in *in vivo* conditions besides the mechanical tension, also hypoxia and activation of coagulation contribute to the formation of the fibrotic response leading to PAs formation (78).

CELLULAR PLAYERS OF PERITONEAL FIBROSIS

Stromal Components of the PM: MCs, Endothelial Cells and Stromal Fibroblasts as a Source of Myofibroblasts

Peritoneal MCs constitute a monolayer of cells with an epithelial-like cobblestone shape covering in a continuum the peritoneal cavity. MCs originate from mesoderm during the gastrulation, and their differentiation is controlled by the transcription factor WT1, which is commonly used for lineage tracing experiments (79, 80). Despite their mesodermal origin, MCs show a cobblestone morphology and actually coexpress in basal conditions epithelial and mesenchymal markers (51, 59, 81).

MCs express tight and adherent junction related molecules such as ZO-1, occludin, claudins and E-cadherin, which is expressed both in plasma membrane and in cytoplasm (82). Moreover, these cells express epithelial intermediate filament proteins such as cytokeratins (8–18) that play an important role in maintaining cellular structural integrity. At the same time, MCs constitutively express also mesenchymal intermediate filaments such as vimentin and desmin (51).

The coexistence of both epithelial and mesenchymal markers may be linked to the characteristic plasticity and to the ability of these cells to acquire mesenchymal-like features in response to a variety of pro-inflammatory/profibrotic stimuli. Almost all the pro-inflammatory factors described in the previous sections may promote, although with different intensity, induction of MMT in MCs. This dedifferentiation process culminates with the acquisition of morphological and functional features making these cells indistinguishable from myofibroblasts of other origin (see below) (**Table 2**).

The secretion of TGF-β by MCs or by other cells such as MØs is central for a full induction of the MMT program. Once transdifferentiated, MCs may invade the submesothelial stroma where they proliferate and produce cytokines and ECM proteins directly promoting peritoneal fibrosis.

The profibrotic activity of TGF-β1 is counteracted by members of the BMP family, such as IGFBP4, BMP4 and BMP7, secreted by the same MCs (83, 84).

Interestingly, transdifferentiated MCs tend to acquire a new stability. This behaviour is different from that of cells with a stronger epithelial identity that rapidly recover epithelial features once the transdifferentiating stimulus has been removed (107). The maintenance of mesenchymal features in MCs has been linked to epigenetic changes, and epigenetic modulation may both influence mesothelial differentiation and promote the recovery of a "epithelial-like" phenotype from *in vivo* transdifferentiated cells (99, 108).

High throughput experiments have demonstrated that induction of MMT from different stimuli induces the acquisition of common dedifferentiation features characterized by the expression of signatures of profibrotic and pro-inflammatory cytokines such as TGFβ, VEGF, and IL-6 (29, 77, 83, 109). In fact, activated MCs are major producers of TGF-β1, VEGF and IL-6, whose concentrations are elevated especially during peritonitis and have been associated with ultrafiltration decline and protein loss (18, 110). The secretion of these cytokines impacts fibrosis, angiogenesis and the inflammatory response.

The ability of MCs to generate myofibroblasts has been a highly debated topic in the previous years. Lineage tracing experiments performed to demonstrate the mesothelial origin of peritoneal myofibroblasts have given contrasting results, with the more recent studies suggesting the existence of a population of MCs origin invading the submesothelial space (83, 111, 112).

Moreover, the coexpression of *bona fide* MMT markers such as αSMA and fsp1 absent in epithelial-like MCs with mesothelial/epithelial markers has been demonstrated *in vivo* both in peritoneum after PD and in peritoneal adhesion (75, 77, 78, 82).

In the peritoneum of mice exposed to PD fluid, the relative contribution of the myofibroblasts-generating cellular populations, including resident dermal fibroblasts, endothelial cells, bone marrow derived cells and MCs, has been quantified (113). As in other organs, endothelial cells contribute to peritoneal fibrosis through a process of endothelial to mesenchymal transition (EndMT) (82, 114). Also, bone marrow derived progenitors, such as mesenchymal stem cells and fibrocytes may generate peritoneal myofibroblasts (115).

MCs may in different ways influence the fibrotic process. Besides being a main source of TGF-β1, activated MCs produce abundant amounts of fibronectin and collagens, may rearrange the ECM through the expression of contractile proteins (αSMA) and produce various metalloproteinases (MMPs), such as MMP2, MMP9 and MMP14 as well as MMP inhibitors such as TIMP1 and PAI1 (77, 99, 100). Besides directly impacting the fibrotic process, the production of inflammatory cytokines and chemokines stimulates other stromal cells and components of innate and adaptive immunity (see below). Thus, MCs are candidates for cellular interventions aimed at restoring the continuity of the monolayer and to warrant the peritoneal function.

LEUKOCYTE SUBPOPULATIONS IMPLICATED IN THE FIBROTIC RESPONSE

Peritoneum as a Lymphatic Organ: The Role of FALCs

Due to its unique localization in the abdominal cavity and its huge extension, peritoneum is a favorite site for encountering with antigens and for the generation of the subsequent immune response. Recirculating leukocytes patrol the peritoneal cavity in uninflamed peritoneum in addition to stably resident leukocyte populations constituted mainly by macrophages and mast cells.

Besides conventional lymph nodes, peritoneum hosts unique anatomic structures called milky spots or **fat-associated lymphoid clusters** (**FALC**s), which are clusters of leukocytes localized especially in omentum and endowed with the ability to collect fluids, particulates, and cells from the peritoneal cavity. Their frequency and size increase in the omentum of patients undergoing PD (116, 117). FALCs are mainly composed of MØs,

TABLE 2 | Epithelial-like and mesenchymal markers of MCs. The main extracellular regulators of MC plasticity, molecular markers and signaling pathways implicated are shown.

Epithelial-like MCs			
Features and Properties	**Extracellular Mediators**	**Markers**	**Trascription Factors and Signaling Pathways**
	BMP7	E-Cadherin	WT1
	BMP4	Claudins	SMAD1-5-8
	IGFBP4	Occludins	p38 MAPK
Cobblestone-like shape	HGF	ZO-1	
Apical-basal polarity		Desmoplakin	
Monolayer organization		Cytokeratins	
Tight junctions		Calretinin	
Adherens junctions		Vimentin	
Glycocalyx production		VEGFR2	
Immunomodulatory activity		CA125	
		Caveolin-1	
		Hyaluronan	
References	(83, 84)	(51, 77, 82, 85–90)	(79, 80, 82, 91)
Mesenchymal-like MCs (MMT)			
Features and Properties	**Extracellular Mediators**	**Markers**	**Trascription Factors and Signaling Pathways**
	TGFβ1	N-Cadherin	Snail, Twist
	IL-1β	Desmin	SMAD 2-3
	FGF-2	Vimentin	GSK-3β
Spindle-like shape	EGF	Fibronectin	Wnt/β-Catenin
Front-back polarity	AngII	Collagen I/III	TAK1/NF-κB
Junctions dissociation	AGEs	α-SMA	ILK
Cadherin switch	PDGF	FSP-1	PI3-K
Cytoskeleton reorganization	HIF-1α	MMPs (2-9)	ERK 1/2 MAPK
ECM deposition		PAI-1	JNK 1/2 MAPK
Basement membrane degradation		Podoplanin	
Migratory and invasive activity		CTGF	
Proinflammatory activity			
References	(92–98)	(75, 77, 78, 82, 99–101)	(59, 102–106)

MCs and B1 cells. B1 cells consist in a subset of B cells that can be distinguished from conventional B (B2) cells by expression of distinct cell-surface markers and antigen receptors that can bind common bacterial epitopes. B1 cells have the potential to produce natural antibodies that provide a first protection to bacterial infections (118). Intestinal leakage or the intraperitoneal delivery of microorganisms lead to rapid activation of B1 cells and promote T cell-independent antibody responses (119).

The chemokine CXCL13, of mesothelial origin, controls the localization of B1 cells into FALCs (120). Another chemokine, CCL19, is produced by other structural components of FALCs called FALCs fibroblast reticular cells (FALCs FRCs) and it is relevant for monocyte recruitment during inflammation. The cross-talk between CCL19 producing FALCs FRCs and inflammatory monocytes promote T cell dependent-B cell immune responses (121) (**Figure 2A**). Thus, FALCs play a main role both in the regulation of PMN and mononuclear cell recruitment in the first phase of inflammation, as well as in the subsequent induction of the adaptive immunity.

Leukocyte Recruitment During Peritonitis: From Neutrophils to Mononuclear Cells

Infectious peritonitis offers a favorite experimental model to study the interactions between immune system and the peritoneal stroma (122). Infection with Gram positive bacteria such as Staphylococcal spp. or with cell-free components such as LPS or zymosan, mimicking Gram negative or fungal infection, respectively, promotes a first wave of polymorphonuclear neutrophils recruited by chemoattractants of bacterial origin

and by chemokines such as CXCL1 and CXCL8 produced mainly by MCs and omental fibroblasts. Neutrophils can use high endothelial venules present in FALCs to enter the peritoneal cavity under the guidance of CXCL1 (120).

Neutrophil influx causes an initial inflammatory response due to the accumulation of neutrophil-secreted proteases and reactive oxygen species. Once they entered the peritoneum, neutrophils undergo **NETosis,** which consists in the release of necrotic cell DNA forming a net of aggregated neutrophils able to trap and sequester microorganisms in FALCs, thus limiting their spreading (123). Interestingly, HMGB1 produced during the inflammatory response promotes NETosis and trap formation through interaction with TLR4 (124) (**Figure 2B**).

The production of CXCL1 and CXCL8 by the peritoneal stroma is enhanced by inflammatory cytokines such as IL-1β and to a lesser extent, TNFα (125). MCs stimulated by LPS or IL-1β also produce a number of cytokines and chemokines including IL-6, TNFα, CCL2, CCL3, that favor mononuclear cell recruitment and activation (126). The first wave of neutrophils is then replaced by a mononuclear infiltrate.

Neutrophils take part in this process secreting a shed form of IL-6 receptor, IL-6Rα. Through a mechanism called **transignaling,** the local increase of IL-6Rα promotes an IL-6-mediated neutrophil clearance subsequent to mononuclear cell recruitment (127, 128). Apoptotic neutrophils are phagocytosed by MØs and to a lesser extent by the same MCs (129). Necrotic neutrophils and NETs promote the infiltration of mature MØs recruited also by locally produced chemokines such as CXCL8 and CCL2 (130).

Neutrophil influx and clearance are also regulated by two other cytokines, IL-17 and IFN-γ. IL-17 is secreted mainly by various leukocyte subpopulations, including neutrophils, Th17

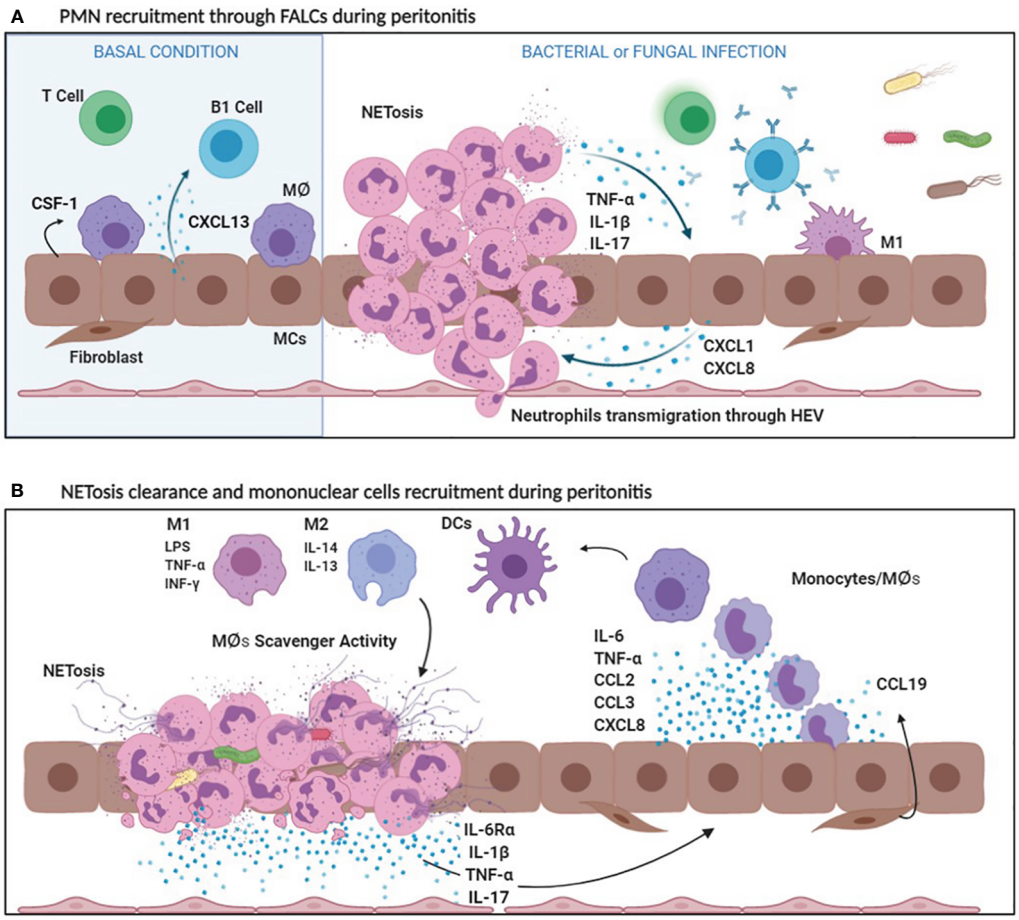

FIGURE 2 | **(A)** PMN recruitment through FALCs during peritonitis. In basal conditions, MCs secrete CXCL13, which attracts B1 cells in FALCs and CSF1, a specific MØ growth factor. Bacterial and fungal infections stimulate the production of CXCL1 and CXCL8, by MCs. Bacterial products, CXCL1 and CXCL8 promote the recruitment of a first wave of PMNs entering the peritoneal cavity through FALCs. PMNs cause an initial inflammatory response secreting inflammatory cytokines (IL-1ß, TNF-α). Afterwards, NETosis helps in sequestering microorganisms in FALCs. **(B)** NETosis clearance and mononuclear cell recruitment during peritonitis. Bacterial products, as well as IL-1ß, stimulate the production of IL-6, TNF-α, CCL2, CCL3, and CXCL8 by MCs. IL-6Ra shedding by PMNs promotes a peripheral IL-6 response (transignaling). Cytokines and chemokines released during the inflammatory process favor mononuclear recruitment and differentiation. Mononuclear phagocytes differentiate in Macrophages (MØs) and dendritic cells (DCs). Among MØs, M1 subtype is endowed with pro-inflammatory and cytotoxic properties, whereas M2 MØs have an anti-inflammatory activity. Moreover, M2 MØs play a key role in the clearance of neutrophils debris due to scavenger activity.

and $\gamma\delta$ T lymphocytes and its expression correlates with the duration of the PD treatment and with the extent of peritoneal inflammation and fibrosis (131, 132). IFN-γ production by Th1 lymphocytes and NK cells (see below) contributes with IL-6 in favoring an initial neutrophil recruitment and subsequent clearance (133).

IL-17 promotes CXCL1 production by MCs through expression and activation of the transcription factor Sp1, whereas IFN-γ through STAT1 activation limits Sp1 induced CXCL1 production. Breaking of this homeostatic cross regulation may lead to excessive or ineffective recruitment of neutrophils during peritonitis with subsequent damages in the PM (131).

Inflammation, Scavenging and Antigen Presentation: Monocytes/MØs and Dendritic Cells

Tissue mononuclear phagocytes, comprised mainly of MØs and dendritic cells (DCs), are key tissue-resident components of the peritoneal immune system. Their roles include induction of the inflammatory response, pathogen clearance, tissue repair, and antigen presentation.

MØs are the major resident immune population in the PM. At the same time, monocytes/MØs are the predominant cell types found in dialysis effluent (134, 135).

Resident MØs form the first line of defense against peritoneal infection in peritonitis. Once the inflammatory response is initiated, monocytes follow a first wave of leukocytes composed mainly by PMN neutrophils.

MØs are generally classified into two functional subtypes. Classical active MØs, also called M1 MØs (representative markers: iNOS and CD80) are defined by their pro-inflammatory and cytotoxic properties, while M2 MØs (representative markers: CD163, CD206 and Arg1) are characterized by anti-inflammatory and scavenging properties (136). However, MØ M1 and M2 subtypes should be considered as the extreme points of a continuum of different cellular populations acting in in different physio-pathological contexts (137, 138).

M1 polarization typically involves IFN-γ with a TLR agonist, such as LPS. M1 MØs, through production of IL-1β and TNF-α, are capable of amplifying the first phase of the inflammatory process and of recruiting other leukocytes into the peritoneum via the creation of a gradient of chemotactic cytokines, such as CXCL8, CCL2 and CCL5. This process is also facilitated by a cytokine driven up-regulation of adhesion molecule expression (ICAM-1 and VCAM-1) on the surface of MCs. At the same time, the generation of M2 MØs, which is sustained by IL-4 and IL-13, plays a role in the resolution of inflammation through the production of soluble anti-inflammatory mediators, and the clearance of debris such as apoptotic or necrotic products, due to their scavenger activity (137).

In the model of peritonitis induced by zymosan, mimicking fungal infection, an infiltration of both M1 and M2 MØs occurs. In this context, MØs are involved in the clearance of debris resulting from neutrophil apoptosis. Both M1 and M2 MØs recognize and endocytose dead cellular debris through apoptosis inhibitor of macrophage (AIM, also called CD5L), a member of the scavenger receptor cysteine-rich superfamily (22) (**Figure 3A**).

Experimental evidence analysing samples from PD patients demonstrates that the majority of peritoneal MØs phenotypically and functionally resemble *in vitro* polarized M2 MØs (139, 140).

In this context, a dysregulated M2 MØs response may promote the development of fibrosis and the decrease of functionality of the PM through the production of a number of extracellular factors.

The production of TGF-β1 by M2 MØs mediates the induction of MMT in MCs and the proliferation and activation of submesothelial fibroblasts, a process leading to ECM production, rearrangement, angiogenesis, and fibrosis. Moreover, MMP9 and CCL18 secretion is increased in both PD effluents and PM biopsies of PD patients (139). MMP9 plays a role in the activation of latent TGF-β1, whereas CCL18 levels have been associated with poor ultrafiltration capability and with development of EPS (139). M2 MØs may favor a fibrotic response also producing CCL17, which promotes migration, proliferation and collagen production by submesothelial fibroblasts (141).

However, besides M2, some evidence points towards an active role of the M1 subtype in the genesis of peritoneal fibrosis. The inhibition of the protein kinase C beta pathway promotes peritoneal damage and fibrosis *via* M1 MØs polarization in a murine model of PD (142). Also, *in vivo* approaches of MØ chemical deletion followed by reperfusion pointed towards an active role of the M1 subpopulation in the genesis of fibrosis, as suggested also by an *in vitro* study (143, 144).

The presence of MØs in the PM is tightly dependent on a cross talk with MCs. In basal conditions, MØs require and interact with MCs present in FALCs and secreting colony stimulating factor 1 (CSF1), a specific MØ growth factor (145). In inflammatory conditions and during PD, fractalkine (CX3CL1), a chemokine secreted by MCs, recruits and activates MØs expressing CX3CR1 in the peritoneal wall, promoting the fibrotic process. A positive feedback loop is induced where direct interaction with CX3CR1-expressing MØs promotes expression of CX3CL1 and TGF-β1 by MCs. In turn, TGF-β1 upregulates CX3CR1 expression in MØs (146).

Besides MØs endowed with pro-inflammatory or scavenger abilities, migrating mononuclear cells may differentiate in DC subsets, characterized by CD1c positivity and different profiles with respect to CD14 positive cells. In particular, CD1c positive cells have upregulated costimulatory molecules, CD80 and CD86, important for antigen presentation and T-cell activation, and CCR7, favoring migration to secondary lymphoid organs such as local lymph nodes where antigen presentation may occur (134). Interestingly, severe and recurrent episodes of peritonitis were associated with significantly higher numbers of peritoneal neutrophils, MØs as well as higher ratio of MØs to DCs than the successfully treated ones (134). The functional role of MØs has been analyzed with pharmacological but not with genetic or immunological approaches. Chemical depletion of MØs using clodronate attenuated peritoneal thickening and suppressed TGF-β1, VEGF expression and MMT induction in a model of

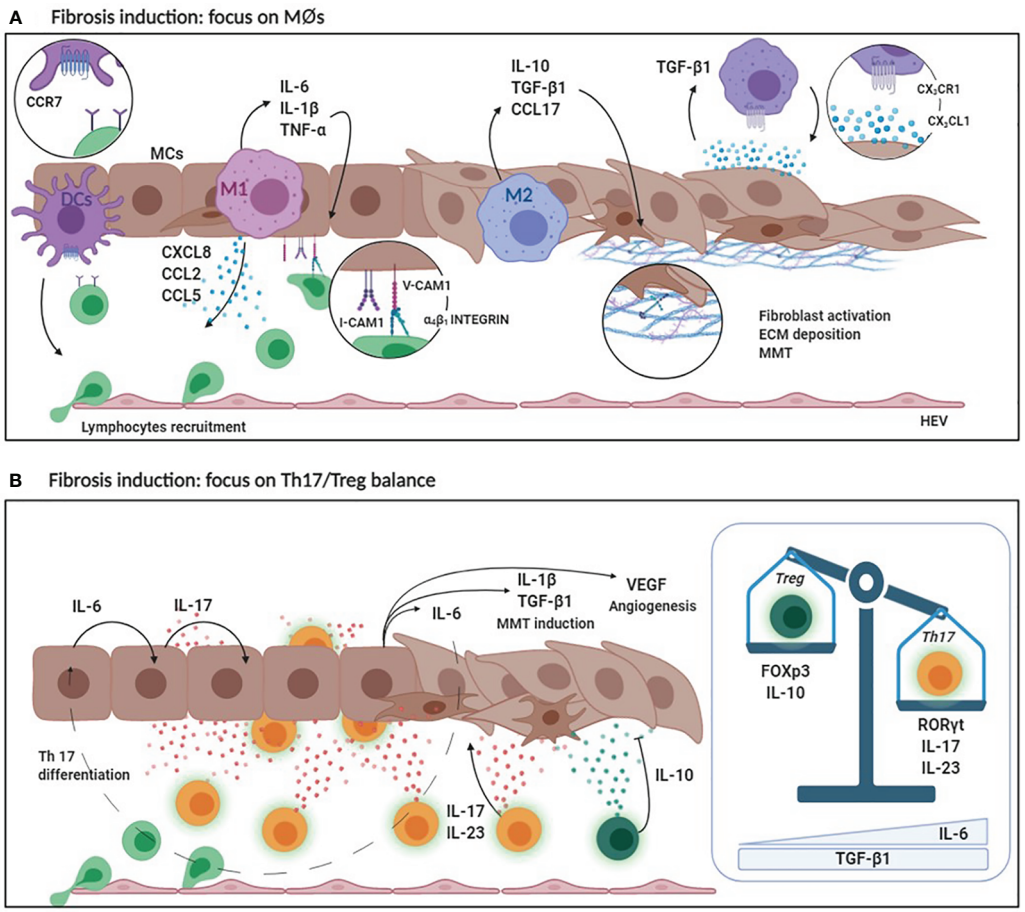

FIGURE 3 | **(A)** Fibrosis induction: focus on MØs. DCs secrete high levels of CCR7 favoring lymphocyte recruitment. Pro-inflammatory M1 MØs secrete CCL2, CCL3 and CXCL8 that are chemoattractant for lymphocytes and monocytes. At the same time, M1 MØs produce inflammatory cytokines such as IL-6, IL-1ß and TNF-α that enhance the expression of adhesion molecules (V-CAM1/I-CAM1) by MCs promoting leukocyte adhesion. M2 MØs produce anti-inflammatory cytokines (IL-10) and lymphocyte chemoattracting chemokines (CCL17). Predominance of M2 MØ response leads to an increased TGF-ß1 secretion that induces MMT of MCs with up-regulation of ECM protein production. Moreover, MCs secreting CX3CL1 recruit MØs expressing CX3CR1. Receptor/ligand interaction determines a positive loop that promote, in turn, CX3CL1 and TGF-ß1 expression. **(B)** Fibrosis induction: focus on Th17/Treg balance. The production of IL-6 by MØs and MCs during the inflammatory process promotes IL-17 production by the peritoneal stroma which, in combination with IL-23, promotes the differentiation of Th17 lymphocytes. IL-17 promotes IL-1ß, TGF-ß1, VEGF and IL-6 production causing MMT induction and neoangiogenesis. Th17 abundance affects the activity of regulatory T lymphocytes. Treg lineage has an anti-inflammatory activity (due to IL-10 production) and protects the peritoneal membrane by mediating tolerance mechanisms. High levels of IL-6 and TGF-*β*1 determine the predominance of Th17 over Treg with consequent peritoneal damage and fibrosis.

peritoneal fibrosis induced by chlorhexidine gluconate in rats (147). Accordingly, depletion of MØs limited fibrosis in a mouse model of PD fluid exposure (143).

Mastocytes

Mastocytes or mast cells are predominantly localized at sites that have direct contact with the external environment, such as the skin, airways, and intestine, where they function as sentinel cells in host defense and as main inducer of type I hypersensitivity and of the allergic response (148).

While mast cells have been implicated in fibrogenesis, angiogenesis, and the immune response against bacteria in various organs such as kidney and in lung, only a few studies have dealt on the role of mastocytes in peritoneal fibrosis (149, 150).

Mechanistically, mast cells secrete various mediators of inflammation such as histamine, platelet-activating factor, prostaglandins, thromboxane, leukotriene, chymase. Moreover, the secretion of cytokines such as TGF-β1 and IL-17 directly contributes to peritoneal fibrosis (149, 151, 152)

The number of mast cells was significantly higher in the fibrotic peritoneum of rats with chronic renal failure (CRF rats). Tranilast, an anti-allergic drug with an activity of mast cell stabilizer, was demonstrated to block the progression of peritoneal fibrosis in CRF rats (153).

An interesting study performed on mast cell-deficient rats demonstrated that mast cells promoted the increase of the omental thickness and omental adhesion formation favoring leukocyte recruitment (154).

Results in human peritoneal disease are controversial. A first study showed reduced numbers of mast cells in samples from PD patients (155), whereas increased mastocytes numbers have been found in samples from different inflammatory and fibrotic peritoneal diseases, including PD and EPS (156). Thus, although evidence suggests that these cells may amplify the inflammatory response during peritoneal damage, their functional role has not so far been demonstrated.

Natural Killer Cells

Natural killer (NK) cells are a specialized lymphocyte subpopulation that play a significant role during viral infections and in tumor immune surveillance through direct killing of virus infected or tumor cells or by production of cytokines and chemokines. NK cells recirculate throughout the peritoneal cavity and are present in the peritoneal fluid. Moreover, in uninflamed peritoneum, a resident NK cell population isolated in mice was able to secrete IFN-γ, GM-CSF, and TNF-α and endowed with killing ability (157).

During an acute inflammatory process such as peritonitis, NK cells produce inflammatory cytokines such as TNFα and IL-6. Moreover, through production of IFN-γ and TGF-β1 these cells may directly orchestrate the fibrotic process.

In other organs, NK cells actively contribute to the genesis of the fibrotic damage. Tubulointerstitial human $CD56^{bright}$ NK cells correlate with loss of kidney function and with induction of fibrosis and chronic kidney disease progression, mechanistically linked to increased NK cell-mediated IFN-γ production (158).

Interestingly, besides its potential in the amplification of the inflammatory response, NK cells appear to have a role in the resolution of inflammation in antigen-dependent peritonitis promoting neutrophil apoptosis (159). Previous results confirm that NK cells are capable of inducing apoptosis of neutrophils (160).

Studies performed in humans are limited to adoptive transfer of activated NK cells in an autologous NK cells setting used in a frame of tumor therapy. It was demonstrated that administration of NK cells in combination with IL-2 in patients with malignancies caused peritoneal fibrosis (161). Thus, although their role is potentially relevant, no definitive information is reported about NK cells in the genesis of peritoneal fibrosis.

T Lymphocytes: A Balance of Mutually Influencing Subpopulations

Besides the components of the innate immunity, the activity of different T lymphocyte subsets is fundamental for the regulation of the inflammatory response in the genesis of peritoneal fibrosis and it could provide molecular targets to control peritoneal damages. The relevance of the adaptive immunity in peritoneal fibrosis is demonstrated by the use of lymphocyte-deficient mice. In RAG-deficient mice, lacking mature T and B lymphocytes, treatment with zymosan induced an exaggerated inflammatory response with increased PMN infiltration (162). Accordingly, the use of another experimental system demonstrated a role of adaptive immunity in limiting PMN and MØ recruitment (163).

More generally, these approaches suggest that a network of mutual interactions occurs between peritoneal stroma, innate and adaptive immunity effectors during the genesis of peritoneal fibrosis.

The composition of peritoneal fluid lymphocytes varies with respect to blood lymphocytes. In particular, B lineage comprises only around 2% of the total fluid, and T leukocyte subpopulations are differently represented (164). Moreover, changes in the consistency of T lymphocyte subpopulations occur during peritoneal inflammation and during the practice of PD.

With respect to the balance between $CD4^+$ T-helper 1(Th1) and T-helper 2 (Th2) subpopulations, it has been demonstrated that during episodes of acute peritonitis, the immune response is predominantly directed to the induction of Th1 cells (165).

On the other hand, the Th2 subset rapidly expands with the practice of PD (166). The Th1/Th2 ratio could be evaluated by measuring IFN-γ (Th1 subset) and IL-4 (Th2 subset) levels both in circulating and peritoneum-derived Th lymphocytes. In PD patients, the IFN-γ/IL-4 ratio is significantly reduced, indicating a negative effect of bioincompatible fluids towards the Th1 cell subset. Interestingly, this effect could be avoided using more biocompatible fluids containing bicarbonate-buffered and icodextrin, that may reestablish a more physiological Th1/Th2 balance and a reduced peritonitis rate (167).

A recently characterized leukocyte subpopulation, Th17 lymphocytes have been demonstrated as the main driver of peritoneal fibrosis (132). The expression and the activity of this lymphocytic subset is linked to the production of IL-17. Besides Th17, other leukocytes, including CD4+ and $\gamma\delta$ T lymphocytes, neutrophils, and mast cells may secrete this cytokine during exposure to PD fluids or during peritonitis (168).

The strong stimulation of Th17 response during these pathological conditions is due to both exogenous and endogenous factors. Exogenous factors are represented by bacteria and their derivatives entering the peritoneal cavity through PD catheter or *via* intestinal translocation. These bacteria stimulate TLR's response by the peritoneal stroma, which leads to an upregulation of IL-6 levels, promoting IL-17 production and subsequent differentiation of Th17 lymphocytes (169). Similarly to bacterial derivatives, also factors related to PD fluid such as AGEs expressed in conventional lactate-based PD solution with low pH and high GDP contents are able to stimulate the Th17 response (170) (**Figure 3B**).

IL-17 contributes to the host defense against bacteria and fungi (171). It promotes neutrophil recruitment favoring the release by MCs of chemotactic factors specifically attracting neutrophils. Moreover, the IL-17 response favors the secretion of a network of cytokines and chemokines including IL-1β, IL-6, CCL2 and TGF-β1. In peritoneum, IL-17 favors through different mechanisms the secretion of VEGF by MCs, promoting angiogenesis (168). Most importantly, repeated intraperitoneal administration of exogenous IL-17 led to increased expression of several fibrosis-related genes, whereas its neutralization with anti-IL-17 alleviated the extent of peritoneal fibrosis (132).

The consistence of Th17 population in the peritoneum directly affects the activity of another T cell subset, regulatory T lymphocytes (Treg) (14). Tregs are suppressors of activated T cell expansion, their activity is anti-inflammatory and favors the

induction of tolerance (172). IL-6, in combination with TGF-β1, is the main cytokine involved in the helper 17/regulatory T (Th17/Treg) balance. The predominance of IL-6 favors the generation of Th17 lymphocytes, which produce inflammatory cytokines. On the other hand, TGF-β1 in the absence of IL-6 promotes the Treg lineage, able to maintain peripheral tolerance and produce anti-inflammatory mediators such as IL-10, which has been linked to protection of the peritoneal membrane from inflammatory damage (173).

Interestingly, the plasma membrane receptor CD69 appears to control Th17/Treg balance. The exacerbated peritoneal fibrosis observed in CD69$^{-/-}$ mice could be alleviated by the blockade of IL-17 (174). Mechanistically, it was demonstrated that CD69 directly interacting with Jak3/STAT5 blocks Th17 differentiation (175).

Besides shaping the immune response, these changes may impact MC plasticity: it was demonstrated that IL-17 itself is able to induce EMT in bronchial cells while inducing peritoneal fibrosis *in vivo* (132, 176). In contrast, low levels of IL-6 may promote Treg differentiation, which is associated to high IL-10 expression, leading to the establishment of an anti-inflammatory state and possibly MMT reversal (14, 177–179).

In case of peritoneal dialyzed patients, the predominassnce of Th17 over Treg favors fibrosis development and PM failure instead of Treg-mediated tolerance. Currently, the modulation of the expression of the cytokines involved in Th17/Treg balance through recombinant antibodies or cytokines is an attracting field for the design of new therapies aimed at counteracting peritoneal MMT and fibrosis.

A bridge between the adaptive and the innate arm of the immune system is constituted by Mucosal-associated invariant T (MAIT) cells. These cells are different from conventional T cells, since they do not react through major histocompatibility complex (MHC) (180). Peritoneal MAITs (pMAITs) provide a marker for systemic inflammation during spontaneous bacterial peritonitis (SBP), since they are configured to respond pro-inflammatory chemotactic signals sensed by CCR5, CXCR3 and CCR6 (181). pMAITs are a source of IL-17 (102). Clinical data indicated a specific immune activation of pMAIT, driven by CD69 expression and correlated to disease severity (181).

CONCLUSIONS

The cellular and molecular mechanisms described above witness the complexity of the physiopathologic response occurring in the inflamed peritoneum.

A study published almost 25 years ago by Topley et al. identified the relationship between MØs and MCs as a key factor in the response of peritoneum to infections, whose dysregulation was causal to ultrafiltration failure and fibrosis in PD patients (182). At that time, it was already clear that MCs under MØ-driven stimuli may produce a number of extracellular mediators including arachidonic acid derivatives, cytokines and chemokines promoting the amplification of the inflammatory response. Since then, the understanding of cellular and molecular mechanisms underlying has evolved considerably. A relevant discovery has been the characterization of different MØ subpopulations implicated in the secretion of pro-inflammatory mediators, phagocytosis, apoptotic debris removal and scavenging activity. Another major breakthrough has been the identification of the ability of MCs and other cells to undergo deep dedifferentiation processes culminating in the generation of myofibroblasts. Moreover, a relevant concept that has emerged in these years is that the relationship between MØs and MCs is not unidirectional: MCs play an active role in influencing MØ recruitment, survival and differentiation due to the synthesis of extracellular mediators acting specifically on MØs. Last, this dialogue is not limited to MØs and MCs: stromal driven signals such as IL-17, IL-6 and TGF-β1 shape the Th17/Treg balance, thus impacting the fibrotic response.

Although the fibrotic process has common marks in all the organs, peritoneum fibrosis has specific features due to the anatomic localization and the cellular components forming this organ. The anatomic localization favors the encounter with microorganisms through unique structures (*i.e.* the FALCs) With regard to peritoneal stroma, the characteristic plasticity of MCs, their ability to transdifferentiate and to become indistinguishable from myofibroblasts, makes the difference with respect to other organs, such as the liver, where parenchyma cells (hepatocytes) may give little direct contribution in the genesis of fibrosis.

These new discoveries related to cellular communication and cellular plasticity may have an impact in future therapeutic strategies. Future directions aimed at improving peritoneal viability and thus duration of PD therapy may be focused at further improving PD fluid biocompatibility, as well at using inhibitory monoclonal antibodies, in line with recent advances with therapy of inflammatory/profibrotic diseases. Also specific therapies aimed at supporting MC viability and regulating peritoneal immune system and immune cell/MC interactions may give a contribution. Deepening the analysis of cellular and molecular mechanisms underlying peritoneal fibrosis may shed light on our understanding of how we can preserve the long-term function of the PM as a dialysing organ, but also to treat other forms of peritoneal fibrosis such as post-surgical adhesions or tumor related-peritoneal fibrosis.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

MTe wrote the manuscript and composed the figures. FT wrote the manuscript and organized the tables. CM wrote the manuscript. MC wrote the manuscript and composed the figures. MTr critically reviewed the manuscript. ML critically reviewed the manuscript and provided a general scheme of

interpretation. RS conceived and wrote the manuscript, and critically reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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