MACROPHAGES ROLE IN INTEGRATING TISSUE SIGNALS AND BIOLOGICAL PROCESSES IN CHRONIC INFLAMMATION AND FIBROSIS

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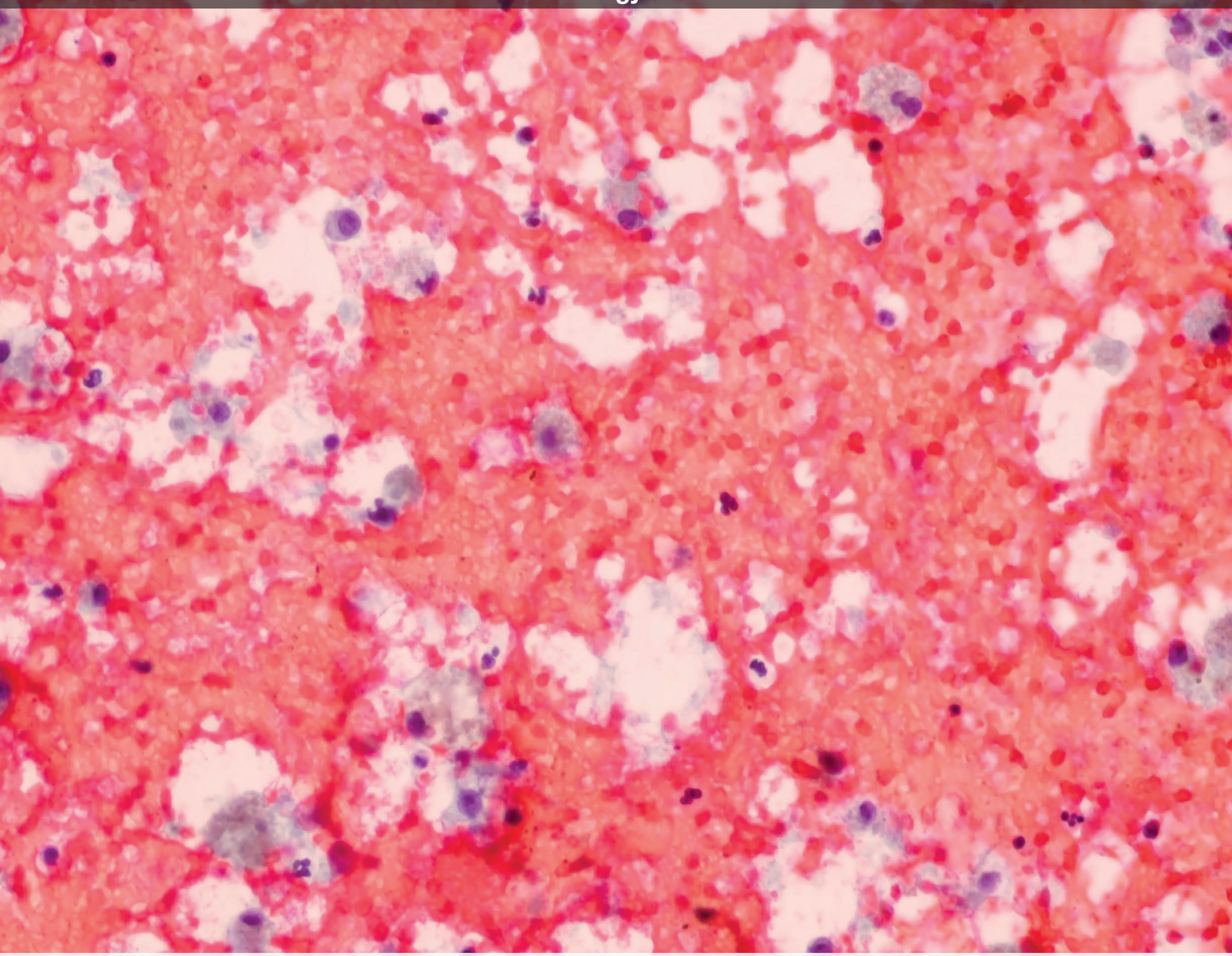

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MACROPHAGES ROLE IN INTEGRATING TISSUE SIGNALS AND BIOLOGICAL PROCESSES IN CHRONIC INFLAMMATION AND FIBROSIS

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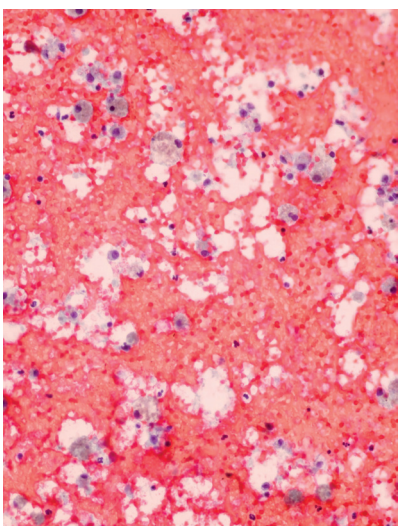

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Macrophages comprehend a heterogeneous mononuclear phagocytic population with wide range phenotypes and roles in homeostasis maintenance and diseases, such as infections, autoimmunity and cancer. Technology improvements enable researchers to track different macrophage populations in different tissues and situations and hypothesize on their role in promoting inflammation or stimulating tissue repair. Through innate immune recognition system macrophages can launch several effector artilleries that culminate in the production of various types of inflammatory mediators as cytokines, chemokines, lipid mediators and oxygen reactive species, which in turn, influence the behavior of other cells. Furthermore, macrophages and interacting cells are also susceptible to metabolic changes that ultimately will define the outcome macrophage signaling and its effect in the tissue. Here, we present a concise series of discussions on the role of macrophages, its response to the microenvironment and effects on other cells during tissue injury and repair. Triggering of inflammasome in macrophage activation and function is of special interest in this issue. We will emphasize the role of different macrophage subpopulations and the plasticity of these cells during fibrotic process in different models of diseases.

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EDITORIAL
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Editorial: Macrophages Role in Integrating Tissue Signals and Biological Processes in Chronic Inflammation and Fibrosis

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Keywords: chronic inflammation, fibrosis, macrophage plasticity, macrophage subtypes, homeostasis maintenance

Editorial on the Research Topic

Macrophages Role in Integrating Tissue Signals and Biological Processes in Chronic Inflammation and Fibrosis

Macrophages comprehend a population with wide range phenotypes and roles in homeostasis maintenance and diseases. Technology improvements enable researchers to track different macrophage populations in different tissues and situations and hypothesize on their role in promoting inflammation or maintaining tissue homeostasis. In the present editorial, we present a concise series of discussions on the role of these cells, its response to the microenvironment, and effects on other cells during tissue injury and repair. We also discuss the themes proposed by the authors on macrophage plasticity during fibrotic processes in the context of the topic subject. M1 macrophages are considered foe cells for the pro-fibrotic process once they are associated with pro-inflammatory functions (Braga et al.), and an exacerbation of tissue inflammation initiates the pro-fibrotic process (1). On the other hand, M2 macrophages have anti-inflammatory properties due to its ability to secrete IL-10, arginase, and TGF-β (2). However, when the insult is persistent, excessive M2 macrophage activation leads to continuous TGF-β production, promoting increased extracellular matrix deposition (3). In this scenario, despite its friendly behavior against the exacerbated fibrosis development, M2 becomes foe cells in the tissue repairing. Macrophages are also able to influence innate lymphoid cells (ILCs) during the fibrotic process (Hams et al.). Repetitive cycles of epithelial damage and repair are able to generate fibrosis through the release DAMPs and alarmins by epithelium (4). Among the alarmins, IL25, IL33, and TSLP are able to polarize ILCs to the ILC2 phenotype. ILC2 can enhance Th2 responses and collagen deposition (5, 6), either indirectly *via* IL13-mediated dendritic cell priming or directly through CD4-T cells interaction (*via* MHCII-CD4) (7, 8). In addition, ILC2 produces IL4 and IL5 and induces tissue collagen deposition in pulmonary and hepatic models of fibrotic diseases (9, 10). In turn, deficiency of IL25 and IL33 or their receptors, IL17RB and ST2, respectively, leads to decreased collagen deposition (5, 9). However, the apparent redundancy of these alarmins may be due to different ligand and receptor expression at different anatomical sites (11).

ILC2s interact with macrophages on the improvement of obesity-induced insulin resistance (Castoldi et al.). Different subtypes of macrophages are related to the maintenance of adipose tissue (AT) homeostasis during the lean state, obesity, and insulin resistance (Castoldi et al.). It has been known that the microenvironment in a lean AT is composed of macrophages subtypes in a ratio of

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4:1 M2:M1 (12). To maintain AT homeostasis in this lipid-rich microenvironment, macrophages present increased adiposity (13) and increased expression of fatty acids transporters (13). However, obesity status triggers the accumulation of M1 macrophages, although it was reported that the secretion of pro-inflammatory cytokines in AT is dependent on peroxisome proliferator-activated receptor gamma (PPAR-γ), an M2 marker (14). Inflammatory factors present in obesity context lead to insulin resistance, characterized by decreased phosphorylation of insulin receptor substrate-1 and -2, decreased phosphorylation of Akt (15, 16) and activation of the mammalian target of rapamycin signaling pathway (17), a sensor of nutrients able to alter the cellular metabolism. In obesity, nutrient sensing by mTOR regulates the switch of ATMs from M2 to M1 (18). However, obesity can be controlled through the production of large amounts of anti-inflammatory cytokines and the induction of uncoupling protein 1 expression in AT, a process called "beiging" or "browning" (19). In line with the relationship between AT and inflammation, it has been reported high levels of inflammatory mediators in the context of cachexia (de Matos-Neto et al.), a health problem present especially in cancer patients (20). Weight loss, the most visible feature of cachexia, is accompanied by increased production of CCL2, CCL3, TNFα, and IL1β and reduced relative numbers of M2 macrophages in the tumor environment (de Matos-Neto et al.).

Macrophages directly influence the metabolic status of the organism (21). Different sterile inflammation, in special type 1 diabetes (T1D) can be triggered by leukotriene B4 (LTB4) (Filgueiras et al.). Filgueiras et al. wonder if LTB4 could be targeted in new therapy strategies for treating T1D once LTB4 could either increase pro-IL1β expression or potentiate the IL1R activation by modulating MYD88. Previously, the same group has demonstrated that low insulin concentrations are able to induce LTB4 production, which triggers systemic inflammation through MyD88 and its transcriptional effector STAT-1 (signal transducer and activator of transcription 1) (22). On the other hand, insulin-treated mice showed less LTB4 in the blood and reduced Myd88 and Stat1 expression in macrophages. In addition, diabetic mice lacking 5-lipoxygenase or the receptor for LTB4 produced less pro-inflammatory cytokines (22). Mitochondrial DNA (mDNA) derived from diabetic mice is also implicated in the activation of NLRP3 and IL1β in the context of T1D (Carlos et al.). It has been known that NLRP3 deficiency plays a protective role against T1D (23) and that polymorphisms in NLRP3 are associated with T1D (24), however, the precise mechanisms by which NLRP3 is triggered in the context of T1D was poorly explored. Besides demonstrating the importance of NLRP3 for the development of T1D, Carlos et al. also took advantage of a sub dosage model of disease that is not able to induce T1D, unless mDNA was given concomitantly with streptozotocin. However, it is still puzzling the fact that only mDNA from diabetic mice activates the NLRP3 inflammasome.

Besides homeostasis-altering compounds, exogenous molecules can also alter the macrophage status of activation (25). Crystalline silica reduces the activation of macrophages by reducing TLR2 expression (Beamer et al.). Previous studies established that the scavenger receptor CD204 is important for the binding/uptake of silica (26, 27). It has been also demonstrated that silica crystals activate NLRP3 inflammasome and induce IL1β production (28), a mechanism dependent of the first signal triggered by the TLR4 agonist, LPS. Beamer et al. demonstrated, on the other hand, that silica crystals leads to less IL1β production after Pam3CSK4 and Pam2CSK4 stimulus, lipopeptides recognized by the TLR2/1 and TLR2/6 heterodimer, respectively (Beamer et al.). Tissue-resident intestinal macrophages can also contribute to the gut homeostasis by eliminating invading pathogens without inducing a robust inflammatory response (Kühl et al.). Bone marrow-derived monocytes are the precursor cells of tissue-resident intestinal macrophages (29) and in the context of ulcerative colitis (UC) and Crohn's diseases (CD), increased numbers of M1 macrophages are observed despite monocyte infiltration. In addition, lesions of UC, but not CD, are characterized by impaired bacterial clearance, formation of granulomas, inflamed mesenteric fat tissue, and pronounced fibrosis.

The prevention of damage that would be caused by macrophage prolonged activation is achieved by changes in their transcriptional program (Hamidzadeh and Mosser). ATP and adenosine can diminish the production of inflammatory cytokines by macrophages (30). In an inflammatory scenario, TLR-stimulated macrophages undergo metabolic alterations that result in an increase rate of aerobic glycolysis and production of ATP. This nucleotide is rapidly hydrolyzed to adenosine on the macrophage surface by CD39 and CD73 (30). Following TLR stimulation, macrophages dramatically upregulate their expression of receptors for adenosine, in a physiological self-regulating program. In addition, it has been demonstrated that IFNγ sustains macrophage inflammatory responses, by attenuating their sensitivity to extracellular adenosine (31). This decreased macrophage sensitivity to adenosine delays the transition of macrophages to a regulatory phenotype, allowing them to sustain macrophage activation for the duration of an adaptive immune response. IFNγ-mediated adenosine sensitivity signals through STAT1 (31); however, the exact mechanism whereby IFNγ affects the macrophage activation remain to be enlightened. However, when not controlled, blood-borne infections change the splenic microenvironment and can ultimately lead to splenomegaly (32). Splenic architecture and differences among red pulp (RpMΦs), marginal metallophilic (MMMΦs), and marginal zone macrophages (MZMΦs) were described by Borges da Silva et al. CD47, a self-molecule ubiquitously expressed on many cell types, function as an inhibitory signal for phagocytosis (33) and red blood cells expressing a modified isoform of CD47 are phagocytized by RpMΦs (34). MZMΦs and MMMΦs populate the interface between the bloodstream and lymphocyte-rich zones, and for this reason they are candidate cells to bridge innate and adaptive immunity. In this collection of articles, the authors show how macrophages influence chronic inflammatory diseases, and how the understanding of their biology can contribute to improved scenario for balance the homeostasis. We hope this collection can help further studies on the development of new therapies and in the better understanding of the biology of these cells.

AUTHOR CONTRIBUTIONS

TB wrote the manuscript. IM, AL, and NC helped to evaluate and edit the manuscript.

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REVIEW
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Purinergic Signaling to Terminate TLR Responses in Macrophages

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Macrophages undergo profound physiological alterations when they encounter pathogen-associated molecular patterns (PAMPs). These alterations can result in the elaboration of cytokines and mediators that promote immune responses and contribute to the clearance of pathogens. These innate immune responses by myeloid cells are transient. The termination of these secretory responses is not due to the dilution of stimuli, but rather to the active downregulation of innate responses induced by the very PAMPs that initiated them. Here, we describe a purinergic autoregulatory program whereby TLR-stimulated macrophages control their activation state. In this program, TLR-stimulated macrophages undergo metabolic alterations that result in the production of ATP and its release through membrane pannexin channels. This purine nucleotide is rapidly hydrolyzed to adenosine by ectoenzymes on the macrophage surface, CD39 and CD73. Adenosine then signals through the P1 class of seven transmembrane receptors to induce a regulatory state that is characterized by the downregulation of inflammatory cytokines and the production of anti-inflammatory cytokines and growth factors. This purinergic autoregulatory system mitigates the collateral damage that would be caused by the prolonged activation of macrophages and rather allows the macrophage to maintain homeostasis. The transient activation of macrophages can be prolonged by treating macrophages with IFN-γ. IFN-γ-treated macrophages become less sensitive to the regulatory effects of adenosine, allowing them to sustain macrophage activation for the duration of an adaptive immune response.

Keywords: adenosine, autoimmunity, ATP, CD39, CD73, glycolysis, IFN-γ

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INTRODUCTION

The central role that macrophages play in host defense has been well described and thoroughly studied. These remarkable cells can change their physiology in response to diverse environmental stimuli and become potent antimicrobial effectors. This property has been loosely called an "activation" response, and the receptors that induce this response are generally called pattern recognition receptors (PRRs) (1). More recently, the role of macrophages in mitigating inflammatory responses and contributing to the resolution of inflammation has become an area of intense study (2, 3). It is clear that the very cell type that could be a potent inducer of inflammatory pathology could be equally effective at reversing this pathology. The remarkable plasticity of macrophages allows this cell to be a primary mediator of homeostasis in the host (4, 5).

Given the remarkable differences in the physiologies of the various macrophage subsets, efforts are underway to characterize each. These characterizations would theoretically allow the

identification of each macrophage subtype in tissue during immunity or immunopathology. However, studies to identify definitive biochemical differences between inflammatory M1 macrophages and anti-inflammatory regulatory macrophages (R-Mϕ) have been surprisingly underdeveloped. The *in vitro* transcriptional responses of the so-called M1 macrophages following their exposure to a variety of TLR ligands, such as LPS or to bacteria themselves, have been reported (6–11). These studies have begun to reveal the molecules that macrophages express and the products they secrete in response to inflammatory stimuli. However, most of these studies lack a careful kinetic analysis of transcriptional responses over time. Therefore, we are left with "snap-shots" of transcriptional responses to stimuli, rather than a motion picture of the sequential transcriptional program these stimuli induce. The transcriptional responses of anti-inflammatory macrophages have also been described (12, 13), but again these studies generally selected only a single time to analyze macrophage transcripts. In this review, we propose that one of the difficulties in identifying definitive biochemical differences between the various macrophage cell populations is due to the transient nature of the inflammatory response of macrophages to stimuli and the compensatory regulatory changes that accompany this activation. We describe an intrinsic program where the metabolic alterations that allow for the production of inflammatory cytokines and mediators are the very alterations that give rise to the anti-inflammatory macrophage phenotype. This autoregulatory response depends on the generation of endogenous ATP by macrophages, which initiates a purinergic signaling cascade to terminate the inflammatory response to innate stimuli, resulting in a transient state of activation. Therefore, the time when one measures the transcriptional responses of macrophages to TLR stimuli is critical. We also propose that this transient state of macrophage stimulation can be prolonged and accentuated in individuals undergoing cell-mediated immune responses. This is due to a novel activity of IFN-γ, which interferes with the stimulus-dependent upregulation of adenosine receptors to block purinergic autoregulatory responses. In this way, IFN-γ prevents the transition to a regulatory macrophage and prolongs the activation response.

METABOLIC ALTERATIONS INDUCED BY THE LIGATION OF MACROPHAGE PATTERN RECOGNITION RECEPTORS

When macrophages encounter pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) they undergo dramatic changes in their metabolism and increase their rate of aerobic glycolysis. An increase in glucose uptake by these cells results in an accumulation of lactate in M1 macrophages (12). In contrast to M1 macrophages stimulated by PAMPs, alternatively activated macrophages exposed to IL-4 or IL-13 undergo oxidative phosphorylation and electron transport. The metabolic alterations associated with M1 macrophage polarization are believed to provide short-term, immediate access to energy for innate immune functions, whereas alternative activation is thought to provide a more stable long-term metabolism to support prolonged processes associated with wound healing (14). Recent work suggests that these metabolic alterations not only accompany differential activation but also promote the polarized responses of M1 and M2 macrophages (15). The rapid alterations in metabolism that M1 macrophages undergo are thought to allow these cells to produce the cytokines and mediators associated with host defense [reviewed in Ref. (14)]. However, the increase in glycolysis by M1 macrophages results in an increase in the production of intracellular ATP by stimulated macrophages. A portion of cytosolic ATP generated by M1 macrophages is released into the extracellular milieu *via* pannexin-1 channels. The addition of inhibitors of either glycolysis or pannexin channels prevents ATP release from macrophages (16). This released ATP is rapidly captured and catabolized to adenosine by M1 macrophages, allowing them to transition from an inflammatory to a regulatory phenotype. Thus, the very metabolic alterations that allow M1 macrophages to promote immune responses can also prevent these cells from causing immunopathology.

THE MACROPHAGE ECTOENZYMES, CD39 AND CD73

Purinergic signaling molecules released as a result of metabolic alterations, cell death, or tissue damage can have profound effects on macrophage activation. ATP concentrations in human plasma are typically in the nanomolar range (17) but can rise to the micromolar range under inflammatory conditions (18). ATP is constitutively released from resting parenchymal cells, and the levels are intrinsic to the tissue in which the cells reside (19). ATP release from resting macrophages is quite low, but this release is substantially increased upon TLR stimulation (16). The ATP that is released by macrophages is catabolized by macrophages in a coordinated two-step process. First, ATP is hydrolyzed to AMP by the macrophage surface ectoenzyme CD39 (E-NTPDase1) in a Ca^{2+}- and Mg^{2+}-dependent manner (20). AMP is then rapidly converted to adenosine by the surface Ecto5′NTase, CD73 (21). The expression of these two enzymes by macrophages can therefore determine the concentration of adenosine in the extracellular milieu immediately surrounding the macrophage.

CD39 and CD73 expression on macrophages can change depending on the macrophage activation state. In hypoxic conditions, CD39 and CD73 function is enhanced approximately sixfold (22), whereas prolonged cultivation of macrophages in complete medium appears to downregulate CD73 expression. CD39 is more highly expressed than CD73 on bone marrow derived macrophages, and this expression pattern remains relatively constant after a brief exposure of these cells to LPS. M1 macrophages have been reported to exhibit a modest decrease in the expression of both CD39 and CD73 (23), while M2 macrophages express higher levels of both (23). These results suggest that macrophages may regulate the catabolism of ATP in order to modulate their inflammatory profile. It has also been shown that CD39 is transcriptionally regulated by the cAMP/CREB second messenger pathway that can be induced following GPCR ligation (24, 25). This suggests a positive feedback loop where adenosine signaling upregulates CD39 to generate more adenosine. Overall,

this work suggests that the increased expression of either/both of these cell surface enzymes can result in an amplification of the purinergic signaling pathway in macrophages.

We recently demonstrated that the addition of exogenous adenosine or ATP to macrophages can induce these cells to assume an anti-inflammatory phenotype (16) characterized by a decreased production of inflammatory cytokines and an increased expression of angiogenic factors and anti-inflammatory cytokines (12). We further demonstrated that the hydrolysis of self-released (endogenous) ATP *via* macrophage CD39 allows that cell to transition from an inflammatory to an immunoregulatory state (16). Macrophages derived from CD39 knockout bone marrow fail to catabolize ATP following LPS stimulation. As a result, the production of inflammatory cytokines is sustained for up to 24 h poststimulation, whereas wild-type macrophages stop synthesizing these cytokines after a few hours (16). Similarly, the pharmacological inhibition of CD39 activity, using the chemical inhibitor POM-1, made macrophages hyperinflammatory with increased TNF and IL-12p40 production over the course of at least 16 h (16). It appears that of the two ectoenzymes involved in ATP hydrolysis, CD39 has more profound effects than CD73, presumably because the conversion of AMP to adenosine can occur in the absence of CD73. It was recently demonstrated that an inhibitor of CD73 did not have a substantial role in macrophage polarization (26).

The ability of macrophages to transition to an immunoregulatory state is key in controlling pathology in an LPS model of endotoxemia. Our lab results and others have shown that CD39 on myeloid cells can decrease mortality in mouse models of sepsis (16, 27), and that the addition of CD39 knockout macrophages can increase mortality in this model (16). CD73 has also been demonstrated to be protective in mouse models of sepsis (28).

THE RECEPTORS FOR ADENOSINE

Macrophages respond to adenosine *via* four transmembrane G-protein-coupled receptors: A1R, A2aR, adenosine 2b receptor (A2bR), and A3R (29). The A1 and A3 receptors are coupled to the G_i family of proteins resulting in decreased cAMP upon stimulation. A2a receptors are high affinity $G\alpha_s$-coupled receptors that increase intracellular cAMP (30, 31). Similarly, the low-affinity A2b receptors can signal through $G\alpha_s$ or G_q proteins, also resulting in increased cAMP (30, 32). When coupled to TLR stimulation, adenosine promotes the transition from an inflammatory to a regulatory macrophage (4). Adenosine is known to be immunosuppressive in macrophages as adenosine treatment leads to increased IL-10 production and decreased TNF and IL-12 production (16). We recently performed high-throughput RNA sequencing on macrophages stimulated with LPS in the presence or absence of adenosine. Macrophages stimulated with LPS in the presence of adenosine upregulated 501 transcripts relative to LPS alone and downregulated 610 transcripts. Many of the genes that were upregulated were involved in cell growth and neovascularization, whereas genes involved in inflammation were most potently downregulated by the presence of adenosine (12, 13). Adenosine signaling through its $G\alpha_s$-coupled receptors also leads to increased IL-10 production *via* posttranscriptional mechanisms (33). Adenosine is thought to inhibit the production of the inflammatory cytokine TNF by signaling through both the A2a and A2b receptors (34).

Although signaling through these GPCRs modulates levels of cAMP within cells, the role of the cAMP/PKA pathway in the regulation of inflammatory cytokines by adenosine receptor signaling remains somewhat unclear. One group has indicated that the decrease in macrophage TNF production after exposure to adenosine is due to a cAMP/PKA-independent pathway, which likely involves phosphatases (35). However, others have shown that cAMP/PKA levels are inversely correlated with TNF production (36). Thus, it is possible that while cAMP itself, mainly investigated in the form of 8-bromo-cAMP, can downregulate TNF production in macrophages, adenosine may also work by additional mechanisms that have not yet been fully defined. It was shown that the A2bR interacts with NF-κB in order to inhibit it, and that A2bR knockout macrophages secrete less IL-10 and more IL-12 and TNF (37).

The adenosine receptors have been implicated in the pathology of a variety of diseases. These receptors are widely expressed in the brain, heart, spleen, muscle, and lung (38, 39). In fact, their widespread expression is one of the challenges of developing therapeutics targeting the receptors with specificity. Studies have implicated a role for both A2aR and A2bR in diabetes as they are involved in gluconeogenesis and glucose homeostasis as a result of increased cAMP (40–42). There is also therapeutic anti-inflammatory potential for A2aR agonists in ischemia reperfusion injury (43). In atherosclerosis, A2aR and A2bR both play a role in reducing foam cell formation, which is a feature of this disease (44, 45). However, it has been shown that the lack of A2aR has a protective effect in a mouse model of hypercholesterolemia because macrophages remain inflammatory and are able to reduce atherosclerotic lesions (46). Adenosine receptors also play a role in wound healing and contribute to cytokine production by macrophages of patients with chronic obstructive pulmonary disease (29, 47).

IFN-γ AND THE PROLONGATION OF THE MACROPHAGE ACTIVATION RESPONSE

Priming macrophages with IFN-γ prior to TLR stimulation results in profound changes in their physiology and dramatically accentuates their inflammatory responses (48, 49). Macrophages exposed to IFN-γ not only make greater amounts of inflammatory cytokines but also produce them for prolonged periods of time (50). In this way, IFN-γ prolongs the activation response to promote host defense against intracellular pathogens (51). The activation of macrophages, however, comes at a cost. Inflammatory macrophages exhibiting an "IFN signature" are observed in rheumatoid arthritis, multiple sclerosis, and many other autoimmune diseases, indicating that IFN-γ contributes to autoimmune pathogenesis by promoting chronic macrophage activation (52, 53). Although the ability of IFN-γ to enhance the inflammatory potential of TLR-activated macrophages is a well-known phenomenon, the mechanism(s) whereby IFN-γ affects the intrinsic regulation of macrophage activation remain to be

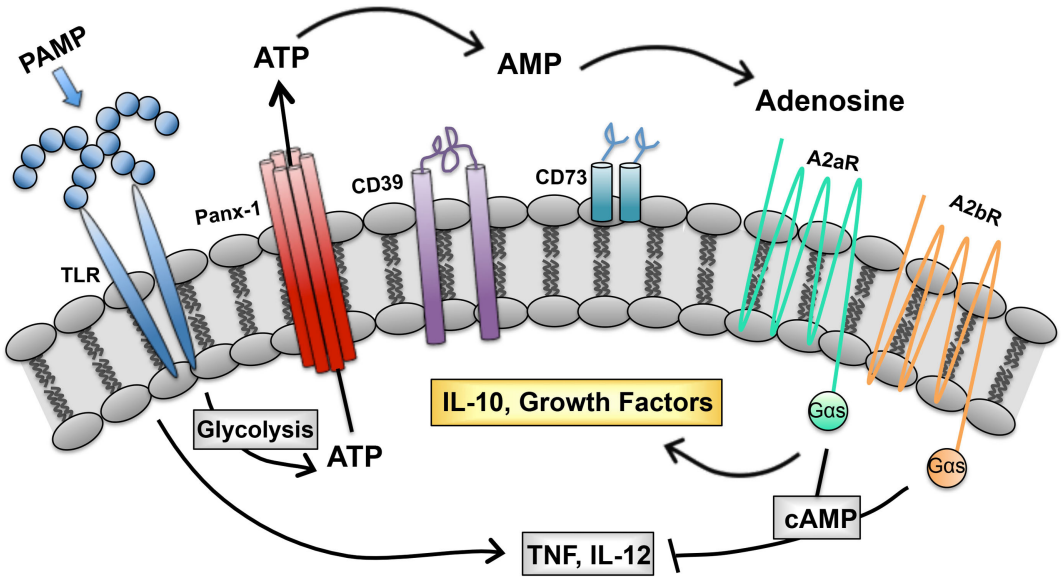

FIGURE 1 | Purinergic autoregulatory signaling in macrophages. Intracellular ATP, generated in response to pathogen-associated molecular patterns (PAMPS), is released from macrophages and converted to adenosine by the concerted action of CD39 and CD73. Adenosine signals through seven transmembrane receptors to terminate the production of inflammatory cytokines and to promote the production of IL-10 and growth factors (54).

determined. We recently identified a novel mechanism whereby IFN-γ sustains macrophage inflammatory responses, by attenuating their sensitivity to extracellular adenosine (50).

Following TLR stimulation, macrophages dramatically upregulate their expression of receptors for adenosine. The A2bR, in particular, is upregulated more than 20-fold in response to virtually any of the TLR ligands (50). The molecular mechanism(s) of A2bR upregulation remain to be determined, but the upregulation of adenosine receptors in response to TLR stimulation enhances macrophage sensitivity to adenosine and leads to the induction of the immunoregulatory phenotype. IFN-γ priming of macrophages signals through STAT1 to prevent adenosine receptor induction. This decreases macrophage sensitivity to adenosine and delays the transition of macrophages to a regulatory phenotype. This prolongs the production of inflammatory cytokines such as TNFα and IL-12. Thus, we propose a novel mechanism whereby IFN-γ contributes to host defense, by desensitizing macrophages to the immunoregulatory effects of adenosine. This mechanism overcomes the transient nature of TLR activation and prolongs the antimicrobial state of the classically activated macrophage.

SUMMARY

We describe a purinergic-based autoregulatory program that terminates inflammatory responses of TLR-stimulated (M1) macrophages. When macrophages are so stimulated, they undergo metabolic alterations that result in ATP generation and release through pannexin channels. Extracellular ATP is rapidly hydrolyzed to adenosine by CD39 and CD73, two ectoenzymes on the macrophage surface. Adenosine generated in this way binds to macrophage adenosine receptors to initiate a signaling pathway that terminates the synthesis of many inflammatory cytokines and induces the synthesis of regulatory transcripts (**Figure 1**). In this way, the overexuberant activation of macrophages is avoided. We propose that this program is in place to prevent the pathological consequences associated with chronic macrophage activation. We suggest that there are many ways to exploit this program to manipulate the phenotype of macrophages. The overexpression of CD39 and CD73 would be predicted to accelerate adenosine production by macrophages and promote a rapid regulatory transition. Drugs to prevent ectoenzyme downregulation may represent a new class of anti-inflammatory therapeutics. Similarly, drugs to induce adenosine receptor upregulation or prevent their downregulation may be developed as a way to interrupt macrophage-mediated inflammation. Conversely, targeting macrophage CD39 would be predicted to prevent this regulatory transition and promote the more efficient killing of intracellular pathogens by macrophages.

AUTHOR CONTRIBUTIONS

The ideas expressed in this review were developed by both authors who contributed equally to the writing of this review.

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Acute Exposure to Crystalline Silica Reduces Macrophage Activation in Response to Bacterial Lipoproteins

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Numerous studies have examined the relationship between alveolar macrophages (AMs) and crystalline silica (SiO_2) using *in vitro* and *in vivo* immunotoxicity models; however, exactly how exposure to SiO_2 alters the functionality of AM and the potential consequences for immunity to respiratory pathogens remains largely unknown. Because recognition and clearance of inhaled particulates and microbes are largely mediated by pattern recognition receptors (PRRs) on the surface of AM, we hypothesized that exposure to SiO_2 limits the ability of AM to respond to bacterial challenge by altering PRR expression. Alveolar and bone marrow-derived macrophages downregulate TLR2 expression following acute SiO_2 exposure (e.g., 4 h). Interestingly, these responses were dependent on interactions between SiO_2 and the class A scavenger receptor CD204, but not MARCO. Furthermore, SiO_2 exposure decreased uptake of fluorescently labeled Pam_2CSK_4 and Pam_3CSK4, resulting in reduced secretion of IL-1β, but not IL-6. Collectively, our data suggest that SiO_2 exposure alters AM phenotype, which in turn affects their ability to uptake and respond to bacterial lipoproteins.

Keywords: lung, inflammation, mouse, scavenger receptor, toll-like receptor, bacterial susceptibility

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INTRODUCTION

Silicon dioxide, also known as silica, is one of the most common elements on earth, yet its inhalation can result in acute lung injury and ongoing inhalation can result in permanent lung damage due to deposition of particles in the lung. Silicosis is a progressive, disabling, and often-fatal lung disease resulting from the inhalation of crystalline silica (SiO_2) particles over prolonged periods of time. Silicosis occurs as the result of exposure through occupation (e.g., construction, mining), recreation (e.g., pottery), or environment (e.g., soil). Inhalation of SiO_2 particles causes a granulomatous inflammatory response that progresses to interstitial fibrosis as well as systemic immune deficits (1–5). There is no cure for silicosis, and treatment options are limited. Although significant efforts have been made through industrial hygiene standards to control ambient dust in the workplace, silicosis remains a prevalent health problem throughout the world, particularly in developing nations (6).

In addition to its importance as an occupational hazard, inhalation of SiO_2 predisposes workers to bacterial infections, impairs lung defense mechanisms, and significantly shortens worker

lifespans – particularly in less-advanced countries and among disadvantaged persons in developed nations (1, 7). In particular, SiO_2-exposed workers, with or without silicosis, are at increased risk for tuberculosis and non-tuberculous mycobacteria-related diseases (5, 8, 9). Previous studies suggest that the acute and accelerated forms of silicosis exhibit the highest prevalence of silicotuberculosis (1), and that the development of *Mycobacterium tuberculosis* (Mtb) infection is directly dependent on the collective SiO_2 exposure (5, 8, 10). Indeed, SiO_2 exposure results in a threefold or greater risk of developing pulmonary Mtb infections. Similarly, recent studies demonstrate that acute exposure to silica nanoparticles increases the susceptibility of mice to *Pseudomonas aeruginosa*-induced pneumonia (11).

Alveolar macrophages (AMs) play a critical role in the ongoing cross-talk between innate and adaptive immune responses in the lung and are the typical host cell for an array of pathogens such as bacterial infections (e.g., Mtb) and many airborne particulates (e.g., SiO_2). In macrophages, formation and activation of the NLRP3 inflammasome are an important mechanism mediating the inflammatory response to numerous particulates, including nanoparticles, silica, MSU crystals, asbestos, and urban particulate matter, resulting in promotion of IL-1β release (12–15). Macrophages not only initiate the inflammatory process to SiO_2 (16) but also play an important role in host resistance to bacterial infections, including Mtb (17). Moreover, numerous adverse effects on macrophage function have been described following exposure to SiO_2 (18–27), suggesting that SiO_2-mediated macrophage injury might impair host defense and increase susceptibility to infection. The current view is that SiO_2 "damages" macrophages or alters their phenotype, thereby inhibiting their ability to phagocytose and kill bacteria (19, 28); however, the molecular mechanisms underlying this predisposition remain unknown.

Alveolar macrophages sense bacterial pathogens through pattern recognition receptors (PRRs) (29, 30) *via* the detection of highly conserved molecular structures, designated pathogen-associated molecular patterns (PAMPs) (31–33). Numerous families of PRRs exist, all of which recognize a different repertoire of PAMPs, including C-type lectin receptors, scavenger receptors (SRs), toll-like receptors (TLRs), NOD-like receptors, and RIG-I-like receptors. Of these PRRs, TLRs 1/2, 4, and C-type lectin receptors have all been shown to mediate the *in vitro* recognition of Mtb and the cytokine response of macrophages is lower in the absence of these TLRs (34). It is clear that macrophages contribute to the lung response to SiO_2 and to bacterial infections independently, and that these effects may be mediated through PRRs. Therefore, we investigated whether SiO_2 exposure alters the expression of select PRRs on alveolar and bone marrow-derived macrophages and assessed the ability of SiO_2-exposed macrophages to uptake and respond to bacterial lipoproteins acting at TLR2/1 and TLR2/6.

MATERIALS AND METHODS

Mice

Breeding pairs of C57BL/6 (C57BL/6J, stock #000664) mice were originally purchased from The Jackson Laboratory (Bar Harbor, ME, USA); whereas breeding pairs of $MARCO^{-/-}$ and $SRA^{-/-}$ mice on C57BL/6 background were kindly provided by Dr. Lester Kobzik (Harvard School of Public Health, Boston, MA, USA), and caspase 1-deficient ($casp1^{-/-}$ B6N.129S2-$Casp1^{tm1Flv}$/J, stock #016621) mice (for experimental use) were kindly provided by Dr. Andrij Holian (University of Montana, Center for Environmental Health Sciences). All mice were maintained in the University of Montana Specific Pathogen-Free (SPF) Laboratory Animal Facility and both sexes used at 6–8 weeks of age. All animal use procedures were in accordance with NIH and University of Montana IACUC guidelines.

Experimental Instillations

Crystalline silica (SiO_2, 1.5–2 μm) (Pennsylvania Glass Sand Corporation, Pittsburgh, PA, USA) was acid washed, dried, and determined to be free of endotoxin (data not shown). Mice were anesthetized with isoflurane and instilled *via* the intranasal (i.n.) exposure route with 25 μl sterile saline (vehicle) or 1 mg SiO_2 suspended in 25 μl of sterile saline (35, 36). Mice were then returned to their cages and monitored until mobility returned. Whole lung lavage samples were collected at 4, 24, and 72 h, as well as 7 days following the initial instillation, as previously described (37, 38).

Flow Cytometry

Single cell suspensions from either whole lung lavages or bone marrow-derived macrophages were washed and re-suspended in 100 μl of purified rat anti-mouse CD16/CD32 diluted 1:100 in PBS with 1% bovine serum albumin and 0.1% sodium azide (PAB) for 15 min on ice to block non-specific Ab binding. Monoclonal Abs specific to CD11c redFluor 710 (clone #N418, Tonbo Biosciences), F4-80 FITC (clone BM-8), DC-SIGN PE (clone # 5H10), TLR2 eF450 (clone # 6C2 eBiosciences), TLR4 PE-Cy7 (clone # SA15-21, Biolegend), TLR5 AF647 (clone # ACT5), and TLR6 (clone # 418601, R&D Systems) to identify cell surface receptor density on live, F4-80$^+$CD11c$^+$ AMs (23). Following titration of the individual antibodies in preliminary experiments, 1 μg of each Ab was added per 10^6 total cells and allowed to incubate for 30 min in the dark on ice, with agitation two to three times. Finally, cells were washed twice with PBS and re-suspended in 0.3 ml PAB on ice. Immediately before acquisition, 5 μl of propidium iodide solution (BioLegend) was added per 10^6 total cells and allowed to incubate for 15 min prior to analysis. Cell acquisition and analysis were performed on a FACS Aria flow cytometer using FACS Diva software (version 6.1.2, Becton Dickinson), with the exception of **Figure 4** – where cell acquisition and analysis was performed on a Attune NxT Accoustic Focusing Cytometer using Attune NxT software (version 2.2, Thermo Fisher Scientific). In the multicolor staining panels, positive/negative gates were set based on fluorescence minus one (FMO) controls and checked against single stained controls. Compensation of the spectral overlap for each fluorochrome was performed using compensation control beads (BD Biosciences).

Generation and Stimulation of Bone Marrow-Derived Macrophages

Bone marrow macrophages (BMM) were generated using murine recombinant macrophage colony-stimulating factor

(50 ng/ml, U.S. Biological, Swampscott, MA, USA), as previously described (37, 39). By 7 days, cells were fully differentiated, >75% confluent, and immune-positive for macrophage characteristics (F4-80$^+$CD11b$^+$ MHC class II^{low}), as assessed by flow cyometry (data not shown). Viability was determined to be >90% by trypan blue exclusion staining prior to experimental manipulations. BMM were seeded at 10^6 cells/ml/well of a 6-well plate, immediately exposed to media alone (vehicle) or 50–100 μg/ml SiO_2, and allowed to incubate for 4 or 24 h at 37°C. By 24 h at these exposure levels, ~20% of the BMM exhibited signs of apoptosis and/or cell death using trypan blue exclusion and/or live dead dyes during flow cytometry experiments. Following stimulation, BMM were lightly scraped within the spent culture media, centrifuged, and the supernatant and cells separated for analysis.

Confocal Microscopy and Quantification of Uptake of Rhodamine TLR Ligands

Bone marrow macrophages (1×10^6 cells/eppendorf microfuge tube) were exposed to media alone (vehicle) or 50–100 μg/ml SiO_2 on a rotisserie for 24 h at 37°C. Macrophages were subsequently exposed to 0.5 μg/ml rhodamine conjugated Pam_2CSK_4 (TLR2/6 ligand) or Pam_3CSK4 (TLR2/1 ligand) (Invivogen) for 2 h. Cells were washed twice with PBS and were cytospun (1×10^5) onto glass slides, coverslipped with Prolong Gold with Dapi, and images collected on an Olympus Fluoview Confocal Imaging System. NIH Image J software or flow cytometry was used to analyze mean fluorescence intensity (MFI) and side scatter (SSC) properties of the BMMs to measure TLR ligand or SiO_2 uptake (40).

Cytokine ELISAs

IL-1β, TNFα, IL-6, and IL-10 were measured in tissue culture supernatants using murine ELISA kits according to the manufacturer's instructions and assay procedure (R&D Systems). Color development was assessed at 450 nm on a plate reader.

Statistical Analysis

For each parameter, the values for individual mice were averaged and the SD and SE calculated. The significance of the differences between the exposure groups was determined by *t*-test, one-way, or two-way ANOVA, in conjunction with Tukey's test for variance, where appropriate. All ANOVA models were performed with Prism software, version 4. A p-value of <0.05 was considered significant.

RESULTS

Differential Pattern Recognition Receptor Expression following Acute Silica Exposure

Previous studies established that SiO_2 alters the phenotype and function of AM, bone marrow-derived dendritic cells, and macrophages, and freshly isolated interstitial macrophages and dendritic cells (23, 39, 41); however, these studies did not evaluate PRR expression on AM in response to SiO_2. To test whether exposure of AMs *in situ* resulted in altered expression of PRRs, C57Bl/6 wild-type mice were instilled with either saline (vehicle control) or 1 mg SiO_2. Four hours after encountering SiO_2 in the alveolus, flow cytometry confirmed that live (PI negative) F4-80$^+$CD11c$^+$AMs had taken up SiO_2 particles *via* changes in SSC properties, and simultaneously downregulated their expression of TLR2 and TLR6, but not DC-SIGN, TLR4, or TLR5 (**Figure 1**, inset). Representative histograms illustrate the relative change in fluorescent intensity between AMs lavaged from the airways of saline (black line) vs. SiO_2 (silver line) exposed C57Bl/6 wild-type mice, compared to unstained controls (dashed line) (**Figure 1**).

In the murine model, TLR-2 in particular plays a crucial role in the cellular response to bacterial pathogens. Therefore, we evaluated whether SiO_2 exposure downregulated TLR2 expression levels on live, F4-80$^+$CD11c$^+$ AMs in a time-dependent manner in wild-type C57BL/6 mice. SiO_2 exposure reduced TLR2 expression on the surface of AMs by 86.4 and 32.9% relative to their corresponding saline controls at 4 and 24 h, respectively. By contrast, SiO_2 exposure increased TLR2 expression on the surface of AMs by 47.8% relative to the saline control at 72 h. By 7 days after silica exposure, TLR2 expression on live, F4-80$^+$CD11c$^+$ AMs had returned to baseline (**Figure 2A**). Representative histograms illustrate the relative change in fluorescent intensity between saline (black line) and SiO_2 (silver line) exposed mice at the indicated time point following exposure (**Figure 2B**).

Silica-Induced Changes in TLR2 Expression Are Dependent on CD204, but Not Inflammasome Activation

Previous studies from our laboratory established that the class A SRs CD204 and MARCO were important for the binding/uptake of SiO_2 and subsequent inflammatory response (35, 40). Therefore, we investigated the relationship between CD204 and MARCO, and SiO_2-induced changes in TLR2 expression. Using bone marrow-derived macrophages as a model system (41), we demonstrate that SiO_2-induced loss of TLR2 expression on F4-80$^+$CD11b$^+$ macrophages was dependent on CD204, but not MARCO at 4 h (**Figure 3A**) and 24 h post-exposure (data not shown). Representative histograms illustrate the relative change in fluorescent intensity of TLR2 on the cell surface of media (black line) and SiO_2 (silver line)-treated bone marrow-derived macrophages (**Figure 3A**). These results were confirmed using AMs lavaged from saline and SiO_2-exposed C57Bl/6 wild-type and CD204$^{-/-}$ mice, at 4 h post-exposure (**Figure 3B**). Representative histograms illustrate the relative change in fluorescent intensity of TLR2 on the cell surface of live, F4-80$^+$CD11c$^+$ AMs between saline (black line) and SiO_2 (silver line) exposed mice (**Figure 3B**).

NLRP3 inflammasome activation and resultant IL-1β production by AMs is recognized as a significant mechanism underlying silicosis (13, 24, 26, 42). Because activation of the NLRP3 inflammasome converges on caspase 1, which then contributes to the production and secretion of mature IL-1β, we examined the contributions of inflammasome activation to SiO_2-induced changes in TLR2 expression using caspase 1-deficient (caspase 1$^{-/-}$) mice. Four hours following exposure, we show that SiO_2-induced loss of TLR2 expression on live, F4-80$^+$CD11c$^+$ AMs occurs independent from NLRP3 inflammasome activation and secretion of IL-1β

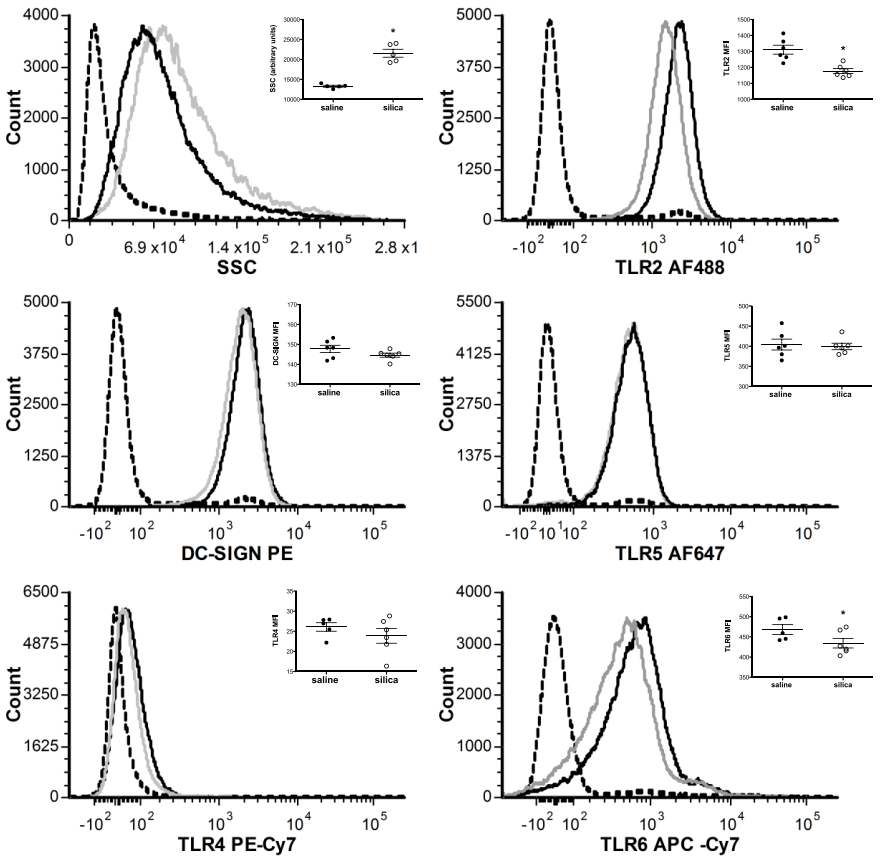

FIGURE 1 | Acute silica exposure selectively reduced TLR2 and TLR6 expression on F4-80$^+$CD11c$^+$ alveolar macrophages. C57Bl/6 wild-type mice were exposed to saline (25 μl, black line) or silica (1 mg, gray line) through intranasal aspiration. After 4 h, whole lungs were lavaged and cells immunostained. Representative histograms from the flow cytometric analysis demonstrates concomitant uptake of SiO_2 particles through increases in side scatter (SSC) measurements and decreased expression of TLR2 and TLR6 expression on alveolar macrophages (AMs) relative to saline control. By contrast, no change was observed in the cell surface expression of DC-SIGN, TLR4, or TLR5 on live, F4-80$^+$CD11c$^+$AMs in response to SiO_2. Scatter plots (inset) show the raw data in graphical form. Results are means ± SEM ($n = 6$). *$p < 0.05$ compared to saline.

(**Figure 4A**). Representative histograms demonstrate the relative change in fluorescent intensity of TLR2 on the cell surface of live, F4-80$^+$CD11c$^+$ AMs between saline (black line) and SiO_2 (silver line) exposed C57Bl/6 and caspase $1^{-/-}$ mice (**Figure 4B**).

Effects of Silica Exposure on the Uptake of TLR2/1 and TLR2/6 Ligands

Because TLR2 cooperates with TLR6 in response to diacylated mycoplasmal lipopeptide and associates with TLR1 to recognize triacylated lipopetides, we next examined the ability of SiO_2-exposed bone marrow-derived macrophages to take up fluorescently labeled bacterial cell wall components recognized by the TLR2/1 heterodimer (Pam_3CSK_4) and TLR2/6 heterodimer (Pam_2CSK_4) using a combination of flow cytometry and confocal microscopy. Flow cytometry demonstrated that SiO_2 exposure reduced the uptake (e.g., MFI) of both the synthetic triacylated and synthetic diacylated lipoproteins recognized by TLR2/1 and TLR2/6 heterodimers, respectively, in both C57Bl/6 and CD204$^{-/-}$ derived cells (**Figure 5A**). This response was slightly dampened in CD204$^{-/-}$ cells vs. C57Bl/6 cells. Moreover, simultaneous measurements of SSC characteristics revealed comparable levels of SiO_2 uptake across all exposure groups and mouse strains (**Figure 5A**), indicating similar levels of SiO_2 exposure and uptake across treatment groups. Representative images collected *via* confocal microscopy support the observation of reduced uptake of fluorescently labeled TLR ligands in response to SiO_2 exposure (**Figure 5B**). These changes were quantified by image analysis using NIH Image J and showed a similar reduction in uptake of fluorescently labeled diacylated and triacylated lipopetides into SiO_2-exposed cells (data not shown).

Silica Exposure Reduced IL-1β Levels in Response to Synthetic Triacylated and Synthetic Diacylated Lipoproteins *In Vitro*

Given that the observed reduction in TLR expression correlated with a decrease in lipoprotein uptake, we next determined if this resulted in functional changes by analyzing the inflammatory response of BMM to SiO_2 plus or minus synthetic triacylated and diacylated lipoproteins. We chose to focus on the trifecta of innate immune cytokines because of both lipoproteins are

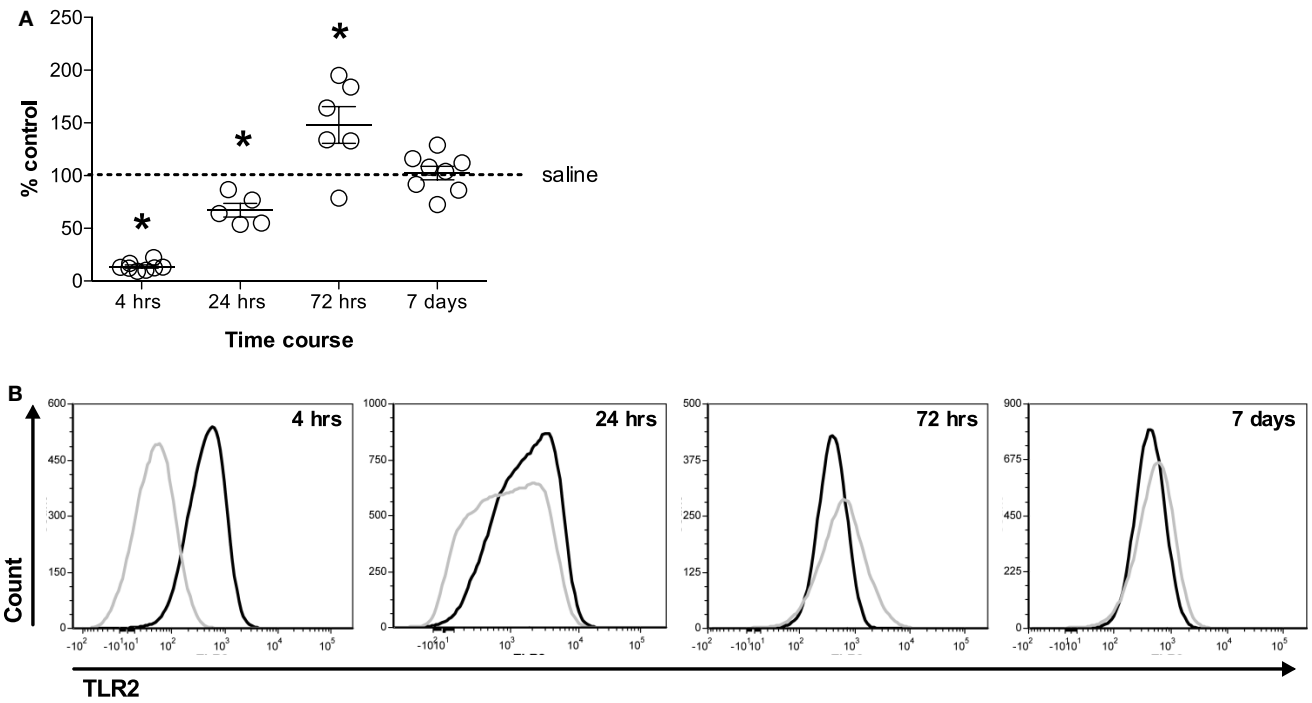

FIGURE 2 | Silica exposure altered TLR2 expression on F4-80$^+$CD11c$^+$ alveolar macrophages. C57Bl/6 wild-type mice were exposed to saline (25 μl) or silica (1 mg) through intranasal aspiration. After 4, 24, 72 h, and 7 days, whole lungs were lavaged and cells immunostained. **(A)** Flow cytometric analysis demonstrates decreased expression of TLR2 on live, F4-80$^+$CD11c$^+$ alveolar macrophages (AMs) at 4 and 24 h, whereas TLR2 expression was increased on AMs at 72 h. By contrast, no change was observed in the cell surface expression of TLR2 on AMs 7 days following exposure to SiO_2. Results are presented as mean percent of control ± SEM (n = 6-8). *$p < 0.05$ compared to saline control. **(B)** Representative histograms from the flow cytometric analysis demonstrate changes in the cell surface expression of TLR2 as a function of time.

anticipated to induce the maturation and release of IL-1β, and to trigger the release of TNFα and IL-6 (likely *via* the activation of NF-kb signaling pathways). As anticipated, bone marrow-derived macrophages recognized and responded to synthetic triacylated and diacylated lipoproteins by increasing levels of IL-1β, TNFα, and IL-6 found in the tissue culture supernatant relative to media alone, whereas exposure to SiO_2 alone resulted in little to no change (**Figure 6**). Although SiO_2 exposure reduced the levels of IL-1β and TNFα present in the tissue culture supernatant in response to stimulation with either Pam_2CSK_4 or Pam_3CSK4, it had no effect on the secretion of IL-6 (**Figure 6**). Furthermore, as a positive control, exposure to SiO_2 plus 10 ng/ml LPS appears to have activated the Nlrp3 inflammasome, thus resulting in enhanced IL-1β secretion relative to either stimulus alone (**Figure 6**) and supporting the finding that TLR4 expression remains unchanged by SiO_2 exposure. Finally, although we analyzed the tissue culture supernatants for the presence of IL-10, the levels detected were at or below the limit of detection of the assay.

DISCUSSION

Silicosis, the most prevalent of the pneumoconioses, is caused by inhalation of crystalline SiO_2 particles. In addition to its importance as an occupational disease, silicosis or even exposure to SiO_2 without established disease is associated with increased risk of developing many pulmonary and systemic comorbidities: chronic obstructive pulmonary disease, lung cancer, tuberculosis, non-tuberculous mycobacteria-related diseases, glomerulonephritis, rheumatoid arthritis, scleroderma, and other systemic autoimmune diseases. Although the epidemiological link between silicosis and tuberculosis has been acknowledged for decades, the cellular and molecular mechanisms underlying this increased risk remain largely unknown. As the first line of defense in the alveolar spaces, AM recognize and respond to inhaled pathogens and particulates, likely through interactions with PRRs, resulting in activation of NLRP3 inflammasome among many other signaling pathways. The primary objectives of this investigation were to (1) uncover if SiO_2 modifies the profile of select PRRs expressed on macrophages and (2) examine the interactions between SiO_2-exposed macrophages and Pam_3CSK_4 and Pam_2CSK_4: synthetic diacylated and triacylated lipopeptide ligands, which mimic bacterial cell wall components recognized by TLR2/6 and TLR2/1, respectively. The results from this study suggest that SiO_2 interferes with the ability of macrophages to appropriately respond to bacterial ligands by downregulating the expression of TLR2 in a CD204-dependent, but inflammasome-independent manner.

The lung is constantly exposed to potentially harmful pathogens, including airborne particulates and microorganisms.

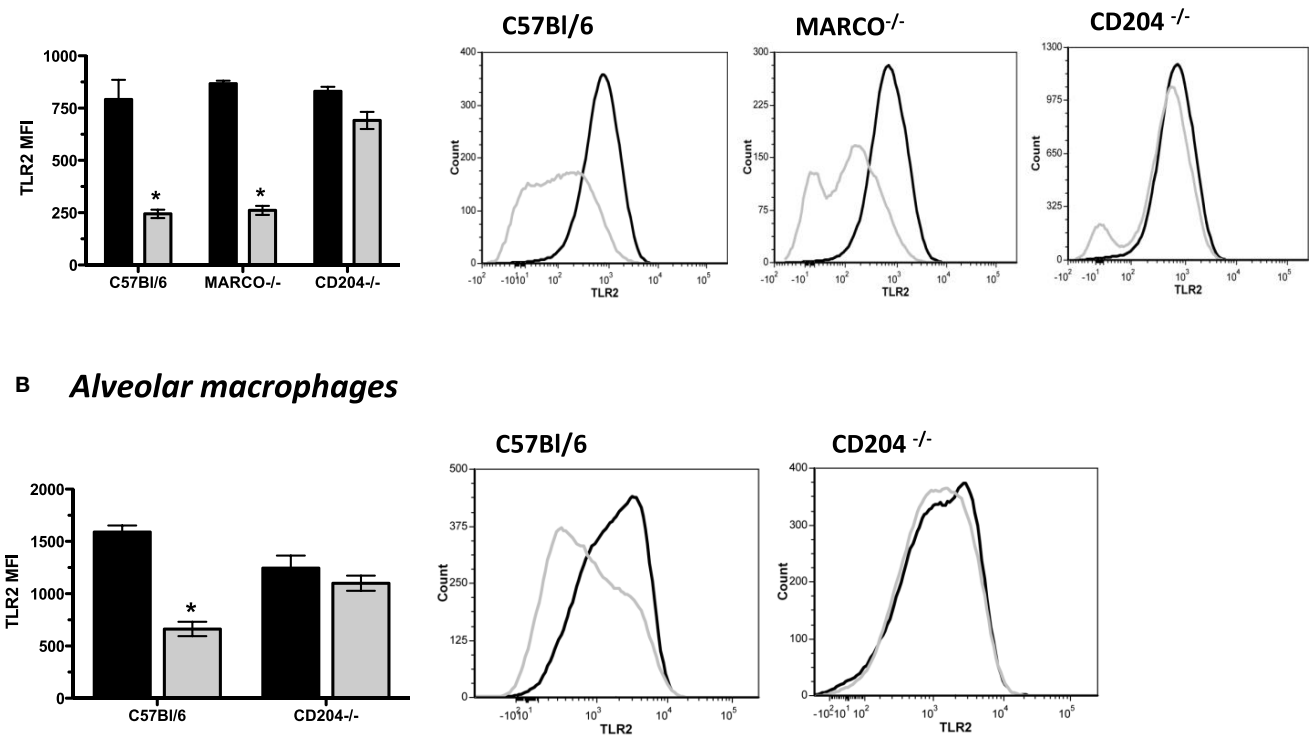

FIGURE 3 | Silica-induced decrease in TLR2 expression is dependent on the scavenger receptor CD204 *in vitro* and *in vivo*. (A) Macrophages were derived from the bone marrow of wild-type C57Bl/6, CD204$^{-/-}$, and MACRO$^{-/-}$ mice and subsequently exposed to media or silica (100 μg/ml). As anticipated, flow cytometric analysis demonstrates decreased expression of TLR2 on *live*, F4-80^{+}CD11b^{+} macrophages 4 h following exposure to SiO_2. This reduction in the cell surface expression of TLR2 was dependent on the presence of the scavenger receptor CD204, but not MARCO. Results are means ± SEM (n = 3–5). *$p < 0.05$ compared to saline. Representative histograms from the flow cytometric analysis display changes in the cell surface expression of TLR2 as a function of mouse strain. **(B)** Wild-type C57Bl/6 and CD204$^{-/-}$ mice were exposed to saline (25 μl) or silica (1 mg) through intranasal aspiration. After 4 h, whole lungs were lavaged and cells immunostained and analyzed by flow cytometry. Flow cytometry shows decreased expression of TLR2 on *live*, F4-80^{+}CD11bc^{+} AMs 4 h after SiO_2, which is dependent on the presence of the scavenger receptor CD204. Results are means ± SEM (n = 4–5). *$p < 0.05$ compared to saline. Representative histograms from the flow cytometric analysis display changes in the cell surface expression of TLR2.

Numerous studies have established that macrophages (alveolar and interstitial) are key orchestrators of pulmonary immunity and the prototypical host for diverse pathogens – including SiO_2 and bacterial pathogens. In the steady state, the ability of macrophages to generate an inflammatory response is tightly regulated to ensure that lung injury does not occur, thus preserving alveolar physiology and gas exchange. By contrast, in response to insult, macrophages are responsible for the uptake and clearance of a wide variety of environmental contaminants (e.g., crystalline silica), as well as phagocytizing and eliminating bacteria (e.g., Mtb). Macrophage responses to airborne particulates and microorganisms ranges from ingestion and clearance with minimal inflammation to massive secretion of inflammatory mediators (e.g., cytokines and reactive oxygen species) and recruitment and/or activation of other innate and adaptive immune cell types. Although freshly isolated AMs most closely represent the *natural* state, bone marrow-derived macrophages are a widely used and accepted model system because of the relative simplicity of the isolation procedure, the high numbers of cell yielded, and the consistency of the cellular response to immune activation. In this study, both freshly isolated alveolar and bone marrow-derived macrophages were utilized to verify findings based on assay specific needs.

Alveolar macrophages are the first line of defense in the alveolar spaces against inhaled pathogens, and also serve to limit inflammation and minimize injury to preserve lung function. Because PRRs, such as C-type lectin receptors, SRs, TLRs, NOD-like receptors, and RIG-I like receptors, play a prominent role in the activation of AMs and subsequent cross-talk with innate and adaptive immune cells, how a macrophage reacts to a given stimulus depends greatly on the diverse range of PRRs expressed on the cell's surface (43, 44). In the case of concomitant or sequential exposure to two distinct pathogens, the capacity of macrophages to recognize, phagocytose, and appropriately respond to a second stimuli may be compromised by stimulant-induced changes in the profile of PRRs (45) – thus altering susceptibility to disease. TLRs, which recognize microbial molecules, are major triggers of innate responses (e.g., enhanced costimulatory molecule expression,

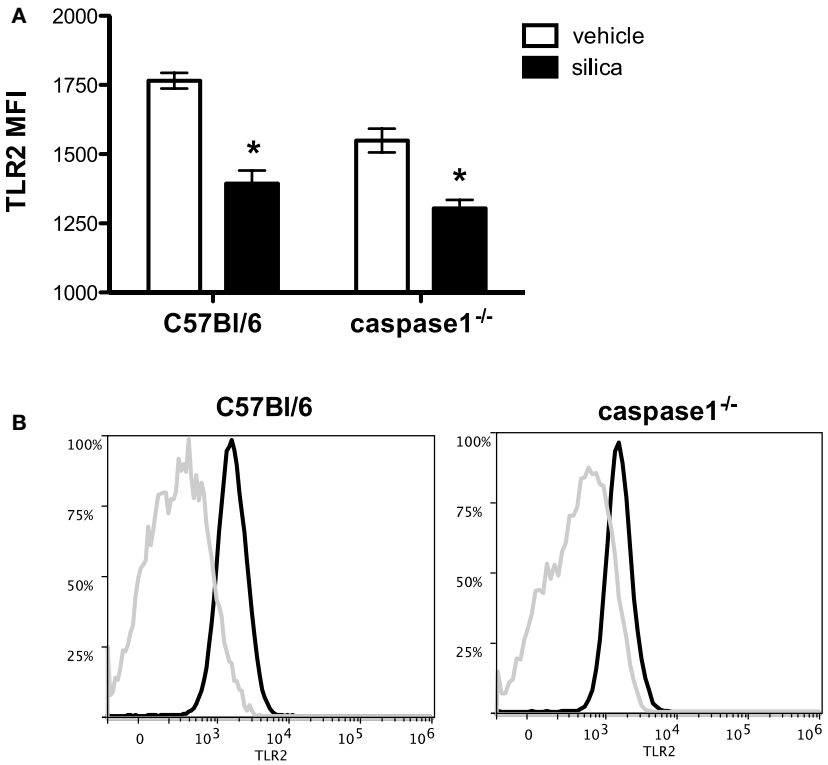

FIGURE 4 | Silica-induced reduction in TLR2 expression occurs independently of inflammasome activation. Wild-type C57Bl/6 and caspase $1^{-/-}$ mice were exposed to saline (25 μl) or silica (1 mg) *via* intranasal aspiration. After 4 h, whole lungs were lavaged and cells immunostained and analyzed by flow cytometry using the Attune NxT acoustic focusing flow cytometer. **(A)** Flow cytometry corroborates decreased expression of TLR2 on *live*, F4-80$^+$CD11c$^+$ AMs isolated from C57Bl/6 mice 4 h after SiO_2 and further demonstrates that this decrease is not dependent on the NLRP3 inflammasome and IL-1β secretion. Results are means ± SEM (n = 3–4, repeated twice). *$p < 0.05$ compared to saline. **(B)** Representative histograms from the flow cytometric analysis demonstrate changes in the cell surface expression of TLR2 as a function of time.

cytokine secretion, production of reactive oxygen species, and antimicrobial mediators) and thus modulate adaptive immunity by influencing macrophage functions. TLRs are important for host responses to Mtb. In particular, TLR-2 activation has been shown to play a prominent role in eliciting appropriate immune responses to *Mycobacterium avium* or Mtb, as well as to bacterial products, such as lipoarabinomannan, lipoprotein, and phosphatidylinositol mannosides (28, 46–51). Furthermore, associations between TLR2 gene polymorphisms and tuberculosis have been reported for a range of different human populations (52, 53), suggesting that changes in TLR2 expression may be involved in susceptibility to disease. In this study, we tested the hypothesis that exposure to SiO_2 triggers phenotypic changes in AMs – recognized as differences in the PRR profile. Following acute SiO_2 exposure (≤4 h) in C57BL/6 mice, we ascertained that live F4-80$^+$CD11c$^+$ AMs downregulate the expression of TLR2 using multi-color flow cytometry. These results were confirmed using F4-80$^+$CD11b$^+$ murine bone marrow-derived macrophages, and more importantly shown to be dependent on the presence of the class A SR, CD204, but not the NLRP3 inflammasome. These findings support the importance of interactions between SiO_2 and CD204 (40) and further link the SiO_2–CD204 interface in macrophages to the inflammatory response to ligands acting at TLR2. Of note, Chávez-Galán et al. recently reported that human monocyte derived macrophages and a human macrophage cell line (THP-1) also respond to SiO_2 by downregulating the expression of TLR2 in a dose-dependent manner (54). Although this study did not explore the role of SRs in this process, their results suggest that SiO_2 may impair the ability of human macrophages to control intracellular bacterial growth (54). These results led us to assess whether SiO_2 altered the expression of other PRRs (e.g., TLR4, TLR5, TLR6, and DC-SIGN) involved in innate immunity to various pathogens. Although acute exposure to SiO_2 decreased TLR6 expression on AMs, we have not yet examined whether this reduction in protein expression is dependent on CD204. These data suggest that interaction between SiO_2 and CD204 may regulate the responsiveness of antigen-presenting cells to TLR2 activation. Previous studies have also observed connections between CD204 and TLR4 signaling (55, 56); although little is known about the biochemical nature of such interactions.

Recognition of TLR ligands results in immune activation, which can be measured as enhanced costimulatory molecule expression, cytokine secretion, production of reactive oxygen species, and antimicrobial mediators. Previous studies from our laboratory group demonstrated that SiO_2 downregulates the expression of costimulatory molecules on murine bone

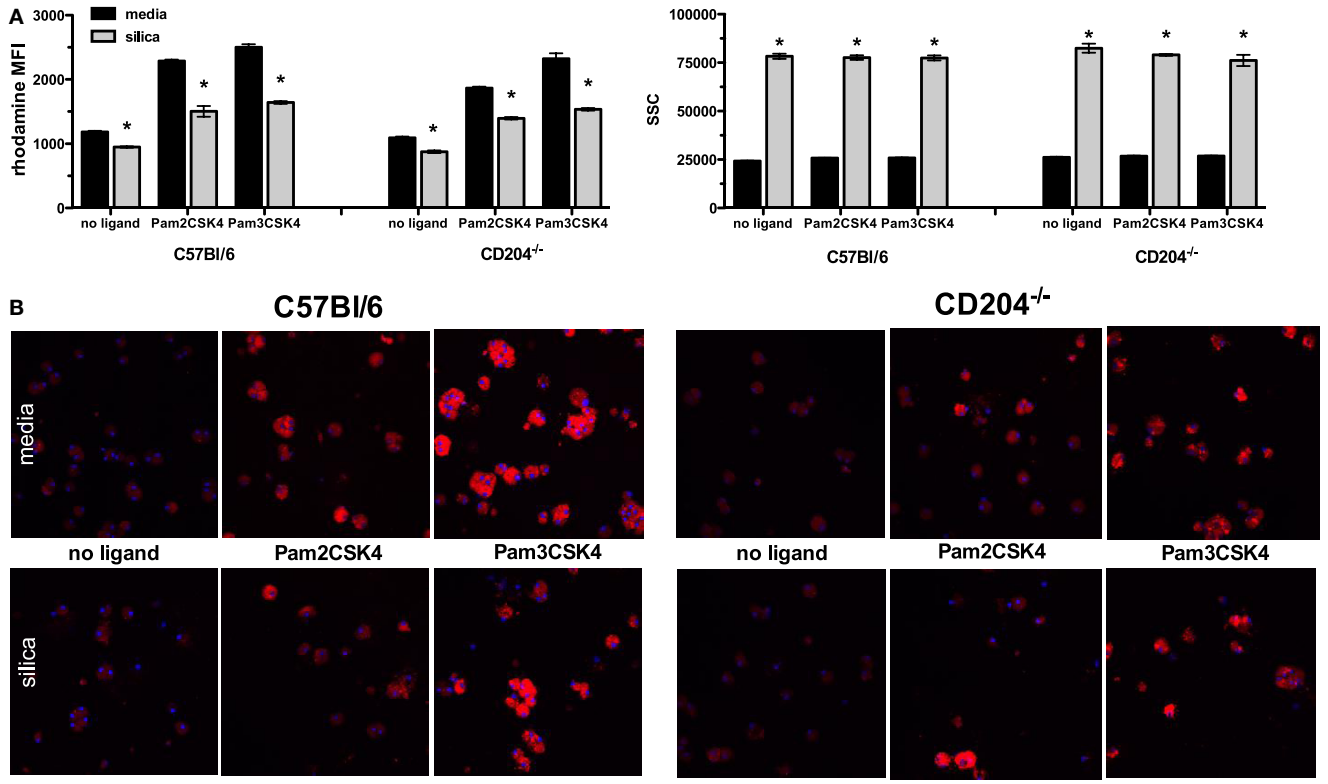

FIGURE 5 | **Silica exposure decreased uptake of fluorescently labeled synthetic diacylated and triacylated lipoproteins *in vitro***. Macrophages derived from the bone marrow of wild-type C57Bl/6 and CD204$^{-/-}$ mice were exposed to media or silica (100 μg/ml) for 24 h and subsequently treated with fluorescently labeled Pam_2CSK_4 and Pam_3CSK4 for 2 h. **(A)** Flow cytometry revealed uptake of the rhomadine-labeled lipoproteins into macrophages as measured by an increase in the mean fluorescence intensity (MFI), which was attenuated in the SiO_2-exposed macrophages. This increase in MFI was not due to changes in the uptake of SiO_2 into the macrophages, as shown by an increase in SSC. **(B)** Representative images from confocal microscopy showed increased fluorescence in the macrophages exposed to fluorescently labeled lipoproteins vs. media alone. Moreover, this fluorescence was diminished in SiO_2-exposed macrophages. Results are means ± SEM ($n = 5$). *$p < 0.05$ compared to media.

marrow-derived dendritic cells. These results lead us to examine whether SiO_2-induced changes in TLR2 expression may result in aberrant response to bacterial ligands *in vitro*. Furthermore, SiO_2 exposure reduced uptake of fluorescently labeled synthetic diacylated and triacylated lipoproteins recognized by TLR2/6 and TLR2/1 in both C57Bl/6 wild-type and CD204$^{-/-}$ derived macrophages. Several possibilities arise from this incongruity that CD204$^{-/-}$ macrophages do not downregulate TLR2 expression, yet exhibit reduced ability to uptake rhodamine-labeled Pam_2CSK_4 and Pam_3CSK_4. CD204$^{-/-}$ macrophages may have enhanced expression of other PRRs (e.g., biological compensation) (57), the reduced levels of TLR2 may not be responsible for changes in diacylated and triacylated ligand uptake, and SiO_2 may alter signaling molecules down stream of the receptor, resulting in the same net effect. Interestingly, we also observed attenuated levels of the inflammatory cytokines IL-1β and TNFα, but not IL-6, in the culture supernatants. These results are intriguing and suggest that SiO_2 disrupts more downstream signal transduction events pertinent to the maturation and secretion of cytokines.

In summary, uptake of SiO_2 downregulates the expression of select PRRs on AMs, as well as their ability to recognize, uptake, and respond to specific ligands. We hypothesize that these changes in AM phenotype may play a role their ability to appropriately respond to a secondary pathogen such as mycobacteria following SiO_2 exposure. Our data suggest that SiO_2, interacting with CD204, does not indiscriminately alter expression of all PRRs, but rather may amend signaling components involved in macrophage activation. Moreover, our data identify CD204 as an important partner for TLR2 on macrophages for the production of inflammatory mediators in response to bacterial stimuli. Future experiments may shed light on the relationship between reduced TLR2 expression and immunity to MTB infection later on.

AUTHOR CONTRIBUTIONS

CB designed the study, coordinated the experiments, prepared the figures, and composed the manuscript. BS performed the tissue culture, flow cytometry, and ELISA experiments under the direction of CB. FJ contributed data relative to **Figures 4** and **5**. GB and DS contributed to the manuscript preparation and critical revision. All authors have read and approved the final version of the manuscript.

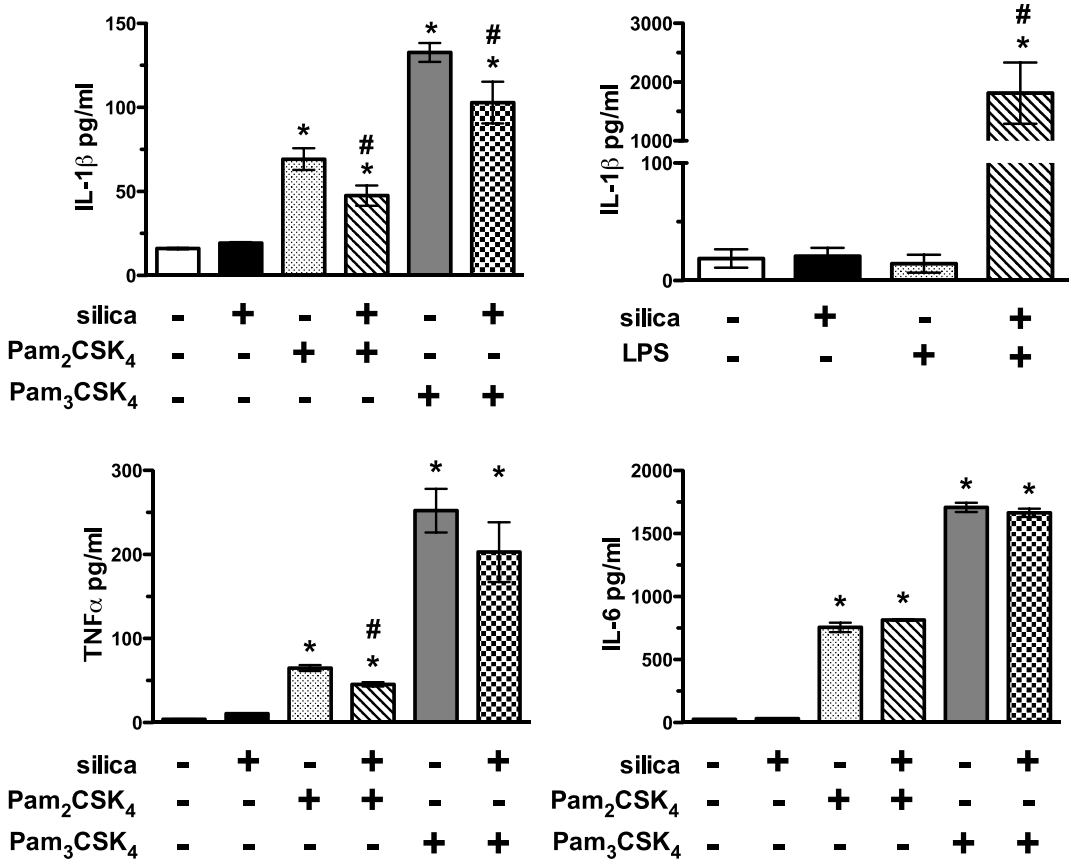

FIGURE 6 | Silica reduced IL-1β, but not IL-6, levels in response to synthetic diacylated and triacylated lipoproteins *in vitro*. Macrophages derived from the bone marrow of wild-type C57Bl/6 mice were exposed to media or silica (100 μg/ml) for 24 h and subsequently treated with fluorescently labeled Pam_2CSK_4 and Pam_3CSK4 for 2 h. Cell-free supernatants were analyzed for the presence of the inflammatory cytokines IL-1β and IL-6. As expected, activation of naïve macrophages with Pam_2CSK_4 and Pam_3CSK4 upregulated IL-1β and IL-6 levels. SiO_2 alone induced a slight, but not significant, increase in IL-1β. Although SiO_2 reduced the levels of IL-1β, it had no effect on the levels of IL-6 induced by TLR activation. Results are means ± SEM ($n = 4$). *$p < 0.05$ compared to media.

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REVIEW
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The Macrophage Switch in Obesity Development

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Immune cell infiltration in (white) adipose tissue (AT) during obesity is associated with the development of insulin resistance. In AT, the main population of leukocytes are macrophages. Macrophages can be classified into two major populations: M1, classically activated macrophages, and M2, alternatively activated macrophages, although recent studies have identified a broad range of macrophage subsets. During obesity, AT M1 macrophage numbers increase and correlate with AT inflammation and insulin resistance. Upon activation, pro-inflammatory M1 macrophages induce aerobic glycolysis. By contrast, in lean humans and mice, the number of M2 macrophages predominates. M2 macrophages secrete anti-inflammatory cytokines and utilize oxidative metabolism to maintain AT homeostasis. Here, we review the immunologic and metabolic functions of AT macrophages and their different facets in obesity and the metabolic syndrome.

Keywords: obesity, adipose tissue, insulin resistance, macrophage, adipokines, macrophage polarization, adipose tissue inflammation

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INTRODUCTION

Obesity is a prevalent metabolic disease characterized by excess accumulation of white adipose tissue (AT) due to increased food intake and changes in lifestyle (1, 2). Obesity leads to the development of a low-grade systemic chronic inflammatory state (3–6). According to the World Health Organization (WHO), 39% of adults over 18 years of age are overweight and 13% are clinically obese, translating to approximately 2 billion overweight adults where more than half a billion are obese (7).

A major player in systemic low-grade chronic inflammation in obesity is the increased numbers of AT pro-inflammatory macrophages and deregulated production and function of AT hormones and cytokines (2, 4). Besides its role in storing energy, AT is an important endocrine organ (8, 9), such that its dysfunction strongly contributes to the initiation and exacerbation of type 2 diabetes (T2D) (8, 10).

Insulin resistance is defined as a reduced response to insulin in liver, muscle, and AT. This impairment is due to the inhibition of the insulin-signaling pathway, leading to hyperglycemia. Insulin resistance is commonly associated with obesity and may precede the onset of T2D (11–13). One hypothesized reason for impaired insulin signaling has been thought to be due to the chronic systemic low-grade inflammation in obesity (14).

The finding that infiltration of monocytes, which differentiate into macrophages, is augmented in obesity is fundamental (15, 16). This results in pro-inflammatory macrophage and polarization leading to AT inflammation and insulin resistance (15, 17). Importantly, macrophages are crucial

for regulating the immune system, specifically by restoring and maintaining AT homeostasis (18, 19).

In this review, we highlight the different functions of AT macrophages (ATMs) in the maintenance AT tissue homeostasis during lean, obese, and insulin resistant states.

ADIPOSE TISSUE MACROPHAGES

The mechanisms by which inflammation increases during obesity are not fully understood. Increased pro-inflammatory cytokine secretion contributes to insulin resistance in obesity. Among these cytokines, tumor necrosis factor-α (TNF-α) was the first cytokine identified to be capable of inducing insulin resistance in adipocytes *in vitro*. In AT, the secretion of TNF-α is primarily derived from macrophages (20–22), and the accumulation of these immune cells in obesity contributes to the development of insulin resistance (23). This supports a key role for inflammation in the regulation of systemic metabolic homeostasis.

Macrophages make up to 40% of all AT cells in obese mice compared to 10% in lean mice (23). These cells are increased in AT during obesity due to increased amounts of several factors, including free fatty acids (FFAs), cholesterol, and lipopolysaccharide (LPS). Serum levels of LPS are elevated in obesity and, this cell wall component from Gram-negative bacteria, is linked to changes in the gut microbiota (metabolic endotoxemia) (24). LPS binds to and activates toll-like receptor 4 (TLR4) and its downstream signaling pathways in AT resident cells. These activated macrophages secrete cytokines and chemokines, such as monocyte chemoattractant protein-1 (MCP-1), and express C–C motif chemokine receptor-2 (CCR2) and CCR5, which in turn augment the recruitment of more monocytes and other leukocytes into AT (25–27). Macrophages share the same differentiation and recruitment molecules with other myeloid cells in many inflammatory conditions (28). As observed during bacterial inflammation (29), in obesity, macrophage activation is dependent on I kappa B kinase-β (IKK-β) (30). Arkan et al. showed that IKK-β activation in macrophages is sufficient for the development of insulin resistance, and mice with loss of IKK-β function only in myeloid cells are protected from obesity development and insulin resistance (30). These findings demonstrate the importance of macrophages in the context of insulin resistance development.

In addition to the activation and inflammatory profile of macrophages in the obese state, ATMs are highly adaptive to its lipid-rich environment. To maintain AT homeostasis in this lipid-rich microenvironment, macrophages increase their adiposity by activating lysosomal lipid metabolism (31). This may be a physiological response to buffer the increase in lipid concentrations released by adipocytes during obesity. This process does not classically activate ATMs, but it activates an immune cell differentiation program where high concentrations of lipids and FFAs induce a macrophage phenotype distinct from differentiated bone marrow macrophages (BMDM) (31). This phenotype is characterized by lipid accumulation in ATMs and increased expression of fatty acids transporters, such as CD36 and the lipid scavenger receptor Msr1 (31).

Several immune cells regulate AT inflammation, insulin resistance (32), and macrophage recruitment and differentiation (19, 33–35). There are two distinct macrophage populations found in AT. In healthy/lean AT, alternatively activated macrophages (M2) that express CD206 and CD301 on their surface and secrete anti-inflammatory cytokines predominates. On the other hand, obesity triggers the accumulation of classically activated macrophages (M1) characterized by CD11c surface expression, and expression of pro-inflammatory cytokines (17, 36), although this pan-classification spans a broad range of macrophage subtypes.

However, Kratz et al. recently described a different subtype of macrophage (37). They observed that treating macrophages with a mix of glucose, palmitate, and insulin ("metabolic activation") generates a unique macrophage pro-inflammatory phenotype that is different from M1. This type of macrophage secretes pro-inflammatory cytokines, such as interleukin-1β (IL-1β) and TNF-α, whereby the secretion is dependent on peroxisome proliferator-activated receptor gamma (PPAR-γ) and p62 expression. *In vivo*, this phenotype is due to continuous and excessive exposure of ATMs to FFAs, such as palmitate, in a microenvironment that is saturated with glucose and insulin. In obesity, this differentiated macrophage subtype indicates the importance and the necessity to identify differentiated profiles of immune cells. Since there is a large spectrum of ATMs that have different immune profiles, we choose to focus on M1 and M2 subtypes of ATMs to better understand how metabolic alterations in ATMs impact obesity and insulin resistance.

M1 MACROPHAGES: AN OVERVIEW

M1 macrophages are associated with a pro-inflammatory profile. These macrophages are generally stimulated by T-helper 1 (Th1) type of cytokines, such as interferon-γ (IFN-γ), or by pathogen-associated molecular patterns (PAMPs), such as LPS (38). In turn, M1 macrophages secrete cytokines, including IL-6, TNF-α, IL-1β (39), IL-12, and IL-23 (40). M1 macrophages can also induce Th1 responses (41, 42). In general, these cells express high levels of major histocompatibility complex class II (MHC-II), CD80 and CD86 costimulatory molecules and CD68 (43). Moreover, M1 macrophages express Th1 cell-attracting chemokines, including CXCL9 and CXCL10 (44).

In addition to IFN-γ and LPS, there are several other molecules involved in M1 macrophage polarization, such as interferon regulatory factor (IRF), signal transducers and activators of transcription (STAT), and suppressor of cytokine signaling (SOCS). IRF5 is involved in M1 polarization by inducing the transcription of interleukin-12 subunit p40 (IL-12p40), IL-12p35, and IL-23p19, and by repressing the transcription of IL-10 (45). M1 macrophages express SOCS3, which promotes nitric oxide (NO) production (46). SOCS3 controls nuclear factor-κB (NF-κB) and phosphatidylinositol 3-kinase (PI3K) activity, favoring NO production in macrophages (46). The induction of inducible nitric oxide synthase (iNOS), another important molecule induced in M1 macrophages is dependent on TLR ligands, such as LPS, and activation of NF-κB, PI3K, and IFN-γ secretion (47, 48) (**Table 1**). Furthermore, myeloid differentiation primary response gene 88 (MyD88)-dependent pathway is also important for M1

TABLE 1 | Differential requirement for stimuli and differential expression of transcription factors, cytokines, chemokines, and other molecules by M1 and M2 macrophages.

	M1	M2
Classical stimuli	LPS/GM-CSF/IFN-γ/TNF-α	PPAR-γ agonists/IL-4/IL-10/IL-13
Membrane markers	MHCII/CD80/CD86/CD11c/CCR7/Ly6C^{high}/CD11b/CD62L/CCR2^{high}/CX$_3$CR1^{low}/CCR5	Dectin-1/CD206/Scavenger receptor/CD163/CCR2^{low}/CXCR1/CXCR2/Ly6C^{low}/CD11b/CX$_3$CR1^{high}
Classical transcription factors	STAT1/IRF5	STAT6/FIZZ1/Ym1/PPARα/β/γ
Cytokines and chemokines	IL-6/TNF-α/IL-1β/IL-12/Il-23/IFN-γ/CXCL9,10,11,13/CCL8, 15, 19, 20	TGF-β/IL-10/CCL17, 18, 22, 24
Other classical molecules	SOCS3/iNOS	*Arg1*

polarization (49). The expression of TLR4/TLR2 is significantly higher in M1 when compared to M2 macrophages (50). The absence of TLR4 drives macrophages toward an M2 phenotype (51), indicating that activation and polarization of macrophages is, at least, in part dependent on TLRs.

In contrast to M1 macrophages generated *in vitro*, which do not express CD11c, M1 ATMs express CD11c concomitant with F4/80 and CD11b (17, 52–54). Interestingly, the expression of CD11c *in vitro* by BMDM can be induced if BMDMs are differentiated in the presence of adipocytes (31, 37). This indicates the importance and requirement of adipocytes in orchestrating the functional phenotype of ATMs.

The recruitment of monocytes, which in AT gives rise to CD11c$^+$ ATMs, is dependent on CCR2, CCR5, and MCP-1 (55, 56). Nagareddy et al. demonstrated that ATM-derived IL-1β promotes monocyte release from the bone marrow (57) and MCP-1 induces M1 ATM proliferation in AT (58). These processes are important to promote macrophage accumulation in the AT during obesity and sustain AT inflammation and insulin resistance (58).

POLARIZING M1 ATMs: HOW THEY INDUCE INSULIN RESISTANCE

Obesity-associated insulin resistance correlates with elevated levels of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6 (42, 59–62). These cytokines are secreted by both adipocytes and ATMs due to increased levels of pro-inflammatory factors released during obesity development. These factors include FFA, triglycerides, resistin, leptin, retinol-binding protein 4 (RBP4), IL-6, TNF-α, and IL-1β, among others (31, 63, 64).

Secretion of these factors activates several inflammatory signal transduction pathways in macrophages and adipocytes, which are required for obesity-induced insulin resistance. The stress-responsive c-Jun NH_2-terminal kinase (JNK 1 and 2) (65), inhibitor of κB kinase (IKK) (66), extracellular signal-regulated kinase 1 and 2 (ERK 1 and 2) (67), and mitogen-activated protein kinase p38 (p38 MAPK) are responsible for alterations in the insulin receptor signaling pathway (68). These alterations lead to decreased tyrosine phosphorylation of insulin receptor substrate (IRS-1 and -2), PI3K activation followed by a decreased serine phosphorylation of Akt and consequently insulin resistance (66, 68–72). There is a crosstalk between the two isoforms of JNK (JNK1 and JNK2) that contributes to obesity-induced insulin resistance development. The balance between these two molecules determines the total activity of JNK in fat tissues (73). Hematopoietic activation of JNK1 is a major player in obesity-induced inflammation and insulin resistance (74). Corroborating this, Han et al. verified that knockdown of both JNK 1 and 2 in macrophages protect mice from HFD-induced insulin resistance and AT inflammation (65). Similarly, Vallerie et al. showed that myeloid JNK1 is a regulator of cytokine expression in AT during the late, but not early states of obesity development (75).

Toll-like receptors and inflammasomes are activated in obesity by damage-associated molecular pattern molecules (DAMPs), such as high-mobility group box 1 (HMGB1) and oxidized low-density lipoprotein (Ox-LDL), RBP4 or PAMPs, such as LPS (24, 76–80). TLRs and inflammasomes modulate macrophage polarization due to activation of NF-κB, STAT1, and caspase-1 to induce IL-1β production (81, 82). Upon activation, these receptors contribute to low-grade chronic inflammation in obesity, leading to M1 polarization of ATMs. Importantly, TLR4 expression is increased in ATMs during obesity (83). Thus, many studies have investigated the role of TLR4 and nod-like receptor protein 3 (NLRP3) in knockout mouse models in HFD-induced obesity (17, 23, 51, 84).

Toll-like receptor 4 deficiency in HFD-fed mice ameliorates AT inflammation, insulin resistance, and adiposity (83, 85, 86). The reduction in inflammation is due to decreased macrophage infiltration and a switch from M1 to M2 macrophage profile (51, 83, 85, 87).

Nod-like receptor protein 3 inflammasome also plays a key role in the development of AT inflammation and insulin resistance (88, 89). Expression of NLRP3, apoptosis-associated speck-like protein containing CARD (ASC), caspase-1, and IL-1β are all upregulated in AT of obese mice, as well as the mature form of IL-1β (82, 90). The secreted IL-1β binds to IL-1R and activates NF-κB and MAPK pathways, thereby impairing insulin signaling through the activation of IRS-1 in adipocytes leading to insulin resistance (82, 91).

Functional deletion of NLRP3 and caspase-1 ameliorate HFD-induced insulin resistance and AT inflammation (82, 90, 92). Moreover, weight loss and insulin sensitivity in patients with T2D is associated with decreased AT expression of NLRP3 and IL-1β (82). Protection from insulin resistance and inflammation following loss of functional NLRP3 may be due to a shift in macrophage

polarization, since NLRP3-knockout mice have decreased M1 and increased M2 gene expression profiles in AT (84).

In addition to these important signaling pathways, the mammalian target of rapamycin (TOR) has an important function in insulin resistance. It is able to sense nutrients and respond by altering the cellular metabolism in different kind of cells, including ATMs (93). Insulin, glucose, leptin, and other growth factors and cytokines activate mTOR pathway via PI3K–Akt signaling pathway (94). The protein kinase Akt phosphorylates and inhibits TSC2 and, consequently, activates mTORC1 (95, 96). Activation of these metabolic sensors, mainly PI3Kγ, is important for immune cell functions. PI3Kγ activation in hematopoietic cells contributes to the development of obesity and insulin resistance. PI3Kγ activity in the non-hematopoietic compartment is critical during obesity (97). Moreover, the catalytic subunit of PI3Kγ, p110γ, was shown to be activated during obesity. Absence of functional p110 improved insulin sensitivity with reduced infiltration of pro-inflammatory macrophages and inflammatory marker expression in AT. In addition, specific depletion of PI3Kγ in bone marrow cells as well as pharmacological blockade also inhibited macrophage infiltration during obesity and insulin resistance (98). Together, these data indicate that activation of metabolic sensors in immune cells during obesity is essential for inflammation and insulin resistance development.

Defects in mTORC1 regulation can lead to metabolic dysfunction, including T2D (93). Deletion of mTORC1 in macrophages diminishes AT inflammation and protects mice against HFD-induced insulin resistance (99, 100). mTORC1 disruption suppresses HK1-dependent glycolysis, caspase-1 activation, IL-1β, and IL-18 secretion *in vitro* and *in vivo* and induces M2 polarization (100). In accordance, Jiang et al. showed that mTORC1 depletion in macrophages protects mice against HFD-induced AT inflammation and insulin resistance through the inhibition of IRE1α/JNK/NF-κB pathways (99).

In 2013, Horng et al. demonstrated *in vitro* and *in vivo* that TSC1 deletion (Tsc1 deficiency, thereby mTORC1 is constitutively active) in macrophages leads to a marked defect in M2 polarization in response to IL-4, although LPS stimulation induced inflammatory responses in an mTOR-dependent manner (101). Moreover, in obesity, nutrient sensing by mTORC1 regulates the switch of ATMs from M2 to M1 (12).

More recently, Zhu et al. proposed that TSC1 deletion in macrophages intensifies the M1 polarization (102). TSC1 inhibits M1 polarization by suppressing the Ras GTPase/Raf1/MEK/ERK signaling pathway in an mTOR-independent manner, whereas TSC1 promotes M2 properties by mTOR-dependent CCAAT/enhancer-binding protein-β pathway (102). These findings indicate a critical role for TSC1 in orchestrating macrophage polarization via mTOR-dependent and -independent pathways (102) (**Figure 1**).

Increased M1 activation in AT is involved in activation of the adaptive immune response through the recruitment and activation of T cells. Increased recruitment of $CD4^+$ T cells correlates with increased M1 polarization. Also, M1 polarization appears to be dependent on AT Th1 polarization (42, 103–106). In addition, during obesity, the activation of Th1 responses in AT are mediated by mTORC1, since this molecule is necessary for polarization of T lymphocytes toward a Th1 phenotype (107). Moreover, circulating leptin, which is elevated during obesity, activates mTOR pathway, and also induces Th1 responses (108, 109). Thus, Th1 polarization is dependent on M1 polarization, and it is critical for the development of insulin resistance (104).

Together, several pathways mediate the induction/activation of ATMs to maintain AT homeostasis, which can also be affected by changes in systemic and cellular metabolism.

M2 MACROPHAGE: AN OVERVIEW

M2 macrophages are associated with tissue remodeling and inflammation resolution (110). M2 macrophages have immunosuppressive properties, have high phagocytic capacity, and secrete extracellular matrix components, angiogenic and chemotactic factors, anti-inflammatory cytokines, and growth factors, such as IL-10 and transforming growth factor β (TGF-β) (111, 112). M2 macrophages are characterized by upregulated expression of Dectin-1, CD206, scavenger receptor A, scavenger receptor B-1, CD163, CCR2, CXCR1, CXCR2, and MgL 1/2 (36). Moreover, the expression of arginase-1 (*Arg1*), PPAR-γ, and transcription factor found in inflammatory zone 1 (FIZZ1), which is specific of murine M2 macrophages, are necessary for collagen synthesis, further supporting the role of these cells in tissue remodeling (44) (**Table 1**).

In vitro, M2 macrophages appear to be a heterogeneous population induced by a variety of stimuli. M2a is induced by IL-4 or IL-13 express high levels of CD206 and has immunoregulatory functions (38, 113–115). M2b is induced by immune complexes and TLRs or IL-1R agonists. Both M2a and M2b have an immunoregulatory role through down-regulation of IL-12, IL-6, and TNF (116). M2c is induced by IL-10 and glucocorticoids. It has an immunosuppressive phenotype and participates in tissue remodeling. M2c secretes pro-fibrotic factors, such as TGF-β, CCL17, and CCL22 (38, 116). In addition, expansion of M2c macrophages is negatively regulated by PPAR-γ, which is expressed in M2 ATM (117). Although significant progress has been made in characterizing M2 subpopulations, it still not completely understood how these cells behave *in vivo*.

M2 ATMs AND INSULIN SENSITIVITY

The microenvironment in a lean AT is composed of a 4:1 M2:M1 ratio (118). The presence of eosinophils and regulatory T cells (Tregs), which secrete the cytokines IL-4/IL-13 and IL-10, respectively, polarizes ATMs toward an anti-inflammatory phenotype (119–121). In lean AT, adipocytes secrete higher levels adiponectin compared with obese AT. Adiponectin enhances insulin sensitivity and increases M2 macrophage polarization (121). These cells and their secretome maintain the positive balance of M2 macrophages in lean AT.

Obesity inversely correlates with AT Tregs (122, 123). Moreover, Tregs can induce M2 macrophage differentiation in mice through IL-10 and TGF-β (124). In lean AT, these cells

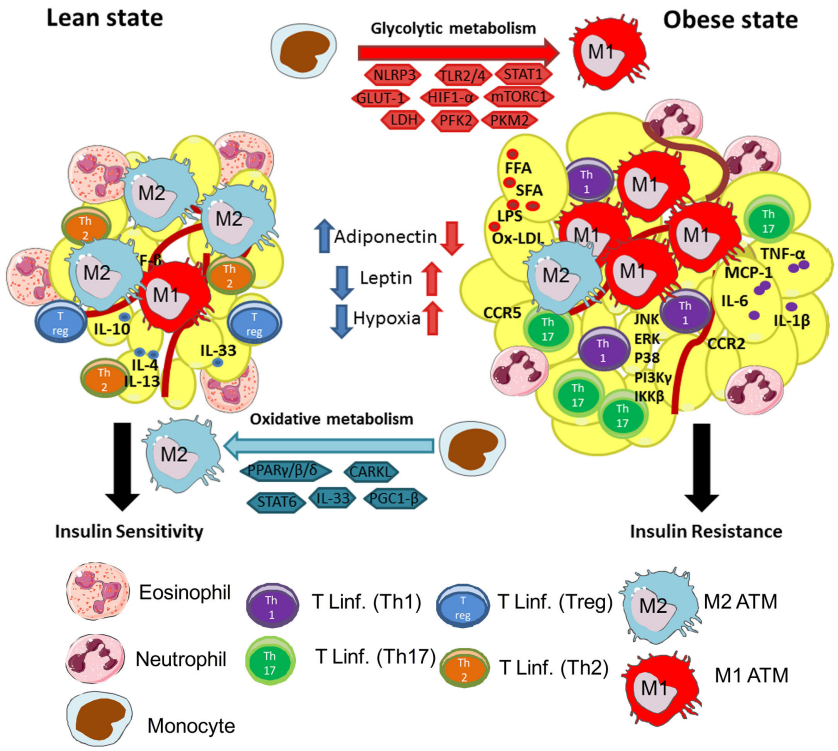

FIGURE 1 | Macrophages are central players in lean and obese states. Lean adipose tissue is abundant in immune cells, such as eosinophils, Th2 T cells, ILC2, regulatory T cells (Treg), and M2 (anti-inflammatory) macrophages. These cells are known to secrete anti-inflammatory cytokines, such as IL-10, IL-4, IL-13, and IL-33, to maintain AT homeostasis and controlling insulin sensitivity. M2 macrophages use oxidative metabolism through PPARγ/β/δ, CARKL, STAT6, and PGC-1β. These events are central to maintain a healthy environment in adipose tissue. In the other hand, during obesity, AT is characterized by infiltration of several immune cells, such as monocytes, neutrophils, Th1 and Th17 lymphocytes, and M1 (pro-inflammatory) macrophages. The increased secretion of FFA, SFA, Ox-LDL, and LPS in obesity activates resident macrophages and adipocytes leading to secretion of pro-inflammatory cytokines, such as TNF-α, IL-6, IL-1β, and chemokines MCP-1, CCR2, and CCR5. This process will instigate the recruitment of monocytes and differentiation of M1 macrophages in AT. Besides, activation of pro-inflammatory signaling pathways downstream to TLRs, such as JNK, ERK, p38, IκB, IKKβ, and Pi3Kγ, inhibit insulin receptor signaling, leading to insulin resistance. Moreover, in obese AT, M1 macrophages use glycolytic metabolism and require activation of intracellular molecules, such as NLRP3, TLR2/4, STAT1, GLUT-1, HIF-1α, mTORC1, PFK2, and PKM2, and conversion of pyruvate to lactate by LDH. Activation of glycolysis in macrophages is central to maintain their pro-inflammatory profile.

are involved in the regulation of tissue homeostasis and help to maintain the M2 macrophage population (122).

Recently, new regulatory players in AT homeostasis have been identified: innate lymphoid type 2 cells (ILC2s) and IL-33. ILC2s are a regulatory subtype of ILCs. These cells were divided into three distinct populations, ILCs 1, 2, and 3 (125–127). These subpopulations of ILCs are analogous to the largely known $CD4^+$ T helper subsets: Th1, Th2, and Th17, respectively, with respect to cytokine profile expression (128). However, ILCs do not have T-cell receptors and respond to antigenic signals in the absence of antigen specificity (128). ILCs are activated by the cytokine IL-33 and produce large amounts of the type 2 cytokines: IL-5 and IL-13 (129).

Interleukin-33 is constitutively present in humans and mice, mainly in specialized populations of epithelial and endothelial cells (130, 131). Its receptor (ST2) is highly expressed in ILC2s and Th2 lymphocytes, and it is also found in eosinophils, mast cells, dendritic cells, basophils, myeloid-derived suppressor cells, and Tregs (132).

Interleukin-33, as well as ILC2s, has been in the spotlight due to their putative contributions in the improvement of obesity-induced insulin resistance. Upon binding to its receptor, IL-33 induces the production of large amounts of anti-inflammatory cytokines by AT ILC2s and also the polarization of ATMs toward an M2 phenotype (133). This results in AT mass reduction and improves insulin resistance (133, 134). Han and colleagues investigated ST2 expression in murine Tregs in lean and obese visceral AT. AT Tregs from lean mice express higher levels of ST2 compared to AT Tregs from obese mice. Moreover, treatment with IL-33 restored the ST2-positive Treg population, reduced AT inflammation, and improved insulin resistance (133).

In this context, Brestoff et al. demonstrated that IL-33 plays an important role in the maintenance of ILC2s in AT, promoting energy expenditure, and reducing adiposity in mice (135). This decrease in adiposity was due to caloric expenditure upon the induction of uncoupling protein 1 (UCP1) expression in AT, a process called "beiging" or "browning" (136, 137). UCP1 protein

is limited to beige and brown adipocytes and regulates caloric expenditure (135). In agreement with Artis et al., Chalwa's et al. found that IL-33 promoted the accumulation and activation of ILC2s in mouse AT, leading to the biogenesis of beige fat, which is crucial for AT metabolic homeostasis (138) (**Figure 1**).

Taken together, these studies demonstrate the importance of alternatively activated macrophages to maintain the tissue homeostasis, especially in AT. Moreover, the discovery of new alternative pathways for the polarization of ATMs toward an M2 phenotype is necessary to better understand the mechanisms by which insulin sensitivity in obesity.

MACROPHAGE METABOLISM AND ITS ROLE IN INSULIN SENSITIVITY

In addition to cytokines, the availability of substrates in tissues orchestrates macrophage function. Cellular metabolism is not static but is rather a dynamic process that allows cells to adapt to the microenvironment (139). The type of nutrient substrate is critical for ATM function. Saturated fatty acids (SFAs) are pro-inflammatory and induce M1-like phenotype, while certain types of unsaturated fatty acids (UFAs), such as omega-3 and branched fatty acid esters of hydroxy fatty acids (FAHFA) (140), are anti-inflammatory and induce an M2-like phenotype (141).

M1 macrophages preferentially metabolize glucose as an energy substrate (142). During activation, macrophages alter its metabolism to support survival and cellular functions. The metabolism of M1 macrophages upon activation is characterized by induced aerobic glycolysis with increased glucose uptake and the conversion of pyruvate to lactate by lactate dehydrogenase (LDH) (143). This activation in aerobic glycolysis decreases respiratory chain activity due to increased ROS levels (144). This metabolic switch is necessary for NO production, an important effector of immune microbicidal activity and pro-inflammatory M1 macrophage responses (144).

In addition, the expression of glucose transporter-1 (GLUT-1) drives the pro-inflammatory phenotype of M1 macrophages, increases glucose uptake, and, subsequently, augments glucose metabolism (145).

One important molecule regulating glycolysis and macrophage activation is hypoxia inducible factor-1α (Hif-1α). Hif-1α induces a pro-inflammatory phenotype in macrophages (146) via TLR4 activation, which involves the PI3K/Akt signaling pathway (147). Low oxygen (O_2) tension and inflammatory responses increase TLR4 expression in macrophages (148). Moreover, M1 macrophages co-localize with AT hypoxic areas in obese mice and are associated with increased inflammatory responses (147–149). Because these macrophages need to adapt to the obesity-induced hypoxic tissue environment, activating anaerobic glycolysis under these circumstances best serves these immune cells to support their rapid and demanding energy requirements (143).

Activation of macrophages with LPS also results in increased levels of succinate and malate (150). Succinate, in particular, drives IL-1β production, which is dependent on Hif-1α activation (150). In addition, pyruvate kinase M2 (PKM2), a critical determinant of macrophage activation by LPS, promotes inflammatory responses (151). Activation of PKM2 plays a key role in stabilizing Hif-1α and Hif-1α-dependent genes, such as IL-1β expression. LPS induces dimerization of PKM2 that in turn complexes with Hif-1α. This complex directly binds to the IL-1β promoter, an event that is inhibited by the activation of tetrameric PKM2, which induces M2 macrophage differentiation and attenuates LPS-induced M1 macrophages (151). Thus, PKM2 in its dimeric form is required for glycolytic reprograming in response to LPS. The dimeric form of PKM2 plays role in Hif-1α function, whereas the tetrameric form of PKM2 impairs the ability of PKM2 to promote transcriptional activity of Hif-1α and LPS-induced IL-1β expression (151).

Nonetheless, the microenvironment rich in LPS and IFN-γ also enhances M1 macrophage polarization and glycolysis activation independently of Hif-1α. This occurs upon 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK2) induction (152).

In contrast to M1 glycolytic metabolism, M2 macrophages utilize oxidative metabolism (142). The induction of oxidative metabolism in M1 macrophages shifts their phenotype toward an M2 profile (152). Moreover, the overexpression of carbohydrate kinase-like protein (CARKL), which regulates the production of sedoheptulose-7-phosphate (S7P), an intermediate of the pentose phosphate pathway (PPP) (153) results in decreased production of pro-inflammatory cytokines, which suggests a shift toward an M2 macrophages phenotype (154).

Besides CARKL, the coactivator protein PPAR-γ-coactivator-1β (PGC1-β) induces mitochondrial respiration as well as mitochondrial biogenesis. This is a key player in the metabolic switch of macrophages from M1 to M2 phenotype (142, 144). Blocking PGC1-β results in impaired M2 macrophage metabolism and function (142). Thus, identifying mechanisms that modulate the metabolism of macrophages may dampen the onset and exacerbation of inflammatory processes.

Adipose tissue-derived IL-4 and IL-13 signals through IRF/STAT to activate STAT6 in M2 macrophages (44, 113). STAT6 induces the expression of transcriptional regulators, such as PPAR-γ (44). PPAR-γ maintains the metabolic switch toward oxidative metabolism and promotes M2 gene expression (*Arg1*) to amplify the effector phenotype of M2 macrophages (collagen synthesis) (31, 155, 156). Other members of the PPAR family, PPARβ/δ, appears to differentially influence macrophage activation, along with IL-4 and IL-13, and promotes an alternative M2 macrophage phenotype (156). Myeloid deletion of PPARβ/δ leads to glucose intolerance and insulin resistance (27), indicating that expression of PPARs transcription factors is crucial to maintain the M2 phenotype through the secretion of Th2 cytokines (**Figure 1**).

Hypoxia inducible factor-2α has been shown to regulate the transcription of *Arg1*, which is expressed by M2 macrophages (157). However, Hif-2α also controls IL-1β production and NF-κB activity, which is associated with an M1 phenotype (150, 157). Thus, although Hif-2α appears to have a role in macrophage polarization, more studies are needed to better understand the

importance of this transcription factor for macrophage phenotype, metabolism, and function.

It is still unclear how M2 macrophages metabolism is regulated during obesity and the role of M2 macrophage metabolism for the development of insulin resistance. Nevertheless, in lean state, they have an oxidative metabolism, which may shift to glycolytic metabolism, during obesity, due to a pro-inflammatory environment and further studies are needed to better understand their role in obesity.

CONCLUSION

Macrophages are central mediators of obesity-induced AT inflammation and insulin resistance. They also are key cells for maintenance of AT homeostasis. Recently, several reports described the importance of these cells as regulators of insulin sensitivity, which involves the activation of innate immune receptors, transcription factors, and intracellular metabolism to support the either pro- or anti-inflammatory AT phenotype. Thus, macrophages have a dual role, changing their status to support immune responses, obesity development, and related diseases.

AUTHOR CONTRIBUTIONS

AC and CN have contributed equally in the writing of this manuscript. NC and PM-V contributed in the writing and corrections of this manuscript. AC and CN have contributed equally to this review.

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Systemic Inflammation in Cachexia – Is Tumor Cytokine Expression Profile the Culprit?

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Cachexia affects about 80% of gastrointestinal cancer patients. This multifactorial syndrome resulting in involuntary and continuous weight loss is accompanied by systemic inflammation and immune cell infiltration in various tissues. Understanding the interactions among tumor, immune cells, and peripheral tissues could help attenuating systemic inflammation. Therefore, we investigated inflammation in the subcutaneous adipose tissue and in the tumor, in weight stable and cachectic cancer patients with same diagnosis, in order to establish correlations between tumor microenvironment and secretory pattern with adipose tissue and systemic inflammation. Infiltrating monocyte phenotypes of subcutaneous and tumor vascular-stromal fraction were identified by flow cytometry. Gene and protein expression of inflammatory and chemotactic factors was measured with qRT-PCR and Multiplex Magpix® system, respectively. Subcutaneous vascular-stromal fraction exhibited no differences in regard to macrophage subtypes, while in the tumor, the percentage of M2 macrophages was decreased in the cachectic patients, in comparison to weight-stable counterparts. CCL3, CCL4, and IL-1β expression was higher in the adipose tissue and tumor tissue in the cachectic group. In both tissues, chemotactic factors were positively correlated with IL-1β. Furthermore, positive correlations were found for the content of chemoattractants and cytokines in the tumor and adipose tissue. The results strongly suggest that the crosstalk between the tumor and peripheral tissues is more pronounced in cachectic patients, compared to weight-stable patients with the same tumor diagnosis.

Keywords: cancer cachexia, inflammatory cells, tumor-adipose tissue crosstalk macrophages

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INTRODUCTION

Cachexia is a multifactorial and multi-organ syndrome characterized by continuous and involuntary weight loss and by systemic inflammation (1, 2). This syndrome was described about 2000 years ago by Hippocrates and is a common feature of several diseases, such as chronic obstructive pulmonary disease, chronic heart failure, chronic infection, and cancer (3).

In cancer, cachexia is present in approximately 50% of all patients and in up to 80% of patients with advanced disease, reducing tolerance to treatment, therapeutic response, and quality of life and survival (4). Among 22–40% of all cancer deaths are directly caused by cachexia (5), and its incidence varies among the different types of cancer, being of around 80% in pancreas and gastrointestinal cancer patients, and of 60% in lung cancer patients (6).

An important feature of cachexia is chronic systemic inflammation and, paradoxically, immunosuppression (7). Mediators produced by both the tumor and the host induce intracellular changes directly associated with persistent inflammation (8). The sources of the inflammatory factors in cachexia are plenty, including tumor cells, tumor infiltrating cells along with peripheral tissue parenchymal cells and associated infiltrating cells (9). Thus, an intricate tumor–host interaction is established, promoting an imbalance that favors the pro-inflammatory over the anti-inflammatory status (10, 11).

Solid tumors often present infiltrating immune cells and release cytokines into surrounding tissues and into the bloodstream (12). The immune cells within tumor microenvironment consist of various phenotypes, among which myeloid-derived suppressor cells, dendritic cells, natural killers, T cells, and macrophages (13). The infiltrate contributes to tumor growth and also to microenvironment remodeling; while the release of cytokines into the bloodstream promotes tissue and organ functional impairment as a result of systemic inflammation (12). Studies with models have shown that the host's tissues play a key role in sustaining systemic inflammation and inducting cachexia (14–17).

However, as far as we know, there are no reports in the literature comparing the cytokine secretory profile of tumors of cachectic and non-cachectic cancer patients matched for tumor type and stage. It is very possible that inflammatory factors secreted by the tumor are the culprit, eliciting secondary tissue inflammation, will as a consequence, fuel systemic inflammation. Argilés et al. review the large number of cytokines that might be responsible for the metabolic changes associated with cancer wasting (18). We have consistently found that WAT (white adipose tissue) is a contributor to systemic inflammation, as both adipocytes and infiltrating immune cells are capable of releasing cytokines in animal models of cachexia. Nevertheless, the mechanisms that trigger adipose inflammation in cancer cachexia are not fully elucidated. We hypothesize that differences in tumor microenvironment and secretion pattern in patients with the same diagnosis and tumor stage could be associated with the presence or absence of cachexia-related peripheral tissue inflammation.

The aim of the present study was therefore, to examine the secretory profile of tumors of cachectic and non-cachectic patients with matched tumor diagnosis and relate to the results with local white adipose tissue and systemic inflammation.

MATERIALS AND METHODS

Subjects

Twenty-three cancer patients (60.53 ± 13.08 years old) participated in the study. The study was approved by the University of São Paulo Biomedical Sciences Institute Ethics Committee (1004/CEP) and by the University Hospital Ethics Committee (CEP-HU/USP: 752/07) in accordance to the *Declaration of Helsinki* (2013). All participants signed an informed consent prior to engaging in the study. The inclusion criteria were: not having received anticancer or continuous anti-inflammatory treatment and willingness to participate. The exclusion criteria were: liver failure, renal failure, AIDS, inflammatory diseases of the bowel, and autoimmune disorders. Patient group division was based on the criteria proposed by Evans et al. (19). Characteristics of the subjects are summarized in **Table 4**.

Realtime PCR

Total RNA was isolated from samples, with Trizol® reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's recommendations, and then homogenized. RNA concentrations were determined by measuring the absorbance in 260/280 nm in Synergy H1 Multi-Mode Reader (Thermo Fisher Scientific Inc., Waltham, MA, USA). Complementary DNA synthesis was carried out using the high capacity cDNA reverse transcription kit (Life Technologies, Grand Island, NY, USA), which consisted of an assay mix containing 1 μg total RNA, 2 μL 10× RT Buffer, 0.8 μL 25× dNTP mix (100 mM), 2 μL 10× Random primers, 1 μL MultiScribe™ Reverse Transcriptase, and 4.2 μL of nuclease-free water in a final volume of 20 μL. The thermal cycler conditions were: 25°C for 10 min, then 37°C for 120 min followed by 85°C for 5 min. Then, 20 ng of cDNA was mixed with 2× SYBR Green fast PCR master mix – and primers (**Table 1**) (Life Technologies, Grand Island, NY, USA) – in a final volume of 10 μL for qPCR, performed in the Quantstudio 12K Real Time Systems (Life Technologies, Grand Island, NY, USA). The mRNA levels were determined by the comparative Ct method. For each sample, a ΔCt value was obtained by subtracting RPL-27 or HPRT1 gene values from those of the gene of interest. The average ΔCt value of the control group was then subtracted from the sample to derive a −ΔΔCt value. The expression of each gene was evaluated by 2−ΔΔCt, according to Livak and Schmittgen (20).

Multiplex Analysis of Sample Protein Content

Samples of the tumor and subcutaneous adipose tissue from the experimental groups were incubated with the mixture of Magplex microspheres and covered with the specific antibodies for 2 h. The detection of target antigens bound to the microspheres was performed with a mixture of biotinylated capture antibodies after incubation for 1 h followed by incubation with streptavidin labeled with phycoerithrin for 30 min. The microspheres were then analyzed with the phycoerithrin Magpix® instrument (Life Technologies, Grand Island, NY, USA). Each cytokine value

was corrected to total protein concentration. The table below describes all analyzed cytokines (**Table 2**).

Immunophenotyping by Flow Cytometry

Preparation of Adipose Tissue and Tumor Cells for Flow Cytometry

Fractions of subcutaneous adipose tissue and tumor were obtained, any lymph nodes were carefully removed, and the tissues were placed in either DMEM (Dulbecco's Modified Eagle Medium) or HBSS (Hank's Balanced Salt Solution). The tissue fragments were then digested for 40 min at 37°C in these culture media containing collagenase type I (280 U/ml) (Sigma Aldrich) under agitation. The samples were filtered through fine plastic mesh and washed with respective media.

TABLE 1 | List of primers.

Gene (species)	Sequence 5′→3′
CCL-2 (*Homo sapiens*) (NM 002982.3)	Fw: TCA GCC AGA TGC AAT CAA TG Rev: ACA CTT GCT GCT GGT GAT TCT
IL-1β (*Homo sapiens*) (NM 000576.2)	Fw: AGC CAA TCT TCA TTG CTC AAG T Rev: AGT CAT CCT CAT TGC CAC TGT
IL-6 (*Homo sapiens*) (*NM* 000600.3)	Fw: CAG CCC TGA GAA AGG AGA CAT Rev: AGC CAT CTT TGG AAG GTT CA
IFN-γ (*Homo sapiens*) (NM 000619.2)	Fw: TGG AAA GAG GAG AGT GAC AGA A Rev: TTG GAT GCT CTG GTC ATC TTT A
TNF-α (*Homo sapiens*) (NM 000594.3)	Fw: CTC TCT CCC CTG GAA AGG AC Rev: ATC ACT CCA AAG TGC AGC AG
IL- 10 (*Homo sapiens*) (NM 000572.2)	Fw: TGTCATCGATTTCTTCCCTGT Rev: TGC CTT TCT CTT GGA GCT TAT T
RPL-27(*Homo sapiens*) (NM 000988.3)	Fw: CCG AAA TGG GCA AGT TCA T Rev: CCA TCA TCA ATG TTC TTC ACG A
IL-8 (*Homo sapiens*) (NM 000584.3)	Fw: AGC TCT GTG TGA AGG TGA T Rev: TTT GGG GTG GAA AGG TTT G
ZAG (*Homo sapiens*) (NM 001185.3)	Fw: CCA GGA GAA CCA AGA TGG TC Rev: CTG CTT CCA ATC CTC CAT TC
PIF (*Homo sapiens*) (NM 005268627.1)	Fw: AGG AAG CAG AGA TCC AGC CT Rev: GGC TCC TTT ACC CAC GCT TT
HPRT1(*Homo sapiens*) (NM 000194.2)	Fw: TGG CGT CGT GAT TAG TGA TG Rev: CTT GAG CAC ACA GAG GGC TA

TABLE 2 | Cytokine analysis.

Cytokine	Abbreviation
Tumor necrosis factor alpha	TNF-α
Tumor necrosis factor beta	TNF-β
Interleukin 6	IL-6
Interleukin 7	IL-7
Interleukin 10	IL-10
Interleukin 13	IL-13
Interferon alpha	IFN-α
Interferon gamma	IFN-γ
Interferon gamma-induced protein 10	IP-10
Monocyte chemotactic protein1	MCP1/CCL2
Macrophage inflammatory protein-1α	MIP-1α/CCL3
Macrophage inflammatory protein-1β	MIP-1β/CCL4
Chemokine(C–C motif) ligand 5	RANTES/CCL5

Finally, cells of vascular-stromal fraction were separated by centrifugation at 500 *g* for 5 min. The cells of the stromal-vascular fraction of adipose tissue were resuspended and washed twice with culture medium and centrifuged again at 500 *g*, for 5 min. The cells were resuspended in 500 μL of FBS and dimethyl sulfoxide (DMSO) and stored in liquid nitrogen until processing for flow cytometry.

Cell Surface Antigens for Flow Cytometry

The samples were rapidly thawed in a water bath at 37°C, washed with culture medium, and pelleted at 600 g for 10 min at 4°C. Compensation of the flow cytometer (FACSCanto II – BD Biosciences) was performed with compensating beads and then the gates were determined for the analysis of cell populations of interest (Figure S1 in Supplementary Material).

The fluorochrome conjugated antibodies (listed in **Table 3**) of the macrophage panels were added to the samples, and these were incubated for 30 min at 4°C, in the dark. The labeled cells were washed, centrifuged 400 *g* for 5 min, resuspended in 500 μL of DMEM, and detected by BD FACSCanto™ II cytometer.

Statistical Methods

Data are expressed as mean ± SE or median [first quartile; third quartile]. First, a Gaussian distributions test was employed for all samples (D'Agostino-pearson omnibus test, Shapiro–Wilk test, Kolmogorov–Smirnov Test). Student's *t*-test or Mann–Whitney test with multiple comparisons was employed for parametric and non-parametric data, respectively. The significance level was set at $p < 0.05$. Graphpad Prism 5.0 was adopted for the analysis. All statistical procedures were performed with the assistance of the Institute of Biomedical Sciences/University of Sao Paulo, under the supervision of Ms. Rosana Duarte Prisco.

RESULTS

General Characteristics of Patients

The general characteristics of patients are illustrated in **Table 4**. No statistical differences were found in regard to age and height between the groups. Body mass in the 12 months before engagement in the study, as informed by the patients at moment of the recruitment interview, showed no statistical differences between groups, while baseline body mass of the cachectic cancer group was lower (in average 11%), when compared with the weight-stable cancer group, although not statistically significant ($p = 0.07$). When comparing the difference between previously

TABLE 3 | Panels of fluorochrome-conjugated antibodies for flow cytometry.

Panel	Antibody	Fluorochrome	Catalog no.
Macrophages (M1 and M2)	CD45	FITC	555482
	CD206	PE	555954
	CD14	PERCP-Cy5.5	562692
	CXCR4	PE-Cy7	560669
	CD86	APC	555660
	CD11b	APC-Cy7	557657
	CCR7	BV421	562555

informed body mass and current body mass, marked weight loss (both in terms of absolute and relative weight) was found for CC, in relation to the weight-stable cancer (WSC) group, in accordance with the proposed by Evans et al. (19) (weight loss >5% over past 6 months – in absence of simple starvation). The body mass index (kg/m^2) of CC, although greater than 20 kg/m^2 (considered the cutoff point for cachexia), was significantly lower than that of WSC. C-reactive protein, albumin, hemoglobin, and IL-6, biochemical markers of cachexia, were also evaluated. CRP plasma content – the most widely accepted index of systemic inflammation – was higher in CC than in WSC ($p = 0.0026$). Similarly, plasma IL-6 levels were significantly higher in cachectic cancer patients (CC) ($p = 0.0119$). Additionally, serum hemoglobin levels of CC were consistently lower when compared with WSC ($p = 0.0064$). Serum albumin levels were not significantly different between groups ($p = 0.316$).

TABLE 4 | General characteristic of patients.

	WSC (weight-stable cancer)	CC (cachectic cancer)	*p*
N	17	19	
Male/female (*n*)	10/7	12/7	
Age (years)	59.2 ± 3.69	61.7 ± 2.55	0.582
Height (m)	1.65 ± 0.024	1.65 ± 0.018	0.936
Previous body mass as informed (kg)	74.1 ± 3.13	72.3 ± 3.21	0.695
Current body mass (kg)	70.5 ± 3.17	62.5 ± 2.86	0.07
Weight loss (kg)	0.00 [0.00; 6.50]	10.00 [5.00; 13.00][a]	0.0009
Weight loss (%)	0.00 [0.00; 9.00]	12.0 [8.00; 16.0][a]	0.0006
BMI (kg/m^2)	25.9 ± 1.04	22.8 ± 0.76[a]	0.0195
Tumor stage (*n*)			
I-II	4	7	
III-IV	13	12	
CRP (mg/L)	3.95 [0.90; 8.03]	11.7 [7.15; 13.5][a]	0.0026
Albumin (g/dL)	4.32 ± 0.18	4.04 ± 0.21	0.316
Hemoglobin (g/dL)	13.4 ± 0.50	11.2 ± 0.57[a]	0.0064
IL-6 (pg/mL)	2.67 ± 0.65	9.84 ± 2.02[a]	0.0119

Data expressed as mean ± SE or as median [first quartile; third quartile].
[a]Significant difference CC vs. WSC group.
BMI, body mass index; CRP, C-reactive protein; IL-6, interleukin 6.

Tumor Gene Expression Analysis

Gene expression of the pro-inflammatory cytokines TNF-α and CCL2 in the tumor were increased in CC compared to WSC, $p = 0.020$ and $p = 0.0354$, respectively (**Figures 1A–B**). No statistically significant difference in mRNA concentration of VEGF (angiogenesis factor), IL-6, IL-1β, IFN-γ, PIF, ZAG, IL-10, between WSC and CC could be detected, as shown in **Table 5**.

Subcutaneous Adipose Tissue Gene Expression Analysis

As previously described, we found that gene expression of TNF-α, IL-1β, and MCP-1/CCL2 were significantly higher in cachectic cancer patients when compared with WSC. IL-6 and IFN-γ gene expression showed no differences among the groups.

Tumor Protein Expression Analysis

Protein expression of chemoattractant factors in tumor tissue CCL [(chemokine (C–C motif) ligand)]-2, CCL4, CCL5 was not significantly different between the groups as shown in **Table 6**. However, CCL3, also known as macrophage inflammatory protein 1 alpha, was higher in CC in relation to WSC ($p = 0.043$) (**Figure 2A**).

The protein concentrations of different pro- and anti-inflammatory cytokines and cachexia-related factors in cachectic and non-cachectic cancer are shown in **Table 6**. Among the pro-inflammatory cytokines, IL-1β was increased in CC compared to WSC ($p = 0.041$) (**Figure 2B**). Protein concentration of IP-10, a chemokine secreted by interferon stimulated cells was not significantly different but showed a tendency to be significantly higher in CC ($p = 0.092$). Other inflammatory cytokines such as IFN-γ and IL-6 were not significantly different between the groups. Members of the tumor necrosis factor family TNF-α and TNF-β were also not statistically different in CC compared to WSC. The protein concentration of anti-inflammatory interleukins IL-10 was not different ($p = 0.9652$) between groups, yet that IL-13 ($p = 0.007$) was lower in CC in compared WSC (**Figure 2C**).

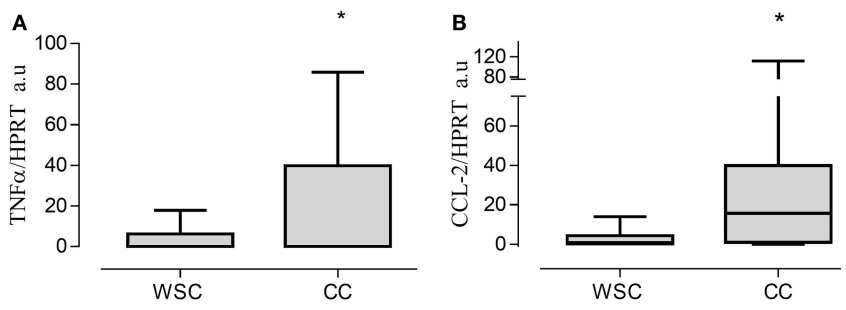

FIGURE 1 | Gene expression in tumor tissue. Data expressed as mean ± SE or as median [first quartile; third quartile]. *Significant difference between WSC vs. CC. Expression of target genes was normalized to the reference HPRT1. TNF-α, tumor necrosis factor α **(A)**; CCL2, chemokine (C–C motif) ligand 2 **(B)**; Arbitrary units, AU. WSC ($n = 10$) and CC ($n = 14$).

TABLE 5 | Tumor gene expression of cytokines and cachexia-related factors (AU).

qRT-PCR (A.U)	WSC (weight-stable cancer)	CC (cachectic cancer)	*p*
VEGF	1.275 [0.446; 8.270]	0.557 [0.069; 3.28]	0.410
IL-6	1.395 [0.368; 2.509]	1.163 [0.537; 8.330]	0.683
IL1-β	2.545 [0.430; 16.07]	0.791 [0.185; 7.893]	0.524
IFN-γ	1.317 [0.313; 5.095]	27.65 [0.420; 80.16]	0.151
PIF	0.711 [0.154; 9.012]	9.706 [0.023; 101.1]	0.571
ZAG	2.029 [0.374; 3.501]	0.716 [0.369; 2.766]	0.497
IL-10	0.728 [0.152; 10.93]	34.12 [0.141; 54.02]	0.398

Data expressed as median [first quartile; third quartile]. Target gene expression was normalized to the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT-1).
Arbitrary units (AU). WSC (n = 10); CC (n = 14).

TABLE 6 | Inflammatory factors in tumor samples.

Pico gram per milligram of total protein	WSC (weight-stable cancer)	CC (cachectic cancer)	*p*
CCL2	230.5 [96.08; 373.1]	261.89 [124.1; 546.4]	0.431
CCL4	9.32 [3.92; 13.41]	16.62 [6.77; 55.84]	0.060
CCL5	649 ± 99.69	977.8 ± 272.2	0.306
IFN-α	20.34 [5.65; 51.66]	10.95 [7.76; 52.70]	0.791
IL-10	0.363 [0.22; 1.58]	0.441 [0.16; 2.42]	0.725
IL-6	1.034 [0.245; 1.92]	2.097 [0.724; 8.33]	0.194
IP-10	243.7 [151.0; 352.2]	1263 [179.8; 2822]	0.092
TNF-α	0.352 [0.202; 0.908]	0.724 [0.339; 1.55]	0.169
TNF-β	2.306 ± 0.567	2.435 ± 0.601	0.878

Data expressed as mean ± SE or as median [first quartile; third quartile], p = significance of Mann–Whitney test. Cytokine concentration was normalized to total protein. WSC (n = 11); CC (n = 12).

Subcutaneous Adipose Tissue Protein Expression Analysis

Data of protein expression of chemoattraction factors are shown in **Table 7**. We found no statistical difference for CCL2, CCL3 and CCL5 in subcutaneous adipose tissue (**Table 7**). CCL4 protein expression was higher in CC, when compared with WSC (**Figure 3A**).

Anti- as well as pro-inflammatory cytokines (IFN-α, IL-10, IL-13, IL-6, IP-10, and TNF-α) did not exhibit differences between the two studied groups (**Table 7**). The pro-inflammatory IL-1β and TNF-β cytokines protein expression presented higher levels in CC in relation to WSC (**Figures 3B,C**, respectively).

Immunophenotyping by Cytometry

The characterization of the different phenotypes within the total population of infiltrating macrophages in the tumor microenvironment is shown in **Figure 4**. The incidence of macrophages with anti-inflammatory profile (M2 macrophages – CD11b CD14++ CXCR4+) was significantly lower in CC, compared to WSC ($p = 0.007$). Macrophages with inflammatory profile (M1 macrophages – CD11b+ CD14++ CCR7+) were found in similar numbers in the tumors of both groups.

The analysis of the stromal-vascular fraction of the subcutaneous adipose tissue yielded no statistic difference in concern to M1M2 macrophage (CD11b $CD14^{++}$ $CCR7^{+}$ $CXCR4^{+}$), M1 macrophage ($CD11b^{+}$ $CD14^{++}$ $CCR7^{+}$) and M2 macrophage ($CD11b\ CD14^{++}CXCR4^{+}$) population percentage (**Figures 5A–C**, respectively).

Correlations Analysis

Non-parametric correlation (Spearman) analysis between chemokine (C–C motif) ligand (CCL)-3 and CCL-4 with the

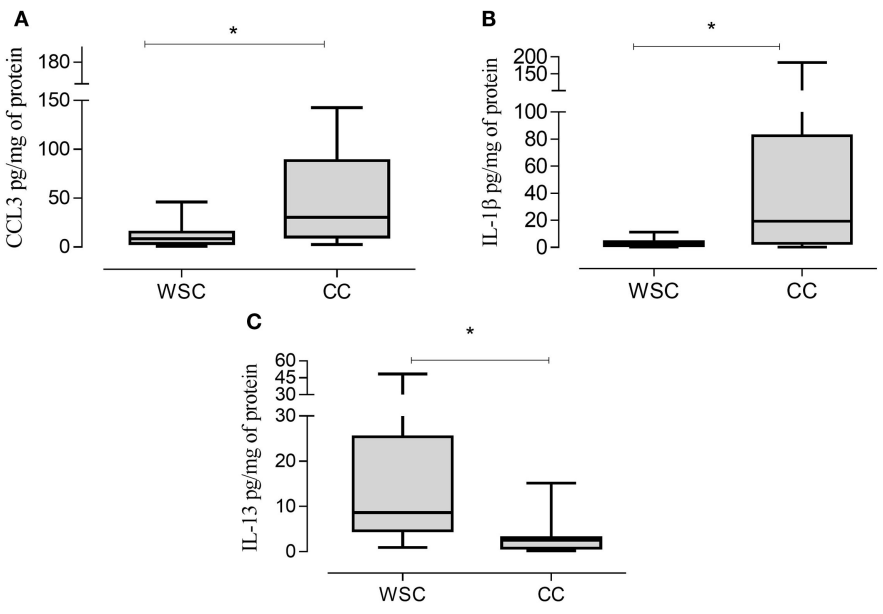

FIGURE 2 | CCL3, IL-1β, and IL-13 protein expression in tumor samples. Data expressed as median [first quartile; third quartile]. *Significant difference between WSC vs. CC. CCL3, chemokine (C–C motif) ligand 3 **(A)**; IL-1β, interleukin 1β **(B)**; IL-13, interleukin 13 **(C)**. WSC ($n = 11$) and CC ($n = 12$).

protein expression of the cytokine anti-inflammatory cytokine IL-13 in the tumor of cachectic patients was found to be significant ($p = 0.0089$); while the relationship between CCL4 and IL-13 ($p = 0.147$) was not (**Figures 6D,H**). Analysis of correlation of CCL3 with the protein expression of the inflammatory cytokine IL-1B showed positive relationship (CCL3/IL-1β) ($p = 0.0059$) (**Figure 6E**). Whether the CCL4/IL-1β correlation ($p = 0.0897$) (**Figure 6F**) nor of CCL3 with %macrophages were found to be significant (**Figures 6A–C**).

When non-parametric correlation (Spearman) analysis was carried out in regard to macrophages and CCL4 in the subcutaneous adipose tissue, no statistical correlations were observed for M1M2 macrophages not for M1 macrophages, or M2 macrophages (**Figures 7A–C**, respectively). Furthermore, non-parametric correlation for CCL4 and IL-1β was found not to be significant, whereas that between CCL4 and TNF-β was significant (**Figures 7D,E**, respectively).

Finally, we performed non-parametric correlation (Spearman) analysis for CCL4 in the subcutaneous adipose tissue and for CCL3 in the tumor, having found a statistically significant positive correlation ($p = 0.0448$) only for the cachectic patients (**Figure 8A**). When the relationship of TNF-α in the subcutaneous adipose tissue and TNF-β in the tumor was analyzed, no statistical significance was found for CC ($p = 0.0892$) (**Figure 8B**). A tendency for positive correlation between IL-10 in subcutaneous adipose tissue and in the tumor ($p = 0.0978$) (**Figure 8C**).

TABLE 7 | Inflammatory factors in the subcutaneous adipose tissue.

Pico gram per milligram of total protein	WSC (weight-stable cancer)	CC (cachectic cancer)	*p*
CCL2	38.0 ± 7.20	20.3 ± 5.26	0.0646
CCL3	13.0 [4.06; 59.4]	3.38 [0.010; 68.6]	0.3725
CCL5	157 ± 31.0	121 ± 30.6	0.4219
IFN-α	0.210 [0.135; 3.68]	2.12 [0.228; 4.73]	0.2883
IL-10	0.070 [0.060; 0.123]	0.100 [0.060; 0.330]	0.2275
IL-13	0.190 [0.110; 1.63]	0.500 [0.170; 0.680]	0.6480
IL-6	0.0711 ± 0.004	0.101 ± 0.024	0.2668
IP-10	9.19 ± 2.42	3.63 ± 0.919	0.0522
TNF-α	0.050 [0.040; 0.0525]	0.055 [0.030; 0.103]	0.5140

Data expressed as mean ± SE or as median [first quartile; third quartile], p = significance of Mann–Whitney test. Cytokine concentration was normalized to total protein. WSC (n = 11); CC (n = 12).

DISCUSSION

Cancer cachexia remains a major health problem worldwide as prevalence of cancer is on the rise. This syndrome is frequently undiagnosed and rarely treated, resulting in compromising of treatment and shortened survival (1, 10). Weight loss is the most visible feature of cachexia, yet some early metabolic and inflammatory changes precede the establishment of the most evident symptoms. The cachectic patients in the study, beyond presenting severe weight loss in the previous 6 months, exhibited systemic inflammation and anemia (CRP >5.0 mg/L, IL-6 >4 pg/mL, Hb <12 g/dL), in accordance to that proposed by Evans et al. (19), but no alterations of circulating albumin levels.

Cachexia-associated inflammation is the result of many alterations acting in concert, among which, the secretion of inflammation-promoting factors by the tumor itself. This, on the other hand, may elicit tissue and organ local sustained inflammation, in a vicious cycle. One such mechanism has been proposed to exist in cancer patients (2, 21).

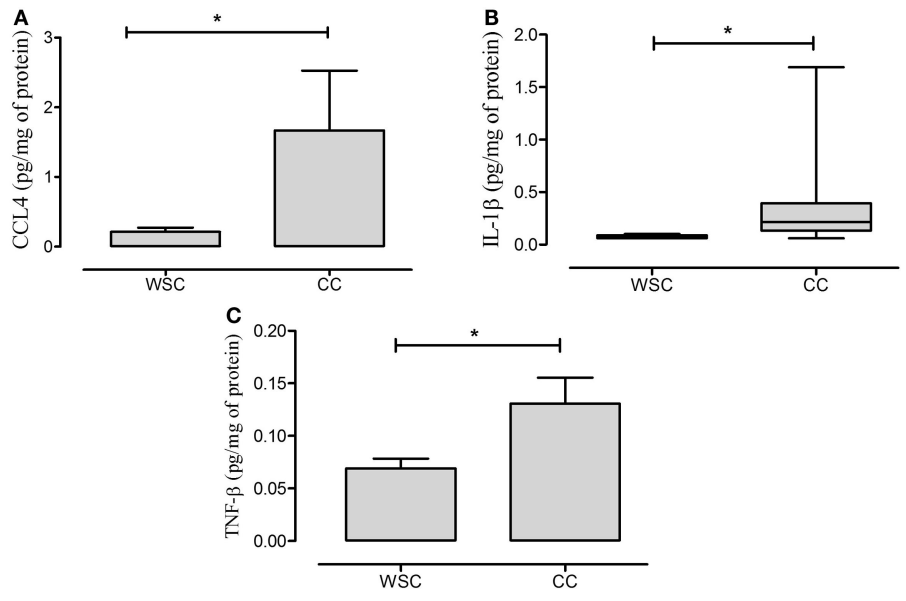

FIGURE 3 | CCL4, IL-1β, and TNF-β protein expression in subcutaneous adipose tissue. Data expressed as mean ± SE. *Significant difference CC vs. WSC group. CCL4, chemokine (C–C motif) ligand 4 **(A)**; IL-1β, interleukin 1β **(B)**; TNF-β, tumor necrosis factor β **(C)**. WSC ($n = 11$) and CC ($n = 12$).

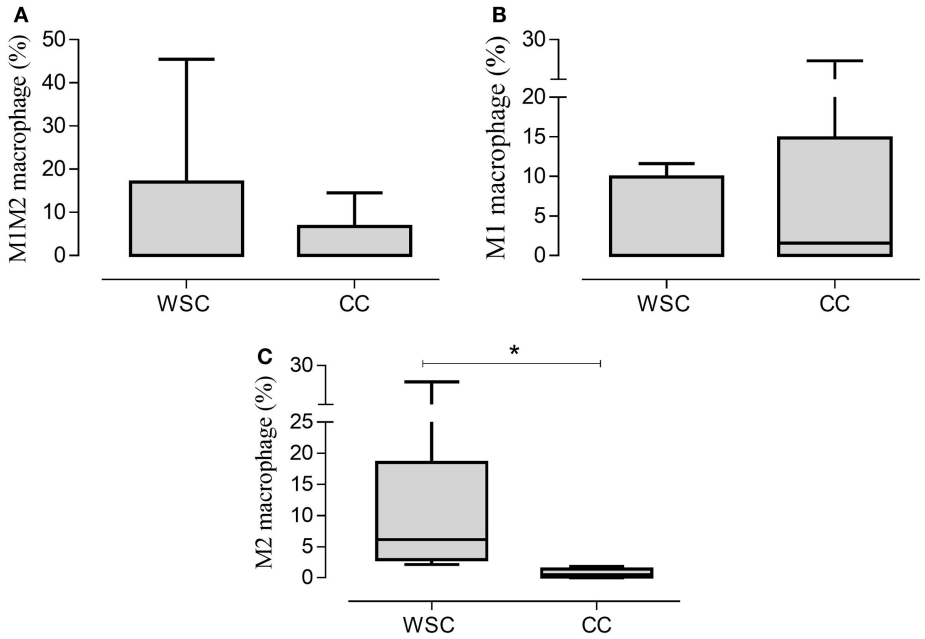

FIGURE 4 | Percentage of the phenotypes of macrophage populations in the tumor microenvironment. Data expressed as median [first quartile; third quartile] or median ± SE. *Significant difference between WSC and CC. Tumor samples WSC and CC (n = 5). M1M2 macrophage **(A)**; M1 macrophage **(B)**; M2 macrophage **(C)**.

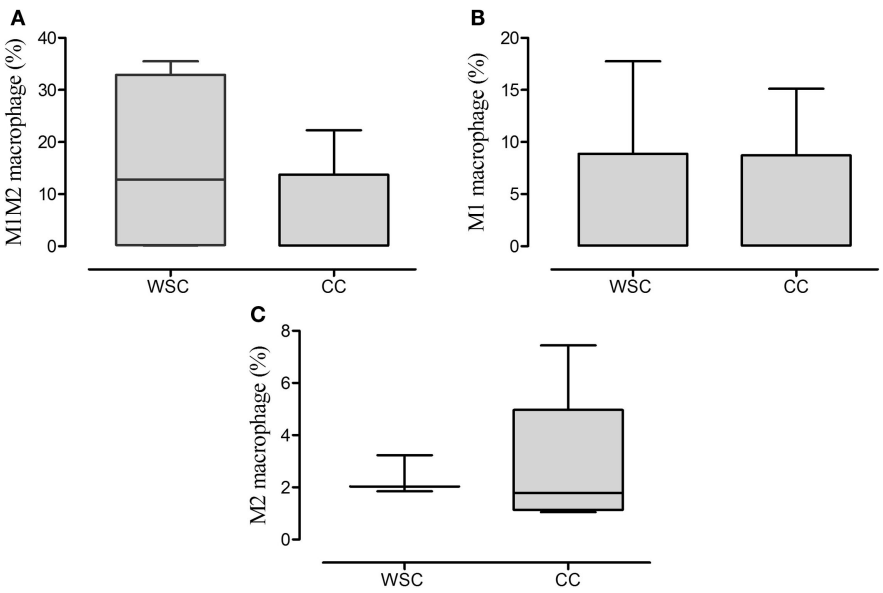

FIGURE 5 | Percentage of the phenotypes of macrophage in subcutaneous adipose tissue. Data expressed as median [first quartile; third quartile]. Stromal-vascular fraction of subcutaneous adipose tissue: WSC (n = 4) and CC (n = 5). M1M2 macrophage **(A)**; M1 macrophage **(B)**; M2 macrophage **(C)**.

Obesity research has provided solid evidence that the adipose tissue is an important player in the onset and maintenance of systemic inflammation (22). Indeed, the adipose tissue produces numerous bioactive molecules as TNF-α, IL-1β, IL-6, CCL2, to cite a few; all of which are able to act in an autocrine, paracrine, and endocrine manner, hence reaching the blood stream and promoting the crosstalk with other tissues (23).

In cancer cachexia, we have previously shown evidence that the white adipose tissue is a potential contributor for systemic inflammation, as it suffers comprehensive rearrangement and immune cell infiltration, in association with robustly increased

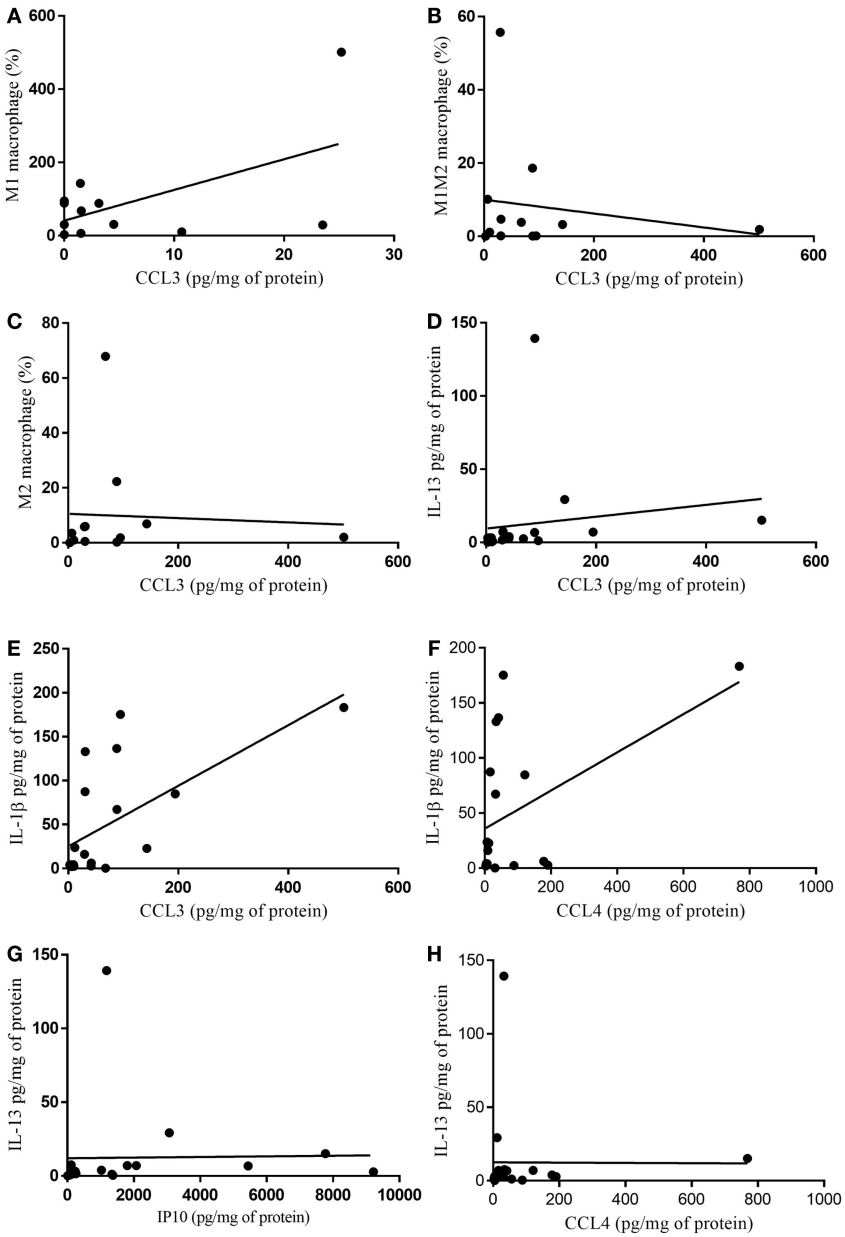

FIGURE 6 | Correlation of cytokine protein expression and % of infiltrating immune cells in tumor. **(A)** CCL3/M1 macrophage (%) $p = 0.938$; **(B)** CCL3/M1M2 macrophage (%) $p = 0.956$; **(C)** CCL3/M2 macrophage (%) $p = 0.342$; **(D)** CCL3/IL-13 $p = 0.0089$; **(E)** CCL3/IL-1β $p = 0.0059$; **(F)** CCL4/IL-1β $p = 0.089$; **(G)** IP10/IL-13 $p = 0.057$; **(H)** CCL4/IL-13 $p = 0.147$.

secretion of inflammatory factors (15, 24–26). Furthermore, the white adipose tissue of Walker 256 tumor-bearing rats was found to be infiltrated with monocytes (24), and we recently reported immune infiltration in cachectic cancer patients (25).

In another recent study employing the animal model of cachexia, we found up-regulation of IL-1β expression and activation of NF-κB and of the inflammasome pathways in adipocytes, and evidence of a major contribution of the vascular-stromal fraction of the retroperitoneal adipose tissue to tissue inflammation (26). In the current study, we have similarly found a population of infiltrated macrophages in the subcutaneous adipose tissue of cachectic patients, despite lack of statistical difference between the cachectic and non-cachectic groups in regard to the predominance of different macrophage phenotypes (M1M2, M1, and M2).

We also previously reported that NF-κBp65 gene expression is increased in the subcutaneous white adipose tissue of cachectic cancer patients, concomitantly to up-regulation of its inflammatory target genes IL-1β, TNF-α, CCL2/MCP-1, and IκB-α. Haugen et al. also found alterations in gene expression, including of TNF-α and CCL2, in the intra-abdominal adipose tissue, which

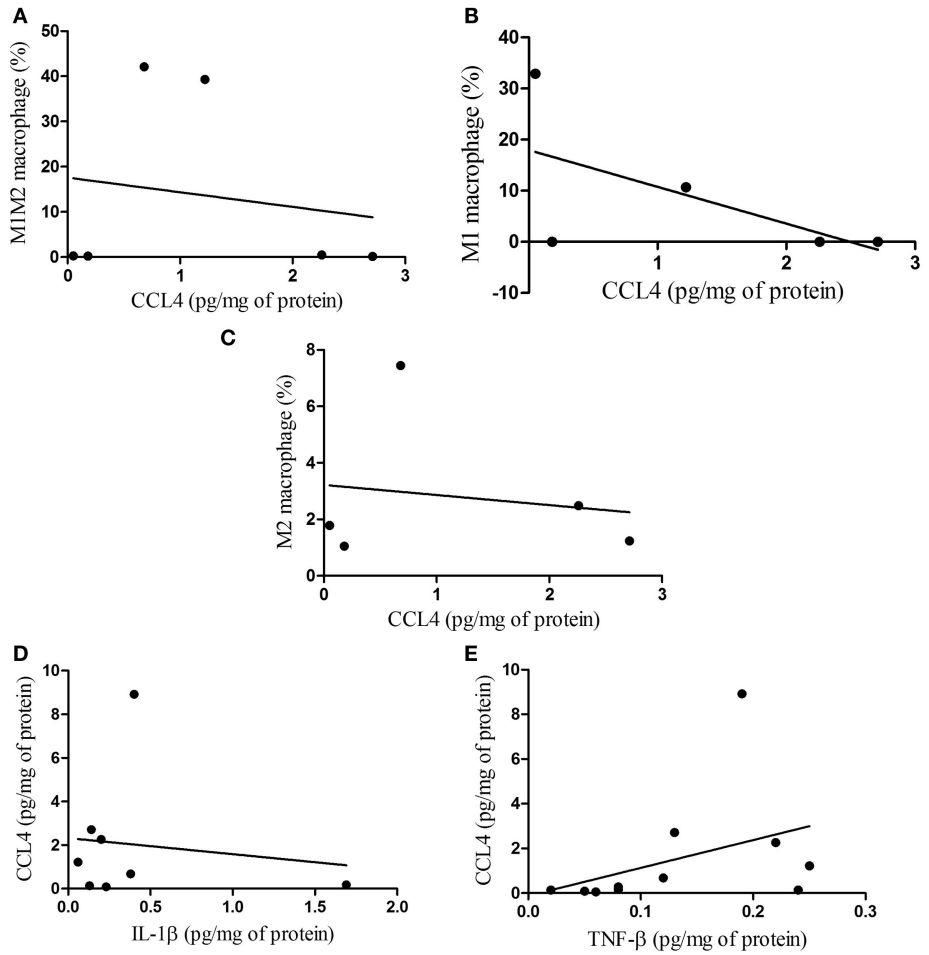

FIGURE 7 | Correlations between macrophage phenotypes and CCL4 protein, and between CCL4 and IL-1β, TNF-β in subcutaneous adipose tissue. **(A)** M1M2/CCL4, $p = 0.787$; **(B)** M1/CCL4, $p = 0.321$; **(C)** M2/CCL4, $p = 0.790$ and correlations between CCL4 protein and IL-1β, TNF-β **(D)** CCL4/IL-1β, $p = 0.955$; **(E)** CCL4/TNF-β, $p = 0.041$.

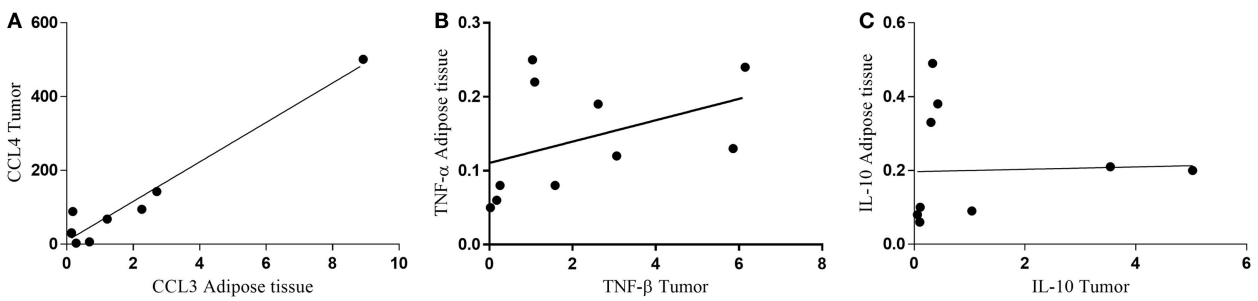

FIGURE 8 | Correlation between protein expression of inflammatory factors in subcutaneous adipose tissue and tumor. **(A)** CCL4 tumor/CCL3 adipose tissue; **(B)** TNF-α adipose tissue/TNF-β tumor; **(C)** IL-10 adipose tissue/IL-10 tumor.

was associated with reduced fat mass in patients with pancreatic cancer (27, 28).

To our knowledge, we are the first to show that the subcutaneous adipose tissue of cachectic patients presents higher CCL4 protein content in relation to WSC with matched tumor diagnosis. Increased CCL4 gene expression was found by Wu et al. (29) in the adipose tissue of obese mice, with concomitant augment of the number infiltrating leukocytes. In the present study, increased IL-1β and TNF-β protein expression was also detected, corroborating our previous findings (27).

However, what are the stimuli inducing adipose inflammation? The group of Michael Tisdale has approached, in several

studies (10, 30–32), the role of tumor-derived factors in the onset of cachexia. Therefore, the main aim of the present study was to address the eventual differences in tumor microenvironment in cachectic and weight-stable cancer patients that could be possibly linked to the presence of cachexia. For that purpose, we evaluated gene and protein expression of inflammatory markers in tumor tissue, along with the profile of infiltrating macrophages in the tumor microenvironment. The first aspect examined was the expression of the tumor-derived factors described to take part in cachexia. Much to our surprise, it was actually the weight-stable group who presented higher values for lipid mobilizing factor (ZAG), while proteolysis inducing factor (PIF) was higher in cachectic patients. The literature provides evidence that these factors are present in cachexia, but no study, has to our knowledge, compared patients with matched tumor diagnosis with and without cachexia. Therefore, it is not impossible to speculate that tumor-derived factors actually have a role in inducing a better immune and metabolic regulatory response to the presence of the tumor. More studies are, nevertheless, required to further elucidate the importance of specific tumor-originated factors.

The microenvironment of solid tumors consists of tumor cells, infiltrating immune cells and matrix components (33, 34). In whole tumor tissue samples, we found higher TNF-α and CCL2 gene expression, along with higher CCL3 protein expression in cachectic patients, as compared to WSC. Billingsley et al. have reported similar results with *in vitro* studies in regard to TNF-α, IL-6, and leukemia inhibitory factor (LIF), in which co-culture of TNF-α with tumor cells augmented significantly cytokine production (35).

We have presently analyzed inflammation-related factors in whole tumor samples, having found that the pro-inflammatory cytokine IL-1β and the anti-inflammatory cytokine IL13 expression was altered (higher and lower, respectively) in cachectic cancer patients, as compared to WSC. The classical studies regarding tumor progression were initially driven to understand intrinsic changes in malignant cells (23). In the recent years, aspects related with the tumor microenvironment and to the host's response to tumor progression have received more attention, and specially, the infiltrating immune cells, as their presence is associated with persistent inflammatory states (36, 37).

In order to establish whether tumors from cachectic patients and from WSC were different in terms of infiltration macrophage populations, we employed specific markers to identify macrophage sub-phenotypes. The results show fewer M2 macrophages in tumors of cachectic cancer patients, as compared with the weight stable group. Weber et al. demonstrated in patients with oral squamous cell carcinoma that increased polarization of macrophages toward a M2 phenotype is potentially correlated with a negative influence on tumor biology, resulting in more aggressive tumors (38). We failed to encounter studies in the literature that associate tumor infiltrating macrophage population with the presence of cachexia.

Considering that several inflammatory signaling pathways work in concert in promotion of inflammation, we performed Spearman's correlation tests for tumor and subcutaneous adipose tissue data. The results show that CCL3 protein levels present a positive correlation with the expression of pro-inflammatory IL-1β protein in the patients' tumors. In the subcutaneous adipose tissue, we report a positive correlation between CCL4 and TNF-β. These data corroborate the idea of complex and active interaction between the tumor and peripheral tissues, with major involvement of infiltrating immune cells.

The limitations of the study should be acknowledged. The previous body mass was informed by patients, and thus inaccuracies regarding this parameter are possible. Owing to human tissue sample implicit variation, some of the analyses were not performed with the total number of patients formerly enrolled, as some samples fell out of the detection range of the assays. The relative contribution of infiltrating monocytes for tissue inflammation was not assessed. Experiments with isolated cell populations are now being conducted.

CONCLUSION

The results provide evidence that tumors from cachectic and weight stable cancer patients with same diagnosis show different secretory profile in regard to inflammatory factors and different macrophage phenotype percentage. An association between tumor-originated factors and adipose tissue inflammatory changes is proposed, as a positive correlation was found between tumor and adipose tissue-derived cytokines and inflammatory factors.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2015.00629

Figure S1 | Gating strategy for determination of macrophage-infiltrating subpopulations in tumor and adipose. Specific gating strategies: **(A)** FSC-H vs FSC-A to exclude doublets. **(B)** FSC vs SSC to gate out the debris. **(C)** CD45+ to include all leukocytes. **(D)** CD14+ or CD11+ macrophages can be identified by markers such as CD14+ or CD11b+. **(E)** Unlabeled sample. **(F)** Labeled sample CCR7 (subpopulation M1), CXCR4+ (subpopulation M2), and double positive CCR7+ CXCR4+ (subpopulation M1–M2).

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MINI REVIEW
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Diversity of Intestinal Macrophages in Inflammatory Bowel Diseases

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Macrophages as innate immune cells and fast responders to antigens play a central role in protecting the body from the luminal content at a huge interface. Chronic inflammation in inflammatory bowel diseases massively alters the number and the subset diversity of intestinal macrophages. We here address the diversity within the human intestinal macrophage compartment at the level of similarities and differences between homeostasis and chronic intestinal inflammation as well as between UC and CD, including the potential role of macrophage subsets for intestinal fibrosis. Hallmark of macrophages is their enormous plasticity, i.e., their capacity to integrate signals from their environment thereby changing their phenotype and functions. Tissue-resident macrophages located directly beneath the surface epithelium in gut homeostasis are mostly tolerogenic. The total number of macrophages increases with luminal contents entering the mucosa through a broken intestinal barrier in ulcerative colitis (UC) as well as in Crohn's disease (CD). Although not fully understood, the resulting mixtures of tissue-resident and tissue-infiltrating macrophages in both entities are diverse with respect to their phenotypes and their distribution. Macrophages in UC mainly act within the intestinal mucosa. In CD, macrophages can also be found in the muscularis and the mesenteric fat tissue compartment. Taken together, the present knowledge on human intestinal macrophages so far provides a good starting point to dig deeper into the similarities and differences of functional subsets and to finally use their phenotypical diversity as markers for complex local milieus in health and disease.

Keywords: intestinal macrophages, gut homeostasis, inflammatory bowel diseases, fibrosis, diversity

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INTRODUCTION

The gastrointestinal tract is the largest immune compartment of the human body. The major function of the intestinal immune cells is to maintain the integrity of the body at the huge interface between external stimuli that include food components and the intestinal microflora. Chronic inflammation in inflammatory bowel diseases (IBD) profoundly alters the composition of all local immune-cell compartments. Macrophages are part of the innate immune system and instrumental in controlling the barrier function in the small and the large intestine. The macrophages integrate signals

Abbreviations: ALDH, aldehyde dehydrogenase; CD, Crohn's disease; CCL, C–C chemokine ligand; CLR, C-type-lectin-like receptor; CX3CR1, chemokine (C–X3–C motif) receptor 1; HLA, human leukocyte antigen; IBD, inflammatory bowel disease; Ig, immunoglobulin; MMP, matrix metalloproteinase; NF-κB, nuclear factor kappa-light-chain enhancer of activated B cells; NOD, nucleotide-binding oligomerization domain; NLR, NOD-like receptor; PRR, pattern recognition receptors; TLR, toll-like-receptor; TREM, triggering receptor expressed on myeloid cells; UC, ulcerative colitis.

from their environment, thereby changing their phenotype and function. The present knowledge about intestinal macrophages is predominantly based on mouse studies. Even the finding of the gut as the largest reservoir of tissue-resident macrophages within the body (1) remains to be verified for men. This minireview deliberately restricts to systematic human studies. Only if such data were lacking, we included findings from animal models that might be relevant for the human mucosal surface. Differences in between mice and men will be highlighted. Non-inflamed tissue areas neighboring the inflamed areas in ulcerative colitis (UC) and Crohn's disease (CD), the main forms of IBD, represent rather homeostatic conditions. Hence, the diversity within the human intestinal macrophage compartment at the level of similarities and differences between homeostasis and chronic intestinal inflammation as well as between UC and CD, including the potential role of macrophage subsets for intestinal fibrosis, will be discussed.

INTESTINAL MACROPHAGES IN GUT HOMEOSTASIS AND IN IBD

In terms of a first-line defense, tissue-resident intestinal macrophages contribute to the gut homeostasis by eliminating invading pathogens without inducing an inflammatory response of the lymphocytes within the lamina propria. Positioned directly beneath the surface epithelium, the macrophages in intestinal tissues are the first immune-cell population encountering foreign material, e.g., luminal bacteria or food antigens randomly passing the epithelial barrier (**Figure 1A**). Whether human macrophages are able to sample luminal antigen by extending their dendrites between the epithelial cells reaching into the gut lumen as shown for mouse macrophages (2, 3) is unknown. On the one hand, intestinal macrophages are tolerant toward foreign matter by down-regulation of recognition receptors (4). On the other hand, intestinal macrophages that recognize food-derived antigens or commensal microbiota present the processed antigens in a tolerizing manner in the absence of co-stimulatory signals (5). Also to fulfill the task of protecting from unwanted immune responses and different from peripheral monocytes, stimulation via pattern recognition receptors (PRR) on resident macrophages results in low cytokine secretion and strong bactericidal activity (6). This increased bacterial clearance is associated with increased metallothionein expression, which is regulated by nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) and by caspase-1 (7).

Precursors of tissue-resident intestinal macrophages are bone marrow-derived monocytes, which circulate through the blood before recruitment into the intestinal mucosa by interleukin (IL)-8 and transforming growth factor (TGF)β (8). These freshly recruited monocytes exhibit an inflammatory phenotype and exert inflammatory functions. Signals from the intestinal mucosa subsequently polarize them into inflammation anergic macrophages, e.g., by stromal TGFβ-induced inhibition of NF-κB activation (9). Additionally, TGFβ and IL-10 induce down-regulation of triggering receptor expressed on myeloid cells (TREM)-1 on intestinal macrophages, a receptor that potently amplifies inflammatory

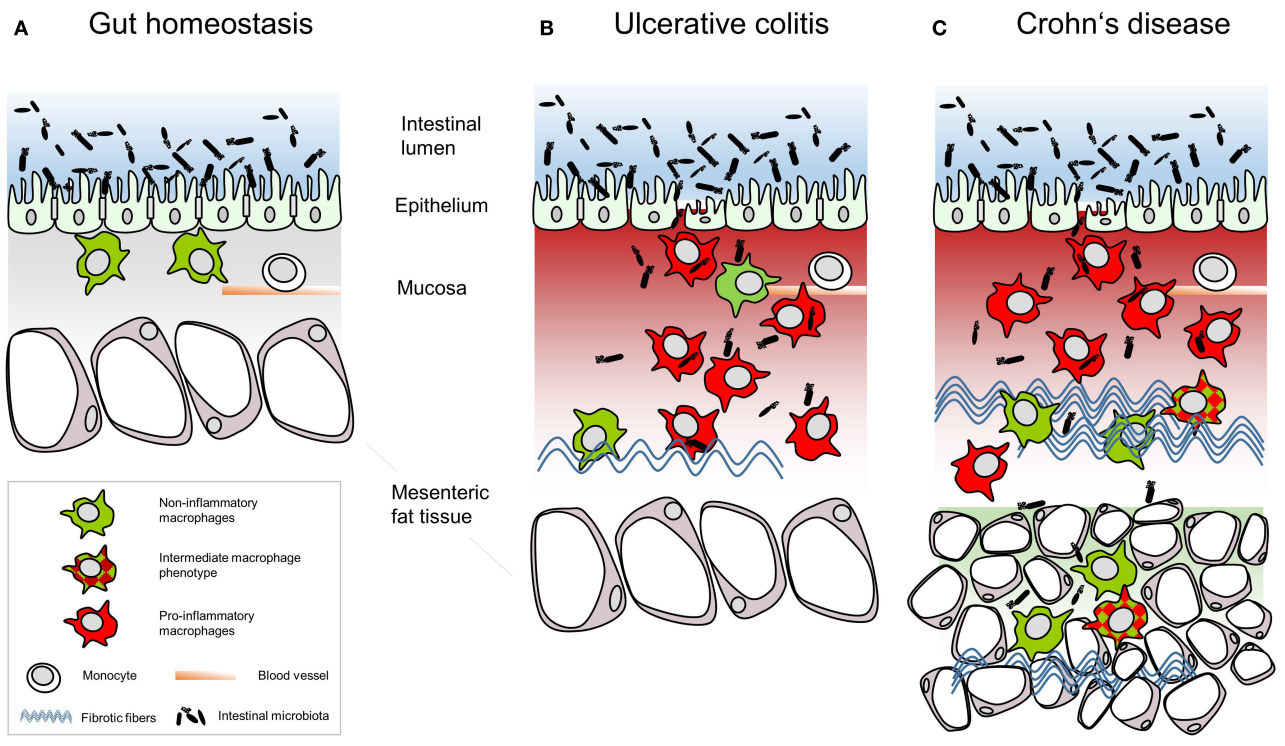

FIGURE 1 | Schematic summary of the relative intestinal macrophage-subtype distribution in (A) gut homeostasis or (B) ulcerative colitis and (C) Crohn's disease.

reactions (10). A minority of tissue-resident intestinal macrophages express CD14 as well as CD11c involved in sensing of bacterial lipopolysaccharides (LPS) and are considered to be differentiation intermediaries (11). Blood monocytes have a life span of 3–4 days, while the life span of intestinal macrophages is unknown. In mice, intestinal macrophages lost upon senescence or apoptosis are constantly replenished by newly recruited blood monocytes and by cell division *in situ* (12). While mouse macrophages replenish in the intestine by recruitment of circulating cells and proliferation (12, 13), human intestinal macrophages fail to do so (8). Again in mice, mucosal tolerance is mediated by intestinal macrophages secreting IL-10, thereby expanding regulatory T cells (Tregs) (14). By contrast, human macrophages isolated from healthy jejunum and stimulated, e.g., with LPS, *Helicobacter pylori* urease, heat-killed *Staphylococcus aureus*, interferon (IFN)γ or phorbol myristate acetate *in vitro* did not produce IL-10 (6).

A hallmark of macrophages is their plasticity as well as the ability to change phenotype and function according to the immediate environment. This has been demonstrated systemically by recent work from Xue and colleagues who defined a core transcriptome network for human and murine macrophages (15).

Hence, it is not surprising that small intestinal macrophages are different from large intestinal macrophages. These two organs have a distinct architecture, exert different functions, and host diverse microbiota. For example, macrophages from healthy jejunum show high expression of human leukocyte antigen (HLA)-DR and very low expression of CD14 and the low-affinity human immunoglobulin (Ig)G receptor CD16 (6), whereas in colonic macrophages low levels of CD14 and CD16 are accompanied by moderately expressed HLA-DR (16). Very early work, e.g., uses the activities of acid phosphatase and nonspecific esterase to distinguish macrophage subtypes (17). Here, tissue-resident intestinal macrophages directly underneath the epithelium differ from macrophages positioned deeper in the lamina propria with no implication that these cells abandon their tolerogenic potential (17).

Following the M1–M2 paradigm, which mirrors the polarization of T helper cells, macrophages are classified as pro-inflammatory M1 macrophages and anti-inflammatory M2 macrophages (18). Adhering to this model, tissue-resident macrophages are considered to be M2 macrophages (19, 20). In IBD, macrophages massively infiltrate the intestinal mucosa and present phenotypes and distribution distinct from tissue-resident macrophages in homeostasis. In CD patients, macrophages also infiltrate the muscular layer and the mesenteric fat (17, 21). At first sight, large numbers of $CD68^+$ macrophages massively infiltrate the intestinal mucosa in IBD and diffusely spread throughout the thickened mucosa and submucosa but differ with regard to the subset composition and function in UC (**Figure 1B**) and CD (**Figure 1C**). Analyses of blood monocytes derived from CD patients reveal a reduction of classical monocytes ($CD14^{hi}CD16^-$), while intermediate monocytes ($CD14^{hi}CD16^+$) were increased (22, 23). Extensive migration of classical monocytes toward the C-C chemokine ligand (CCL)2 *in vitro* and massively enhanced $CD14^{hi}$ macrophages in the ileal and the colonic mucosa of the CD patients led to the conclusion that peripheral classical monocytes immigrated into the intestinal mucosa (23). These newly recruited macrophages express high levels of CD33, of the high-affinity human IgG receptor CD64 and of the G-protein-coupled fractalkine receptor CX3CR1 but were $HLA\text{-}DR^{dim}$ (23). Infiltrating intestinal macrophages are distinct in phenotype and function from their resident counterparts. For example, tissue-infiltrating intestinal macrophages strongly express CD14 (24), TREM-1 and the human myeloid IgA Fc receptor CD89 (25) as well as activated NF-κB (26). Additionally, tissue-infiltrating intestinal macrophages secrete pro-inflammatory cytokines such as TNF, IL-6, IL-8, IL-23, IL-1β, and IFNγ as well as the chemokine CCL2 attracting monocytes (25, 27). This pro-inflammatory macrophage phenotype might result from polarization of any monocytic cell entering the pro-inflammatory environment of the inflamed intestinal mucosa. In line with this, the conditioning of newly recruited monocytes toward inflammation anergic M2 macrophages might be disturbed in IBD patients due to defective TGFβ signaling (28). In IBD, a broken epithelial barrier allows luminal content to enter the lamina propria, thereby triggering the inflammatory response of the lamina propria leukocytes. For recognition of microbiota, macrophages up-regulate PRR, including membrane-bound toll-like-receptors (TLR) and C-type-lectin-like receptors (CLR) as well as cytoplasmic nucleotide-binding oligomerization domain-containing protein (NOD)-like receptors (NLR) and retinoic acid-inducible gene-I-like receptors. Human PRR show less variants than those in mice; 10 TLR and 22 NLR are known in men compared to 13 TLR and 34 NLR in mice. Tissue-infiltrating macrophages in the inflamed colon mucosa predominantly express TLR2, TLR4, and TLR5 responding to bacterial peptidoglycans, LPS, and bacterial flagella (29). CLR bind a variety of carbohydrate ligands but only collectins function in terms of PRR (30). NOD2 recognizing muramyl dipeptide on Gram-positive and -negative bacteria is expressed in monocytes and Paneth cells but not in intestinal macrophages (31). *In vitro* studies showed that NOD2 level declined during differentiation of monocytes into macrophages (31). *CARD15* coding for the caspase-recruitment domain of NOD proteins is highly up-regulated in colonic macrophages of CD patients (32). So far it is not clear whether in chronic inflammation in CD the down-regulation of NOD2 in monocytes infiltrating the colon mucosa is affected or whether resident macrophages up-regulated NOD2 expression. A missense mutation in the coding sequence of *NOD2* was found in 17% of CD patients and in 4% of UC patients (33). As over 200 genes have been linked to IBD (34) and many of them are associated with macrophage functions (35–39), these immune cells present one cell population contributing to the pathogenesis of UC and CD.

DIVERSITY WITHIN INTESTINAL MACROPHAGE COMPARTMENTS IN ULCERATIVE COLITIS AND CROHN'S DISEASE

Above, we highlighted differences in the macrophage compartments and differentiated between tissue-resident and tissue-infiltrating macrophages in gut homeostasis and IBD. Additionally, the composition and functions of intestinal macrophages also differ in the inflamed gut of UC and CD patients, while overall macrophage

numbers are comparable. So the question arises whether distinct macrophage subpopulations and distributions of these subtypes within the inflamed tissue areas might explain the overall different outcome in CD and UC. As for similarities in the local distribution, monocytes and M1 macrophages directly contribute to the defect of the barrier in IBD and large numbers of pro-inflammatory macrophages reside in the inflamed mucosa (40).

Over a decade ago, CD has even been referred to as a macrophage primary immunodeficiency (41). While this statement might simplify the overall interaction of immune cells in the mucosa, several facts add to this hypothesis. Thus, impaired bacterial clearance in CD has been attributed to defective cytokine secretion by macrophages (42). *E. coli* is commonly found within intestinal macrophages in CD (43), a dysfunction not reported for UC. On the contrary, macrophages of UC patients exuberantly and protractedly respond toward bacteria (44). This difference in bacterial clearance is also reflected by the formation of granulomas in CD but not UC (45, 46). Granulomas are formed when the effective eradication of invading pathogens fails.

Tissue-resident intestinal macrophages express the scavenger receptor CD163 that also recognizes Gram-positive and -negative bacteria (47, 48). While CD163 was initially thought to be exclusively expressed on noninflammatory M2 macrophages (49, 50), CD163 is expressed on resident macrophages of all normal tissues except on splenic white pulp macrophages and on germinal center macrophages (51). $CD163^+$ macrophages are enriched in the peripheral blood as well as in the colonic mucosa of IBD patients (52–54). As CD163 is cleaved by metalloproteinases (MMPs) and shed from macrophages upon activation, soluble CD163 is an appropriate marker for macrophage activation (55). Compared to healthy controls, sCD163 is increased in UC and CD patients (56). In line with comparable numbers of macrophages in the intestinal mucosa in CD and UC, sCD163 levels are comparable in both entities (56). Upon successful treatment with glucocorticoids or TNFα-antibodies, histomorphologically reflected by reduced macrophages in colon biopsies (57), serum sCD163 levels are reduced (56, 58).

No differences were found regarding the numbers of $TREM\text{-}1^+$ macrophages triggered to high production of pro-inflammatory cytokines (25) or in the expression of the co-stimulatory molecules CD80 and CD86 (5).

Aldehyde dehydrogenase (ALDH) is involved in the release of retinoic acid, which has immunomodulatory properties and is mandatory in the induction of forkhead-box protein 3^+ Tregs (59, 60). Directly relating to Treg numbers in the colonic mucosa, $ALDH^+$ macrophages are reduced in the intestinal mucosa of UC but not of CD patients (61). While Treg numbers are generally increased in intestinal tissues from IBD patients compared to those of healthy controls, the numbers are lower in UC compared to CD (62, 63). Taking into account that the composition of macrophage subpopulations might mirror the local environment, these findings suggest rather pro- than anti-inflammatory macrophage subpopulations involved in UC.

Specific for CD and relying on the presence of numerous M2 macrophages, the hyperplastic mesenteric fat tissue beyond the transmural inflammation could be defined as a second protective barrier from invading luminal contents (21). In the liver, macrophages are the master regulators of fibrosis (64). Large numbers of macrophages are found in fibrotic lesions of CD patients (65). Gene polymorphisms associated with the fibrostenotic phenotype in IBD like the V249I polymorphism of *CX3CR1* and the T300A mutation in the autophagy-related *ATG16L1* link to macrophage functions (66, 67). An indication for the involvement of distinct macrophage subpopulations in IBD is the development of fibrosis that is more pronounced in CD than in UC (68–70). Fibrosis and subsequent fibrotic strictures result from excessive wound-healing processes. Intestinal wound healing involves various steps with macrophages involved in all of these steps. In the early phase, inflammatory macrophages clear the wound from bacteria and cellular debris; in later phases, wound-healing M2 macrophages promote tissue remodeling. Tissue-resident intestinal macrophages express matrix MMP-2 (71) that takes part in the breakdown of extracellular matrix. In fibrotic CD, MMP2 is increased in the mucosa compared to that of healthy persons (72). The tyrosine-protein kinase Hck, a master regulator for human M2 macrophages (73) regulates myeloproliferation in mice (74). Other studies in mice showed that noninflammatory macrophages are involved at many levels in the whole wound-healing process, i.e., in wound closure, in formation of granulation tissue, in angiogenesis, in collagen synthesis, and in the production of growth factors (75). The pleiotropic cytokine IL-13 was also identified as a pro-fibrotic factor in CD (72). In combination with TNFα, IL-13 induces TGFβ production in macrophages (76).

Macrophages carrying the mannose receptor CD206 and considered wound-healing macrophages (77) are increased in the injured mucosa of UC patients (78). The expression of the proto-oncogene protein Wnt1 by $CD206^+$ macrophages enhanced the proliferation of stem cells in response to the epithelial injury in UC (78). Relating to the increased risk of cancer development upon long-standing IBD, large numbers of $CD206^+$ macrophages are found in colorectal cancer (79).

Taken together, many open questions remain with regard to specifics of the involvement of different subpopulations of human macrophages in the pathogenesis and the chronicity of UC and CD. Further dissecting the diversity and the local distribution of functional macrophages in human gut tissues will help to define the clinical relevance of the macrophage subset.

AUTHOR CONTRIBUTIONS

AK, UE, LK, and BS summarized the content of the manuscript. AK and UE wrote the manuscript, and BS and LK discussed and edited the manuscript.

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Macrophages During the Fibrotic Process: M2 as Friend and Foe

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Macrophages play essential activities in homeostasis maintenance during different organism's conditions. They may be polarized according to various stimuli, which subsequently subdivide them into distinct populations. Macrophages with inflammatory activity function mainly during pathological context, while those with regulatory activity control inflammation and also remodel the repairing process. Here, we propose to review and to present a concise discuss on the role of different components during tissue repair, including those related to innate immune receptors and metabolic modifications. The scar formation is directly related to the degree of inflammation, but also with the appearance of M2 macrophages. In spite of greater numbers of macrophages in the fibrotic phase, regulatory macrophages present some characteristics related to promotion of fibrosis but also with the control of scar formation. These regulatory macrophages present an oxidative metabolism, and differ from the initial inflammatory macrophages, which in turn, present a glycolytic characteristic, which allow regulatory ones to optimize the oxygen consumption and minimizing their ROS production. We will emphasize the difference in macrophage subpopulations and the origin and plasticity of these cells during fibrotic processes.

Keywords: polarization, macrophage activation, cell metabolism, myofibroblasts, fibrosis

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INTRODUCTION

Macrophages are cells of the innate immune system highly heterogeneous, involved in the primary response to microorganisms, in inflammatory responses, homeostasis, and tissue regeneration (1). Several evidences show that initial infiltration of macrophages culminates with pro-inflammatory cytokines and reactive oxygen species (ROS) production, which exacerbates inflammatory diseases such as diabetes mellitus, kidney disease, heart, and liver disease. Conversely, macrophages in the later phase of diseases have been associated with release of anti-inflammatory molecule and growth factors, which attenuate inflammation and promote tissue regeneration (2). However, there are macrophage dysfunction, which can impair the proper regenerative process, and otherwise, promote the development of fibrosis, deposition of type I and III collagen, and myofibroblasts activation. Emerging evidence demonstrates that both inflammatory and regulatory macrophages may participate in the pro-fibrotic processes, and this event may be dependent on the macrophage origin and the intrinsic aspects of the pathology (3, 4). Below, we will discuss the differences in macrophage subpopulations characteristics and their ontogeny with emphasis in the fibrotic process.

ONTOGENY OF MACROPHAGES

Since the description of macrophages in 1888 by the renowned scientist Elie Metchnikoff (5) considerable accumulating knowledge about their biology, development, and origin were generated, re-evaluated, and placed on discussion as a result of advances in biology technology such as conditional deletion and colored-labeled-monocytes, that unquestionably enable us to better understand these cells (6–9). One example is that for years we assumed that all resident macrophages come from circulating monocytes derived from a single myeloid precursor in bone marrow (7). However, nowadays we know resident macrophages are heterogeneous cells that can develop from different sources, including embryonic progenitor cells, bone marrow hematopoietic cells or local proliferation (6–9).

Embryonic hematopoiesis begins on the eighth day after conception in the yolk sac (10, 11). Progenitors migrate to fetal liver to establish a temporary hematopoiesis (7, 12). Macrophages with embryonic origins may be regulated by CSF1R and their corresponding ligands IL-34 and CSF1 (13–15). Studies on CSF1R ablation verified CSF1R is important for the generation of resident macrophages once deletion of this receptor compromises the development of resident macrophages in brain, bone, skin among other tissue (15).

Monocytes derived from bone marrow myeloid progenitors also give rise to both dendritic cells and macrophages. Two different types of monocytes are described in mice: $Ly6C^{+}CCR2^{high}$ and $Ly6C^{-}CX3CR1^{high}$. $Ly6C^{+}CCR2^{high}$ are called "inflammatory" monocytes and are considered to be recruited to inflamed lymph nodes and tissues where typically differentiate into DC or inflammatory macrophages. In contrast, $Ly6C^{-}CX3CR1^{high}$ present low CCR2 expression, are smaller and are known as "resident-monocyte," responsible by surveillance in homeostatic conditions, an essential task to accomplish the cleaning oxidized lipids, dead cells, and possible pathogens (16–18). Besides, these cells are also related to reduce inflammation and promotion of tissue repair (16). A schematic origin of macrophage is shown in **Figure 1**.

MACROPHAGE POLARIZATION

Resident and infiltrating macrophages may be polarized according to the microenvironment stimuli (6, 8). They may be considered M1, also known as classical or pro-inflammatory, and M2 also known as alternative macrophages, but with intermediate states of activation (19, 20).

Classically activation is acquired in presence of IFN-γ, derived from natural killer cells and Th1 lymphocytes, and LPS from pathogens. Such activation increases the phagocytic capacity of macrophages along with the expression of class II MHC and costimulatory molecules such as CD80/CD86 (21). This biological event makes the macrophage a cell specialized to present antigens, along with the production of inflammatory cytokines (TNF-α, IL-12, and IL-23), besides recruiting Th1 and Th17 lymphocytes. Consequently, the adaptive immune system maintains activation of macrophages in order to provide a stable defense against any pathogen. The role of M1 macrophages is associated with microbicide capacity, antigen presentation, antitumor activity, and they are related to inflammatory diseases (2, 21). M1 macrophages also express ROS and chemokines such as CCR7, CXCL9, and CXCL10 (2, 22).

M2 macrophages, in turn, present different properties, sometimes opposite, to M1 macrophages. They secrete anti-inflammatory factors, which help to diminish the inflammation (2, 20). The polarization of M2 cells is mainly promoted by Th2 cytokines such as IL-4 and IL-13. The profile of chemokines and cytokines are also different between both cases. M2 macrophages produce chemokines that recruit Th2 lymphocytes and T regulatory cells such as CCL17, CCL22, and CCL24 (23). Other features that characterize M2 macrophages are the expression of Arg1, Ym1, and Fizz, secretion of angiogenic factors such as IL-8, VEGF, and EGF4, increased mannose receptor (CD206), besides reduced expression of pro-inflammatory cytokines and ROS. M2 macrophages carry out the clearance of apoptotic cells, combat intestinal parasites, stimulate tumor growth, and promote the regeneration of organs (24, 25).

THE ROLE OF MACROPHAGES IN THE PROGRESSION OF FIBROSIS

Defining Fibrosis

The repair tissue damage is a fundamental biological process that allows the orderly replacement of damaged or dead cells due to some injury, an essential mechanism for survival. The damage tissue can result from various stimuli, acute or chronic, including infections, autoimmune reactions, mechanical injury, or any stimulation of the immune response. The repair process typically involves two distinct stages: a regenerative phase, in which the damaged cells are replaced by cells of the same type without bringing any evidence of harm; and a phase called fibroplasia or more commonly called fibrosis, in which connective tissue replaces normal parenchymal tissue. Although initially beneficial, the healing process becomes pathological when it becomes continuous, resulting in substantial remodeling of the ECM and formation of permanent scar. In some cases, this can lead to organ failure (26).

Unlike acute inflammatory reactions that are characterized by fast vascular changes, edema, and neutrophil infiltration, fibrosis usually originates from chronic inflammatory responses, defined as a response that persists for several weeks or months, and which inflammation and tissue destruction process repair occur simultaneously. Although different etiologies and clinical distinction, most fibrotic disorders have in common a persistent inflammation which maintains production of growth factors, proteolytic enzymes, angiogenic factors, and pro-fibrotic cytokines, which together stimulate the deposition of connective tissue elements remodel or progressively destroy normal tissue architecture (27, 28).

Irrelevant of the initial cause, the development of interstitial fibrosis is characterized by the appearance of activated fibroblasts, positive for α-smooth muscle actin (α-SMA), also called myofibroblasts. In renal parenchyma, the deposition of ECM products is largely attributed to these cells (29).

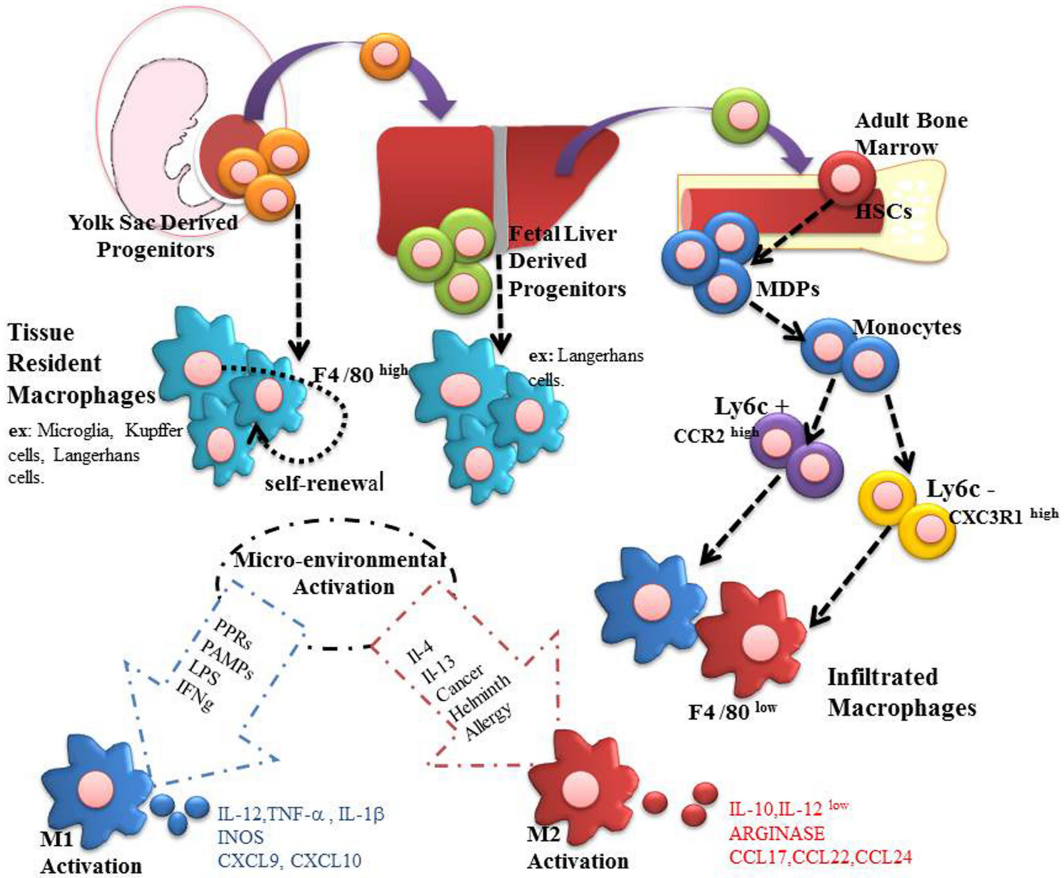

FIGURE 1 | Macrophages are present in all mammalian tissues, contributing to homeostasis and organ disease. Most tissue macrophages have an embryonic origin, and they are not fully derived from circulating monocytes. From embryonic day 6.5–8.5, resident macrophages can be generated in yolk sac. These macrophages can be identified as being regulated by CSF1R, and they are independent of the factor myb. Subsequently, during day E 8.5 to E 10.5 hematopoietic stem cells (HSCs)-derived aorta-gonad-mesonephros can migrate to fetal liver and establish a temporary hematopoiesis, giving rise, for example, to Langerhans cells and alveolar macrophages. In addition, resident macrophages derived from fetal liver may originate both HSCs precursors and mature erythro-myeloid cells. Finally, during the perinatal period, HSCs migrate to the bone marrow to establish itself a definitive place of hematopoiesis that will last until the adulthood. On this point, they are produced as Ly6C$^+$CCR2^{high} and Ly6C$^-$CX3CR1^{high} monocytes capable of infiltrating organs and differentiate into macrophages. Both infiltrating and residents macrophages can be polarized to M1 and M2 according to the microenvironment stimuli.

Myofibroblasts as Effector Cells in Fibrosis

Myofibroblasts are recognized as the effector cells of fibrogenesis (30). These cells are recognized by synthesizing large amounts of ECM, a substance which is mainly comprised of fibers of type I and III collagen, fibronectin, laminin, and other basal membrane proteins that are the source of scar tissue (31, 32). In addition, myofibroblasts are characterized by generating contractility, and distort the architecture organs, a property that is due to the expression of smooth muscle proteins as α-SMA (33). It has been identified at least three different sources for myofibroblasts (34). The first origin is related to the activation of local stromal cells such as fibroblasts and pericytes in the presence of pro-fibrotic factors (35). The second myofibroblasts source is from circulating fibrocytes. These cells originate in the bone marrow and express markers such as CD34, CD45RO, 25F9, S100A8/A9, and type 1 collagen (36). They can be recruited by inflammatory chemokines, and its importance is related to the role they have in lung, skin, heart, liver, and kidney fibrosis process (37). Other sources of myofibroblasts are epithelium or endothelium to mesenchymal transition (EMT and EndoMT), a reported process that occur in tubular cells in the presence of TGF-β in which such cells may adopt mesenchymal characteristics (38, 39). During EMT and EndoMT, renal tubular cells lose their phenotype and thus transdifferentiate into myofibroblast cells expressing α-SMA and type I collagen. The EMT/EndoMT process involves four key events: (1) loss of epithelial adhesion properties, (2) new α-sMA expression and actin reorganization, (3) increased permeability of the tubular basement membrane, and (4) increased migration and invasion ability (40).

TGF-β is the only factor described as participating in the four events of EMT and two molecules: hepatocytes growth factor (HGF) (41) and bone morphogenic protein-7 (BMP-7) (42) have been demonstrated as being capable of reversing the process of

EMT due to inhibition of TGF-β and hence decreasing renal fibrosis.

Macrophages and Fibrosis

Since embryonic stages, it has been shown that CSF1R$^+$ macrophages participate in the homeostasis and architectural remodeling of tissue (43). However, it has also been shown that the degree and severity of damage and fibrosis correlates with infiltrating macrophages (44). Depletion of resident macrophages by clodronate or CCL2 blockade improves kidney injury and reduce the pro-fibrotic process (45, 46). Interestingly, Nishida et al. showed that there are apparently infiltrating macrophages with opposing functions, once angiotensin II type 1 receptor (AGTR1$^+$) macrophages have an anti-fibrotic role. In fact, it was observed that AGTR1$^{-/-}$ animals have a more pronounced fibrosis (47). This suggests that there are diverse populations of macrophages that infiltrate the kidney, with pro- and anti-fibrotic capacities which could be related to the time the injury happens.

M1 macrophages are known to predominate during the onset of injury (48–50). They release pro-inflammatory cytokines that exacerbate the injury, amplify the inflammatory response and contribute to myofibroblasts proliferation and recruitment of fibrocytes (4, 32). M1 have been associated with the release of metalloproteinases that degrade ECM and promote EMT/EndoMT (51). It was shown that blocking MMP-9 or MMP-2 results in reduction of fibrosis in the UUO model of disease (52). Oppositely, Lutz et al., have demonstrated that inhibition of MMP-2 in chronic allograft nephropathy results in a more severe fibrosis (53), which suggests that MMPs are also important enzymes for the control of fibrosis and scarring area limitation. Macrophage secretion of MMP-9, MMP-12 and MMP-13 in the liver is related to ECM degradation and resolution of fibrosis (54, 55). Also, it has been identified a Ly6C^{low} macrophage population that secrete MMPs and have anti-fibrotic role in the liver (56). However, by transcriptional analysis, such macrophage population could not be classified as M1 or M2. In the liver, as in the kidney, macrophages have an important role in fibrosis progression. For example, there is strong evidence showing that Kupffer cells activate hepatic stellate cells to promote their transdifferentiation into myofibroblasts (57). These cells are the main source of ECM in the liver and they are responsible for the progression of cirrhosis.

When the acute phase of inflammation finishes, Th2 cytokines are produced to promote the polarization and recruitment of M2 macrophage (58). Added to this, apoptotic cells are recognized and phagocytosed by macrophages M1, an event that also promotes macrophage alternative activation (59). M2 macrophages are intended to create an anti-inflammatory environment and promote healing and regeneration of wounds. However, when the lesion is persistent, M2 macrophages take an important pro-fibrotic role and these cell population are known to secreting large amounts of pro-fibrotic factors such as TGF-β and Galactin-3 (60). The latter is a protein that is widely associated with cardiac fibrosis and atrial fibrillation (61). Preclinical studies have shown that infusion of recombinant galectin-3 activates cardiac fibroblast proliferation, leading to ventricular dysfunction (62). Furthermore, it has been observed that patients with paroxysmal atrial fibrillation have elevated levels of galectin-3 (63). Therefore, some authors believe that galectin-3 could act as heart failure and fibrosis biomarker. Furthermore, Braga et al. showed that in the absence of IL-4, mice underwent UUO are associated with improved parameters and decreased renal fibrosis (64).

M1 and M2 in the Context of Myofibroblasts Activation

There are growing evidences showing the relationship between macrophages and myofibroblasts activation during inflammation by different ways (50). M1 macrophages generate cytokines that activate myofibroblasts, either by the production of pro-inflammatory cytokines such as TNF-α and IL-1β, or chemokine production, such as CCL2 that assist in fibrocytes recruitment (65). Fibrocytes can migrate to the site of the inflammation through the expression of receptors as CCR2, CCR3, CCR7, and CXCR4 (37, 66).

M2 macrophages contribute to the control of inflammatory process through the release of IL10, arginase, TGF-β and HO-1, a process which promotes controlled wound healing and tissue regeneration (67, 68). However, the healing process depends on whether the initial insult persists or not (69). In this sense, if the insult persists, chronic activation of M2 leads to an opposite effect. M2 can activate resident fibroblasts through the release of TGF-β, PDGF, VEGF, IGF-1, and Galactin-3 (50, 57, 70). This evidence demonstrates that the exacerbation of fibrosis could depend on the type of macrophage polarization and persistence of the inflammatory insult, as shown in **Figure 2**.

Macrophage Metabolism Regulation in Fibrosis

Recently, a large amount of data has been coming in focus concerning metabolism and macrophage plasticity (71–73). We know M1 macrophages present an glycolytic cellular metabolism (74). It has been shown LPS, in an M1 polarization context, induces the transcriptional factor HIF-1α, which, in turn, transcriptionally couples glycolytic metabolism to macrophages' inflammatory and microbicidal programs (74). HIF-1α is stabilized by succinate, an effect that is inhibited by 2-deoxyglucose, a glycolytic pathway inhibitor (75). A metabolomic screen of LPS-stimulated macrophages revealed not only the expected activation of the Warburg effect but also an unexpected accumulation of intermediates of the tricyclic acid cycle, in particular succinate (75, 76). In M1 macrophages, it was also identified a metabolic break at the enzyme that converts isocitrate to α-ketoglutarate, providing mechanistic explanation for tricyclic acid cycle fragmentation (76).

On the other hand, M2 polarization was found to activate glutamine catabolism (76). Given that M2 macrophage activation and chronic diseases are energetically demanding, both in terms of intensity and duration, Vats et al., demonstrated that distinct substrates and pathways might meet the metabolic demands of M2 (77). Microarray analysis of M2 revealed that genes important in fatty acid oxidation were preferentially expressed in such cells. Metabolic studies further verified that M2 present increased mitochondrial amount and function. Accordingly, inhibition of

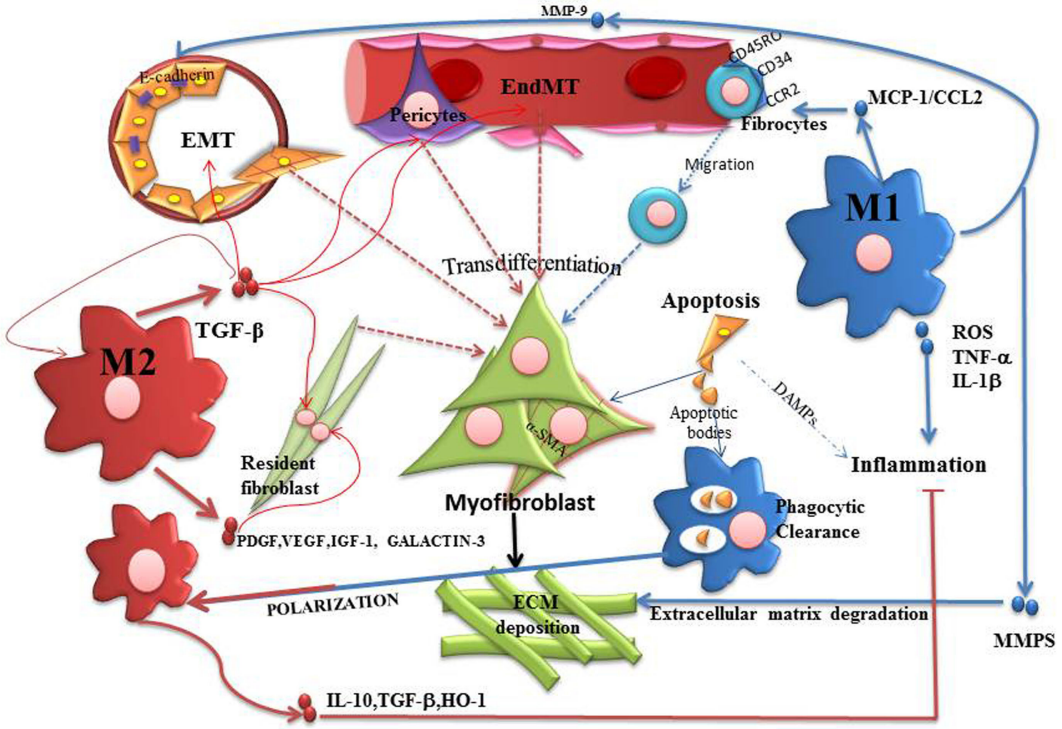

FIGURE 2 | Participation of M1 and M2 macrophages in the process of fibrosis. The activation of myofibroblasts is a physiological process generated in order to repair and restore tissue homeostasis. However, if the insult persists, fibrosis progress with proliferation of myofibroblasts and deposition of ECM which replaces functional tissue, leading to scar tissue formation. In this context, M1 macrophages represent the starting point of pro-fibrotic process. Therefore, M1 releases pro-inflammatory cytokines and chemokines that indirectly promote the proliferation of myofibroblasts. M1 can release CCL2, assisting in the recruitment of fibrocytes and also MMP-9, that promotes EMT/EndoMT. M1 has also been associated with an anti-fibrotic effect by releasing MMPs that degrade ECM. M2 macrophages can be generated by phagocytosis of apoptotic bodies or Th2 cytokines stimulation. M2 is initially anti-inflammatory cells through the release of IL10, arginase, TGFβ and HO-1. But when the damage persists, M2 activation leads to EMT/EndoMT as well as proliferation of fibrocytes due to the release of several growth factors. In this sense, macrophage modulation is the central axis of the exacerbation or control of fibrosis.

oxidative phosphorylation by metabolic inhibitors dramatically diminished the expression of M2 markers (77). It is also known that IL-4 and IL-13 induce oxidative metabolism by inhibiting mTOR, via activation of its upstream negative regulators TSC1 and TSC2 (78). Inhibition of mTOR can also lead to a decrease in HIF-1α levels, and therefore could result in reduced HIF-1α-dependent glycolytic and inflammatory gene expression (79).

There is a clear distinction in metabolism between macrophage subtypes, otherwise, the relevance of these observations and the implications for fibrosis are not fully understood. It is known pulmonary fibrosis development is related to mutations in maternally inherited mtDNA encoding for key genes of mitochondrial energy-generating oxidative phosphorylation, rather than Mendelian nuclear genetic principles (80, 81). Mitochondrial ROS are also responsible by death of alveolar epithelial cells in the context of fibrosis originated from fibrogenic dusts, such as asbestos and silica (82). Also, liver kinase B1 (*Lkb1*), an upstream regulator of fatty acid metabolism, has been implicated in chronic kidney disease (CKD) development (83). Loss of *Lkb1* impaired metabolic signaling and caused intracellular lipid accumulation, impaired fatty acid oxidation, and decreased glycolysis compared to control cells. Subcellular analyses of the mutant cells also identified a distorted mitochondrial structure, which negatively impacted upon cellular ATP content (83). Besides fatty acid, glucose metabolism has been implicated in CKD. High glucose concentrations may play important role in fibrosis development once leads to up-regulation expression of TGFβ, Smad3, Smad7, and CTGF (84).

However, much is expected in order to correlate macrophage metabolism and fibrosis formation. We still do not understand the scar formation in the context of drugs capable to modulate the metabolism in cells. It is known that chronic ethanol consumption disturbs several hepatic enzymes, including those related to cellular metabolism, such as PGC-1α (85), in a cirrhosis model of disease, meanwhile new studies in fibrotic models that do not are related to metabolites ingestions are needed.

CONCLUSION

Macrophages represent a heterogeneous cell population that can develop from different sources. M1 macrophages are associated with pro-inflammatory functions, and an exacerbation of tissue inflammation initiates the pro-fibrotic process (69). In this direction, M1 activates myofibroblasts through the release of MMPs

that promote EMT/EndoMT and fibrocytes recruitment through CCL2 secretion. On the other hand, M2 macrophages have anti-inflammatory properties due to the ability to secrete IL-10, arginase, TGFβ, and HO-1 (65, 68). In this point of view, M2 becomes friend of the tissue repairing. However, when the insult is not controlled and there is a persistent activity of M2 macrophages, these cells act as an enemy for tissue homeostasis. Excessive M2 macrophage activation leads to the continuous production TGFβ and growth factors that promote proliferation of myofibroblasts, activation of EMT/EndoMT and ECM deposition (34). In this scenario, M2 represents a break point between wound healing and exacerbation of pro-fibrotic process. Recently, much has been studied about macrophages metabolism. We know, for example, that pro-inflammatory cells present a glycolytic metabolism while anti-inflammatory ones are characterized by an oxidative metabolism. Otherwise, more studies are needed in order to identify macrophages components responsible by fibrosis triggering and different intervention manners in fibrotic process.

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REVIEW
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Macrophage and Innate Lymphoid Cell Interplay in the Genesis of Fibrosis

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Fibrosis is a characteristic pathological feature of an array of chronic diseases, where development of fibrosis in tissue can lead to marked alterations in the architecture of the affected organs. As a result of this process of sustained attrition to organs, many diseases that involve fibrosis are often progressive conditions and have a poor long-term prognosis. Inflammation is often a prelude to fibrosis, with innate and adaptive immunity involved in both the initiation and regulation of the fibrotic process. In this review, we will focus on the emerging roles of the newly described innate lymphoid cells (ILCs) in the generation of fibrotic disease with an examination of the potential interplay between ILC and macrophages and the adaptive immune system.

Keywords: fibrosis, macrophages, innate lymphoid cells, Th2 cells, epithelial-derived cytokines

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INTRODUCTION

Fibrosis is a characteristic pathological feature of an array of chronic diseases. The development of fibrosis in distinct tissues and organs is associated with numerous conditions, for example, idiopathic pulmonary fibrosis (IPF), cystic fibrosis (CF), systemic sclerosis, non-alcoholic steatohepatitis (NASH), primary biliary cirrhosis, cancer, and atherosclerosis. In these diseases, the chronic development of fibrosis in tissue can lead to marked alterations in the architecture of the affected organs and subsequently cause defective organ function. As a result of this process of sustained attrition to organs, many diseases that involve fibrosis are often progressive conditions and have a poor long-term prognosis. Indeed, due to the limited understanding of the mechanisms underlying the generation of fibrosis and the heterogeneity of fibrotic disease, there is currently a paucity of effective treatment strategies, contributing to the poor prognosis. The processes that underlie fibrosis are a tightly controlled natural mechanism of repair; however, dysregulation in the wound healing mechanism can result in aberrant fibrosis. Inflammation is often a prelude to fibrosis, with innate and adaptive immunity involved in both the initiation and regulation of the fibrotic process. In different organs, the insult to distinct cells, for example, bronchial epithelial cells in the respiratory tract, can lead to cell damage and release of various mediators, such as damage-associated molecular patterns (DAMPs), as well as proinflammatory and profibrotic factors. The mediators released can, depending on prevailing stimuli and local cellular environment, initiate a cascade within the cellular milieu in a tissue that leads to the accumulation of extracellular matrix components (ECM), rich in fibrillar collagens, fibronectin, and hyaluronic acid culminating in the deposition of fibrous connective tissue (1, 2). In this review, we will focus on the development of pulmonary fibrosis and the emerging roles of the newly described innate lymphoid cells (ILCs) in the generation of fibrotic disease with an examination of the potential interplay between ILC and macrophages.

INFLAMMATION AND FIBROSIS

While chronic injury is a prominent factor in many fibrotic diseases, acute inflammatory reactions may also play an important role in the initiation of fibrosis. Using experimental models involving acute lung injury, such as bleomycin-induced pulmonary fibrosis, where cellular apoptosis and necrosis are the underlying causative mechanisms, acute inflammatory responses initiated via activation of DAMP signaling cascades, results in a profibrotic response. While most chronic fibrotic diseases have an underlying inflammatory cause in many cases, for example, IPF, the causative mechanisms are not fully understood. Indeed, IPF is not responsive to anti-inflammatory steroid treatment, conversely treatment appears to exacerbate disease (3). However, in certain fibrotic disorders where the inflammatory cause has been identified, the use of anti-inflammatory therapies, such as ibuprofen to reduce the symptoms of CF (4, 5), demonstrate the potential roles of inflammation in chronic fibrotic diseases.

A loss of membrane integrity of cells, through injury, apoptosis, or necrosis, results in uncontrolled release of cellular contents, some of which can act as DAMPs, initiating an inflammatory response to clear cellular debris and initiate wound healing. In addition, DAMPs can be further synthesized and released in response to local cellular damage. The receptors for DAMPs, the pattern recognition receptors, including the Toll-like receptor (TLR) family, can in addition to recognizing pathogen-associated molecular patterns (PAMPs) identify fragments of ECM, such as hyaluronic acid and fibrinogen cleavage products (6). Indeed, effective danger signaling is implicated in the generation of fibrosis, with TLR2-, TLR3-, TLR4-, and TLR9-deficient animals demonstrating exacerbated collagen deposition in experimental disease models (7). The excessive synthesis and release of DAMPs underlies "sterile inflammation," with innate immune cells promoting inflammation in the absence of an active infection (8). Apoptotic and necrotic epithelial cells are a primary source of DAMPs, in particular ATP, IL-33, and uric acid that can initiate fibrosis (7). Release of uric acid, which crystallizes locally, can activate the NALP3 inflammasome in macrophages resulting in the release of IL-1β (9). Inflammasome activation leads to an increase in a number of other proinflammatory and profibrotic cytokines and chemokines, such as CXCL1, platelet-derived growth factor (PDGF), and transforming growth factor β1 (TGF-β1), linking innate immune activation and generation of fibrosis (10). Due to the requirement for inflammasome activation in the processing of IL-1β and IL-18 and the upregulation of other profibrotic mediators, inflammasome activation may play a critical role in wound healing; however, further investigation is required to address the potential for therapeutics targeting the inflammasome as beneficial in fibrotic disease.

THE EPITHELIAL BARRIER IN WOUND HEALING AND FIBROSIS

The epithelium serves as the initial defense against insult, providing both a physical and mechanical barrier, and is therefore a crucial interface to orchestrate both the innate and adaptive immune responses. Proinflammatory mediators released by damaged and dying epithelial cells, as well as recruited leukocytes, activate mesenchymal precursor cells in tissues and induce their trans-differentiation to ECM-producing myofibroblasts (1). The fibrosis cascade progresses following the insult to cells and subsequent release of mediators, such as IL-13, connective tissue growth factor (CTGF), and TGF-β, that operates downstream of initial cellular injury (6, 11). The mature epithelium in the lung is non-proliferative; however, in response to injury or inflammation, it is vital that the damage to the epithelium is repaired to ensure it remains an effective physical barrier. The signaling pathways activated in the process of repairing epithelial damage are similar to those initiated during development, with the dysregulation of these developmental pathways underlying the generation of fibrosis (12).

Transforming growth factor-β is the major profibrotic cytokine; it has central roles in promoting the activation and proliferation of fibroblasts, upregulates α-smooth muscle actin (α-SMA) and collagen I synthesis by myofibroblasts and promotes epithelial-to-mesenchymal transition (EMT) (6). The Wnt signaling pathway has also been implicated in EMT, with overexpression of the WNT-1 inducible signaling protein regulating the expression of profibrotic markers, such as MMP7 and plasminogen-activator inhibitor 1 (PAI-1), thus promoting EMT locally (6). CTGF is a matricellular protein, which can mediate the activities of a number of other profibrotic and angiogenic factors, such as TGF-β, bone morphogenic protein (BMP) 4, and vascular endothelial growth factor (VEGF) (13, 14). CTGF has been implicated in fibrosis in the liver, lung, skin, and kidney and acts synergistically with TGF-β to promote chronic fibrosis inducing ECM expression and collagen production by fibroblasts (6). Indeed, trials of antibodies targeting CTGF are currently ongoing in patients with IPF and liver fibrosis (15). Repetitive cycles of epithelial damage and repair are required for the generation of fibrosis (16, 17), with factors that damage the epithelium and initiate DAMPs and alarmin responses being actively pursued as potential therapeutic targets.

EPITHELIAL-DERIVED CYTOKINE MEDIATORS OF FIBROSIS

In addition to the "classic" profibrotic mediators, such as TGF-β and CTGF, recent research has focused on epithelial-derived type 2 cytokines as potential therapeutic targets for fibrosis. In response to epithelial cell injury, the alarmin cytokines IL-25, IL-33, and TSLP are released and are responsible for the initiation of a cascade of inflammatory responses. These cytokines have important roles in type 2 immunity, in particular in helminth infection and allergy (18). In the context of fibrosis, all three epithelial cell-derived cytokines have individually been shown to be involved in different aspects of fibrosis and are dysregulated in patients with fibrotic diseases (**Table 1**).

IL-25

IL-25, also known as IL-17E, is a member of the IL-17 family of cytokines and is secreted by many immune cells including activated Th2 cells, eosinophils, mast cells and macrophages, in addition to epithelial cells. IL-25 binds to IL-17RB, which forms a receptor complex with IL-17RA, activating the NF-κB pathway and

TABLE 1 | Expression of selected epithelial-derived cytokines in human fibrotic diseases.

Cytokine	Disease	Observation	Reference
IL-25	IPF	Increased IL-25 detected in BAL fluid of IPF patients' levels positively correlate with fibrotic marker periostin	Hams et al. (21)
	Asthma	Rhinovirus-induced IL-25 exacerbates asthma attacks	Beale et al. (25)
	Systemic sclerosis	Increased IL-25$^+$ cells in the skin of SSc patients	Lonati et al. (23)
IL-33	IPF	IL-33 is elevated in the lungs and BAL of IPF patients	Luzina et al. (34)
	Asthma	Increased IL-33 in the serum and sputum of patients with allergic asthma	Hamzaoui et al. (31) Guo et al. (32)
	Hepatitis	IL-33 is increased in the endothelial cells from livers of patients with hepatitis B, hepatitis C, and cirrhosis	Marvie et al. (33)
	Systemic sclerosis	Serum IL-33 is increased in SSC patients Serum IL-33 positively correlates with skin lesions	Yanaba et al. (30)
TSLP	Asthma	Bronchial and BAL expression of TSLP increased in asthmatics	Ying et al. (41)
		TSLP promotes airway remodeling in lung fibroblasts	Wu et al. (42)
	Systemic sclerosis	TSLP is upregulated in the skin of patients with SSc	Christmann et al. (43)

initiating Th2-mediated inflammation. IL-25 has been implicated in both experimental models of fibrosis and has been detected in samples from patients with chronic lung conditions and in the skin of patients with systemic sclerosis (19–23). Mice deficient in IL-25, or its functional receptor IL-17RB, show impaired collagen deposition in response to bleomycin-induced lung injury or *S. mansoni* egg-induced granulomatous pulmonary inflammation (21). Furthermore, intranasal administration of IL-25 induces collagen deposition and TGF-β and CTGF expressions in the lungs (21, 22). IL-25 is also upregulated in asthma and has been shown to play a role in airway remodeling and angiogenesis both *in vitro* and in *in vivo* models (24, 25). Treatment with an anti-IL-17RB antibody, thereby blocking IL-25-mediated signaling, improves airway hyper-responsiveness in a mouse model of allergic lung inflammation (26, 27). The therapeutic benefits of inhibiting IL-25 in conditions, such as allergic lung inflammation, where airway remodeling is a key event, suggest that IL-25 is an important mediator of tissue regeneration and consequently fibrosis in conditions, such as asthma.

IL-25-dependent fibrosis elicited in the lungs has been attributed to a downstream pathway involving IL-25-mediated expansion of ILC2 within the lungs with subsequent induction of fibrosis via an IL-13-dependent mechanism (21). Further mechanistic studies have demonstrated that in addition to activating ILC2, IL-25 can also directly drive polarization of bone marrow-derived macrophages *in vitro* toward a type 2 phenotype, with increasing surface expression of M2 marker CD206, in synergy with coadministered IL-4 (28). In addition, IL-25 can directly bind to human pulmonary fibroblasts through its receptor IL-17RB and can promote proliferation and differentiation to a myofibroblastic phenotype (22). These data suggest that IL-25 is an important mediator of fibrosis with roles in human fibrotic disease and, as such, is an exciting therapeutic target.

IL-33

IL-33 is the functional ligand for the IL-1 receptor family member ST2 in a complex with IL-1R accessory protein (IL1RAP) (29). IL-33 is not normally secreted, instead it is found localized to heterochromatin in the nucleus; however, it is released upon cell damage as an alarmin. IL-33 and ST2 have been causally linked with fibrotic conditions, including Crohn's disease, pulmonary, and liver fibrosis (**Table 1**) (30–32). In mouse studies, *Il33*$^{-/-}$ and *Il1rl1*$^{-/-}$ mice demonstrate decreased collagen deposition in models of lung, liver, and intestinal fibrosis (33–37). Interestingly, only the full length but not the proteolytically cleaved mature IL-33 is implicated in the pathogenesis of the bleomycin-induced model of pulmonary fibrosis (34, 38). Mechanistically, IL-33 initiates a local inflammatory response through the recruitment and activation of type 2-associated effector cells including eosinophils, basophils, mast cells, and ILC2, resulting in the release of Th2 cytokines and activation of macrophages, thereby potentially contribution to the downstream development of fibrosis. Indeed, in the liver and lung, the profibrotic effects of IL-33 are closely linked with increased IL-13 production from ILC2 (35, 39, 40).

TSLP

TSLP is secreted predominantly by keratinocytes but is also found in the small airway and intestinal epithelium, and signals via a heterodimeric receptor comprising one chain of IL-7Rα and one chain of TSLPR. TSLP has also been implicated in several models of fibrosis [**Table 1** (41–43)], with diminished pulmonary and skin fibrosis in mice deficient in the receptor for TSLP (44, 45).

While it is evident that these epithelial alarmin cytokines individually contribute to the generation of fibrosis, there is overlap and functional redundancy in IL-25, IL-33, and TSLP potentially due to the ability of all three cytokines to activate ILC2, as reported by Locksley and colleagues, with respect to chitin-elicited pulmonary inflammation (46). However, this apparent redundancy may be due to different ligand and receptor expression at different anatomical sites and a hierarchy of action at each tissue, although this speculation would need experimental clarification.

INNATE LYMPHOID CELLS

Innate lymphoid cells are a recently described group of innate cells of a lymphoid lineage that do not express antigen-specific receptors. These cells have important roles in the innate response, regulation of homeostasis and inflammation, and interplay

with adaptive immunity. While relatively rare in the systemic circulation in comparison to other hematopoietic cells, ILCs are enriched at epithelial barrier surfaces and act as regulators of chronic inflammation and tissue remodeling, acting to bridge innate and adaptive immunities.

Mature ILCs can be identified by a lack of markers associated with cells of a lymphoid lineage; however, they share expression of Thy1, the common gamma chain (γc), and IL-7Rα (47). ILC develops from common lymphoid progenitors (CLPs) in the fetal liver and adult bone marrow, relying upon the transcription factors' inhibitor of DNA binding 2 (Id2), nuclear factor interleukin-3 regulated (NFIL3), promyelocytic leukemia zinc finger protein (PLZF), and thymocyte selection-associated mobility group box (Tox) (47–52). Expression of Id2 is essential for the development of ILCs; however, PLZF is only transiently expressed in the early ILC precursor populations, with levels barely detectable in mature ILCs, suggesting that its importance in the early development of ILCs (49). Expression of NFIL3 and Tox is detected earlier than Id2 in the development cascade of ILC; however, these transcription factors do not appear to be as critical as Id2 for ILC development, with only minimal effects observed in the ILC repertoire in mice deficient in either NFIL3 or Tox (48, 51). These precursor cells differentiate to NK precursors or common helper innate lymphoid precursors, which, under the influence of additional transcription factors and cytokines give rise to mature ILC subsets (**Figure 1**) (53, 54).

Innate lymphoid cells can be divided into distinct subsets based on the cytokines they produce and the transcription factors necessary for their development and function: group 1, which produces interferon (IFN) γ and includes NK cells; group 2, which produces Th2-associated cytokines; and group 3, which produces IL-17 and IL-22 (**Figure 1**). Expression of the transcription factors T-bet, GATA3, and RORγt is required for the development of ILC1, ILC2, and ILC3 respectively (**Figure 1**). While GATA3 is required for the maturation of all ILC subsets, it is expressed at much higher levels in ILC2. The transcription factors RORα and Bcl11b are also required for effective function of ILC2, deficiency in either RORα or Bcl11b diminishes the generation of mature ILC2 (55–58). Expression of the aryl hydrocarbon receptor (Ahr) appears critical for ILC3 function, with reduced IL-22 production and decreased presence of ILC3 in the intestines of *Ahr*-deficient mice (59). There is some plasticity between ILC subsets, ILC3 can downregulate RORγt expression, allowing T-bet to become the prominent transcription factor, and ILC3 cells can take on a more ILC1 phenotype associated with increased IFNγ expression (47). A recent study has also demonstrated that $CD14^+$ DCs in the intestine of Crohn's disease patients promote polarization of ILC3 to $CD127^+$ ILC1 (60). An IL-25-elicited ILC2 population also has been detected, which has been shown to transition to produce IL-17 (61). Furthermore, in the absence of the T cell-associated transcription factor Bcl11b in ILC2, there is an increase in the expression of RORγt, and the cells take on an ILC3-like phenotype (57, 58, 62). While each ILC subset has unique roles in host defense and development, the plasticity between groups suggests that ILC subtypes may change depending on the tissue environment.

Innate lymphoid cells play an important role in orchestrating acute inflammation in response to infection and also chronic inflammation and wound healing. While ILC2 is commonly associated with chronic tissue inflammation and fibrosis, ILC1 has not yet been formally implicated in the pathogenesis of fibrosis, while ILC3 is also associated with the development of fibrosis and is elevated in the bronchoalveloar lavage (BAL) fluid of asthma patients (63, 64). ILC3 is an important source of IL-17, which may mechanistically underlie a role for ILC3 in fibrosis. IL-17A has been implicated in the generation of fibrosis, with elevated levels detected in patients with IPF and CF (65, 66). Furthermore, IL-17A has a critical role in the generation of bleomycin-induced pulmonary fibrosis, which is dependent on TGF-β, suggesting codependent roles for IL-17A and TGF-β in the pathogenesis of fibrosis (65). Therefore, as a source of IL-17 in mucosal tissues, ILC3 may represent an important cell subset in the progression of IL-17-mediated fibrosis. The relative roles of ILC subsets may have further implications in the pathogenesis of lung inflammation. Indeed, a recent study has identified both Th2-high and Th17-high clusters of asthma patients, which are inversely correlated (67). Experimental models have shown that therapeutically targeting one cluster promotes the other subtype and that combination therapy may prove more effective (67). This study clearly demonstrates the interplay between Th2-cytokine-producing cells and IL-17-producing cells and the potential implications for inflammatory and fibrotic diseases.

TYPE 2 INNATE LYMPHOID CELLS, CHRONIC TISSUE INFLAMMATION, AND FIBROSIS

ILC2 is characterized by their ability to produce the Th2 cytokines IL-4, IL-5, IL-9, IL-13, and amphiregulin (**Figure 1**) (68–71). They rely upon the transcription factors GATA3 and RORα for their development and the cytokines IL-25 and IL-33 for their maturation and recruitment (55, 69, 72). Recently, it has been reported that ILC2 can be further classified into two distinct subtypes: the IL-33-elicited $Lin^-T1/ST2^+$ "natural ILC2" (nILC2) and the IL-25-elicted Lin^-KLRG1^{hi} "inflammatory ILC2" (iILC2) (61). While ILC2 has been implicated in the pathogenesis of fibrosis, the relative functions of nILC2 and iILC2 with regards to inflammation, tissue repair, and fibrosis has yet to be fully elucidated.

ILC2 is implicated in the effective resolution of helminth infection, and in the development of allergic inflammation (73). Furthermore, ILC2 has been shown to play an important role in wound healing, tissue repair, and consequently chronic tissue inflammation and fibrosis (74). Studies have demonstrated that while the pathogenesis of ILC2 in fibrosis is associated with IL-13 release (21, 56), ILC2-mediated wound healing and tissue regeneration in the lung are promoted by release of amphiregulin by ILC2 (70, 71). ILC2 is associated with tissue fibrosis in experimental models, and dysregulated ILC2 responses have been detected in samples from patients with chronic inflammatory diseases, including IPF, atopic dermatitis, chronic rhinosinusitis, and asthma (21, 75–78). Furthermore, depletion of ILC2 in experimental models of fibrosis attenuates collagen deposition; conversely, transfer of ILC2 can induce tissue collagen deposition (21, 39).

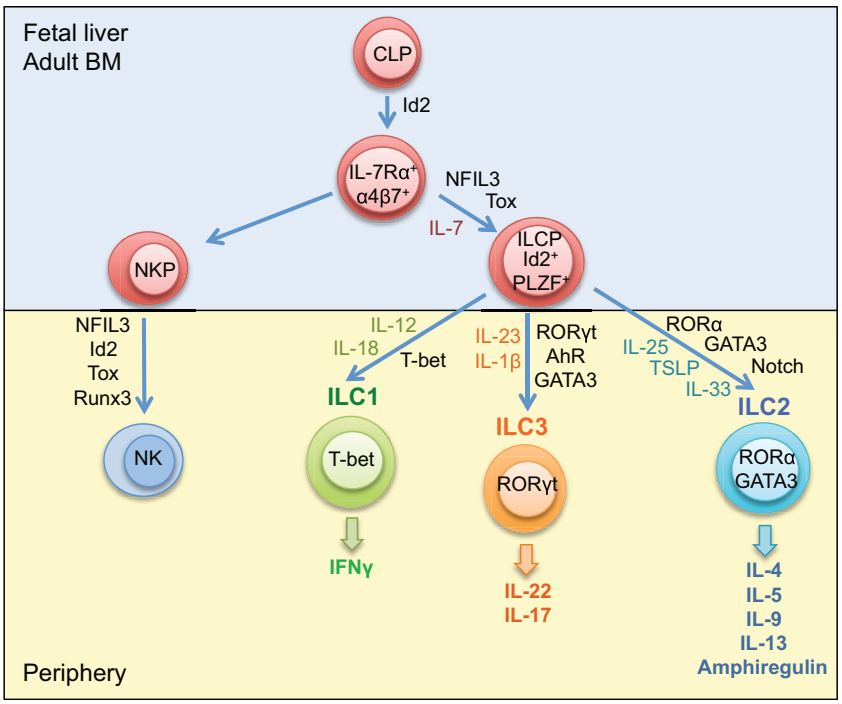

FIGURE 1 | Development of innate lymphoid cells. Innate lymphoid cells differentiate from common lymphoid progenitors in the fetal liver or adult bone marrow. The ILC precursor develops from CLP under the influence of the transcription factors Id2, PLZF, NFIL2, and Tox. ILC1, ILC2, and ILC3 differentiate from ILCP dependent on T-bet, RORα, and GATA3, and RORγt, respectively. Maturation and activation of ILC1 requires IL-12 and IL-18; ILC2 requires IL-25, IL-33, and TSLP and the influence of the Notch signaling pathway; ILC3 requires IL-23 and IL-1β and the additional influence of the transcription factor AhR. NK cells develop from NK precursors in the bone marrow under the influence of the transcription factors Id2, NFIL3, Tox, and Runx3. CLP, common lymphoid progenitor; ILCP, innate lymphoid cell progenitor; NKP, natural killer cell progenitor; Id2, inhibitor of DNA binding 2; PLZF, promyelocytic leukemia zinc finger protein; RORα, RAR-related orphan receptor α; AhR, aryl hydrocarbon receptor.

Increased localized expression of IL-25 and IL-33 is associated with expansion of ILC2 that may thereby promote tissue fibrosis through a number of mechanisms (**Figure 2**). ILC2-derived IL-5 can recruit and activate eosinophils, contributing to tissue inflammation (79). ILC2 can also enhance Th2 responses, either indirectly via IL-13-mediated DC priming or directly through major histocompatibility complex class II (MHCII) interaction with TCR on $CD4^+$ T cells (56, 80, 81). ILC2-derived IL-13 can activate macrophages toward a profibrotic phenotype and can also induce collagen deposition from fibroblasts (21). These studies clearly demonstrate an important pathogenic role for ILC2 in the generation of fibrosis. This suggests that targeting ILC2 and the associated signaling pathways offers the possibility for therapeutic exploitation.

TYPE 2 RESPONSES IN FIBROSIS

$CD4^+$ Th1 and Th2 cells and the cytokines they produce are important mediators in the inflammatory phase of fibrosis. While Th1-derived IFNγ inhibits fibrosis, the Th2 cytokines IL-4, IL-5, and IL-13 have been linked to a number of fibrotic conditions. Both IL-4 and IL-13 can promote polarization of macrophages to an alternatively activated profibrotic phenotype, recruit innate cells, such as basophils and eosinophils, and can directly act on fibroblasts to induce myofibroblast differentiation and collagen deposition (82, 83). Indeed, transgenic mice overexpressing IL-13 spontaneously develop tissue fibrosis with significant collagen deposition (84). IL-5 release by Th2 cells can also recruit and activate eosinophils, which are a potent source of the profibrotic cytokines TGF-β, PDGF, and IL-13 (85).

Studies using IL-4- and IL-13-deficient mice (*Il4*$^{-/-}$, *Il13*$^{-/-}$, *Il4ra*$^{-/-}$, and *Il-13ra1*$^{-/-}$) demonstrate a prominent role for IL-13 over IL-4 in the Th2-induced generation of fibrosis (86–89). Using IL-13-deficient mice, a profibrotic role for IL-13 was shown in *S. mansoni* egg-induced fibrosis in the livers of infected mice as well as in the lungs of egg-injected animals (87, 90). As reported first by Wynn and colleagues using soluble IL-13Ralpha2-Fc (86), a specific role for IL-13 in fibrosis was identified with anti-IL-13 antibodies now in clinical trials for fibrotic diseases (91). The functional receptors for IL-13, IL-4Rα, and IL-13Rα1 are expressed on fibroblasts, fibrocytes, and myofibroblasts (92). IL-13 can directly induce inhibition of the matrix metalloproteinase synthesis and can drive the differentiation of resident fibroblast and circulating fibrocytes to myofibroblasts, resulting in enhanced collagen deposition (83, 93, 94). These studies clearly demonstrate the importance of Th2 cells and specifically the associated cytokines, IL-4 and IL-13, in the pathogenesis of fibrosis.

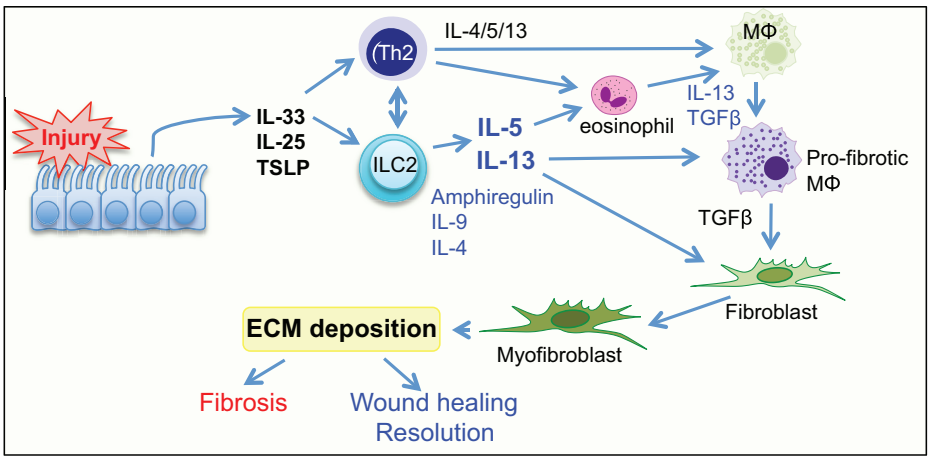

FIGURE 2 | Group 2 innate lymphoid cells have a central role in wound healing and fibrosis. Tissue injury initiates the release of the alarmin cytokines IL-25, IL-33, and TLSP from the epithelium. This activates ILC2 and Th2 cells to release the cytokines IL-4, IL-5, IL-13, also amphiregulin, and IL-9. The release of Th2 cytokines actively promotes activation of resident macrophages to a profibrotic phenotype, induces eosinophils to release the profibrotic cytokines IL-13, PDGF, and TGFβ, and can also directly influence differentiation of fibroblasts to myofibroblasts. TSLP, thymic stromal lymphopoetin; TGFβ, transforming growth factor β; ILC2, group 2 innate lymphoid cell; MΦ, macrophage.

Recent studies have identified crosstalk between the innate and adaptive immune responses as integral in the initiation and maintenance of type 2 immunity (**Figure 3**). ILC2 is able to activate Th2 cells via MHCII-mediated antigen presentation, whereas MHCII expressing ILC3 suppresses T cell activation due to the lack of costimulatory molecules (80, 95). Antigen-specific interaction between ILC2 and Th2 cells leads to the production of IL-4, IL-13, and also IL-2 by the Th2. Notably, Th2-derived IL-2 interacts with CD25 expressed on ILC2 activating ILC2 to release IL-13 (80). Furthermore, in addition to directly producing IL-13, ILC2 produces IL-5, which activates eosinophils, which are also potent producers of IL-13 and TGF-β (79). These cytokines are all able to activate recruited and resident macrophages to a profibrotic phenotype, as well as directly inducing trans-differentiation of fibroblasts. This interplay between innate ILC2 cells and adaptive $CD4^+$ T cells to induce macrophage activation and myofibroblast differentiation provides interesting mechanistic insight and identifies pathways that could potentially be exploited by novel therapeutics.

MACROPHAGES SUBTYPES, INFLAMMATION, AND FIBROSIS

Macrophages are phagocytic cells, which are integral in homeostasis, development, and immunity and are found in all tissues where they display distinct anatomical and functional diversity. A brief overview of the central role that macrophages play in fibrosis is provided, as there have recently been a series of comprehensive reviews focused on macrophages (1, 96–99). Resident macrophages regulate tissue homeostasis by responding to changes in the local environment. If required, circulating monocytes are recruited to the site of insult and activated to the desired phenotype or resident cells may proliferate locally in response to tissue injury (100). Macrophages can exist in many activation states dependent upon the inflammatory environment or stimulation used (98). Macrophages were commonly broadly divided into two subtypes: those associated with a type 1 response, termed "classically" activated or "M1," which are generally proinflammatory, and "alternatively" activated or "M2," which are typically associated with type 2 responses and wound healing. These two macrophage subtypes are defined experimentally by *in vitro* responses to IFNγ and the TLR4 agonist lipopolysaccharide (LPS) and the Th2 cytokines IL-4 and IL-13, respectively, with macrophages differentially generated having a unique gene profile and distinct functions. However, it is now accepted that the broad M1 versus M2 dichotomy terminology does not adequately describe the diverse phenotypes of macrophages. Therefore, newer and broader characterization of subtypes based on the activation of the macrophages under experimental conditions has been proposed (**Figure 4**) (98). Macrophages have a key role in the generation of fibrosis with distinct subtypes temporally activated and expanded in damaged tissue contributing to aspects of both the development of fibrosis and its subsequent resolution (97). Studies specifically depleting $CD11b^+F4/80^+$ macrophages, using *Cd11b*-DTR mice, have demonstrated that macrophages are crucial for the maintenance of type 2 immunity and also the associated generation of fibrosis (101, 102).

When tissues are damaged following infection or injury, circulating $Ly6C^+$ monocytes are recruited and differentiate into proinflammatory macrophages as they migrate through the affected tissue (103). Proinflammatory macrophages elicited via STAT1 in response to localized release of IFNγ or TLR agonists are a potent source of the cytokines tissue necrosis factor (TNF)-α, IL-6, IL-12, and IL-23, and reactive oxygen species (ROS), which act to kill invading pathogens (96). To counteract the damaging effects of macrophage-derived reactive oxygen and nitrogen species to the local tissue, macrophages undergo apoptosis or switch to an anti-inflammatory phenotype, which dampens the immune

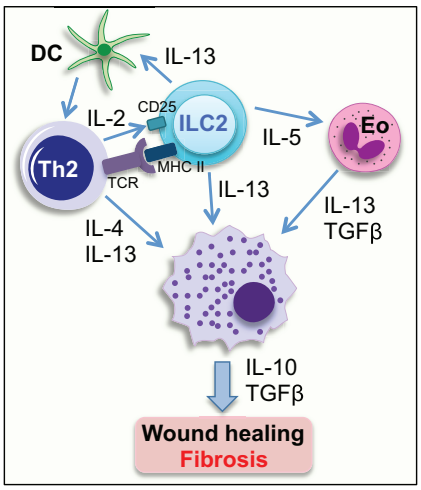

FIGURE 3 | Group 2 innate lymphoid cells can activate other cell types to initiate a wound-healing response. ILC2 can activate Th2 cells directly through interaction between MHC class II, expressed on the surface of ILC2, and the TCR on CD4+ Th2 cells, and indirectly via IL-13-mediated activation of DCs. IL-2 release from Th2 cells activates IL-13-producing ILC2s via interaction with CD25 expressed on ILC2. IL-5 release from ILC2 can activate eosinophils to release IL-13 and TGFβ. IL-4 and IL-13 released from activated Th2 cells, and also ILC2- and eosinophil-derived IL-13 can activate tissue resident and infiltrating macrophages to initiate a wound-healing response, which if excessive, can result in the generation of a fibrotic lesion. Eo, eosinophil; DC, dendritic cell; TCR, T cell receptor; MHCII, major histocompatibility complex class II.

response and facilitates tissue repair (96). If the causal insult is not removed, as is the case in a number of chronic inflammatory diseases, the resulting aberrant activation of macrophages can lead to fibrosis. Indeed, macrophages play a crucial role in the pathogenesis of most chronic fibrotic diseases.

Activation of macrophages by proinflammatory stimuli causes a metabolic switch from oxidative phosphorylation to glycolysis, similar to the Warburg effect originally identified in tumors (104, 105). This switch occurs in response to inflammatory stimuli, such as LPS and type I interferon, as well as hypoxic conditions and activation of hypoxia-inducible factor-1α (HIF-1α) (105). Indeed, the metabolic status of macrophages is closely linked to their function. Aerobic glycolysis is initiated upon activation of proinflammatory macrophages, increasing the uptake of glucose and attenuating the activities of the respiratory chain allowing for the generation of ROS, this provides the cell with a rapid release of energy essential for the removal of pathogens (106). Conversely, anti-inflammatory macrophages have a more sustained role requiring a slower release of energy and thus rely on fatty acid oxidation and oxidative metabolism (107). There is a clear distinction in metabolism between macrophage subtypes; however, the relevance of these observations and the implications for diseases, such as fibrotic disease, are, as yet, not fully understood.

The development of anti-inflammatory macrophages within a type 2 immune environment in response to IL-4 and IL-13 via STAT6 signaling has specific functions in wound repair and resolution (99). Macrophages elicited by IL-4 and IL-13 have a distinctive expression profile characterized by high expression of Arginase (Arg) 1, chitinase-like protein Ym1 and RELMα, and release of the chemokines CCL17, CCL22, and CCL24 (**Figure 4**). Macrophages can also be activated by IL-10, via STAT3, which results in autocrine production of IL-10; these macrophages are characterized by expression of IL-4Rα [**Figure 4** (108)]. Indeed, IL-4/IL-13-primed macrophages expressing Arg1 have been shown to inhibit IL-13-mediated fibrosis, via suppressing the activation of $CD4^+$ T cells and suppressing myofibroblasts by competing for arginase in the local environment (109, 110). Conversely, IL-13-elicited macrophages are also implicated in the pathogenesis of fibrosis (102). There is clearly a balance between the pro- and antifibrotic roles of macrophages in inflammation; however, IL-13-elicited profibrotic macrophages (PFMs) are associated with the release of TGF-β and are considered profibrotic in most chronic inflammatory diseases.

Distinct from the pro- and anti-inflammatory macrophage populations a $CD11b^{low}$ non-phagocytic macrophage population that does not express Arg1, termed resolution-promoting macrophages (Mres), has been identified in the lymphoid organs and adipose tissue (111). These macrophages appear to be antifibrotic and immune regulatory, secreting low levels of inflammatory cytokines and IL-10 and therefore may play an important role in the localized and systemic termination of an inflammatory response (112).

Recently, the epithelial-derived cytokines IL-25, IL-33, and TSLP discussed above have been shown to activate macrophages, both directly and indirectly, by promoting expansion of IL-13-expressing ILC2 (113–115). Indeed, IL-13 production from ILC2 and also eosinophils and Th2 cells has been shown to induce and maintain localized tissue macrophage activation both in the lung and in the adipose tissue (21, 115, 116). This interplay between ILC2 and Th2 cells in the maintenance of potentially PFMs at tissue sites could have implications in fibrotic disease.

Given the heterogeneity of macrophages (**Figure 4**), studies have focused on characterizing the PFM populations. These include IL-4-elicited proangiogenic PFMs that express a number of factors that are key mediators in the tissue repair process including TGF-β, PDGF, VEGF, as well as a number of matrix metalloproteinases (MMPs) (96). These factors contribute to the fibrotic cascade via recruitment of tissue fibroblasts, circulating fibrocytes and bone marrow-derived myofibroblasts, activation of resident myofibroblasts, and differentiation of epithelial cells into myofibroblasts through EMT. Indeed, in fibrotic tissue, macrophages localize in close proximity to myofibroblasts, suggesting the importance of macrophages and macrophage-derived mediators in the progression of fibrosis (2). Macrophages are clearly important regulators of wound healing and therefore also fibrosis. The heterogeneity in macrophage populations (**Figure 4**) highlights the extent of further mechanistic investigation needed to address the relative roles of macrophage populations in the fine balance between wound healing and fibrosis.

CONCLUSION

In this article, we have expanded on the potential roles of innate cells in fibrosis with a focus on the interplay between the epithelial-derived cytokines, ILC2, and macrophages. We have also explored

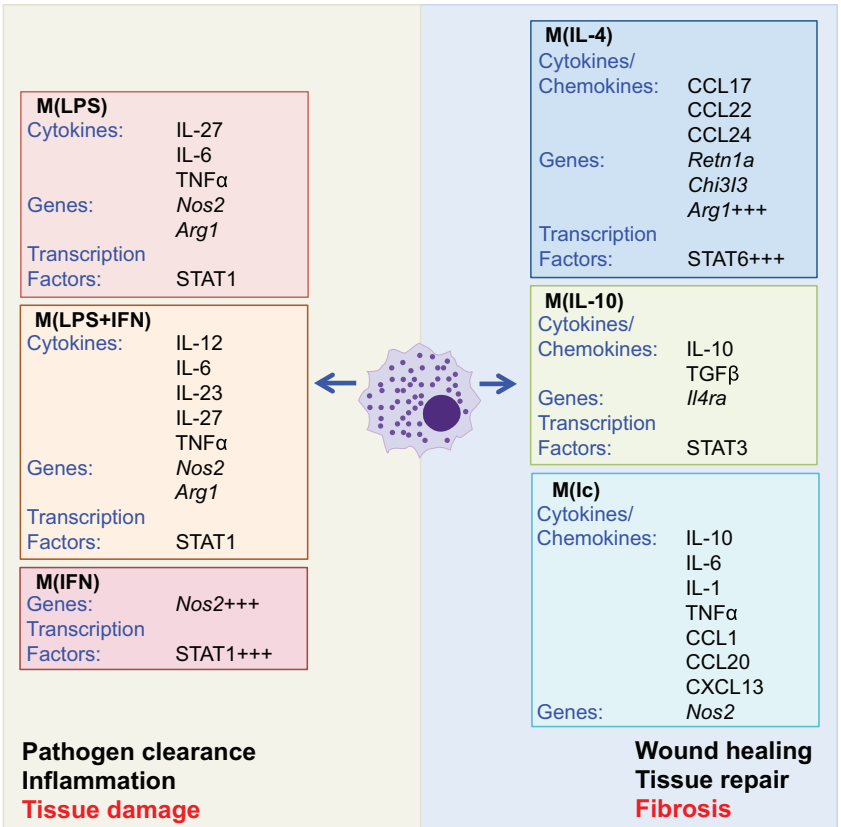

FIGURE 4 | Activated macrophage subtypes. Macrophage subtypes can be described by the experimental stimulus used to activate the cells. Although there are multiple macrophage subtypes, these can broadly be split into two groups associated with inflammation and pathogen clearance or wound healing and tissue repair. Exacerbated activation of both groups has the potential to cause pathological tissue damage or fibrosis (outlined in red text). Macrophages activated with lipopolysaccharide (LPS), interferon (IFN) γ, or a combination of the two (LPS + IFN) are associated with increased expression of Nos2 and production of proinflammatory cytokines. Conversely, activation with IL-4 and IL-10 increases expression of IL-10, TGFβ, and arginase (Arg1), while activation with immune complexes (Ic) gives a phenotype similar to that elicited by LPS. However, activation by IL-4, IL-10, and immune complexes is associated with tissue repair and wound healing (98).

the role of ILC2 in bridging the innate and adaptive immune system in the context of inflammation and fibrosis. Dysregulation of macrophages underlies a majority of inflammatory and fibrotic disease conditions, with a number of therapies targeting macrophages currently under development (97). While the relative roles of macrophages in the induction and resolution of fibrosis have been extensively studied, it is yet unclear whether distinct populations of macrophages control these disparate functions, or whether the phenotype of the local macrophages alters dependent on changes in the tissue microenvironment. Many mechanisms underlying fibrosis are common to multiple organs, which is important for the development of potential therapeutics (117). A key to developing effective therapeutics for tissue fibrosis is the identification of common pathways and, although further studies are needed, the epithelial cytokines and ILC2 axis interplay with macrophages is a promising area for therapeutic intervention.

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Leukotriene B4 as a potential therapeutic target for the treatment of metabolic disorders

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In the last decade, the incidence of metabolic disorders has increased drastically worldwide and is becoming a global health threat. Studies have shown that the pathogenesis and co-morbidities of diseases such as diabetes, gout, and atherosclerosis involve chronic low-grade inflammation and metabolic changes (1). As this inflammation is triggered by endogenous substances, instead of pathogens, it is called "sterile inflammation". Chronic low-grade inflammation can be triggered by the accumulation of metabolic products such as uric acid, glucose, cholesterol, and free circulating fatty acids. These substances can induce inflammation by two distinct mechanisms: (1) engagement of Toll-Like Receptors (TLR), such as TLR-2 (2), TLR-4 (3), and TLR-9 (4) and (2) activation of the intracellular receptor complex known as inflammasome that leads to caspase-1 activation, an enzyme that cleaves pro- interleukin (IL)-1β into its active form (5–7). IL-1β acts on its receptor IL1R1, a member of the TLR family whose activation is dependent on the presence of the adaptor molecule Myeloid Differentiation primary response gene 88 (MyD88). Although TLR-2 signaling is mediated mainly through the MyD88, TLR-4 activates MyD88-dependent and TIR-domain-containing adapter-inducing interferon β (TRIF)-dependent pathways. The MyD88-dependent pathway culminates in the activation of the Nuclear Factor kappa B (NFκB)/Activator Protein (AP) 1 and the TRIF-dependent pathway leads to delayed activation of NFκB associated with Interferon Regulatory Factor (IRF) (8). Thus, NFκB is a transcription factor of several genes involved in inflammation and also regulates its own transcription (9). In metabolic diseases with chronic low-grade inflammation, NFκB is continuously activated (10). Since NFκB can be activated through the adaptor molecule MyD88, modulation of its expression should have important consequences on the inflammatory response.

Leukotrienes are lipid mediators whose production is increased during inflammation. Activated phospholipase A2 releases arachidonic acid from membrane phospholipids. Liberated (soluble) arachidonic acid can be metabolized by 5-lipoxygenase (5-LO) to produce leukotrienes including LTB_4 and cysteinyl leukotrienes, LTC4, LTD4, and LTE4. It is well documented that leukotrienes are mediators of inflammatory events such as edema and leukocyte infiltration and activation and that they have an essential role in acute and chronic inflammatory diseases. Leukotrienes were also shown to mediate resistance to infections by several microorganisms (11). In macrophages, leukotrienes were shown to potentiate phagocytosis and microbicidal activity by affecting the mechanisms involved in actin polymerization and activation of NADPH oxidase, respectively (12).

LTB_4 binds to two distinct G protein-coupled receptors. The Leukotriene Receptor (BLT)1 is the high affinity receptor that induces inflammation, enhances cytokine production, phagocytosis, and mediates antimicrobial effector functions. Through BLT1, LTB_4 was shown to enhance MyD88 expression and potentiate MyD88-dependent stimuli responses while no difference on MyD88-independent stimuli was found (13). BLT2 binds LTB_4 with lower affinity and has

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been much less studied, currently no information is available on BLT2 in the context with metabolic syndrome. It was shown that LTB_4 through both, BLT2 and BLT1 receptors enhances NFκB activation (14).

It can be concluded that LTB_4, by increasing MyD88 expression, would potentiate a TLR/IL-1R dependent sterile inflammation. Considering that metabolic diseases involve sterile inflammation we propose that LTB_4 plays a central role in the development of metabolic diseases and may be considered a target for the development of new therapies. Here, we will highlight recent findings on LTB_4 involvement in Type 1 Diabetes (T1D), Type 2 Diabetes (T2D), and gout.

According to the World Health Organization, diabetes is a syndrome characterized by hyperglycemia with disturbances in protein, lipid, and carbohydrate metabolism due to a deficiency in insulin production (in T1D) or insulin resistance (in T2D). In T1D, both hyperglycemia and insulin deficiency can be responsible for the sterile inflammation (15, 16). We found that mice with T1D exhibited higher serum levels of IL-1β, TNF-α, and LTB_4. Macrophages from type 1 diabetic mice, compared to those from non-diabetics, expressed higher levels of MyD88 mRNA and produced higher levels of pro-inflammatory cytokines and nitric oxide, in response to MyD88-dependent stimuli such as LPS and IL-1β. Inhibition of LT synthesis restored MyD88 expression and cytokines production to similar levels found in macrophages from non-diabetic mice (15). Another important finding in this work was that pharmacologic or genetic inhibition of LTB4/BLT1 protected mice from succumbing to sepsis and this correlated with decreased macrophage MyD88 expression and decreased systemic inflammatory responses in the septic mice. This was an interesting finding because increased susceptibility to sepsis is a characteristic of diabetic patients (17).

In T2D, obesity is one of the largest risk factors for the development of insulin resistance (18, 19). It has been proposed that in obese people and in murine models of obesity, chronic sterile inflammation is triggered by free fatty acids (FFA), which engage MyD88-dependent receptors to produce IL-6 (20) and TNF-α (21). FFA can also activate the inflammasome and induce IL-1β production (7). Macrophages that infiltrate adipose tissue seem to play an essential role in insulin resistance. In diet-induced obesity, adipose tissue macrophages express an activated M1 phenotype (22–24). These results suggest that pro-inflammatory cytokines produced by macrophages have a local effect on adipocytes and a systemic effect on liver and muscle cells impairing insulin signaling.

In obese mice, increased uptake of omega-3-polyunsaturated fatty acids (ω-3-PUFA) led to enhanced insulin sensitivity. This correlated with decreased production of 5-LO products and increased generation of anti-inflammatory lipid mediators such as resolvins and protectins in the adipose tissue (25). Resolvins and protectins are mediators derived from ω-3-PUFA and are associated with the resolution phase of inflammation (26). Resolvin E1 can bind to BLT1, acting as a partial agonist to attenuate LTB_4-induced NFκB activation in polymorphonuclear leukocytes. The effect of resolvin E1 was comparable to that of the BLT1 antagonist, U-75302 (27). Together these results suggest a dominant role for LTB_4 through BLT1 in insulin resistance.

Recently, it was demonstrated that knockdown of the Ltb4r1 gene (the gene that transcribes BLT1) or inhibition of LTB_4 synthesis protected mice from diet-induced insulin resistance (10, 28, 29). In mice fed a high-fat diet, increased amounts of LTB_4 can be found in the white adipose tissue, liver, and muscle (29, 30). In obese animals, LTB_4 promotes NFκB p65 nuclear translocation and production of IL-6 and TNF-α in adipose tissue (10). Moreover, when NFκB activation is increased, LTB_4 could enhance pro-IL-1β expression for subsequent cleavage to the mature form via inflammasome activation.

Another possibility is that in skeletal muscle cells, adipocytes, and hepatocytes, LTB_4 by enhancing MyD88 expression and action would potentiate the IL-1R response, further impairing insulin signaling in insulin target organs. LTB_4 was also shown to decrease insulin signaling in hepatocytes through BLT1 by activating the NFκB pathway and up-regulating inhibitors of insulin pathways such as Phosphatase and Tensin homolog (PTEN) and Protein-Tyrosine Phosphatase 1B (PTP1B) (31). Thus, LTB_4 could promote insulin resistance by enhancing macrophage pro-inflammatory cytokine production, potentiating IL-1β action in insulin target organs and negatively affecting different components of insulin action. Therefore, LTB_4 is an essential mediator in the development of insulin resistance in T2D.

Retinal capillary degeneration is a hallmark of diabetic retinopathy, and there is evidence that LTB_4 is involved in this diabetes co-morbidity. This is supported by studies in animal models of diabetic retinopathy. 5-LO-deficient mice exhibited decreased leukocyte adherence to the vascular wall (the leukocyte subset was not assessed in this study), superoxide generation, NFκB expression and did not exhibit signs of capillary degeneration (32, 33). Both superoxide generation and NFκB expression can be induced by MyD88-dependent events (34). In humans, leukotriene precursor levels were increased in vitreous samples from patients with diabetic retinopathy compared with samples from non-diabetics (35). These results show that the 5-LO pathway is important for the development of diabetic retinopathy in humans.

In gout, joint deposition of monosodium urate (MU), a byproduct of purine degradation, is the disease etiological agent. MU is to activate macrophage NLRP3 leading to IL-1β and IL-18 secretion (caspase-1-dependent), IL-6, CXCL1 and CXCL2 production and inflammatory cell recruitment (36). It has been shown that LTB_4 is produced by macrophages stimulated with MU and in the knee-joint of mice injected with MU crystals. Amaral et al. showed that pharmacologic and genetic inhibition of LTB_4 production or BLT1 antagonism reduced MU-induced IL-1β and CXCL1 production and this correlated with neutrophil migration to the joint. Moreover, the injection of LTB_4 into the joint was sufficient to induce IL-1β production and neutrophil recruitment, suggesting an essential role for this lipid mediator in the pathogenesis of gout (37). In patients, LTB_4 in gouty effusion was found at a higher concentration that in synovial fluid from patients with rheumatoid arthritis or osteoarthritis (38).

In summary, involvement of LTB_4 on sterile inflammation in metabolic diseases is supported by the finding that inhibition of LTs synthesis or BLT1 antagonism: (a) reduced IL-1β and TNF-α serum levels in T1D (15) and MCP-1, IL-6, and TNF-α serum levels in T2D (29); (b) reduced the sterile inflammation in adipose

tissue in obese mice, more specifically the macrophage infiltration (28), pro-inflammatory cytokine production (10), and NFκB activation (10); reduced neutrophil migration and IL-1β production in a murine model of gout (37); prevented diet-induced insulin resistance and steatosis (28, 30), and reduced susceptibility to sepsis in T1D mice (15).

Evidence presented here led us to propose that LTB_4 has a central role in metabolic dysfunctions. By increasing MyD88 expression, LTB_4 enhances macrophage response to TLR/IL1 receptor agonists potentiating the sterile inflammation, a central event in metabolic disease progression. Furthermore, LTB_4 can amplify tissue injury by increasing reactive oxygen and nitrogen species that are known to mediate β-cell destruction, impairing insulin production. Although further studies are required, inhibition of the LTB4/BLT1 axis is a promising therapeutic strategy for the treatment of metabolic disorders. There is a 5-LO inhibitor already approved to treat asthma, and BLT1 antagonists are under development. Reduction in LTB_4 production or activity may reduce sterile inflammation and decrease disease severity.

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REVIEW
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Splenic macrophage subsets and their function during blood-borne infections

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The spleen is one of the major immunological sites for maintaining blood homeostasis. Previous studies showed that heterogeneous splenic macrophage populations contribute in complimentary ways to control blood-borne infections and induce effective immune responses. Marginal metallophilic macrophages (MMMΦs) and marginal zone macrophages (MZMΦs) are cells with great ability to internalize blood-borne pathogens such as virus or bacteria. Their localization adjacent to T- and B-cell-rich splenic areas favors the rapid contact between these macrophages and cells from adaptive immunity. Indeed, MMMΦs and MZMΦs are considered important bridges between innate and adaptive immunity. Although red pulp macrophages (RpMΦs) are mainly considered scavengers for senescent erythrocytes, several data indicate a role for RpMΦs in control of infections such as blood-stage malaria as well as in the induction of innate and adaptive immunity. Here, we review current data on how different macrophage subsets recognize and help eliminate blood-borne pathogens, and, in turn, how the inflammatory microenvironment in different phases of infection (acute, chronic, and after pathogen clearance) influences macrophage function and survival.

Keywords: spleen, macrophages, phagocytosis, pattern-recognition receptors, tissue remodeling

Introduction

Effective control of infections through the immune system is ensured by the well-organized structure of secondary lymphoid organs, which allow capture, processing, and presentation of antigens, ultimately leading to successful elimination of pathogens and induction of adaptive immunity. Among lymphoid organs, the spleen is particularly shaped for clearance of blood-borne pathogens. Microanatomically, the spleen is divided into the white pulp and the red pulp (Rp), separated by the marginal zone (MZ) [reviewed in Ref. (1)]. Rp and MZ have a complex macrophage (MΦ) network with distinct origins and functions in the immune response to infections. RpMΦs form a vast network inside the Rp and are characterized in mice by expression of F4/80^{high}CD68^{+}CD1 1b$^{low/-}$ and intense autofluorescence (2). In turn, inside the MZ, two populations of MΦs can be discerned. The MZMΦs typically express in their surface the C-type lectin SIGN-related 1 (SIGNR1) and a type I scavenger receptor called Macrophage Receptor with Collagenous structure (MARCO), which recognize non-opsonized molecules (3), mainly blood-borne antigens (4). Furthermore, marginal metallophilic MΦs (MMMΦs) are defined, among other molecules, by the expression of Sialic acid-binding Ig-like Lectin-1 (Siglec-1, Sialoadhesin, CD169) and MOMA-1 (5). A general scheme of the spleen structure is depicted in **Figure 1**.

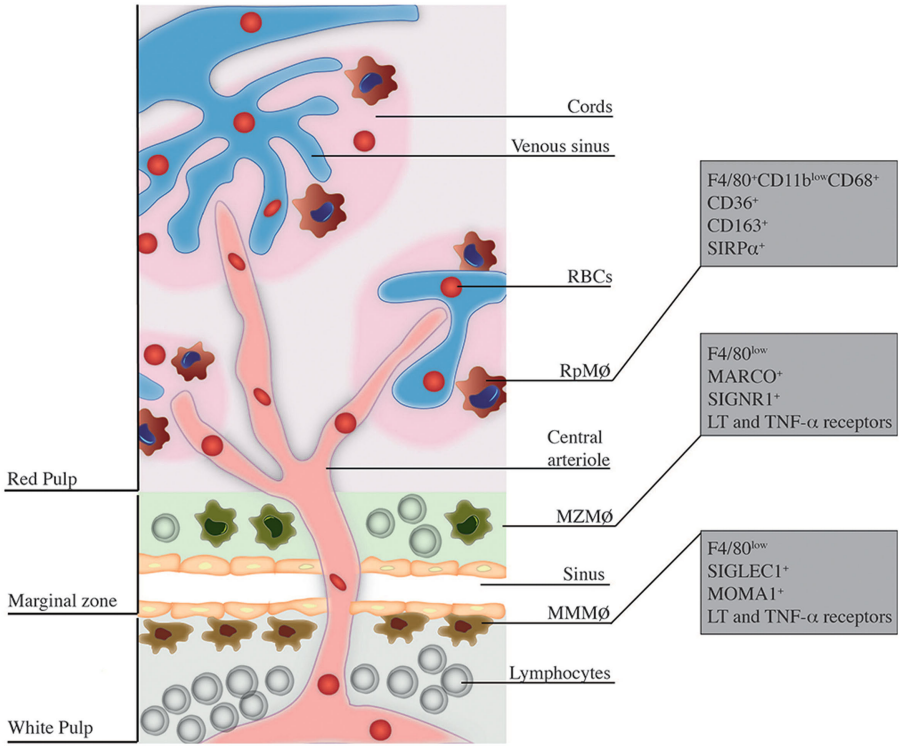

FIGURE 1 | Localization and phenotype of splenic MΦ subsets. This figure is a broad scheme of the positioning of RpMΦs, MZMΦs, and MMMΦs inside spleen and their respective phenotypic markers. RpMΦs (in red) are typically found within cords on the red pulp, allowing direct contact with RBCs and other blood cells/particles passing through venous sinuses. They are better defined by the concomitant expression of F4/80, CD11b (at low levels), and CD68 as well as other receptors that aid in their function. MZMΦs (in green) are found in the marginal zone (MZ) outer layer – they are also in direct contact with blood-borne particles. These cells express in their surface the molecules MARCO and SIGNR1 and other receptors that help in the uptake of blood-borne pathogens. Finally, the MMMΦs (in brown) reside within the inner layer of the MZ, in the contact with the white pulp. They are also specialized in blood-borne particle uptake and express surface markers such as SIGLEC-1 and MOMA-1.

Recent studies led to a growing understanding of the precise roles different splenic MΦs play to maintain blood homeostasis, particularly in infectious diseases, in which pathogen elimination depends on the development of appropriate adaptive immune response. In this review, we addressed the roles of each one of these MΦ subsets, with special focus on blood-borne infections. We described the current knowledge on the effects of splenic microarchitecture and microenvironment on these MΦs and reciprocal influence of these cells on spleen structure and functionality.

How Splenic MΦ Sense Pathogens and Damage-Associated Self-Molecules?

Splenic MΦs have two main protective activities during blood-borne infections. The first and most well characterized is phagocytosis and elimination of pathogens from circulation. However, beyond the task of eliminating blood-borne pathogens, splenic MΦs can play a prominent role in immune system activation. To properly execute these functions, they are provided with a large variety of pattern-recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). Engagement of Toll-like receptor (TLR) 4 by pathogen molecules, such as lipopolysaccharides (LPS) from Gram-negative bacteria is fundamental for the induction of a proinflammatory gene and protein expression signature in MΦs, which ultimately leads to innate immune activation (6). This also holds true for several other interactions such as TLR2 and/or TLR4 with glycosylphosphatidylinositol (GPI) anchors from *Trypanosoma* and *Plasmodium* parasites (7, 8) and TLR9 engagement by CpG motifs found in bacterial (9) and plasmodial DNA (10).

On the other hand, TLRs recognize DAMPs in situations of tissue injury. For example, heat shock proteins (HSPs) are endogenous damage signals (molecules released by cells under stress or necrotic cell death) and bind to TLR2 and TLR4 in MΦs, inducing these cells to produce proinflammatory cytokines and to express costimulatory molecules (11). Release of HSPs to circulation has been reported during sepsis (12) as well as production of HSP homologues by pathogens such as *Plasmodium* parasites (13). Also, TLRs – especially TLR2 and TLR4 – can recognize extracellular matrix components such as fibronectin (14). TLR4 engagement by fibronectin leads to MΦ activation in a similar fashion to what happens after LPS stimulation. Fibronectin is

presumably secreted by fibroblasts inside the spleen. Thus, this molecule may be produced during blood-borne infections such as malaria, where profound changes in splenic microarchitecture following acute infection occur, leading to the accumulation of fibroblasts inside the Rp (15). Expression of fibronectin-binding proteins (FnBPs) by *Staphylococcus aureus* is important to bacterial uptake by MΦs in inflammatory situations through binding of very late antigen 5 (VLA-5) (16). Therefore, it is reasonable to question whether TLR2 and/or TLR4 expressed in MΦs are engaged by fibronectin in those situations. Importantly, *S. aureus* FnBPs are crucial for the development of sepsis (16).

Another DAMP that can induce MΦ activation is the high mobility group box protein 1 (HMGB1), an intracellular DNA-binding protein involved in chromatin remodeling and transcription regulation (17). Extracellular HMGB1 binds to different endogenous ligands that are recognized by receptors such as TLR4, as well as the receptor for advanced glycation end products (RAGE) (18), and triggers inflammatory responses by the innate immune system. Release of HMGB1 by splenic MΦs occurs upon extensive splenic cell apoptosis, a feature commonly observed during sepsis. Indeed, HMGB1 is released into the extracellular milieu during sepsis and neutralization of this protein by monoclonal antibody treatment blocks sepsis development (19). Abundant splenic cell apoptosis is also typical in rodent malaria, at the peak of acute infection (20). In human malaria, endogenous HMGB1 serum levels are significantly higher in patients with severe disease compared to uncomplicated cases (21), suggesting that HMGB1 might also be involved in the development of immunopathology. Thus, it would not be surprising if acute immune response to *Plasmodium* and consequent immunopathology could be suppressed in great extent with neutralization of HMGB1.

Splenic MΦ receptors also include C-type lectin receptors (CLRs), such as dectin-1, mannose receptor, and dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN). CLRs have multiple functions in the immune system, including pathogen recognition and neutralization (22). Additionally, the liver synthesizes mannose-binding protein (MBP) during infectious diseases. This protein activates the complement system in order to form the membrane attack complex (MAC), and, more importantly in the spleen, to opsonize microorganisms such as virus (23) or protozoan parasites such as *Trypanosoma cruzi* (24). Scavenger receptors (SRs), such as SR-A1 and MARCO, are also expressed in splenic MΦs and likewise bind both self and pathogen molecules – more specificities of these receptors will be discussed later in this review. Among class B SRs, CD36 is known to mediate the uptake of oxidized low-density lipoprotein (oxLDL) and apoptotic cells, but also promotes phagocytosis of *S. aureus* bacteria by peritoneal MΦs (25). However, CD36 mediates cytoadherence of *Plasmodium*-infected red blood cells (iRBCs) to microvascular endothelium (26), a process supposed to avoid parasite clearance inside the spleen. The role of CD36 in recognizing this parasite by splenic MΦs still needs to be fully elucidated. Of note, RpMΦs express constitutively this molecule, which implies a possible role for this receptor in antiplasmodial immunity. This is a clear example of a receptor capable of mediating the recognition of both self and non-self molecules, implicating RpMΦs with both blood homeostasis and control of blood-borne infections.

Among cytoplasmic PRRs, splenic MΦs express molecules from the NOD-like receptor (NLR) family (27). For example, disturbance of cellular ionic gradient activates the pyrin subfamily member NLRP3, leading to inflammasome complex formation and in consequence to the release of inflammatory cytokines IL-1β and IL-18. Hemozoin, a disposal product formed from hemoglobin digestion by *Plasmodium* parasites, triggers the NLRP3 inflammasome in bone marrow-derived macrophages (BMDMs) (28), mediating the production of proinflammatory cytokines by these cells. Furthermore, the NLRP3 inflammasome is activated in mouse RpMΦs and human peripheral monocytes during acute malaria – although large amounts of IL-1β are only produced after stimulation with LPS (29). Interestingly, in mice, this process is mediated by the purinergic P2 × 7 receptor which recognizes extracellular ATP. ATP accumulates in *Plasmodium*-iRBCs and is released into the extracellular milieu through ion channels in the erythrocyte membrane or upon iRBC rupture (30).

Role of RpMΦs in Blood-Borne Infections

As stated previously, RpMΦs form a vast network inside the Rp, and although there is no consensus about the origin of RpMΦs, recent data indicate that these MΦs are maintained by local proliferation during physiological conditions (31). Conversely, in some pathological conditions, circulating monocytes are able to differentiate into RpMΦs (32). RpMΦ population comprises macrophage colony-stimulating factor (M-CSF)-dependent and M-CSF-independent cells (33). M-CSF-dependent RpMΦs are efficient phagocytes and produce proinflammatory cytokines such as TNF-α and type I IFNs and are highly responsive to prostaglandin E2 (PGE2). In contrast, M-CSF-independent BMDMs are less efficient phagocytes that produce high amounts of PGE2 (34). If this is a general feature of M-CSF-independent MΦ populations, M-CSF-independent RpMΦs might influence the activity of M-CSF-dependent RpMΦs.

Venous cords and sinuses render the splenic Rp bloodstream in a slow pace. This characteristic allows for the filtering function of the spleen and favors elimination of aberrant red blood cells (RBCs) or *Plasmodium*-iRBCs (35). Of note, development of RpMΦs relies on the expression of the transcription factor Spi-C, which is induced by free heme from RBC degradation (32). Thus, iron homeostasis – which conversely is controlled by RpMΦs – might play a role in RpMΦ development. Splenic structure also facilitates the control of numerous blood-borne infections by RpMΦs. For example, RpMΦs can recognize the capsular polysaccharide glucuronoxylomannan (GXM) from *Cryptococcus neoformans* and subsequently phagocytize the bacteria (36). RpMΦs can also eliminate *Streptococcus pneumoniae* under conditions of splenomegaly (37). However, these MΦs are permissive to intracellular growth of *Salmonella typhimurium* (38).

Red pulp macrophages have also been implicated in the control of blood-stage malaria (35). Nevertheless, in experimental *Plasmodium yoelii* infection, spleen remodeling facilitates iRBC

adherence to the vascular endothelium and, in consequence, allows parasites to escape from phagocytes (15). Interestingly, a proportion of Rp phagocytes exhibit strong labeling for F4/80 and CD11c, a phenotype shared by RpMΦs and DCs (39). This population participates in the early clearance of *Plasmodium chabaudi* parasites, but it sharply declines at the parasitemia peak. RpMΦs have a slow turnover rate and possibly undergo cell death after ingesting *Plasmodium*-iRBCs due to the toxic effects of hemozoin. RpMΦs, which are primarily required to maintain tissue homeostasis, might be substituted by inflammatory phagocytes as well as by MΦs derived from inflammatory monocytes. An alternative explanation is downregulation of the F4/80 molecule upon MΦ activation, as reported during mycobacterial infection (40).

Several mechanisms mediate RBC recognition and clearance by RpMΦs. One of the most studied mechanisms is the antibody binding to altered self components such as Band 3 clusters (41) or phosphatidylserine residues exposed in the outer leaflet of RBC membrane (42). In these cases, natural antibodies and complement system proteins opsonize RBCs though recognition of Band 3 clusters or phosphatidylserine residues. Another important interaction involved in RBC phagocytosis by RpMΦs is the ligation of CD47 to Signal Regulatory Protein alpha (SIRPα) (43). CD47 is a self-molecule important to avoid clearance by phagocytes, which is ubiquitously expressed on many cell types, including RBCs. CD47 expression on RBCs is an inhibitory signal for phagocytosis (44), but RBCs expressing a modified isoform of this molecule are phagocytized by RpMΦs through SIRPα binding (43). Interestingly, the conformation-dependent anti-CD47 antibody 2D3 binds sickle RBCs preferentially (45), which might explain the enhanced phagocytosis of sickle RBCs inside spleen. A recent study showed that *P. yoelii* parasites preferentially infect young RBCs expressing high levels of CD47 and, in consequence, escape from splenic clearance (46). Furthermore, enhanced resistance to *P. yoelii* observed in CD47-deficient mice is associated with a larger population of RpMΦs that ingest more iRBCs than wild-type counterparts. These findings explain why individuals with mild genetic RBC disorders (e.g., sickle cell trait and glucose-6-phosphate dehydrogenase deficiency) are protected from lethal malaria due to enhanced RBC phagocytosis.

Apart from being phagocytized by splenic MΦs, *Plasmodium*-iRBCs are also destroyed intravascularly as a consequence of plasma membrane damage upon release of free merozoites. Hemozoin, a disposal product formed from hemoglobin digestion by parasites, is released from lysed iRBCs. Furthermore, a massive destruction of non-infected RBCs occurs during blood-stage malaria, leading to increased hemoglobin levels in circulation [reviewed in Ref. (47)]. Another example of hemolysis induced by infections is observed in septicemia caused by *Escherichia coli*, which produces exotoxin α-hemolysin (Hlyα) (48). Evidencing RpMΦs crucial role in neutralizing toxic effects of hemoglobin, these MΦs have high levels of intracellular heme due to RBC phagocytosis (2) and of free hemoglobin through the scavenger receptor CD163 (49). The enzyme heme-oxygenase 1 (HO-1) plays an important role in degrading free heme, which in excess causes toxicity to MΦs (50). Importantly, RpMΦs are able to control pathogen burden through control of iron availability. For example, RpMΦs express the natural resistance associated macrophage protein-1 (NRAMP1) that is associated with protection against intraphagosomal pathogens, such as *Mycobacterium bovis* BCG, *Leishmania donovani*, or *S. typhimurium*. This molecule is a pH-dependent metal transporter localized in phagosomal compartments, which reduces intraphagosomal iron levels derived from RBC phagocytosis (51). NRAMP1 synthesis is upregulated in IFN-γ-activated MΦs (52), a condition likely to occur during acute blood-borne infections. RpMΦs also limit pathogen iron uptake through TLR-mediated release of lipocalin-2, which can form complexes with pathogen-secreted siderophores – molecules that help the collection of iron available for pathogens (53). RpMΦs involvement in controlling excessive immune responses is suggested by studies on autoimmune syndromes, while a similar participation in infectious diseases remains to be established. For instance, RpMΦs constitutively express peroxisome proliferator-activated receptor-γ (PPAR-γ), which might be important to curb excessive immune responses to pathogens, in a similar manner to PPAR-γ expressed on lung MΦs upon *S. pneumoniae* infection (54). RPMΦs can also prevent autoimmunity by producing anti-inflammatory cytokines such as TGF-β and IL-10 and by inducing generation of regulatory T (Treg) cells (55). Of note, there are many T cells scattered in Rp (55), and this population participates in acute immune responses to infections, such as blood-stage malaria (39). We present an illustrated scheme of the different roles of RpMΦs in homeostasis and disease in **Figure 2**.

MZMΦs and MMMΦs Role in Blood-Borne Infections

Marginal zone macrophages and MMMΦs have unique characteristics that contribute to rapid phagocytosis of pathogens and other particles. Thus, these MΦs act like scavenger cells, developing pro- or anti-inflammatory responses depending on the nature of the interaction. MZMΦs express SIGNR1 that binds to yeasts and the yeast-derived particle zymosan (4), to bacteria such as *Mycobacterium tuberculosis* (56), *S. pneumoniae* (57), *E. coli*, and *S. typhimurium* (58), and to virus such as human immunodeficiency virus (HIV) (4). This receptor recognizes carbohydrate antigens from blood-borne pathogens and mediates their subsequent internalization into phagosomes (4). Although SIGNR1 in peritoneal MΦs cooperate with dectin-1 in zymosan uptake (59), these innate receptors colocalize poorly in MZMΦs (60). Similar to classical complement pathway activation, but independently of antibodies, SIGNR1 also binds C1q and assembles the complex C4bC2a or C3 convertase that catalyzes C3b opsonin formation (61). This mechanism was shown to provide resistance to intravenous *S. pneumoniae* infection.

Expression of the scavenger receptor MARCO is upregulated in different MΦ populations, especially in MZMΦs and MΦs in the medullary cord of lymph nodes (3). MARCO was firstly reported to bind and mediate uptake of Gram-negative bacteria and also to recognize oxLDL [reviewed in Ref. (62)]. The structure of MARCO is similar to that of the Scavenger Receptor A1 (SR-A1, CD204), which plays a role in bacteria and virus recognition (3). TLR-mediated activation of BMDMs stimulates

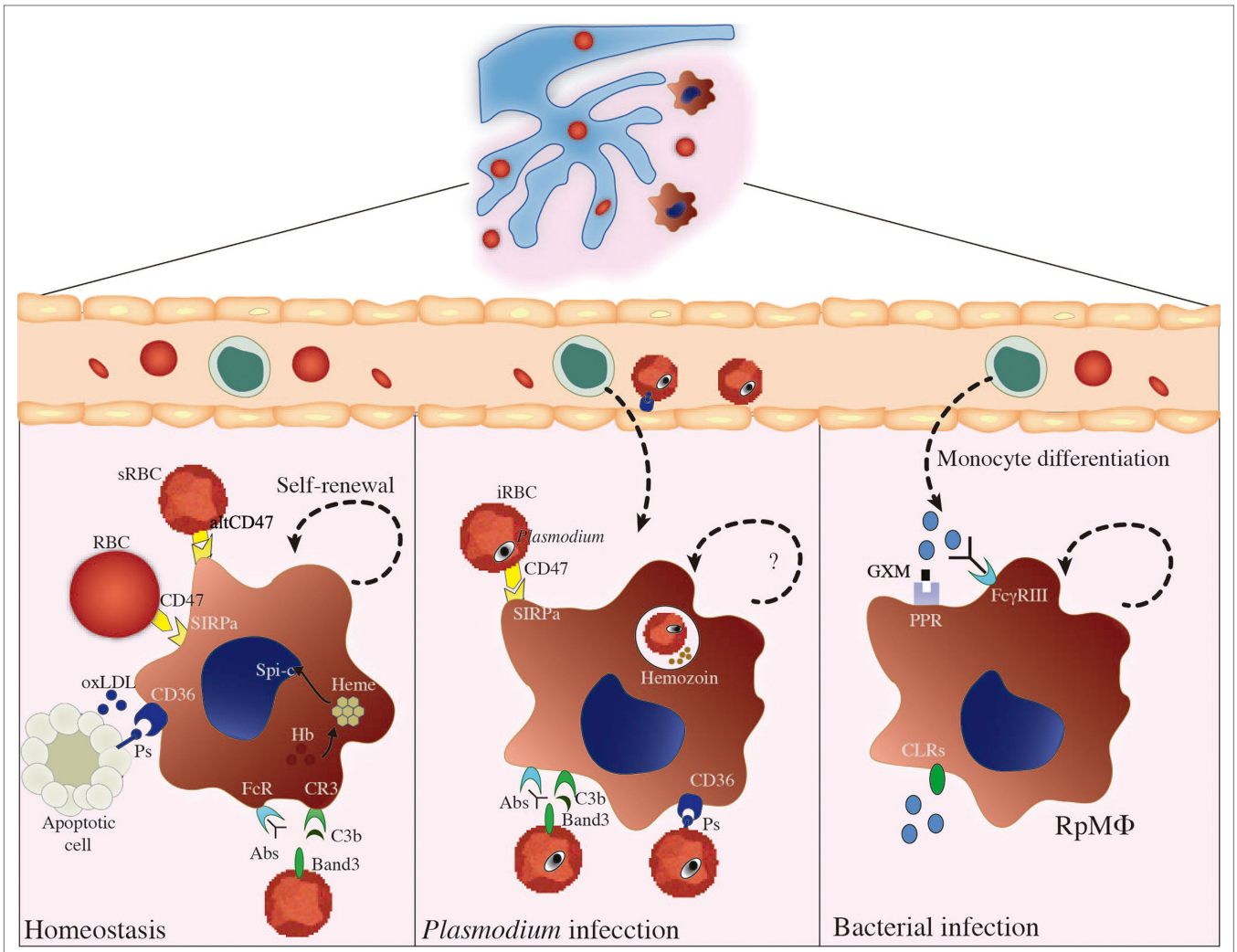

FIGURE 2 | RpMΦ biology during homeostasis and infection. This figure summarizes the different roles of RpMΦs in maintenance of host homeostasis and in the control of different infections. In the absence of infection (left), RpMΦs play important roles in the uptake of apoptotic cells, oxidized LDL (oxLDL), or senescent RBCs (sRBCs) from the circulations, through interaction with receptors such as SIRPα, CD36, CR3, or FcRs. CD47 expression on RBCs is an inhibitory signal for phagocytosis mediated by SIRPα, but sRBCs expressing a modified isoform of this molecule (altCD47) are phagocytized by RpMΦs. CD36 binds to phosphatidylserine (PS) and, alternatively, to oxLDL. RpMΦs are also important for iron homeostasis, and conversely, iron homeostasis seems to control RpMΦ development, through the action of free heme on Spi-C transcriptional factor. In these situations, RpMΦs have the ability of self-renewal by proliferation. Beyond the task of maintaining blood homeostasis, RpMΦs contribute to control blood-borne infections such as malaria (center) or bacterial infections (right) lead to changes in RpMΦ function. *Plasmodium*-infected RBCs (iRBCs) are recognized through the same receptors that recognize sRBCs, such as SIRPα, CR3, FcRs, or CD36, inferring a role for RpMΦs in parasite clearance. However, the adherence of iRBCs to microvascular endothelium through CD36 prevents iRBC clearance inside the spleen. Interestingly, *P. yoelii* parasites preferentially infect young RBCs expressing high levels of CD47 and, in consequence, escape from splenic clearance. RpMΦs also present with other receptors such as CLRs and PPRs, which in conjunct with FcγRIII contribute to recognition and elimination of bacteria from circulation. RpMΦs can recognize the capsular polysaccharide glucuronoxylomannan (GXM) from *Cryptococcus neoformans* and subsequently phagocytize the bacteria. The ability of RpMΦ renewal during infections, however, is poorly understood, and substitution of dead RpMΦs for monocyte-derived RpMΦs is presumable.

MARCO-mediated phagocytic activity (63). Furthermore, MARCO in MZMΦs directly binds and mediates phagocytosis of *E. coli* and *S. aureus* bacteria (3). TLR engagement leads to activation of transcriptional mechanisms that increase phagocytosis and cell activation, and MARCO seems to work in conjunct with TLRs in order to mediate pathogen control (64).

Marginal zone macrophages and MMMΦs are fundamental in the early control of *Listeria monocytogenes* bacteremia, as evaluated by depletion of these MΦs using a low dose of clodronate liposomes (65). T-cell responses are not affected in this experimental model, ruling out the participation of MZMΦs and MMMΦs as antigen-presenting cells. Similar findings were reported during infection with *Neisseria meningitidis* (64), thus it is likely that these MΦs have a direct role in the elimination of bacteria from circulation. Conversely, adenoviruses colocalize with MZMΦs as soon as a few minutes after intravenous injection in mice (66). MZMΦs and MMMΦs play a similar role in lymphocytic choriomeningitis virus (LCMV) infection, corroborating

the importance of these MΦs in first-line antiviral defense (67). On the other hand, localization of MZMΦs and MMMΦs in the interface between the bloodstream and lymphocyte-rich zones makes them suitable to bridge innate and adaptive immunity in several situations. For instance, mice lacking SRs MARCO and SR-A1 show a defective microarchitecture of the splenic MZ and an impaired T-independent type 2 response when challenged with pneumococcal polysaccharide (68). MMMΦs also collaborate in cytotoxic T-cell activation by transferring antigen directly to CD8α$^+$ DCs, which are specialized in cross-presentation to CD8$^+$ T cells (69). This observation supports the use of the MMMΦs antigen-concentrating capacity in therapeutic strategies for the development of antitumor immunity. The different roles of MZMΦs and MMMΦs in blood-borne infections are shown in **Figure 3**.

Reciprocal Influence of Splenic Microenvironment and MΦs

In several aspects, splenic MΦs shape splenic structure and/or microenvironment. The development of splenomegaly is typical in blood-borne infections, and it is characterized by profound changes in splenic microarchitecture, including remodeling of Rp (1). Given this, splenic MΦs are expected to play a prominent role in the recruitment of different cell types during acute immune responses. For example, RpMΦs recruit neutrophils into the spleen during early *Candida* infection by releasing CXCL1 and CXCL2, through autophagy-mediated depletion of the NF-κB inhibitor molecule A20 (70). Another example is the arresting of T cells inside the Rp during acute *Plasmodium* infection (39). RpMΦs may produce CXCR3- and/or CCR5-binding chemokines by a mechanism similar to that observed during early *Candida* infection – CXCR3 and CCR5 are the main upregulated chemokine receptors in splenic CD4$^+$ T cells during acute blood-stage malaria (71). However, splenic MΦs might also act on splenic microenvironment after an acute infection. For example, apoptotic cell uptake induces CCL22 production by MMMΦs, which in turn induces Foxp3$^+$ Tregs and DCs recruitment and accumulation, leading to a state of tolerance (72). Since the accumulation of apoptotic cells is a normal feature after acute blood-borne infections (20), a similar mechanism possibly takes place. RPMΦs can also prevent autoimmunity by producing anti-inflammatory cytokines such as TGF-β and IL-10 and by inducing the generation of Treg cells (55). These cytokines may be important – besides limiting autoimmunity – to curb an excessive immune response that could be dangerous to the host after pathogen clearance.

Conversely, the splenic structure and its microenvironment seem to play pivotal roles in MΦ homing and function. For instance, arrangement of sinusoidal endothelial cells inside Rp hampers the circulation of aging and/or iRBCs (1), facilitating their trapping inside Rp and posterior phagocytosis by RpMΦs. Importantly, the cytokine milieu in the microenvironment – which varies throughout an acute infection – may also dictate RpMΦ function. Classic M1 MΦs have an enhanced capacity to accumulate iron, which positively influences the maintenance of these cells in a pro-inflammatory state. On the other hand, alternative M2 MΦs have an increased ability to release iron, and increased iron availability in the microenvironment seemingly favors tissue remodeling [reviewed in Ref. (73)]. These effects can easily be associated with RpMΦs especially considering their role in iron uptake (1), thus it

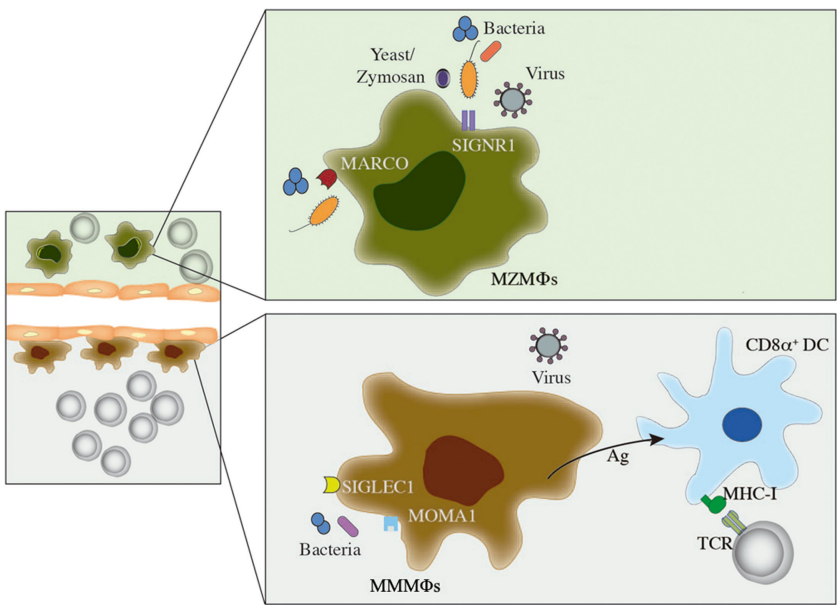

FIGURE 3 | Role of MZMΦs and MMMΦs during infection. In this figure, a brief description on how MZMΦs and MMMΦs are able to recognize and mediate protection against blood-borne pathogens is shown. MZMΦs (above) can recognize bacterial and viral infections by receptors such as MARCO or SIGNR1, which usually induce internalization and further pathogen degradation. A similar feature can be depicted for MMMΦs, where MOMA-1 or SIGLEC can mediate pathogen recognition and elimination from circulation. MMMΦs can also interact with CD8α$^+$ dendritic cells (DCs), which ultimately lead to CD8$^+$ T-cell activation.

TABLE 1 | Overview of splenic MΦ subsets.

MΦ type associated markers	Connection to immune response	Associated pathogens
RpMΦs		
F4/80$^{+/++}$ (2), CD11b^{low} (2), CD68^{+} (2), and SIRPα^{+} (43)	• Uptake of aging or apoptotic RBCs (2) • Limitation of autoimmunity (IL-10 and TGFβ in resolution of inflammation) (55) • Induction of Tregs by IL-10 production (55) • Phagocytosis of blood-borne pathogens (35–38) • Iron homeostasis (1, 50–53)	*Plasmodium* (35), *Cryptococcus neoformans* (36), *Streptococcus pneumoniae* (37), *Salmonella typhimurium* (38)
MZMΦs		
SIGNR1^{+} (3, 4), F4/80$^{+/-}$ (3, 4), MARCO^{+} (3, 4), lymphotoxin, and TNF receptors (75, 76)	• Clearance of modified LDL (1) • TI-2 B cell responses (68) • Phagocytosis of blood-borne pathogens (3, 4, 65, 67)	*Staphylococcus aureus* (3), *Listeria monocytogenes* (65), *Escherichia coli* (3), HIV (4), LCMV (67)
MMMΦs		
SigLec-1^{+} (CD169^{+}) (5), MOMA-1^{+} (5), F4/80$^{+/-}$ (5), lymphotoxin, and TNF receptors (75, 76)	• Indirect activation of CD8^{+} T cells (69) • Phagocytosis of blood-borne pathogens (67)	*Listeria monocytogenes* (65), LCMV (67)

A subdivision of splenic MΦs, detailing RPMΦs, MZMΦs, and MMMΦs associated markers, their connection to the systemic immune response, and associated pathogens. The respective references from each feature are detailed inside the table.

is possible that RpMΦs play distinct roles as M1- or M2-skewing microenvironments may occur during the beginning of an acute blood-borne infection or after pathogen clearance, respectively. Furthermore, the MZ contains a large number of resident cells that apparently depend on each other for their localization, thereby establishing and maintaining MZ integrity (1). For example, studies in which B cells were absent from the time of birth or in which they are depleted led to disappearance of MZMΦs and MMMΦs (74). Thus, the continuous interaction between resident and transmigrating cells inside the spleen MZ is crucial for efficient homing of MZMΦs and MMMΦs as well as for efficient removal and destruction of blood-borne pathogens by these cells. Lymphotoxin (LT) and TNF also influence the dynamics of MZMΦs and MMMΦs. *L. donovani*-infected mice have profound changes in the splenic MZ including loss of MZMΦs, which depend on TNF signaling that may increase MZMΦs susceptibility to parasite-induced cell death (75). These changes block lymphocyte traffic in the white pulp, impairing the development of an appropriate adaptive immune response. In another case, MZ B cells secrete LT-α1β2, and this leads to induction of a range of chemokines that could, in turn, influence lodging and retention of MZMΦs (76).

Concluding Remarks

As discussed above, splenic MΦs (RpMΦs, MZMΦs, and MMMΦs) play important roles in the control of blood-borne infections and shape several aspects of innate and adaptive immune responses (**Table 1**). Thus, a clear concept on the nature of splenic MΦ populations can be drawn, in which their interplay with the splenic microenvironment guarantees efficient control of blood-borne pathogens and maintenance of homeostasis following these infections. At the same time, the splenic structure is likely fundamental for proper localization and function of these MΦs. However, several questions on the nature and function of these cells are still unanswered, especially on (a) the development of splenic MΦs during embryogenesis, (b) the exact signals required for the homeostatic maintenance of these cells, and (c) the extent of how important each of these subsets are for the development of immunity against blood-borne infections. The development of mouse models to accurately study the distinct roles of RpMΦs, MZMΦs, and MMMΦs as well as the development of more detailed studies on signaling pathways and epigenetic modifications on genes involved in the function of these cells will be of great utility to solve these questions.

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Mitochondrial DNA Activates the NLRP3 Inflammasome and Predisposes to Type 1 Diabetes in Murine Model

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Although a correlation between polymorphisms of NOD-like receptor family-pyrin domain containing 3 (NLRP3) and predisposition to type 1 diabetes (T1D) has been identified, the potential function and activation of the NLRP3 inflammasome in T1D have not been clarified. The present study shows that non-obese diabetic mice exhibited increased NLRP3, and pro-IL-1β gene expression in pancreatic lymph nodes (PLNs). Similar increases in gene expression of NLRP3, apoptosis associated speck like protein (ASC) and pro-IL-1β were induced by multiple low doses of streptozotocin (STZ) in C57BL/6 mice. In addition, diabetic C57BL/6 mice also exhibited increased IL-1β protein expression in the pancreatic tissue at day 7, which remained elevated until day 15. Diabetic mice also showed increased positive caspase-1 macrophages in the PLNs, which were decreased in NLRP3$^{-/-}$ mice, but not in ASC$^{-/-}$ mice, after STZ treatment. NLRP3- and IL-1R-deficient mice, but not ASC-deficient mice, showed reduced incidence of diabetes, less insulitis, lower hyperglycemia, and normal insulin levels compared to wild-type (WT) diabetic mice. Notably, these mice also displayed a decrease in IL-17-producing CD4 and CD8 T cells (Th17 and Tc17) and IFN-γ-producing CD4 and CD8 T cells (Th1 and Tc1) in the PLNs. Following STZ treatment to induce T1D, NLRP3-deficient mice also exhibited an increase in myeloid-derived suppressor cell and mast cell numbers in the PLNs along with a significant increase in IL-6, IL-10, and IL-4 expression in the pancreatic tissue. Interestingly, diabetic mice revealed increased circulating expression of genes related to mitochondrial DNA, such as cytochrome *b* and cytochrome *c*, but not NADH dehydrogenase subunit 6 (NADH). Mitochondrial DNA (mDNA) from diabetic mice, but not from non-diabetic mice, induced significant IL-1β production and caspase-1 activation by WT macrophages, which was reduced in NLRP3$^{-/-}$ macrophages. Finally, mDNA administration *in vivo* increased Th17/Tc17/Th1/Tc1 cells in

the PLNs and precipitated T1D onset, which was abolished in NLRP3$^{-/-}$ mice. Overall, our results demonstrate that mDNA-mediated NLRP3 activation triggers caspase-1-dependent IL-1β production and contributes to pathogenic cellular responses during the development of STZ-induced T1D.

Keywords: NLRP3 inflammasome, type 1 diabetes, cytokines, IL-17-producing CD4 T cells (Th17), IL-17-producing CD8 T cells (Tc17), IFNγ-producing CD4 T cells (Th1), IFNγ-producing CD8 T cells (Tc1)

INTRODUCTION

Type 1 diabetes (T1D) results from the autoimmune destruction of insulin-producing pancreatic β cells (1) in genetically predisposed individuals. It is currently known that both innate and adaptive immune responses play a role in the pathogenesis of the disease. Adaptive immunity has been studied thoroughly over the past few years with several therapies, such as anti-CD3 monoclonal antibody therapy (2), anti-CD20 (3), and antithymocyte globulin treatment, reaching clinical trials (4). However, whether the innate immune response triggers T1D remains poorly understood and controversial. In non-obese diabetic (NOD) mice, a deficiency of toll-like receptor (TLR) 2 (5) or the MyD88 adaptor molecule (6) correlated with protection from developing T1D, yet TLR2$^{-/-}$ (7) and MyD88$^{-/-}$ (8) mice are susceptible to T1D induced by multiple low doses of streptozotocin (MLD-STZ).

Although many studies in the literature on innate immunity focused on the TLRs in T1D, the contribution of nucleotide binding and oligomerization domains (NOD), such as nucleotide-binding domain-like receptor (NLR), in the development of T1D needs to be explored. The NOD-like receptor family-pyrin domain containing 3 (NLRP3) is a member of the NLR family. NLRP3 assembles a complex called the inflammasome through oligomerization with apoptosis-associated speck-like protein (ASC) in myeloid cells, such as dendritic cells (9) and macrophages (10). This process leads to the autocatalytic activation of caspase-1 (11), which in turn cleaves pro-IL-1β and pro-IL-18 into mature forms (12). The NLRP3 inflammasome is thought to play an important role as a defense mechanism against pathogens and damage signals called danger-associated molecular patterns (DAMPs), such as uric acid crystals, ATP, high-mobility group box 1, and the heat-shock proteins hsp70 and hsp90 (13).

Other activators, such as pore-forming toxins (14) and UV radiation, also activate the NLRP3 inflammasome by reducing intracellular K+ concentrations or by promoting cytosolic release of lysosomal cathepsins (13). Certain activators, such as ATP, are able to induce mitochondrial dysfunction and apoptosis, which results in the release of oxidized mitochondrial DNA (mDNA) into the cytosol then binds and activates the NLRP3 inflammasome (12). The role of the NLRP3 inflammasome in autoinflammatory diseases, such as type 2 diabetes (T2D) (15), and autoimmune diseases, such as experimental autoimmune encephalomyelitis (EAE) (16), has been recognized. In this context, inhibition of caspase-1 suppresses IL-17 production by CD4 T cells and γδ T cells and the induction of EAE, which suggests that IL-1β induces the Th17 responses in autoimmunity (17). In fact, IL-1β synergizes with IL-6 and IL-23 to trigger the expression of the IRF4 and RORγt transcription factors as well as driving the induction of Th17 cells (18).

Despite this evidence, little is known about the role of the NLRP3 inflammasome in T1D. Interestingly, an association study in Brazil identified two single-nucleotide polymorphisms in NLRP3 that are associated with T1D (19). A recent study has also demonstrated that NLRP3 deficiency plays a protective role against T1D by inhibiting chemokines and chemokine receptors involved in immune cell migration to pancreatic islets (20). However, the activator of the NLRP3 receptor in T1D and the precise immunological mechanisms related to T1D pathogenesis remain elusive. Our data demonstrate that mDNA isolated from diabetic mice displays an intrinsic capacity to activate the NLRP3 inflammasome in macrophages. Furthermore, increased expression of mDNA-related genes was detected in diabetic mice sera. Finally, mDNA administration causes IL-1β production associated with the induction of pathogenic Th17/Tc17/Th1/Tc1 responses in the pancreatic lymph nodes (PLNs), which results in STZ-induced T1D onset.

RESULTS

Diabetic Mice Have Upregulation of NLRP3 Inflammasome Gene Expression and IL-1β Production in PLNs and Pancreas

To investigate the role of the NLRP3 inflammasome in the pathogenesis of T1D, C57BL/6 wild-type (WT) male mice were inoculated intraperitoneally with MLD-STZ (40 mg/kg) for five consecutive days and were assessed for mRNA expression of NLRP3, ASC and pro-IL-β in the PLNs at 7 and 15 days after starting the STZ injections. Increased gene expression of the NLRP3, ASC, and pro-IL-1β genes was found in the PLNs of diabetic mice at 7 and 15 days after STZ treatment compared to non-diabetic mice treated with the vehicle (**Figures 1A–C**). We also observed a peak of protein IL-1β levels at day 7 after STZ-induced T1D, which decreased at day 15, but remained significantly elevated compared to non-diabetic mice (**Figure 1D**). In addition, we detected a significant increase of NLRP3 protein expression on day 7 using a Western blot, which remained increased at day 15 after STZ-induced T1D (**Figure 1E**). In agreement, we noted an increase of caspase-1-positive macrophage percentage at day 7, which slightly decreased at day 15 in the PLNs after STZ-induced T1D (**Figure 1F**). Notably, we observed a significant decline in the caspase-1-positive macrophage percentage in the PLNs of NLRP3-deficient mice, but not in ASC-deficient mice, not only

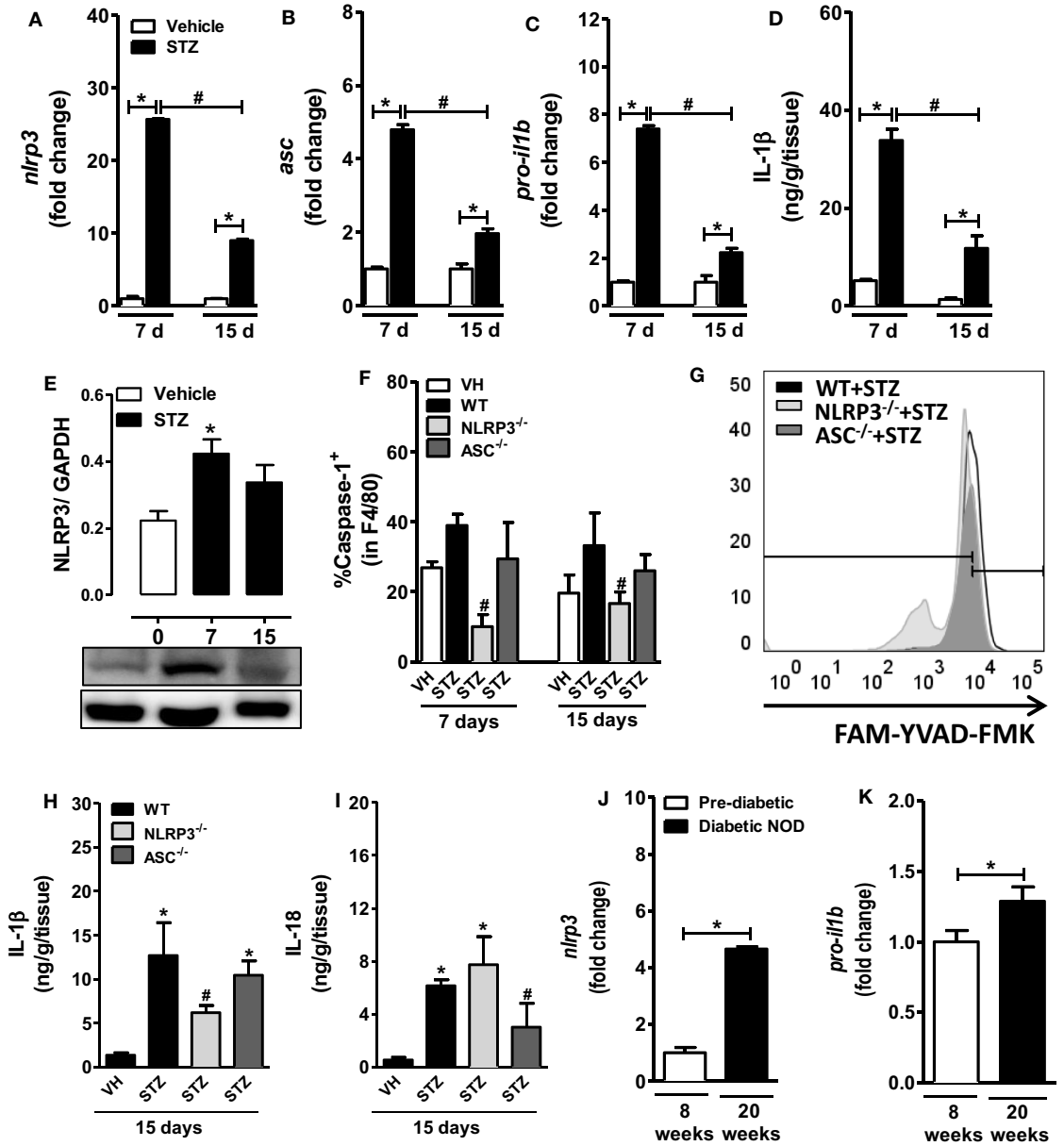

FIGURE 1 | NOD-like receptor family-pyrin domain containing 3 (NLRP3) inflammasome-related protein and gene expression profile during type 1 diabetes. Relative gene expression of NLRP3 **(A)**, apoptosis-associated speck-like protein (ASC) **(B)**, and pro-IL-1β **(C)** by RT-PCR in the pancreatic lymph nodes (PLNs) of streptozotocin (STZ)-induced diabetic or non-diabetic mice (vehicle). The time course of IL-1β production was determined in the pancreatic tissue by an ELISA assay **(D)**. The kinetics of NLRP3 expression was quantified 7 or 15 days after STZ by Western blotting **(E)**. The PLNs were removed, and caspase-1 activation was measured with a fluorescent cell-permeable probe that binds to activated caspase-1 (FLICA), after 7 and 15 days of STZ or vehicle administration **(F,G)**. The concentrations of IL-1β **(H)** and IL-18 **(I)** were determined in the pancreatic tissue by an ELISA assay. The relative gene expression of NLRP3 **(J)** and pro-IL-1β **(K)** was assessed by RT-PCR in the PLNs of prediabetic (8 weeks of age) and non-obese diabetic (NOD) mice (20 weeks of age). The results are expressed as the mean ± SEM ($n = 9$ in the vehicle-injected wild-type (WT) group; $n = 15$ in the STZ-administered WT group; $n = 15$ in the STZ-administered ASC$^{-/-}$ group; $n = 15$ in the STZ-administered NLRP3$^{-/-}$ group; $n = 18$ in the prediabetic group and $n = 12$ in the diabetic NOD group). $^{*}p \leq 0.05$ compared to the vehicle-injected WT group or prediabetic group, $^{\#}p \leq 0.05$ compared to the STZ-administered WT group. Significant differences between two groups were compared by Student's *t*-test or three or more groups by one-way ANOVA followed by Tukey's multiple-comparison test. The results are representative of a single experiment repeated three times.

on day 7 but also on day 15 compared to WT mice after STZ treatment (**Figures 1F,G**). Similarly, the IL-1β protein levels decreased significantly in the pancreatic tissue of NLRP3-deficient mice, but not in ASC-deficient mice after STZ treatment (**Figure 1H**). Conversely, the IL-18 protein levels were reduced significantly only in the pancreatic tissue of ASC-deficient mice compared to

WT mice after STZ treatment (**Figure 1I**). Additionally, genetically determined NOD mice displayed increased gene expression of NLRP3 and pro-IL-1β in the PLNs compared to prediabetic mice (**Figures 1J,K**). These findings demonstrated that NLRP3-dependent IL-1β expression and caspase-1 activation is induced in macrophages in PLNs during T1D.

IL-1R Signaling Confers Susceptibility to the Development of STZ-Induced T1D

Diabetic WT mice exhibited body weight loss compared to non-diabetic mice (**Figures 2A,E**). On the other hand, whereas caspase-1/11$^{-/-}$ mice maintained the body weight loss (**Figure 2A**), IL-1R$^{-/-}$ mice had a very minor body weight loss after STZ treatment (**Figure 2E**). Similarly, 66% of caspase-1/11$^{-/-}$ mice in comparison to 50% of IL-1R$^{-/-}$ mice developed T1D at day 15 after STZ treatment (**Figures 2B,F**). Additionally, blood glucose levels were significantly lower in IL-1R$^{-/-}$ mice (**Figure 2G**), but were not affected in the caspase-1/11$^{-/-}$ mice compared to WT mice (**Figure 2C**). Serum insulin levels increased in IL-1R$^{-/-}$ (**Figure 2H**), but not in caspase-1/11$^{-/-}$ mice (**Figure 2D**) compared to WT mice after 15 days of the STZ treatment, although the increase in IL-1R$^{-/-}$ animals was not significant. The pancreatic islets from non-diabetic WT mice appeared to be structurally normal with no leukocytic infiltration (**Figure 2I**). However, pancreatic islets of diabetic WT mice revealed invasive insulitis (**Figure 2J**), whereas a less extensive inflammatory infiltration was observed in the IL-1R$^{-/-}$ mice after STZ (**Figure 2K**). Moreover, immunostaining showed the islets of non-diabetic mice had a high number of β cells containing insulin (**Figure 2L**). The islets of diabetic WT mice had fewer β cells (**Figure 2M**), while those cells from the IL-1R$^{-/-}$ mice had many more β cells positive for insulin (**Figure 2N**). Collectively, these results suggest that IL-1R signaling contributes to pancreatic islet inflammation, which leads to insulin-producing β cell damage and development of T1D.

IL-1R Signaling Increases Pathogenic Th1/Th17/Tc17 Populations during STZ-Induced T1D

Despite the fact that there was no observable difference in the percentage of CD4$^+$IL-17$^+$ cells (Figure S1A in Supplementary Material), the absolute number of this population was significantly reduced in the PLNs of IL-1R$^{-/-}$ compared to diabetic WT mice after STZ treatment (Figure S1D in Supplementary Material). In addition, the percentage of CD8$^+$IL-17$^+$ cells did not differ among the several experimental groups (Figure S1B in Supplementary Material), but there was a significant decrease in the absolute number of this cell population in the PLNs of IL-1R$^{-/-}$ mice compared to diabetic WT mice (Figure S1E in Supplementary Material). The frequency and absolute numbers of CD4$^+$IFN-γ$^+$ cells in the PLNs were significantly reduced in IL-1R$^{-/-}$ mice compared to diabetic WT mice (Figures S1C,F in Supplementary Material). There was a significant increase in the protein IL-6 levels in the pancreatic tissue of IL-1R$^{-/-}$ mice compared to the diabetic WT mice (Figure S1G in Supplementary Material). Despite the lack of differences in expression levels, IL-17 decreased in IL-1R$^{-/-}$ mice compared to diabetic WT mice after STZ treatment (Figure S1H in Supplementary Material). Despite a trend toward increase, no differences in the IFN-γ or TNF-α expression between the experimental groups were observed (Figures S1I,J in Supplementary Material). In contrast, the IL-4 and IL-10 levels were significantly increased in the pancreatic tissues of IL-1R$^{-/-}$ mice compared to diabetic WT mice after STZ treatment (Figures S1K,L in Supplementary Material). These data support the idea that the IL-1R signaling pathway plays an important role in driving the Th17/Tc17/Th1 immune response after STZ induces T1D.

NLRP3 Activation Is Required for Insulitis and Development of STZ-Induced T1D

To further explore whether NLRP3 and ASC are involved in T1D onset, we used the MLD-STZ model in WT, NLRP3$^{-/-}$, and ASC$^{-/-}$ mice, and disease incidence was monitored. STZ-treated diabetic WT and ASC$^{-/-}$ mice had a cumulative disease incidence of 100%, while the NLRP3$^{-/-}$ mice developed resistance and had a reduction of disease incidence of 40% (**Figure 3A**). As expected, WT, NLRP3$^{-/-}$, and ASC$^{-/-}$ mice did not become hyperglycemic and had normal serum insulin levels after vehicle administration (**Figures 3B–D**). The WT and ASC$^{-/-}$ mice became hyperglycemic at day 15 after STZ treatment, whereas NLRP3$^{-/-}$ had a significant decrease of glycemia levels after STZ treatment (**Figures 3E,F**). Importantly, NLRP3$^{-/-}$ presented higher levels of insulin in the serum, whereas STZ-injected ASC$^{-/-}$ mice maintained unaltered insulin levels compared to WT after STZ administration (**Figure 3G**). Corroborating this observation, these mice displayed increased insulin immunohistochemistry staining in the pancreatic islets, whereas ASC$^{-/-}$ mice had similar staining to WT mice after STZ treatment (**Figures 3H,I**).

In STZ-administrated mice, we found the appearance of more invasive insulitis and reduction of insulin-positive β cells (**Figures 3J,L,O**). Later, we investigate whether the protection observed in NLRP3$^{-/-}$ mice against STZ-induced T1D could be attributed to a reduced pro-inflammatory response into the pancreatic islets. In fact, histological analysis showed that STZ-injected NLRP3$^{-/-}$ mice had milder inflammatory infiltration with less severe insulitis in the pancreatic islets and an increase of insulin-positive β cells (**Figures 3J,M,P**). Non-diabetic WT mice did not have moderate or severe insulitis and showed intense insulin-positive β cells in the pancreatic islets (**Figures 3J,K,N**). Taken together, our data indicate that an NLRP3-dependent mechanism is required for pancreatic islet inflammation, which results in insulin-producing β cell damage and T1D development.

NLRP3 Activation Increases Th17/Tc17 and Decreases the Myeloid-Derived Suppressor Cell (MDSC) Populations during STZ-Induced T1D

The percentage and absolute numbers of CD4$^+$ T cells and CD8$^+$ T cells were unaltered in the PLNs of diabetic WT mice compared to vehicle-treated mice (**Figures 4A–D**). However, the percentage of CD4$^+$ T cells, but not CD8$^+$ T cells, were significantly reduced in the PLNs of NLRP3$^{-/-}$ mice compared to diabetic WT mice (**Figures 4A,C**). In addition, the CD4$^+$IL-17$^+$ cell frequency

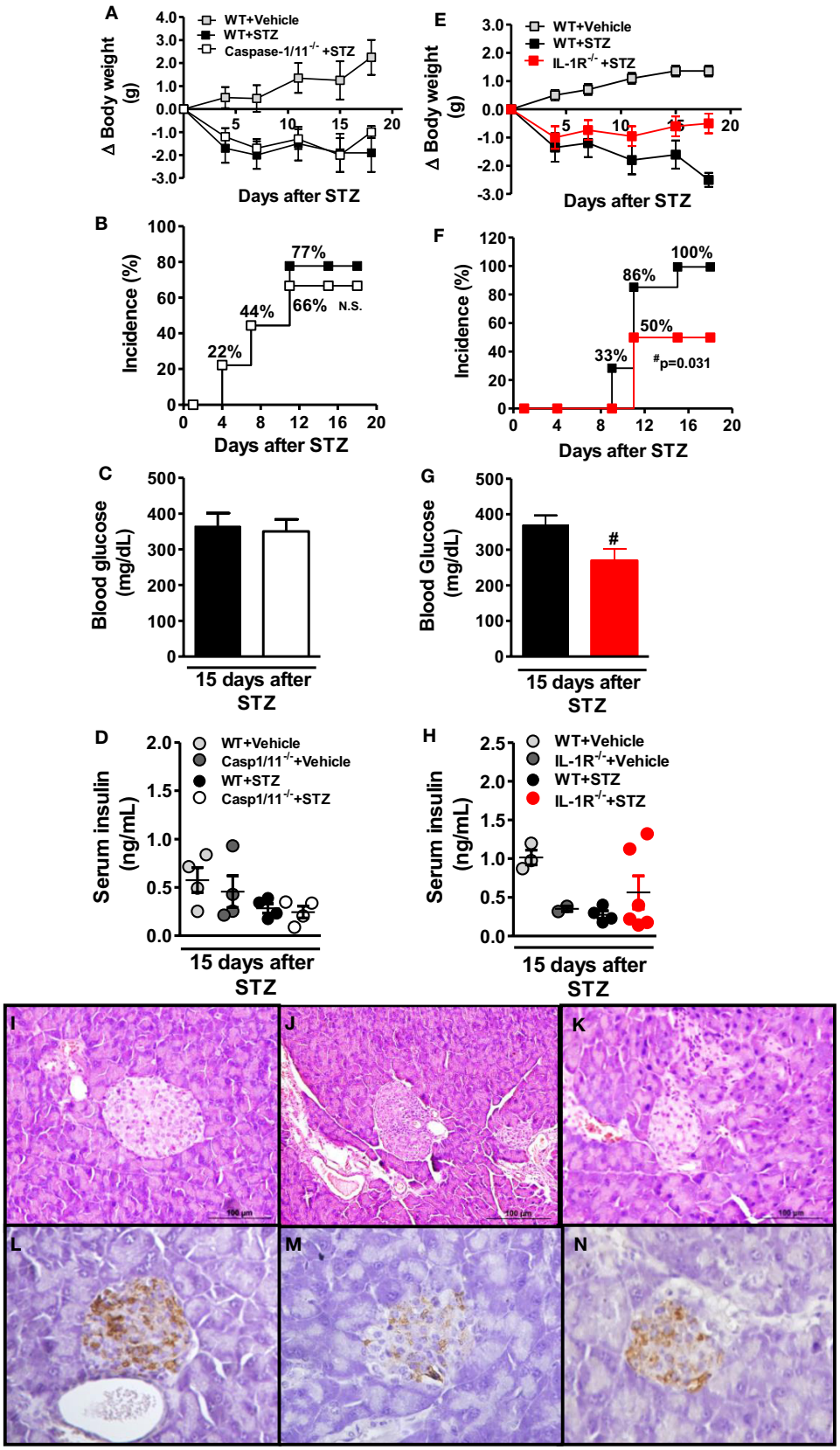

FIGURE 2 | **Continued**

FIGURE 2 | Continued

IL-1R deficiency, but not caspase-1/11, confers resistance to type 1 diabetes development. Body weight variation **(A,E)**, cumulative disease incidence **(B,F)**, and blood glucose levels **(C,G)** were detected in caspase-1/11$^{-/-}$, IL-1R$^{-/-}$, or wild-type (WT) mice. These clinical parameters were monitored 1, 4, 7, 11, 15, and 18 days after the initial streptozotocin (STZ) treatment. The non-diabetic mice only received the sodium citrate (vehicle). The serum insulin concentrations were determined at day 15 after the initiation of STZ or vehicle administration **(D,H)**. Pancreatic tissues from vehicle-injected WT **(I,L)**, STZ-treated WT **(J,M)**, and STZ-treated IL-1R$^{-/-}$ mice **(K,N)** were stained with hematoxylin-eosin (H&E) (upper panels) or immunostained for insulin (lower panels), respectively (original magnification 400×). The results are expressed as the mean ± SEM (n = 12 in the vehicle-injected WT group; n = 18 in the STZ-administered WT group; n = 18 in the STZ-administered caspase-1/11$^{-/-}$ group; and n = 18 in the STZ-administered IL-1R$^{-/-}$ group). $^{\#}p \leq 0.05$ compared to the STZ-administered WT group. Significant differences between the two groups were compared by Student's t-test or three groups by one-way ANOVA followed by Tukey's multiple-comparison test. The results are representative of a single experiment repeated three times. n.s., not significant.

and absolute numbers in the PLNs from NLRP3$^{-/-}$mice were significantly decreased compared to diabetic WT mice (**Figures 4E,F,M**). Similarly, the frequency and absolute numbers of CD8^{+}IL-17^{+} cells were significantly reduced in the PLNs of these mice (**Figures 4G,H**). Despite a trend toward reduction, there was no significant difference between the percentage and absolute numbers of CD4^{+}IFN-γ^{+} and CD8^{+}IFN-γ^{+} cells in the PLNs of NLRP3$^{-/-}$mice and diabetic WT mice (**Figures 4I–L**). In parallel, NLRP3 deficiency significantly increased IL-6 and IL-4 levels (**Figures 5A,D**) without alterations in the IL-17 and IFN-γ levels (**Figures 5B,C**) in the pancreatic tissue after STZ treatment. Surprisingly, ASC-deficient mice had significantly decreased IL-17, IFN-γ, and IL-4 (**Figures 5F–H**), but the IL-6 levels remained unaltered (**Figure 5E**). Our next step was to identify whether the resistance observed in NLRP3$^{-/-}$ mice could be attributed to the induction of tolerogenic cells in the myeloid or lymphoid compartment. Importantly, NLRP3$^{-/-}$ mice had a normalization of frequency and absolute numbers of MDSCs in the PLNs compared to diabetic WT mice (**Figures 5I–K**). On the other hand, the arginase-1 and iNOS gene expressions decreased in the pancreatic tissue of NLRP3$^{-/-}$ mice compared to diabetic WT mice (**Figures 5L,M**), while Foxp3 and TGF-β gene expression was not altered (**Figures 5N,O**). Overall, our results showed that NLRP3 activation promotes IL-1β production, which in turn triggers Th17/Tc17 induction and dampens MDSC expansion in STZ-induced T1D.

Mitochondrial DNA Triggers Caspase-1-Dependent IL-1β Production by Macrophages

Later, we determined the effect of mDNA from non-diabetic mice (cmDNA) or from diabetic mice (dmDNA) on NLRP3 inflammasome activation. To examine whether the inflammasome is activated by mDNA, bone marrow-derived macrophages (BMDMs) were exposed to different concentrations of mDNA after priming with LPS to allow expression of pro-IL-1β. Consistently, IL-1β production was significantly increased in BMDMs from WT mice after stimulation with dmDNA (10 μg/mL) when compared with cells stimulated only with LPS. However, this response was reduced in BMDMs from NLRP3$^{-/-}$ mice. On the other hand, cmDNA stimulation (5 or 10 μg/mL) after LPS priming did not induce IL-1β production (**Figure 6A**). Conversely, cmDNA or dmDNA stimulation (5 or 10 μg/mL) did not change IL-1α production in BMDMs from WT mice when compared to LPS-stimulated cells. Nevertheless, BMDMs from NLRP3$^{-/-}$ mice already presented a significant IL-1α reduction when compared to BMDMs from WT mice, independent of the stimulus that was used (**Figure 6B**).

Because active caspase-1 is crucial for the cleavage of pro-IL-1β to its mature and biologically active form, we determined if dmDNA is able to trigger the activation of caspase-1. The activation of caspase-1 was demonstrated by using the FAMYVAD-FMK fluorescent inhibitor (FLICA), which binds covalently to active caspase-1. Notably, the dmDNA stimulation at a concentration of 10 μg/mL induced a significant increase in the percentage of caspase-1-positive BMDMs compared BMDMs incubated only with LPS, and this effect was inhibited in the BMDMs of NLRP3$^{-/-}$ mice. However, cmDNA stimulation at a concentration of 5 or 10 μg/mL did not promote a significant increase in caspase-1-positive BMDMs compared to the LPS stimulus alone (**Figures 6C,D**). Because nigericin is a potent activator of the NLRP3 inflammasome, we used this compound as a positive control in the Western blot assays. Of fact, we detected an increase of active IL-1β (p17) expression in the supernatant of BMDMs from WT mice exposed to nigericin after LPS priming, which was inhibited in BMDMs from NLRP3$^{-/-}$ mice. Similarly, the immunoblot analysis showed that the active form of IL-1β was being produced in response to dmDNA at a concentration of 10 μg/mL in the supernatant of the BMDMs from WT mice, but this effect was inhibited in BMDMs from NLRP3$^{-/-}$ mice, after priming the cells with LPS. Of interesting manner, the expression of active form of IL-1β was similar by BMDMs from WT mice or NLRP3$^{-/-}$ mice in response to cmDNA at the same concentration (**Figures 6E,F**). These results suggest that the NLRP3 inflammasome senses mitochondrial DNA from diabetic mice in macrophages and causes caspase-1-dependent IL-1β production.

Mitochondrial DNA from Diabetic Mice Precipitates STZ-Induced T1D Onset

The administration of five doses of STZ (40 mg/kg) induces T1D as described before. However, the administration of only four doses does not have this effect (**Figures 7A–D**). This result suggests that four doses of STZ do not induce T1D because the doses are not sufficient to produce robust β-cell damage. Therefore, we tested whether four sub-diabetogenic doses of STZ would cause T1D if they were administered with mDNA from diabetic mice (three doses each at 5 μg i.p.) to C57BL/6 mice on day 1 before and days 6 and 9 after STZ administration. Our findings showed that dmDNA administration predisposes animals to T1D onset, which was confirmed by 83% disease incidence (**Figure 7B**) and

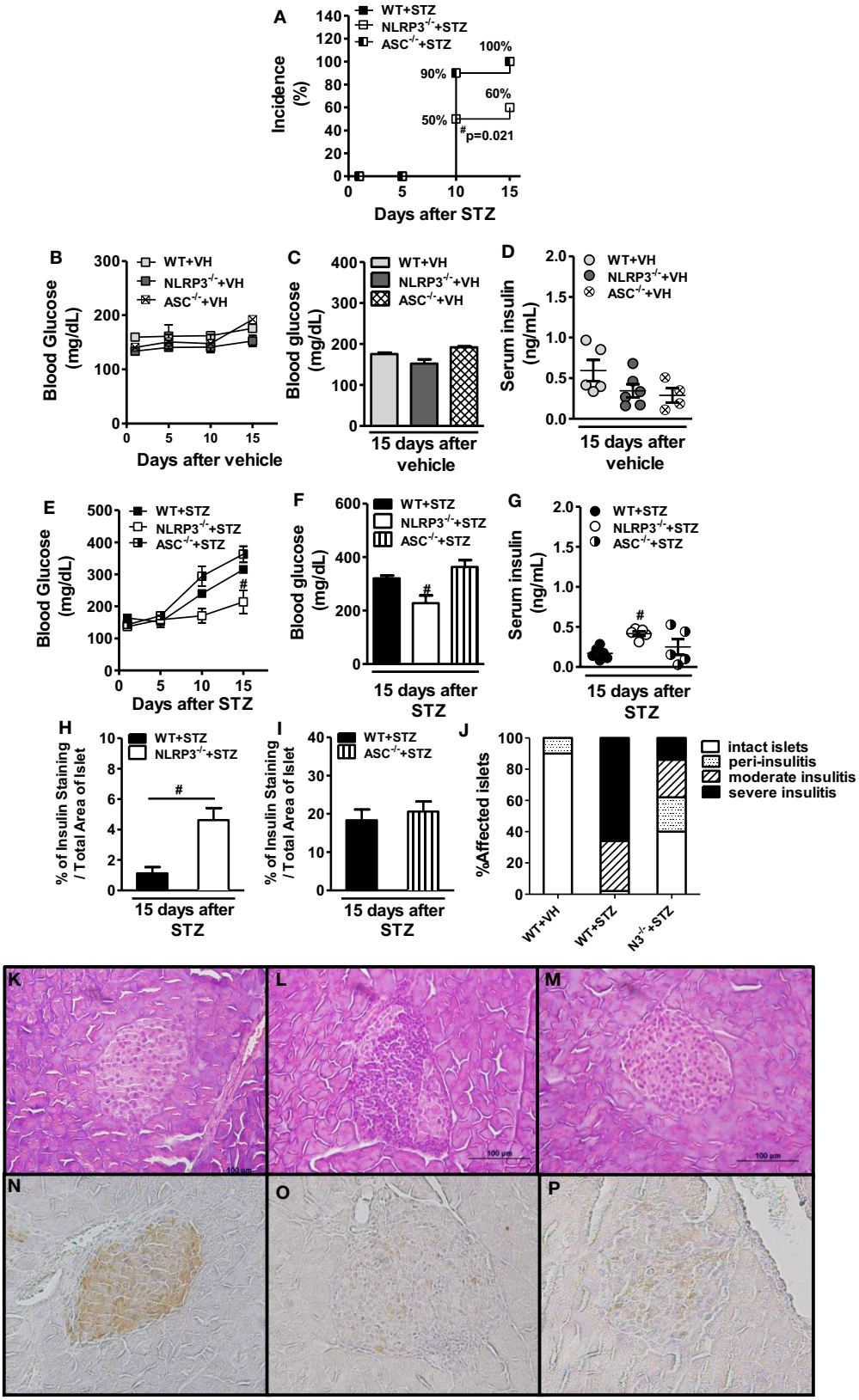

FIGURE 3 | **Continued**

FIGURE 3 | Continued

NOD-like receptor family-pyrin domain containing 3 (NLRP3) deficiency promotes protection against type 1 diabetes development. Cumulative disease incidence was monitored in NLRP3$^{-/-}$, ASC$^{-/-}$, or wild-type (WT) mice after streptozotocin (STZ) administration **(A)**. The time course of glycemia was monitored or blood glucose levels was determined at day 15 after the initiation of vehicle **(B,C)** or STZ administration **(E,F)** by a glucometer system Accu-Chek Active. The serum insulin concentrations were determined at day 15 after the initiation of STZ or vehicle administration **(D,G)**. The quantitative analysis of insulin-staining pancreatic islets **(H,I)** or semiquantitative scale insulitis score **(J)**. Pancreatic tissues from vehicle-injected WT **(K,N)**, STZ-treated WT **(L,O)**, and STZ-treated NLRP3$^{-/-}$ mice **(M,P)** were stained with hematoxylin-eosin (H&E) (upper panels) or immunostained for insulin (lower panels), respectively (original magnification 400×). The results are expressed as the mean ± SEM (n = 12 in the vehicle-injected WT group; n = 24 in the STZ-administered WT group; n = 12 in the vehicle-injected ASC$^{-/-}$ group; n = 24 in the STZ-administered ASC$^{-/-}$ group; n = 12 in the vehicle-injected NLRP3$^{-/-}$ group; and n = 24 in the STZ-administered NLRP3$^{-/-}$ group). $^{\#}p \leq 0.05$ compared to the STZ-administered WT group. Significant differences between two groups were compared by Student's t-test or three or more groups by one-way ANOVA followed by Tukey's multiple-comparison test. The results are representative of a single experiment repeated three times or a compilation of two different experiments (A).

increased blood glucose levels after four sub-diabetogenic doses of STZ (**Figures 7C,D**). Nevertheless, dmDNA administration did not affect body weight loss compared with mice only given four doses of STZ (**Figure 7A**). The dmDNA effects in the disease incidence and hyperglycemia were abrogated in NLRP3$^{-/-}$ mice, since we observed a significant reduction of glycemic levels in these mice compared to diabetic WT mice after four doses of STZ (**Figures 7B–D**). As shown in **Figure 7E**, the group treated only with STZ had a striking proportion of insulitis-free islets (96%) and some infiltrated areas with peri-insulitis (4%). However, more infiltrated areas with peri-insulitis (16%), moderate insulitis (34%), and severe insulitis (18%) were observed in mice treated with four sub-diabetogenic doses of STZ and dmDNA. On the other hand, the pancreatic islets of NLRP3$^{-/-}$ mice revealed more insulitis-free islets (74%) and no moderate or severe insulitis after the same treatments. Similarly, serum insulin levels further decreased in WT mice, but were normalized in NLRP3$^{-/-}$ mice treated with dmDNA after STZ (**Figure 7F**). Thus, these data indicate that NLRP3 activation mediated by mDNA from diabetic mice is required for the pancreatic islet inflammation involved in insulin-producing β cell damage and T1D development.

Mitochondrial DNA Induces Pathogenic Lymphocyte Response and Dampens Mast Cell and MDSC Expansion in STZ-Induced T1D

Considering dmDNA promotes IL-1β production mediated by NLRP3 and the established role of IL-1β in Th17 differentiation (21, 22), we examined whether NLRP3 is involved in Th17 and Tc17 induction in mice treated with mDNA after sub-diabetogenic doses of STZ. The dmDNA administration plus STZ was able to promote a significant increase of CD4^{+}IL-17^{+} and CD8^{+}IL-17^{+} absolute numbers in the PLNs of WT mice (**Figures 7K,L**). Importantly, the frequency and absolute numbers of CD4^{+}IL-17^{+} cells were significantly reduced in the PLNs of NLRP3$^{-/-}$ mice compared to diabetic WT mice treated with STZ and dmDNA (**Figures 7G,K,O**). Although there were no differences in the frequency, the absolute number of CD8^{+}IL-17^{+} cells was significantly decreased in the PLNs of NLRP3$^{-/-}$ mice compared to diabetic WT mice after the same treatments (**Figures 7H,L**). Another important observation is the increased frequency and absolute numbers of CD4^{+}IFN-γ^{+} and CD8^{+}IFN-γ^{+} cells in the PLNs of mice treated with mDNA after sub-diabetogenic doses of STZ (**Figures 7I,J,M,N**). Of note, the frequency and absolute numbers of CD4^{+}IFN-γ^{+} cells were significantly decreased in the PLNs of NLRP3$^{-/-}$ mice compared to diabetic WT mice treated with STZ and dmDNA (**Figures 7I,M,P**). However, only the absolute numbers of CD8^{+}IFN-γ^{+} cells, but not the percentage, were significantly decreased in the PLNs of NLRP3$^{-/-}$ mice compared to diabetic WT mice (**Figures 7J,N**).

Analysis of circulating mDNA genes, such as NADH dehydrogenase subunit 6 (NADH), cytochrome *b* (Cyt B), and cytochrome *c* (Cyt C), demonstrated a significant increase in gene expression of Cyt B and Cyt C, but not NADH, 15 days after STZ in diabetic mice compared to vehicle-treated mice (Figures S2A–C in Supplementary Material). Interestingly, we observed that NLRP3$^{-/-}$ mice treated with STZ and dmDNA exhibited a significant increase in the percentage and absolute numbers of mast cells (Figures S2D,G in Supplementary Material), but not M2 macrophages (Figures 2E,H in Supplementary Material), compared to WT and NLRP3$^{-/-}$ mice after only STZ doses. In addition, NLRP3$^{-/-}$ mice had a trend to increase the percentage and absolute number of monocytic MDSCs compared to WT mice treated with STZ and dmDNA (Figures S2F,I in Supplementary Material). A coadministration of STZ and dmDNA also significantly increased IL-1β levels in the pancreatic tissue of WT mice, but significantly decreased IL-1β levels in NLRP3-deficient mice (Figure S2J in Supplementary Material). Conversely, the NLRP3 deficiency caused a significant increase in IL-6 levels (Figure S2K in Supplementary Material) without affecting the IL-17, IL-23, IFN-γ, and IL-10 levels (Figures S2L–O in Supplementary Material) in the pancreatic tissue after dmDNA and STZ administration. Taken together, our results showed that NLRP3 activation depended on mDNA from diabetic mice for the induction of Th17/Tc17/Th1/Tc1 responses and the suppression of mast cells and MDSCs in STZ-induced T1D.

DISCUSSION

Type 1 diabetes is one of the most prevalent autoimmune diseases in the world. It affects approximately 10–20 million people and develops most frequently in childhood but also can develop in adulthood. Similar to other autoimmune disorders, the etiology of diabetes remains unclear, but it is known that the risk of developing the disease is determined by genetic and environmental factors, including viral infections, food, vaccination, toxins, and stress (23, 24). A strong association between NLRP3

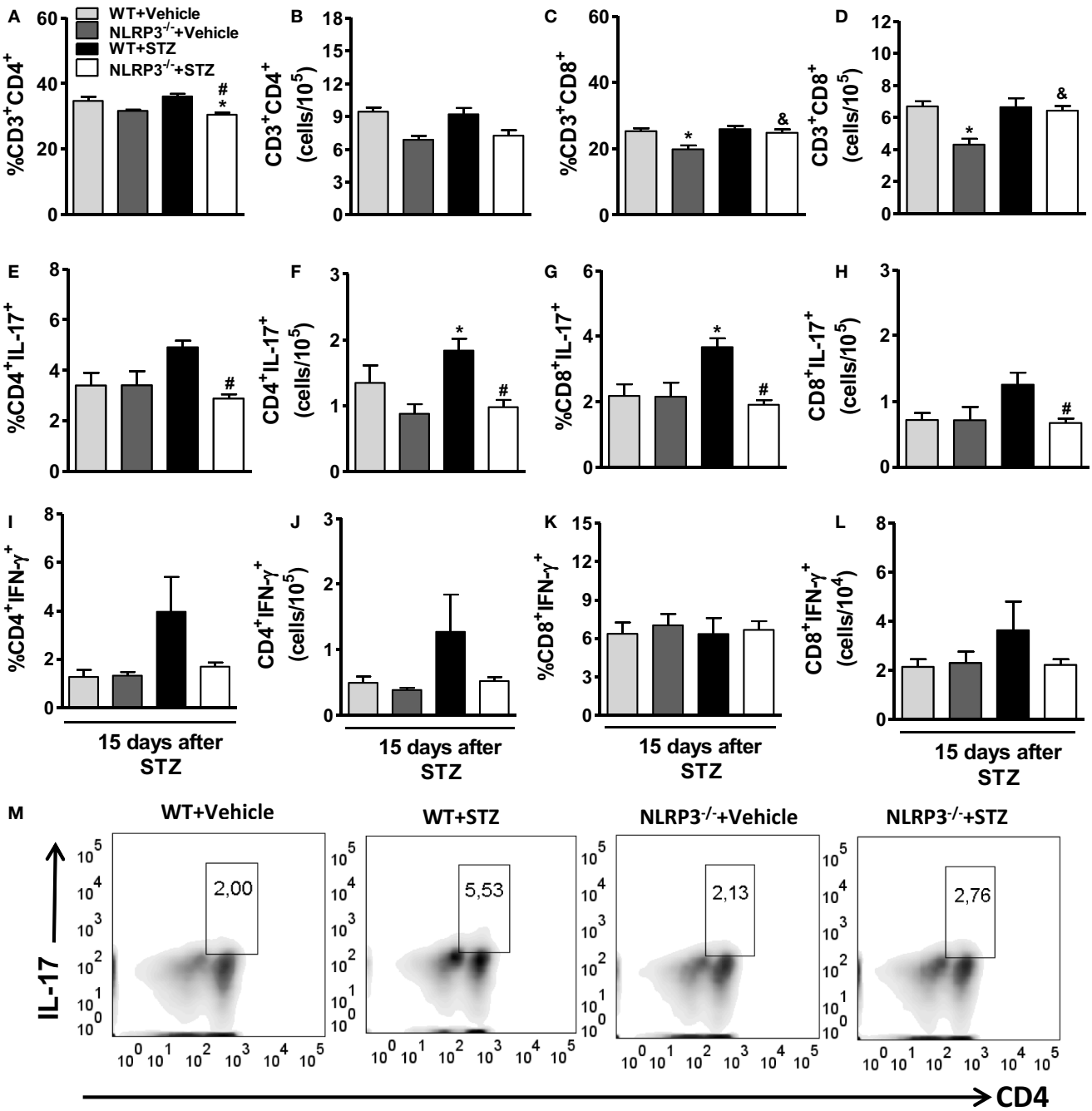

FIGURE 4 | **NOD-like receptor family-pyrin domain containing 3 (NLRP3) deficiency decreases the Th7/Tc17 cell populations in the pancreatic lymph nodes (PLNs) in type 1 diabetes**. PLN cells from vehicle-injected wild-type (WT) (light gray bars), NLRP3$^{-/-}$ (dark gray bars), streptozotocin (STZ)-administered WT mice (black bars), or NLRP3$^{-/-}$ (white bars) mice were harvested 15 days after the initiation of STZ or vehicle administration. The percentage and absolute numbers of $CD3^+CD4^+$ **(A,B)**, $CD3^+CD8^+$ **(C,D)**, $CD4^+IL\text{-}17^+$ **(E,F)**, $CD8^+IL\text{-}17^+$ **(G,H)**, $CD4^+IFN\text{-}\gamma^+$ **(I,J)**, and $CD8^+IFN\text{-}\gamma^+$ **(K,L)** cells were determined in the PLNs by flow cytometry. Th17 percentages in the PLNs are shown in representative dot plots **(M)**. Intracellular cytokine levels were detected after stimulation with PMA plus ionomycin. The gate was set on CD3-positive lymphocytes. The results are expressed as the mean ± SEM ($n = 12$ in the vehicle-injected WT group; $n = 24$ in the STZ-administered WT group; $n = 12$ in the vehicle-injected NLRP3$^{-/-}$ group; and $n = 24$ in the STZ-administered NLRP3$^{-/-}$ group). $^*p \leq 0.05$ compared to vehicle-injected WT group, $^\#p \leq 0.05$ compared to STZ-administered WT group, or $^\&p \leq 0.05$ compared to vehicle-injected NLRP3$^{-/-}$ group. Significant differences between the groups were compared by one-way ANOVA followed by Tukey's multiple-comparison test. The results are representative of a single experiment repeated three times.

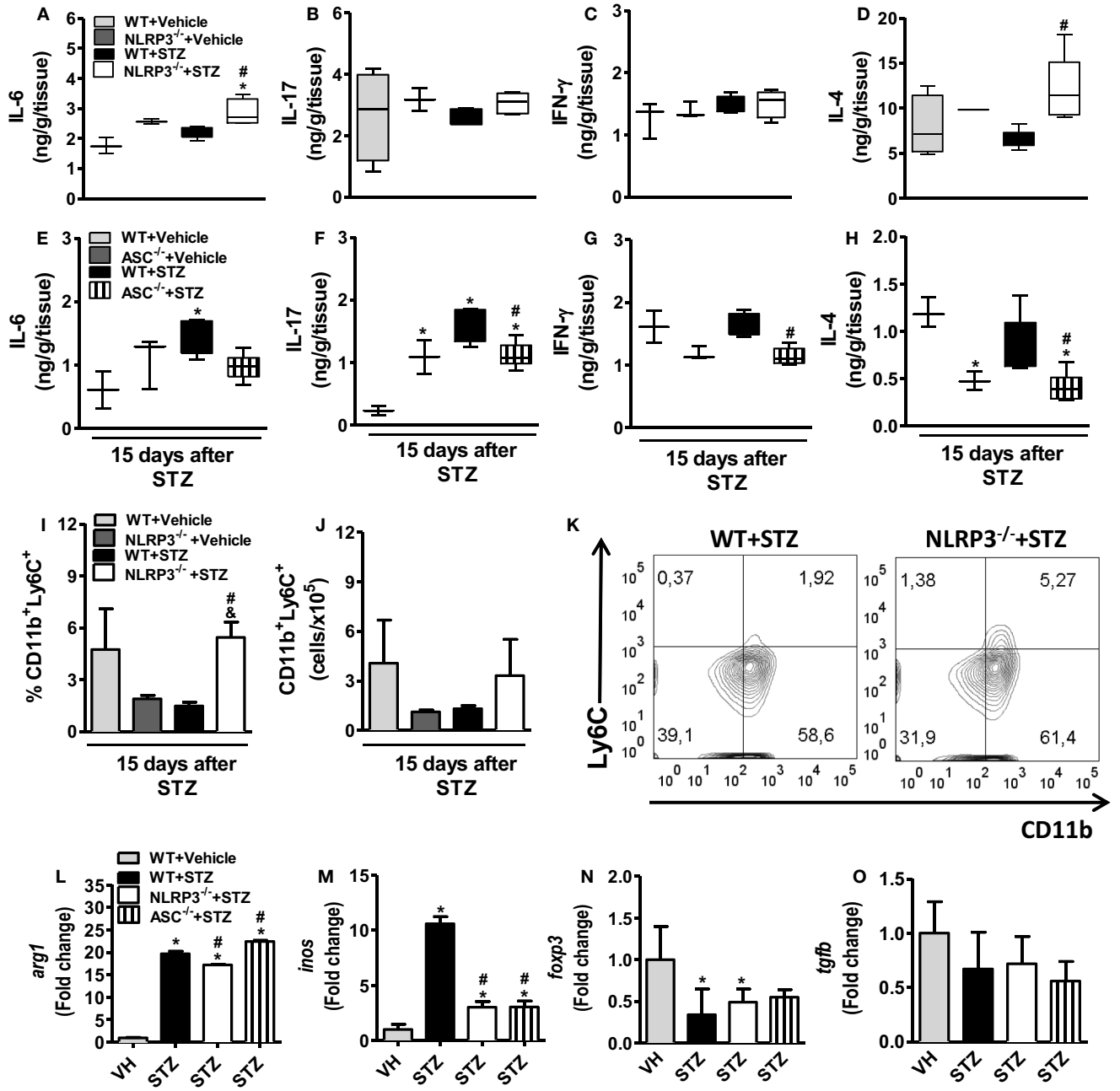

FIGURE 5 | NOD-like receptor family-pyrin domain containing 3 (NLRP3) deficiency potentiates IL-6 and IL-4 production and myeloid-derived suppressor cell (MDSC) expansion in pancreatic lymph nodes (PLNs) in type 1 diabetes. Pancreatic tissue from vehicle-injected wild-type (WT) (light gray bars), NLRP3$^{-/-}$, or ASC$^{-/-}$ (dark gray bars) and streptozotocin (STZ)-administered WT (black bars), NLRP3$^{-/-}$ (white bars), or ASC$^{-/-}$ (striped bars) mice were harvested 15 days after the initiation of STZ or vehicle administration. The concentrations of IL-6 **(A,E)**, IL-17 **(B,F)**, IFN-γ **(C,G)**, or IL-4 **(D,H)** were determined in the pancreatic tissue by an ELISA assay. The percentage and absolute numbers of MDSC cells in the PLNs were determined by flow cytometry **(I,J)**. Monocytic MDSC percentages (CD11b^{+} Ly6C^{+}) in the PLNs are shown in representative dot plots **(K)**. Relative gene expressions of arginase-1 **(L)**, iNOS **(M)**, Foxp3 **(N)**, and TGF-β **(O)** were measured by RT-PCR in the pancreatic tissue. The results are expressed as the mean ± SEM ($n = 12$ in the vehicle-injected WT group; $n = 24$ in the STZ-administered WT group; $n = 12$ in the vehicle-injected NLRP3$^{-/-}$ group; and $n = 24$ in the STZ-administered NLRP3$^{-/-}$ group). $^{*}p \leq 0.05$ compared to the vehicle-injected WT group, $^{\#}p \leq 0.05$ compared to the STZ-administered WT group, or $^{\&}p \leq 0.05$ compared to the vehicle-injected NLRP3$^{-/-}$ group. Significant differences between the groups were compared by one-way ANOVA followed by Tukey's multiple-comparison test. The results are representative of a single experiment repeated three times.

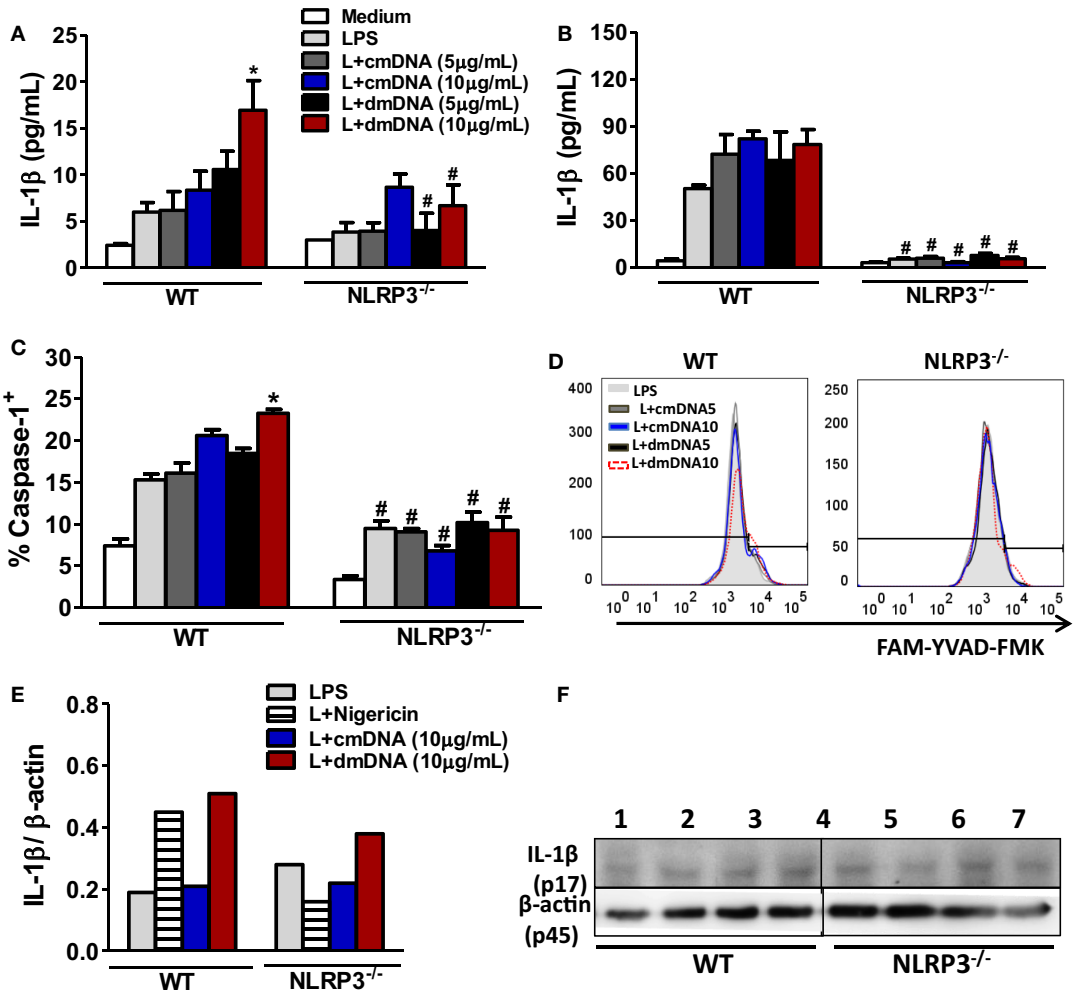

FIGURE 6 | NOD-like receptor family-pyrin domain containing 3 (NLRP3) activation by mitochondrial DNA (mDNA) from diabetic mice induces IL-1β expression and caspase-1 activation in macrophages. Bone marrow-derived macrophages (BMDMs) were primed without (medium alone) or with LPS for 4 h (0.5 μg/mL). After that, they were incubated with nigericin at 20 μM for more 30 min or were incubated with mDNA from control mice (cmDNA) or from diabetic mice (dmDNA) at concentrations of 5 or 10 μg/mL for more 2 h. IL-1β **(A)** and IL-1α **(B)** concentrations in supernatants of BMDMs were measured by an ELISA assay. Caspase-1 activation in macrophages was detected with a fluorescent cell-permeable probe that binds to activated caspase-1 (FLICA) **(C,D)**. Cleaved IL-1β p17 subunit was detected by Western blotting. Bar graph represents the bands quantified by densitometric analysis **(E)**. BMDMs from wild-type (WT) (left panel) or NLRP3$^{-/-}$ mice (right panel) were stimulated with LPS (L) only (1,5), or with LPS plus nigericin (2,6), plus cmDNA (3,7) or plus dmDNA (4,8) in the concentration of 10 μg/mL **(F)**. $^{*}p \leq 0.05$ compared to LPS-stimulated BMDMs or $^{\#}p \leq 0.05$ NLRP3$^{-/-}$ vs. WT BMDMs after respective treatments. Significant differences between the groups were compared by one-way ANOVA followed by Tukey's multiple-comparison test. Four biological replicates per group were used in each *in vitro* experiment. The results are representative of a single experiment repeated three times.

polymorphisms and a greater predisposition to the disease has been reported in diabetic patients (19).

The NLRP3 inflammasome is a molecular platform required for the proteolytic cleavage of caspase-1 and is activated by endogenous and exogenous stimuli, including uric acid crystals and silica, bacterial toxins, β-amyloid particles, and ATP (12–14). After activation, NLRP3 oligomerization and interaction with the adapter molecule ASC resulted in activation of caspase-1 and expression of active forms of IL-1β and IL-18. Our results showed a correlation between increased NLRP3, ASC, and pro-IL-1β gene expression in the PLNs, as well as IL-1β, but not IL-18 protein expression at day 7 in the pancreatic tissue of STZ-induced diabetic mice. In addition, pancreatic IL-1β expression remained elevated at day 15 through a mechanism dependent on NLRP3 inflammasome activation. IL-18 expression, after 15 days of STZ-induced T1D, was not dependent on NLRP3 inflammasome activation. In parallel, we observed an elevated percentage of caspase-1-expressing macrophages in the PLNs of diabetic mice, which was reduced in mice lacking NLRP3. In addition, deficiency of IL-1R and NLRP3 in mice triggered resistance to T1D development. This protection observed in IL-1R$^{-/-}$ mice was associated with smaller IL-17 production in the pancreatic tissue during T1D. NLRP3 deficiency in NOD mice also protected against T1D through inhibition of chemokines CCL5 and

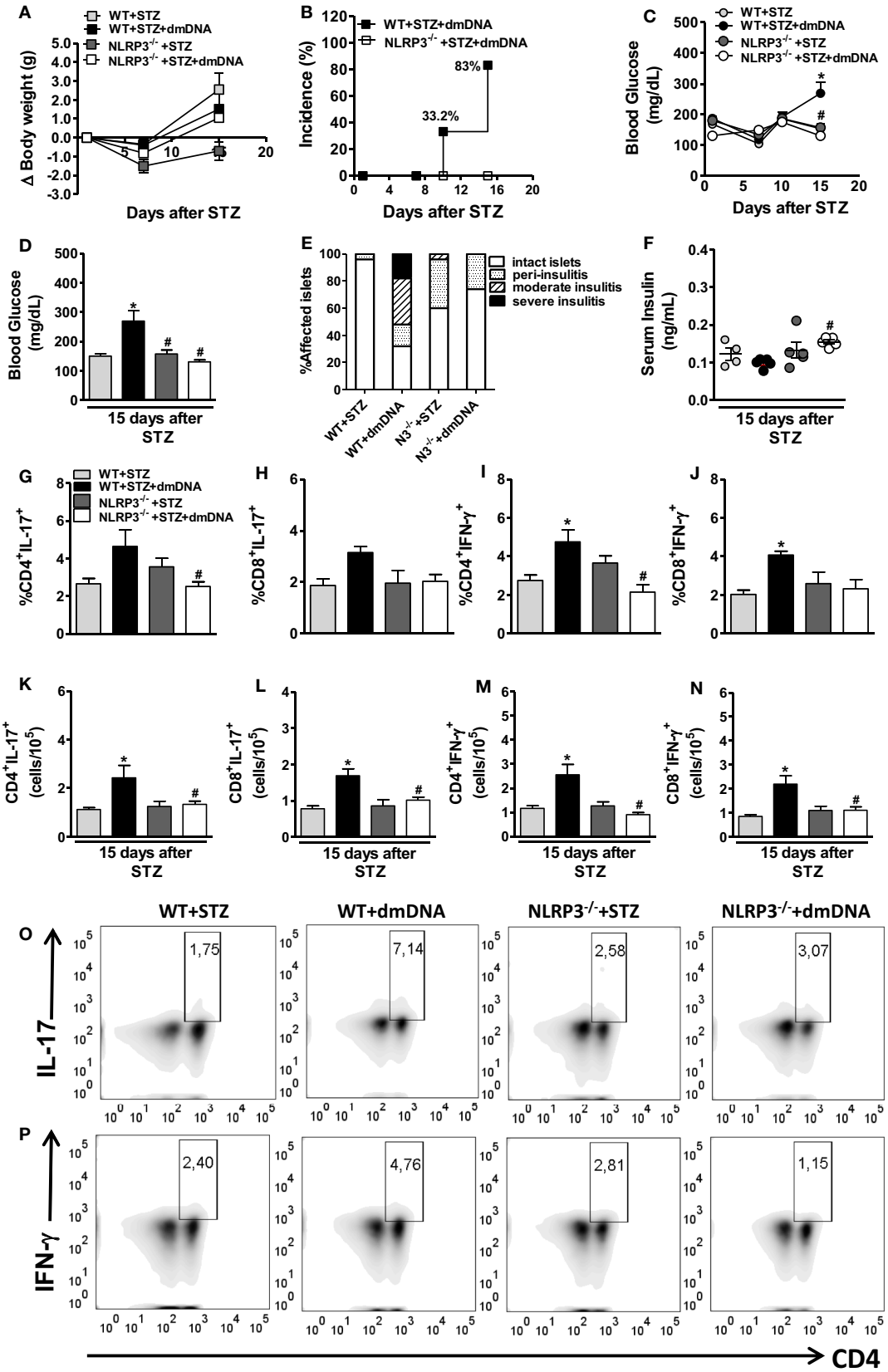

FIGURE 7 | **Continued**

FIGURE 7 | Continued

NOD-like receptor family-pyrin domain containing 3 (NLRP3) activation by mitochondrial DNA (mDNA) from diabetic mice contributes to Th17/Tc17/Th1/Tc1 response and leads to type 1 diabetes onset. Body weight variation **(A)**, cumulative disease incidence **(B)**, and time course of glycemia was monitored, or blood glucose levels were determined after 15 days of streptozotocin (STZ) **(C,D)** in wild-type (WT) mice treated only with four doses of STZ (light gray bars) plus mDNA from diabetic mice (dmDNA) (black bars) or NLRP3$^{-/-}$ mice treated only with four doses of STZ (dark gray bars) plus dmDNA (white bars). These clinical parameters were monitored 1, 7, 10, and 15 days after the initial STZ treatment. The insulitis score was evaluated using a semiquantitative scale **(E)**. The serum insulin concentrations were determined at day 15 after the initiation of STZ **(F)**. The percentage and absolute numbers of CD4$^+$IL-17$^+$ **(G,K)**, CD8$^+$IL-17$^+$ **(H,L)**, CD4$^+$IFN-γ$^+$ **(I,M)**, and CD8$^+$IFN-γ$^+$ **(J,N)** cells in the pancreatic lymph nodes (PLNs) was determined by flow cytometry. Representative dot plots of the Th17 (CD4$^+$IL-17$^+$) and Th1 (CD4$^+$IFN-γ$^+$) percentages in the PLNs, respectively **(O,P)**. Intracellular cytokine levels were detected after stimulation with PMA plus ionomycin. The gate was set on CD3-positive lymphocytes. The results are expressed as the mean ± SEM (n = 12 in the WT group administered only with STZ; n = 18 in the WT group administered with STZ plus mDNA; n = 12 in the NLRP3$^{-/-}$ group administered only with STZ; and n = 18 NLRP3$^{-/-}$ group administered with STZ plus mDNA). $^*p \leq 0.05$ compared to the WT group treated only with STZ, $^\#p \leq 0.05$ compared to the WT group treated with STZ plus mDNA. Significant differences between the groups were compared by one-way ANOVA followed by Tukey's multiple-comparison test. The results are representative of a single experiment repeated three times.

CXCL10 in the pancreatic islets (20). Taken together, these results indicate that NLRP3-dependent IL-1β production accounts for T1D onset in the STZ-induced experimental model.

NLRP3 receptor activation plays a crucial role in the induction of inflammatory responses and in the subsequent polarization of the adaptive immune response. In terms of cellular immunity, T CD4 lymphocytes are related to Th1, Th2, Treg, Th9, Th22, and Th17 according to their profile of cytokine expression and transcription factors (25). The differentiation of these cell subtypes is induced by a differential pattern of anti- or pro-inflammatory cytokines produced by macrophages and dendritic cells (26). It has been reported that IL-18 and IL-1β play an important role in driving Th1 and Th17 cellular responses, respectively (27). Initial studies supported a crucial role for IFN-γ-secreting Th1 cells in T1D pathogenesis (28, 29). However, this notion was altered by the discovery that genetic absence neither IFN-γ nor its receptor protect against T1D in NOD mice (30, 31). Notably, Emamaullee et al. provided strong evidence about the pathogenic role of Th17 in T1D by treating animals with either a neutralizing anti-IL-17 antibody or recombinant IL-25 (32). The deficiency of IL-1R, as well as NLRP3 in mice protected against T1D development and was associated with reduced Th17/Tc17/Th1 populations in the PLNs. Previous studies have also reported that deficiency of IL-1R or NLRP3 results in a lower production of IL-17 and causes resistance to EAE (16, 33). Similarly, another study reported that IL-1Ra-deficient mice spontaneously develop arthritis due to the high expression of IL-17 caused by increased signaling of IL-1 (34).

Mechanisms involving IL-1β-induced Th17 differentiation have been reported. TGF-β induces ROR-γt expression in naïve T cells and triggers IL-23R and IL-1R expression, which makes these cells receptive to IL-23 and IL-1β (35). *In vitro* studies have shown that IL-1β induces the expression of IRF-4, which positively regulates IL-21-mediated STAT-3 and ROR-γt transcription factor expression (18, 36). Additionally, a role for IL-1β on Th17 phenotype induction has been attributed to alternative splicing of Foxp3 (37). However, there are no reports about the direct effect of IL-1β on the induction or expansion of Th1 lymphocytes. More recently, the ability of Th17 and Tc17 lymphocytes to be converted into Th1 lymphocytes in the presence of IL-12 in T1D was also observed (38, 39), demonstrating the considerable plasticity of these cellular subtypes. Therefore, it is possible that the reduction in the Th1 lymphocyte population in NLRP3$^{-/-}$ mice is due to a defect in the differentiation of Th17/Tc17 lymphocytes and their subsequent conversion into Th1 lymphocytes. Taken together, our results demonstrate that NLRP3-dependent IL-1β expression appears to drive Th17/Tc17/Th1 differentiation under inflammatory conditions, such as T1D.

NLRP3 deficiency increased IL-6 and IL-4 protein expression in the pancreatic tissue after STZ administration. A study has shown that the activation of basophils and mast cells induces the secretion of IL-4 and delays the onset of T1D in NOD mice (40). Additionally, coculturing mast cells with MDSC leads to IL-6, IL-13, and TNF-α production, increasing their suppressor activity (41). MDSCs are increased in the blood of patients and experimental models of T1D, but these cells have a defect in their suppressor activity (42). Supporting these findings, we also observed a positive correlation between increased numbers of mast cells and MDSCs in the PLNs of NLRP3$^{-/-}$ mice after STZ administration, which indicates a synergistic effect between these two cell subtypes in protection against T1D. More recently, IL-6 has been involved in the generation of both mouse and human MDSC cells (43, 44). Accordingly, we demonstrated that mast cells play a regulatory role through IL-6-dependent mechanisms during T1D (45). Based on this evidence, we suggest that the increased MDSC population in NLRP3$^{-/-}$ mice is due to elevated IL-6 expression, which in turn inhibits the inflammatory response in the pancreatic islets and prevents the onset of T1D.

Danger-associated molecular patterns are usually found in different compartments within cells and are often modified by proteolytic and oxidative processes associated with cellular injury mechanisms (46). Most DAMPs are released or secreted and exert their biological activity through the activation of TLR and NLR receptors (47). In particular, mitochondrial DAMPs, including mDNA and formylated peptides, stimulate neutrophils *via* TLR9 and FPR-1, respectively, after being released into the extracellular space (48). In humans, the presence of mDNA is detected in the synovial fluid of patients with arthritis, and intra-articular injection of mDNA induces arthritis mediated by the recruitment of macrophages and monocytes. Researchers concluded that oxidatively damaged DNA bases are major contributors to arthritis development (49). Another study also revealed that neutrophil mitochondria guide oxidized mDNA in the steady state into lysosomes for degradation. On the other hand, blood neutrophils from patients with systemic lupus erythematosus (SLE) patients

have mitochondrial retention of oxidized nucleoids, indicating that a defect in degradation of neutrophil oxidized mDNA might contribute to SLE pathogenesis (50).

It is known that excessive oxidative damage to DNA impairs the normal repair mechanisms and induces apoptosis (51, 52).

Considering that alterations in DNA induced by oxidative stress contribute to diabetes progression (53), we addressed the *in vitro* and *in vivo* effects of mDNA released in response to pancreatic damage in T1D in the activation of NLRP3 inflammasome in macrophages. Macrophages primed with LPS and stimulated with mDNA from diabetic mice exhibited increased IL-1β production, caspase-1 expression and cleavage of pro-IL-1β in active IL-1β *in vitro*. In addition, coadministration of mDNA plus four doses of STZ-induced pancreatic islet inflammation and led to T1D, which was abrogated in NLRP3-deficient mice. mDNA plasma levels are significantly elevated in diabetic patients compared with healthy controls (54). Likewise, we also detected increased expression of mDNA-related genes in the serum of diabetic mice. It is plausible that tissue necrosis resulting from beta-cell death leads to extracellular mDNA release. However, it is still puzzling the fact that only mDNA from diabetic mice activates the NLRP3 inflammasome. We speculate that beta-cell death and DAMP release, such as mDNA, occur during the initial phase of diabetes (prediabetic phase). In addition, oxidative stress that contributes to beta-cell death may induce mDNA oxidation, which turns into an immunogenic molecule. In this context, it has been reported that ATP induces mitochondrial dysfunction, apoptosis, and oxidized mDNA release into the cytosol, which activates the NLRP3 inflammasome (12). Thus, it seems likely that the presence of extracellular mDNA exacerbates inflammation by stimulating IL-1β production *via* NLRP3 activation, thereby causing massive β-cell destruction and accelerating T1D onset in this experimental model.

In summary, we conclude that NLRP3 inflammasome activation mediated by mitochondrial DNA from diabetic mice promotes caspase-1 activation and IL-1β production by macrophages, which drives pathogenic Th17/Tc17/Th1 responses and negatively modulates the tolerogenic responses mediated by MDSC and mast cells in the PLNs, and leads to the development of T1D. Thus, alternative therapies using nucleases or drugs that cause extracellular mDNA degradation should be explored in human T1D.

ANIMALS AND METHODS

Animals

This research project was approved by the Animal Research Ethics Committee of the Ribeirao Preto Medical School, University of São Paulo (no. 001/2008). Male NLRP3$^{-/-}$, IL-1R$^{-/-}$, and ASC$^{-/-}$ mice generated on the C57BL/6 background (8–12 weeks old) were obtained from the Isogenic Breeding Unit at Ribeirao Preto Medical School, University of São Paulo, Ribeirao Preto, Brazil. Female NOD/LtJ mice (8–20 weeks old) were obtained from the Jackson Laboratory and housed in the animal facility of the Department of Biochemistry and Immunology, Ribeirao Preto Medical School, at 23–25°C with free access to water and food.

Induction of Diabetes by MLD-STZ

The mice were given daily intraperitoneal injections of 40 mg/kg of streptozotocin (Sigma-Aldrich,) dissolved in 0.1 M sodium citrate (pH 4.5) for five consecutive days. Blood glucose levels, body weight, and diabetes incidence were monitored weekly. Mice were defined as diabetic when glucose levels were ≥230 mg/dL after two consecutive determinations under non-fasting conditions.

Flow Cytometry Analysis of Intracellular and Extracellular Markers

Flow cytometry analysis was performed on samples with 1×10^6 cells/tube in 100 µL of PBS. First, cell suspensions were incubated with 5% normal rabbit serum for 30 min to block non-specific binding. Next, antibodies against CD3, CD4, CD8, CD25, CD117, FcεRI, CD11b, Ly6C, CD206, TLR2, and their control isotypes (BD Pharmingen, San Diego, CA, USA) were added and incubated for 30 min in the dark. IL-17 and IFN-γ production was evaluated after *in vitro* reactivation with PMA (25 ng/mL) and ionomycin (1 mg/mL, Sigma-Aldrich) together with 10 mg/mL monensin (Sigma-Aldrich) as previously described (55). The cells were analyzed using a FACS Canto flow cytometer, and the data were analyzed using FlowJo (Tree Star) software.

Detection of Cytokine Levels in Pancreatic Tissue

Pancreatic fragments (tail portions) were removed, weighed, and placed in a tube containing 700 µL of Complete Protease Inhibitor Cocktail (Roche Diagnostics, Abbott Park, IL, USA). The tissue was homogenized using a Polytron homogenizer (Thermo Fisher Scientific, Waltham, MA, USA) and IL-1∝, IL-1β, IL-18, IL-6, IL-17, IL-23, TNF-α, IFN-γ, IL-10, and IL-4 levels were detected by ELISA using colorimetric kits according to the manufacturer's instructions (R&D Systems). The results were expressed as the mean nanograms ± SEM per gram/tissue (pancreatic tissue) or picograms per milliliter (culture supernatant).

Quantification of Serum Insulin Levels

Serum samples were collected 15 days after MLD-STZ administration of non-fasting mice. The insulin concentration was determined using the Mouse Ultrasensitive Insulin ELISA kit (Alpco Diagnostics) according to the manufacturer's instructions.

Histological and Immunohistochemistry Analysis

Pancreatic fragments (head portion) were removed, fixed in 10% buffered formalin, and embedded in paraffin. Then, 4–5 µm sections were stained with hematoxylin and eosin (Merck, Whitehouse Station, NJ, USA). Immunohistochemistry

reactions were performed as previously described (55). The degree of insulitis was evaluated using a semiquantitative scale: 0, intact islet; 1, peri-insulitis; 2, moderate insulitis (<50% of the islets infiltrated); and 3, severe insulitis (>50% of the islets infiltrated).

Culture of BMDMs

The BMDMs from WT and NLRP3$^{-/-}$ mice were differentiated as previously described (56). Briefly, total bone marrow cells were cultured for 7 days in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Molecular Probes, Carlsbad, CA, USA) and 30% L-929 cell-conditioned media at 37°C and 5% CO_2. The cells (0.5×10^6/well) were stimulated with nigericin (20 μM) for 30 min (Sigma-Aldrich), and then mitochondrial DNA from non-diabetic or control mice (cmDNA) or diabetic mice (dmDNA) at the concentration of 5 or 10 μg/mL for 2 h. Prior to stimulation, BMDMs were prestimulated for 4 h with LPS (0.5 μg/mL) (InvivoGen).

Western Blotting

Fifty micrograms of extracted proteins were loaded directly into sodium dodecyl sulfate (SDS) sample buffer for 10% SDS-polyacrylamide gel electrophoresis. After transferring the samples onto a nitrocellulose membrane (Trans-Blot Transfer Medium; Bio-Rad, Hercules, CA, USA), the membranes were blocked with 5% milk in Tris buffer solution containing 0.1% Tween 20 for 1 h and then incubated with antibodies against IL-1β (Santa Cruz) or NLRP3 (R&D Systems) overnight at 4°C. Next, the cells were incubated with an IgG HRP-conjugated secondary Ab (Cell Signaling) for 1 h at room temperature. After the membranes were rinsed, the immunocomplexes were developed using an enhanced peroxidase/luminol chemiluminescence reaction (ECL Western blotting detection reagents; Pierce Biotechnology) and exposed to X-ray film with autoradiography (Carestream Health). The bands were quantified densitometrically using ImageTool 2.0 software (University of Texas), and the results were expressed as arbitrary units.

Active Caspase-1 Staining

Active caspase-1 was detected using the caspase-1 fluorochrome inhibitor of caspases (FLICA) kit (Immunochemistry Technologies) according to the manufacturer's instructions. Briefly, macrophages or PLN cells were adjusted to a volume of 0.5×10^6/tube or $1\text{-}2 \times 10^6$/tube, respectively. Later, the cells were stained for F4/80 and FLICA for 30 min at 37°C. The cells were then washed two times with PBS containing 10% FBS and analyzed directly with flow cytometry on a FACS Canto flow cytometer.

Mitochondrial DNA Isolation

Pancreata from diabetic and non-diabetic mice (control) were used in protocols for isolating the mitochondria. Briefly, the pancreatic tissue was cut in pieces and added to 50 mL of medium (Hepes 10 mM, saccharose 250 mM e EGTA 1 mM) at pH 7.2 and homogenized for 15 s. Later, the pancreatic tissue was centrifuged at 600 *g* for 5 min, and the supernatant was collected and centrifuged at 2,000 *g* for 10 min. The pellet containing the isolated mitochondria was recovered. The mitochondria were sonicated at an amplitude of 100% (10 sonicagens of 30 s with 30 s intervals). Then, the suspension of lysed mitochondria was centrifuged at 12,000 *g* for 10 min at 4°C followed by centrifugation at 100,000 *g* at 4°C for 30 min. The supernatant from this centrifugation was used for DNA extraction with the phenol–chloroform–isoamyl alcohol mixture (Sigma-Aldrich). Finally, DNA quantitation was determined with a Nanodrop 2000 (Thermo Technologies).

Mitochondrial DNA Quantification

Circulating DNA was extracted and purified using the QIAamp DNA Blood Mini Kit (Qiagen, Germantown, MD, USA). Isolated DNA from mice was amplified and quantified using real-time (RT)-PCR. The primers (Invitrogen, Grand Island, New York, NY, USA) that were used to amplify mDNA were cytochrome *b* (Cyt *b*) (forward 5-ACCTCAAAGCAACGAAGCCT-3′ and reverse 5′-GGTTGGCCTCCAATTCAGGT-3′), cytochrome *c* (Cyt *c*) (forward 5′-GACTTGCAACCCTACACGGAT-3′ and reverse 5′-CCGGTTAGACCACCAACTGT-3′), and NADH dehydrogenase subunit 6 (forward 5′-ATTCCACCCCCTCACGACTA-3′ and reverse 5′-TGTCGTTTTGGGTGAGAGCA-3′). The primer sequences have no homology with DNA found in any bacterial species published on BLAST. The RT-PCR results are presented as the inverse of cycle threshold (CT) for gene amplification as described (57).

RNA Extraction and Quantitative RT-PCR

Total RNA was extracted from the PLNs or pancreatic tissue using Trizol (Life Technologies, Molecular Probes, Carlsbad, CA, USA) following the manufacturer's instructions. cDNA was obtained using a High Capacity reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Quantitative mRNA analysis by RT PCR was performed using the SYBR Green fluorescence system (Applied Biosystems). The following primers were used: β-actin: 5′-AACGAGCGGTTCCGATG-3′, reverse: 5′-GGATTCCATACCCAACAAGGA-3′, NLRP3 forward: 5′-GTGGATGGGTTTGCTGGGAT-3′, reverse: 5′-CCACACTCTACCTAGACGC-3′; IL-1β forward: 5′-TGACAGTGATGAGAATGACCTGTTC-3′, reverse: 5′-TTGGAAGCAGCCCTTCATCT-3′; arginase-1 forward: 5′-GTTCCCAGATGTACCAGGATTC-3′, reverse: 5′-CGATGTCTTTGGCAGATATGC-3′; iNOS forward: 5′-CGTGAGTGGAGTCATACTGGAA-3′, reverse: 5′-CGAAACGCTTCACTTCCAA-3′; TGF-β forward: 5′-TGAACCAAGGAGACGGAATACA-3′, reverse: 5′-GGAGTTTGTTATCTTTGCTGTCACA-3′; Foxp3 forward: 5′-ACAACCTGAGCCTGCACAAGT-3′, reverse: 5′-GCCCACCTTTTCTTGGTTTTG-3′. Specific mRNA expression levels were normalized relative to β-actin mRNA levels using the comparative $2\Delta\Delta C_t$ method.

Statistical Analysis

The data are expressed as the mean ± SEM. The differences observed among the several experimental groups were performed by applying one-way ANOVA followed by the parametric Tukey's test for comparing multiple groups or by Student's *t*-test for comparing two groups. The incidence curve was analyzed by Mantel–Cox log-rank test. All analyses were performed using Prism 5.0 software (GraphPad Software). Statistical significance was set at $p < 0.05$.

AUTHOR CONTRIBUTIONS

DC carried out the experimental design, performed experiments, analyzed the results, and wrote the manuscript; FC, FR, and JY contributed to the analysis and interpretation of data and helped with *in vivo* experiments; CP and GO participated in the acquisition and interpretation of data and helped with *in vitro* experiments; SR supported us with histology and imaging data. FC, RT, and NC edited the manuscript, provided scientific assistance, and revised it critically, and JS provided intellectual support in addition to directing and supervising the study.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00164/full#supplementary-material.

FIGURE S1 | **IL-1R deficiency reduces the Th17/Tc17/Th1 cell population in the pancreatic lymph nodes (PLNs) in type 1 diabetes**. The percentage and absolute numbers of $CD4^+IL\text{-}17^+$ **(A,D)**, $CD8^+IL\text{-}17^+$ **(B,E)**, and $CD4^+IFN\text{-}\gamma^+$ **(C,F)** cells in the PLNs were determined by flow cytometry. Intracellular cytokine levels were detected after stimulation with PMA plus ionomycin. The gate was set on CD3-positive lymphocytes. The concentrations of IL-6 **(G)**, IL-17 **(H)**, IFN-γ **(I)**, TNF-α **(J)**, IL-4 **(K)**, and IL-10 **(L)** were determined in the pancreatic tissue by an ELISA assay. The results are expressed as the mean ± SEM ($n = 9$ in the vehicle-injected wild-type (WT) group; $n = 24$ in the streptozotocin (STZ)-administered WT group; $n = 9$ in the vehicle-injected IL-$1R^{-/-}$ group; and $n = 24$ in the STZ-administered IL-$1R^{-/-}$ group). $^*p \leq 0.05$ compared to the vehicle-injected WT group, $^\#p \leq 0.05$ compared to the STZ-administered WT group. Significant differences between the groups were compared by one-way ANOVA followed by Tukey's multiple-comparison test. The results are representative of a single experiment repeated three times.

FIGURE S2 | **NOD-like receptor family-pyrin domain containing 3 (NLRP3) activation by mitochondrial DNA (mDNA) from diabetic mice decreases the mast cell and myeloid-derived suppressor cell population in the pancreatic lymph nodes (PLNs)**. Relative quantification of circulating mDNA genes, NADH dehydrogenase subunit 6 (NADH), cytochrome *b* (Cyt *b*), and cytochrome *c* (Cyt *c*) measured by RT-PCR of vehicle-injected or streptozotocin (STZ)-administered mice **(A–C)**. The percentage and absolute numbers of $CD117^+Fc\varepsilon RI^+$ **(D,G)**, $CD11b^+CD206^+$ **(E,H)**, and $CD11b^+Ly6C^+$ **(F,I)** in the PLNs were determined by flow cytometry. The concentrations of IL-1β **(J)**, IL-6 **(K)**, IL-17 **(L)**, IL-23 **(M)**, IFN-γ **(N)**, or IL-10 **(O)** were determined in the pancreatic tissue by an ELISA assay. The results are expressed as the mean ± SEM [$n = 12$ in the wild-type (WT) group administered only with STZ; $n = 18$ in the WT group administered with STZ plus mDNA; $n = 12$ in the $NLRP3^{-/-}$ group administered only with STZ; and $n = 18$ $NLRP3^{-/-}$ group administered with STZ plus mDNA]. $^*p \leq 0.05$ compared to the vehicle-injected WT group or treated only with STZ, $^\#p \leq 0.05$ compared to the WT group or treated only with STZ plus mDNA, or $^\&p \leq 0.05$ compared to the $NLRP3^{-/-}$ group administered only STZ. Significant differences between the groups were compared by one-way ANOVA followed by Tukey's multiple-comparison test. The results are representative of a single experiment repeated three times.

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