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The role of monocytes/ macrophages in autoimmunity and autoinflammation

Edited by
Atsushi Kawakami, Keishi Fujio and Naoki Iwamoto

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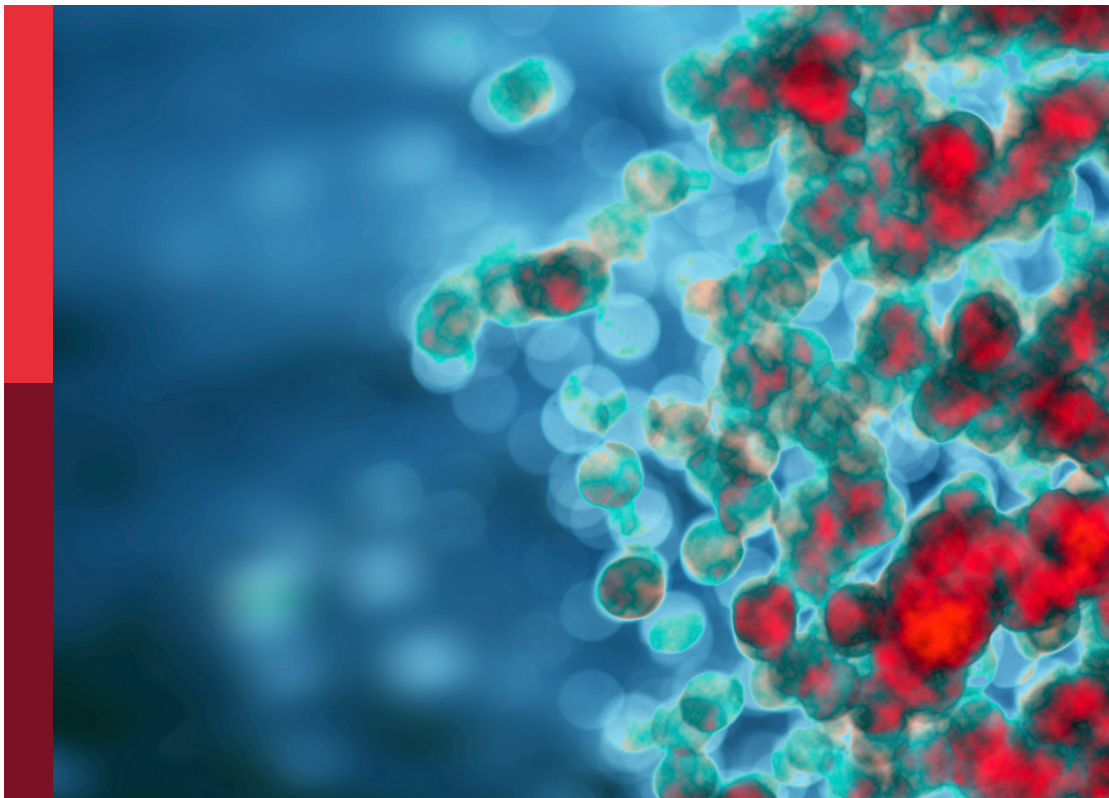

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The role of monocytes/ macrophages in autoimmunity and autoinflammation

Topic editors

Atsushi Kawakami — Nagasaki University, Japan
Keishi Fujio — The University of Tokyo, Japan
Naoki Iwamoto — Nagasaki University Hospital, Japan

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EDITED AND REVIEWED BY
Betty Diamond,
Feinstein Institute for Medical Research, United States

*CORRESPONDENCE
Naoki Iwamoto
naoki-iwa@nagasaki-u.ac.jp
Atsushi Kawakami
atsushik@nagasaki-u.ac.jp

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Editorial: The role of monocytes/macrophages in autoimmunity and autoinflammation

Atsushi Kawakami[1*], Naoki Iwamoto[1*] and Keishi Fujio[2]

[1]Department of Immunology and Rheumatology, Division of Advanced Preventive Medical Sciences, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan, [2]Department of Allergy and Rheumatology, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

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Editorial on the Research Topic
The role of monocytes/macrophages in autoimmunity and autoinflammation

Monocytes and macrophages are widely distributed innate immune cells that play indispensable roles in a variety of physiologic and pathologic processes, including regulation of the initiation, development and resolution of inflammatory disorders. They also actively participate in the development of autoimmune and autoinflammatory diseases. Their active mechanism is essentially to secrete a wide range of cytokine and chemokines, leading to the recruitment of additional specific immune cells.

Abnormal regulation of monocytes and macrophages has been reported in several autoimmune diseases. An example of this is the infiltrating $CD16^+$ are associated with a reduction in peripheral $CD14^+CD16^+$ monocytes in systemic lupus erythematosus (SLE) patients (1). Macrophages from SLE patients overexpress intercellular adhesion molecule (ICAM-1), and this overexpression is mitigated by corticosteroid treatment (2). Unusual expression of inflammatory and regulatory molecules associated with monocytes have also been found in autoinflammatory diseases, such as microRNA-204-3p in familial Mediterranean fever (3).

Furthermore, macrophages can be phenotypically polarized by the surrounding microenvironmental stimuli and signals. Classically activated macrophages (M1) can produce toxic effector molecules such as reactive oxygen species and nitric monoxide, as well as inflammatory cytokines such as interleukin (IL)-1β, tumor necrosis factor (TNF) and IL-6. Alternatively activated macrophages (M2) drive immune regulation, tissue remodeling. It has been suggested that such macrophage and monocyte subset polarization contributes to autoimmune and autoinflammatory diseases, most notably, Rheumatoid Arthritis (RA) and Crohn's Disease (4, 5).

Interestingly, a recent investigation of immune profiling analysis linked to Genome-Wide Association Studies (GWAS) has suggested the importance of monocyte-macrophage lineage cells for the development of autoimmune and autoinflammatory conditions. As mentioned above, there is growing evidence that monocytes and macrophages affect the progression and development of autoimmune and autoinflammatory diseases. Until now, the pathogeneses of these diseases have not been fully elucidated despite the enthusiasm of researchers for this topic; moreover, few treatment options have been available for these diseases. Investigating and understanding the role of monocytes/macrophages in these diseases is likely to improve this situation.

The goal of this Research Topic was to gather Original Research articles and Reviews that have improved our understanding of monocytes and macrophages in autoimmune and autoinflammatory disease.

Eighteen articles were contributed to this edition (8 original research and 10 reviews). The original research focused on monocytes/macrophages in various autoimmune disease such as SLE, RA and vasculitis from a "bench-to-clinic" standpoint.

Murakami et al. and Nomura et al. reported on the roles of monocytes in lupus model mice. Murakami et al. revealed that inhibition of toll-like receptor 7 (TLR7) suppressed the progression of lupus nephritis in lupus-prone NZBWF1 mice. In their study, the anti-TLR7 antibody reduced IgG deposition in the glomeruli, and this reduction was caused by a decrease of patrolling monocytes. NZBWF1 mice had an abundance of $Ly6C^{low}$ patrolling monocytes expressing a high level of TLR7 and upregulated expressions of IL-10, CD115, CD31 and TNF superfamily member (TNFSF)15, which has been associated with nephritis. Further, administration of an anti-TLR7 antibody abolished this abundance of lupus-associated patrolling monocytes. Thus, targeting of TLR7 was revealed as a promising therapeutic option targeting monocytes in lupus nephritis. Interestingly, association of TLR-7 and monocytes in a lupus model was also reported by Nomura et al. Imiquimod, a TLR-7 agonist, induces a lupus like-phenomenon in C57BL/6 and NZB/NZW mice by activating TLR-7 signaling. The authors studied the involvements of different monocytes in these model mice according to site and temporal disease progression. $Ly6C^{hi}$ monocytes were increased in the lymph nodes and upregulated interferon (IFN)-α genes, whereas $Ly6C^{lo}$ monocytes were increased in the late phase, infiltrating tissues and becoming inflammatory cells in the kidneys. In human SLE patients, Zhu et al. reported that the level of ribonuclease A family member 2 (RNASE2), which is known to have antiviral activity and immunomodulatory function, was associated with the proportion of autoreactive B-cells, and expansions of these cells were related with monocyte-derived IL-10 levels. mRNA expression of RNASE2 was elevated in peripheral blood mononuclear cells from SLE patients and was particularly associated with autoreactive B-cell subsets. Moreover, silencing of RNASE2 reduced monocyte-derived IL-10.

Recent years have seen an explosion of research focusing on macrophage polarization in autoimmune disease. We gathered five articles addressing macrophage polarization. Paoletti et al. investigated differences in macrophage polarization capabilities according to treatment. They reported that polarization into M2 macrophage from monocytes of RA patients *in vitro* was decreased compared with that of healthy donors. Interestingly, this *in vitro* defect in monocyte polarization into M2 macrophages was restored in monocytes from RA patients treated with adalimumab, but not in those treated with etanercept. Cutolo et al. reviewed this compelling issue of macrophage polarization in relation to RA synovitis. In the review, the authors suggested that in RA, M1/Th1 activation occurs in an inflammatory environment dominated by TLR and IFN signaling, promoting the abundant production of pro-inflammatory cytokines and matrix metalloproteinases, resulting in osteoclastogenesis and the progression of joint destruction; on the other hand, the activation of M2/Th2 promotes the release of growth factors and cytokines involved in the anti-inflammatory process leading to clinical remission of RA. They reasoned that since the synovial tissue of RA under remission is characterized by a higher presence of M2 macrophages, the regulation of M1/M2 imbalances in favor of anti-inflammatory M2 macrophages might represent a clear therapeutic goal in the management of RA. Bibliometric analysis by Xu et al. quantified the existing research on the roles of macrophages in RA from 2000 to 2021. During that period a total of 7253 original articles related to macrophages in RA were published. In the publications on this subject, "bone loss" and "polarization" were the most frequently used keywords. These authors certainly confirmed that the study of macrophage polarization in RA has become a keen research focus in recent years.

The association of macrophage polarization with inflammatory bowel disease (IBD) was reported by Tian et al. Astragaloside IV (AS- IV), a natural saponin extracted from the traditional Chinese medicine herb *Ligusticum chuanxiong*, attenuated the clinical activity of dextran sulfate sodium (DSS)-induced colitis, which mimics human IBD. That is, AS-IV administration modulated the phenotype change of macrophages, inhibiting pro-inflammatory macrophages and promoting pro-resolution macrophages to ameliorate the progression of DDS-induced colitis *via* the regulation of the STAT signaling pathway. Hirahara et al. reviewed the role of monocytes/macrophages in Behçet's disease (BD), also with a focus on macrophage polarization. They summarized the role of monocytes/macrophages in the pathogenesis of BD, including the issue of genetic factors, and discussed the abnormal macrophage polarization in the context of IL10, also the influence on monocytes of current BD treatments.

He et al. analyzed monocyte subsets in patients with Sjögren's syndrome (SS) and controls. Single-cell RNA sequencing using monocytes from SS patients identified a new

monocyte subset characterized by higher expression of VNN2 and S100A2, and this subset was increased in SS patients. Further, all monocyte subsets from SS patients had increased expression of TNFS10, and the IFN-related and neutrophil activation-associated pathways were also upregulated in SS-derived monocytes.

Regarding vasculitis, we present one original study and one review. Tang et al. reported how extravasation of $CD16^+$monocytes to the kidneys was involved in renal damage in myeloperoxidase ANCA-associated vasculitis (MPO-AAV). An increase of $CD16^+$monocytes in the glomeruli of MPO-AAV patients with renal damage was detected, and this increase was supposed to be caused by the MPO-ANCA promotion of an increase in the C-X3-C motif chemokine ligand 1 (CX3CL1) on glomerular endothelial cells, leading to recruitment of $CD16^+$monocytes. The pathogenic role of monocytes/macrophages in large vessel vasculitis (LVV) was reviewed by Watanabe and Hashimoto They focused on the subpopulation of circulating monocytes and tissue macrophages in LVV and discussed the potential blockades of chemokines or chemokine–receptor interactions that attract circulation monocytes and T cells as a therapeutic option. However, they noted that monocytes might have already been recruited to the vascular tissues and differentiated into macrophages by time LVV has developed.

Because circulating monocytes might migrate to a specific location in the bones and fuse with each other to become mature multinucleated osteoclasts, monocytes are important sources of osteoclasts. We have two review articles addressing this issue. Hasegawa and Ishii reviewed the heterogeneity of osteoclasts involved in inflammatory arthritis. Their single-cell RNA sequence analysis of the osteoclast precursor-containing macrophages in the joints succeeded in specifying the populations that differentiate into mature osteoclasts at the arthritic inflammation site (pannus-bone interface), called "arthritis-associated osteoclastogenic macrophages (AtoMs)". They also discussed the clinical implications of AtoMs, such as factors which most effectively differentiate AtoMs into osteoclasts, specifically receptor activator of nuclear factor-kappa B ligand (RANKL) and TNF. A systematic review by Zuo and Deng summarized the role in osteoclastogenesis of Fc gamma receptors (FcγRs), which are expressed on the surfaces of monocytes and macrophages. The activation of FcγRs is required for RANKL-induced osteoclastogenesis; thus, FcγRs can regulate inflammatory arthritis. The FcγRs may have dual roles in osteoclastogenesis, i.e., both inhibiting and activating, depending on the extent of FcγR occupancy by IgG and RANKL. For example, specific IgG molecules of Fc fragments with a high affinity to FcγRs designed to occupy FcγRI may inhibit osteoclastogenesis.

Kamata and Tada reviewed the roles of dendritic cells and macrophages in the pathogenesis of psoriasis. They focused on plasmacytoid dendritic cells (pDCs) and M1 macrophages in the early phase of psoriasis. pDCs produce IFN-α, causing the maturation of resident dermal DCs and the differentiation of monocytes into inflammatory DCs, which produce the key cytokines of psoriasis. In early-phase psoriasis, M1 macrophages contribute to development of psoriasis by producing TNF-α

Tsuboi et al. reviewed the activation mechanism of monocytes/macrophages into adult-onset Still disease (AOSD). In this review, they discussed monocyte/macrophage activation by several factors such as the pathogen-associated molecular pattern (PAMPs), damage-associated molecular patterns (DAMPs), and neutrophil extracellular traps (NETs)-DNA. These stimulation plays an important role in the pathogenesis of AOSD by causing activation of the nucleotide-binding oligomerization domain and the pyrin domain (NLRP) 3 inflammasomes, which trigger caspase-1 activation, resulting in the conversion of pro-IL-1β and pro-IL-18 into their mature forms. They also identified placenta-specific 8 (PLAC8) as a molecule whose expression is elevated in the monocytes of active AOSD.

A comprehensive overview of current advances in the use of induced pluripotent stem cell (iPSC)-derived monocytes/macrophages for research into autoinflammatory diseases was provided by Tanaka et al. They discussed the possibility of immortalized PSC-derived cell lines produced by introducing *MYC, BML2* and *MDM2* into iPSC-derived floating monocytic cells, and their potential use for research on disease pathogenesis.

The role of monocytes/macrophages in the disease development or progression of autoimmune and autoinflammatory disease can be described as "trained immunity"; for example, some vaccines and microorganisms induce epigenetic changes in monocytes/macrophages, modifying their functional response. In this regard, Funes et al. reviewed the relationship of trained immunity to autoimmune and inflammatory disorders.

Lastly, Nagafuchi et al. summarized their results from transcriptome analysis of various autoimmune diseases. Transcriptome analysis enables researchers to observe the dynamics of gene expression in different cell types. They focused on their recent studies using immuNexUT, a database containing immune cell gene expression data from various immune-mediated diseases and many types of immune cells, in addition to healthy controls. In particular, they discussed how single-cell RNA-seq analysis has provided atlases of infiltrating immune cells in various autoimmune disease lesions, and also how expression quantitative trait locus (eQTL) analysis can help identify candidate causal genes and immune cells.

In conclusion, we are pleased that this issue focusing on the Research Topic "*the role of monocytes/macrophages in autoimmunity and autoinflammation*" has attracted a variety of novel investigations and discussions of current research on monocytes/macrophages that should provide interested readers a wealth of insights on the links between monocyte-macrophage lineage cells and autoimmune/autoinflammatory diseases.

Author contributions

AK, NI and KF have completed this editorial articles. All authors made substantial, direct and intellectual contribution to the work. All authors have given their final approval of the manuscript to be published as presented.

Conflict of interest

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

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SYSTEMATIC REVIEW
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Fc Gamma Receptors as Regulators of Bone Destruction in Inflammatory Arthritis

*Yuyue Zuo and Guo-Min Deng**

Department of Rheumatology and Immunology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

Bone erosion is one of the primary features of inflammatory arthritis and is caused by excessive differentiation and activation of osteoclasts. Fc gamma receptors (FcγRs) have been implicated in osteoclastogenesis. Our recent studies demonstrate that joint-deposited lupus IgG inhibited RANKL-induced osteoclastogenesis. FcγRI is required for RANKL-induced osteoclastogenesis and lupus IgG-induced signaling transduction. We reviewed the results of studies that analyzed the association between FcγRs and bone erosion in inflammatory arthritis. The analysis revealed the dual roles of FcγRs in bone destruction in inflammatory arthritis. Thus, IgG/FcγR signaling molecules may serve as potential therapeutic targets against bone erosion.

Keywords: FcγRs, autoantibodies, osteoclasts, bone erosion, inflammatory arthritis

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Naoki Iwamoto, Nagasaki University Hospital, Japan

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Peng Zhang, Huazhong University of Science and Technology, China
Hyung-Mun Yun, Kyung Hee University, South Korea
Shoichi Fukui, Nagasaki University, Japan

***Correspondence:**
Guo-Min Deng
gmdeng@hust.edu.cn

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INTRODUCTION

Inflammatory arthritis is a group of diseases characterized by joint inflammation and bone damage. About 0.1% of adults develop inflammatory arthritis annually (1). Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by progressive synovitis and bone destruction, causing irreversible joint damage and disability (1–4). Bone erosion is the central hallmark of RA in ultrasonography identification (5, 6). Anti-citrullinated protein antibodies (ACPAs) are considered to be among the leading risk factors for bone destruction in RA (7). Ankylosing spondylitis (AS) and psoriatic arthritis (PsA) are other common inflammatory arthritis diseases with bone destruction (8, 9).

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by multi-organ tissue damage and high levels of autoantibodies in the serum (10). Arthritis is a common clinical manifestation with a prevalence of 69 to 95% in patients with SLE (11). However, only 4 to 6% of patients with SLE arthritis display bone erosion on plain radiographs (12–14). As to ACPA positive SLE patients, which are also called rhupus patients, they often overlapped clinical features and fulfilled American College of Rheumatology (ACR) criteria for RA classification (15, 16). It is still unclear why lupus arthritis without ACPA lacks bone destruction. Recently, FcγRs have been reported to exert a regulatory effect on osteoclastogenesis (17–25). Our recent study demonstrated that joint-deposited lupus IgG triggered arthritis without bone erosion in mice and lupus IgG inhibited osteoclastogenesis induced by receptor activator of nuclear factor kappa-B ligand (RANKL). FcγRI exerted an inhibitory effect of lupus IgG on RANKL-induced osteoclastogenesis (26). Our study suggests that FcγR could function as critical regulators of inflammatory arthritis.

Here, we review the published studies and demonstrate the association between the FcγR and bone erosion in inflammatory arthritis.

Fcγ RECEPTOR FAMILY

FcγRs are receptors for the constant (Fc) region of IgG; these are expressed widely on the surface of immune cells, including monocytes, macrophages, neutrophils, dendritic cells (DCs), B cells, natural killer cells, and mast cells. Four different classes of FcγRs have been identified in mice, namely FcγRI, FcγRIIB, FcγRIII, and FcγRIV (27–29). The human and primate FcγR classifications are more complex. Humans possess six classic FcγRs with different IgG binding capacity and downstream signaling pathways: FcγRI (CD64), FcγRIIA (CD32A), FcγRIIB (CD32B), FcγRIIC (CD32C), FcγRIIIA (CD16A), and FcγRIIIB (CD16B), which are encoded by genes *FCGR1A*, *FCGR2A*, *FCGR2B*, *FCGR2C*, *FCGR3A*, and *FCGR3B*, respectively (**Figure 1**).

The affinity of FcγRs for IgG depends on the type of FcγR and IgG isotypes (30–36). FcγRI is the only known high-affinity FcγR (10^8–10^9 M^{-1}) with a restricted isotype specificity. In contrast, FcγRII and FcγRIII have a low affinity for IgG (about 10^6 M^{-1}) with a broader isotype binding pattern (31, 32). FcγRIV is a novel receptor conserved across all mammalian species with an intermediate affinity (10^7 M^{-1}) and restricted subclass specificity (29, 37). FcγRIIIA is engaged by IgG1 and IgG2, whereas FcγRI and FcγRIV are engaged by IgG2 only (35). The affinity of mouse FcγRs is significantly higher compared with their corresponding human FcγRs (36).

FcγRs are divided into activating and inhibitory receptors and coexpressed on the same cell (38). Activating FcγRs, including FcγRI and FcγRIII, contain an immunoreceptor tyrosine-based activation (ITAM) in intracellular structure and transmit their signals *via* the ITAM, which recruits spleen tyrosine kinase (Syk) (39). FcγRIIB is the only known inhibitory FcγR with an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its intracytoplasmic domain (40). The phosphorylation of ITIM counteracts the signals mediated by activating FcγRs (41–43). FcγRIIB is expressed widely on B cells, macrophages, and mast cells and downregulates several cellular functions, such as B-cell activation and mast cell degranulation (44). The activating-to-inhibitory (A/I) ratio on the same cell acts as the specific checkpoint for the arrest or progression of an immune response. Surprisingly, when monomeric or low-affinity immune complexes bind to activating FcγRs, the normally activating ITAM domain cannot induce co-aggregation of activating receptors, thereby partially phosphorylating the ITAM domain. Thus, partial tyrosine phosphorylation of ITAM by Src family kinases may result in the recruitment of inhibitory SHIP. This is called inhibitory ITAM (ITAMi) signal and is important in maintaining immune homeostasis (45–47).

Unlike other activating FcγRs, FcγRII proteins do not require the common FcR γ-chain for stable expression or function. They all have signaling motifs in their intracellular cytoplasmic domains (48). All the above FcγRs are transmembrane

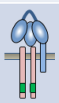
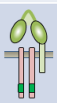
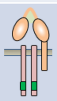
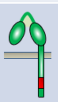
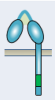
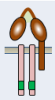
	activating Fc receptors					inhibitory Fc receptors
Mouse						
Name	FcγRI		FcγRIII	FcγRIV		FcγRIIB
Structure						
Gene	Fcgr1		Fcgr3	Fcgr4		Fcgr2b
Affinity	High		Low to medium	Low to medium		Low to medium
Human						
Name	FcγRI (CD64)	FcγRIIA (CD32A)	FcγRIIC (CD32C)	FcγRIIIA (CD16A)	FcγRIIIB (CD16B)	FcγRIIB (CD32B)
Structure						
Gene	FCGR1A	FCGR2A	FCGR2C	FCGR3A	FCGR3B	FCGR2B
Affinity	High	Low to medium	Low to medium	Low to medium	Low to medium	Low to medium

FIGURE 1 | The family of classical Fc receptors for IgG. Schematic representations of FcγRs with respect to the cell membrane (brown bar), in complex with their respective signaling subunits. Mouse and humans have one high-affinity receptor, FcγRI; all other FcγRs have a low-to-medium affinity for the antibody Fc fragment.

glycoproteins, except for human FcγRIIIB, which is expressed on neutrophils and is a glycophosphatidylinositol (GPI)-anchored protein (49, 50). The mechanisms by which FcγRIIIB transduces signals are still unknown (51).

FcγRs AND ARTHRITIS

During autoimmune diseases, such as RA and SLE, the autoantibodies and immune complexes cause inflammation *via* FcR aggregation (52). The altered expression of FcγRs on immune cells in the circulation and synovium of RA patients is the first indication of their involvement in inflammation (53–60). The absence of all FcγRs does not affect the number of osteoclast precursors or their osteoclastogenic potential. However, it reduces joint inflammation and bone erosion during inflammatory arthritis (61). FcγRIIB is particularly critical for maintaining the balance of an efficient inflammatory response or countering unwanted autoimmunity attacks. Multiple clinical studies have shown that FcγRIIB is a reliable biomarker for SLE susceptibility in different ethnic groups. FcγRIIB and its signaling pathways represent a vital checkpoint in peripheral and central tolerance and in controlling the development of autoreactive antibodies (62).

In addition to the altered expression of FcγRs, genetic variants associated with related single-nucleotide polymorphisms (SNPs) in populations with RA and lupus arthritis have been reported. Several genes encoding FcγRs that alter the affinity of FcγRs for IgGs have been described in several RA populations. In particular, some of these, such as the hFcγRIIa-R131 variant, which is related to an increased risk of developing RA, even influence the susceptibility to RA development and the response to treatment (63–70). In addition, an association between lupus arthritis and the FCGR2A as well as FCGR3A low copy number genotypes has been observed in Taiwan patients with SLE. The FCGR3A low copy number genotype was significantly enriched in patients with SLE having arthritis (71–73). Moreover, a meta-analysis revealed the association of the *FcγRIIa-R131* allele with SLE, especially in African Americans, whereas the *FcγRIIIa-F176* allele was associated with SLE in Caucasians and other groups (74). Furthermore, Tsang et al. demonstrated the association between low-affinity FcγR polymorphisms and susceptibility to SLE (75).

Studies using *FcγR* gene-deficient mice have greatly enhanced our understanding of the role of FcγRs in inflammatory arthritis (76, 77). The lack of activating FcγRs alleviates the disease severity in arthritis models (78–81). In different disease phases of inflammatory arthritis, the individual activating FcγRs have different significance (36, 61, 82–86). In the absence of FcγRI, FcγRIIB, and FcγRIIIA, FcγRIV is sufficient to induce arthritis alone (35). In contrast with activating FcγRs, the inhibitory FcγRIIB suppresses inflammation by inhibiting the activating signaling, as well as providing negative feedback on the production of autoantibodies by B cells (87–92).

Autoantibodies and their immune complexes play a central role in shaping a pro-inflammatory environment. Indeed, complexes of ACPA and rheumatoid factor (RF) induce the production of potent inflammatory cytokines (93–96). This effect is predominantly mediated by FcγR signaling on macrophages (51, 97). Tumor necrosis factor (TNF)-α, in combination with cytokines interleukin (IL)-4 and IL-13, downregulates FcγR-mediated function by decreasing the expression of activating FcγRs, suggesting that downregulated activating FcγRs might have an anti-inflammatory effect (98).

The Fc receptors on white blood cells are essential for effective phagocytosis of immune complexes and bacteria. Moreover, FcγRI is upregulated during infection. FcγRI (CD64) has previously been reported to distinguish systemic infections from inflammatory autoimmune diseases and viral infections. Patients without inflammatory and infectious conditions, such as osteoarthritis, have a lower level of neutrophil FcγRI than those with infections (99–104). Oppegaard et al. investigated the use of FcγRI in discerning septic arthritis from inflammatory joint disease and found that FcγRI is highly specific for infectious diseases, including septic arthritis. However, its sensitivity is poor in local infections (104). Although distinct meta-analyses have confirmed this, more large prospective studies need to be conducted to verify several cut-off values reported in the neutrophil FcγRI test in the clinical setting (105, 106).

Human and murine activating FcγRs are not functionally equivalent. A few studies performed in transgenic mice expressing human FcγRs examined their involvement in inflammatory arthritis (107). The results confirmed that the expression of the human FcγRIIA is associated with spontaneous autoimmune inflammation, with a crucial role in autoimmune diseases (92).

FcγR ROLE IN BONE EROSION

Osteoclast Activation and Differentiation

Bone balance depends on a dynamic regulation of bone formation and resorption, which are predominantly mediated by osteoblasts and osteoclasts, respectively (108, 109). Enhanced osteoclast activity could result in severe bone destruction as exemplified in autoimmune inflammatory diseases such as RA, whereas defective osteoblast differentiation causes diseases with a high bone mass, including osteopetrosis. Osteoclasts are the only bone-resorbing cells and play a central role in bone erosion. Osteoclasts are derived from multinucleated progenitors of the monocyte/macrophage family and are the link between immune and bone systems. RANK and RANKL are critical factors that together regulate osteoclast functions. In addition, macrophage colony-stimulating factor (M-CSF) is an essential cytokine in osteoclastogenesis (109–111). RANKL is majorly secreted by osteoblasts, osteocytes, T cells, and endothelial cells. And osteocytes express a much higher amount of RANKL required for osteoclastogenesis than osteoblasts (112, 113). The most important negative regulator of RANKL is the decoy receptor osteoprotegerin (OPG), which inhibits osteoclastogenesis by preventing RANKL–RANK interaction. The RANKL–RANK–OPG system modulates bone homeostasis by regulating

osteoclasts (114). Osteoblasts and osteocytes also produce OPG to suppress osteoclastogenesis by masking RANKL signaling (115, 116). RANKL initiates osteoclastogenesis by inducing nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1), *via* TNF receptor-associated factor 6 (TRAF6) and c-Fos pathways (117) (**Figure 2**). NFATc1 is the master transcription factor for pro-osteoclastogenic genes. In addition, several pro-inflammatory cytokines produced by innate immune cells and T cells, such as TNFα, IL-17, IL-1, and IL-6, stimulate osteoclastogenesis directly or indirectly (118).

FcγRs and Osteoclastogenesis

Apart from the M-CSF and RANKL signaling, an ITAM costimulatory signal provided by the accessory protein for RANKL-RANK is required for osteoclastogenesis (119). Takayanagi et al. first reported that the activation of NFATc1 was insufficient for terminal differentiation of monocytes/macrophages into osteoclasts; calcium signals and calcineurin activation are essential for this process (117, 120). Calcium signals in myeloid cells are provided by the ITAM-bearing proteins, Fc receptor γ subunit, and its functional analog DNAX activation protein of 12 kDa (DAP12). Both the accessory proteins are intracellular adaptor molecules and play a crucial function in transducing the costimulatory signals for RANKL (121). Mice lacking the accessory proteins display a severe osteopetrotic phenotype with deficient osteoclast function (122).

FcRγ-chain is associated with immunoglobulin (Ig)-like receptors, such as osteoclast-associated receptor (OSCAR) and paired Ig-like receptor-A (PIR-A) (**Figure 2**). DAP12 is associated with its signaling counterpart, triggering receptor expressed on myeloid cell-2 (TREM-2), and signal-regulatory protein β1 (SIRPβ1), which are expressed on the cell membrane of osteoclast precursors and are essential for the communication between osteoclast precursors (33, 123). Activation of RANKL-RANK rapidly phosphorylates the ITAM motifs and recruits the protein kinase Syk, subsequently activating multiple downstream signaling cascades, such as phospholipase Cγ (PLCγ) and Bruton's tyrosine kinase (BTK) as well as Tec kinases. They all enhance the effects of RANKL-signaling by augmenting the calcium influx required for the activation of NFATc1. NFATc1 subsequently migrates to the nucleus, where it binds to its gene promoter and triggers an auto-amplifying feedback loop (124, 125).

Osteoclasts and their precursors express FcγR (126), whereas FcγRI, FcγRIIB, and FcγRIIIA are significantly upregulated during human *ex vivo* osteoclastogenesis (127). Blocking of the FcR and deleting the FcγR gene reduce osteoclastogenesis

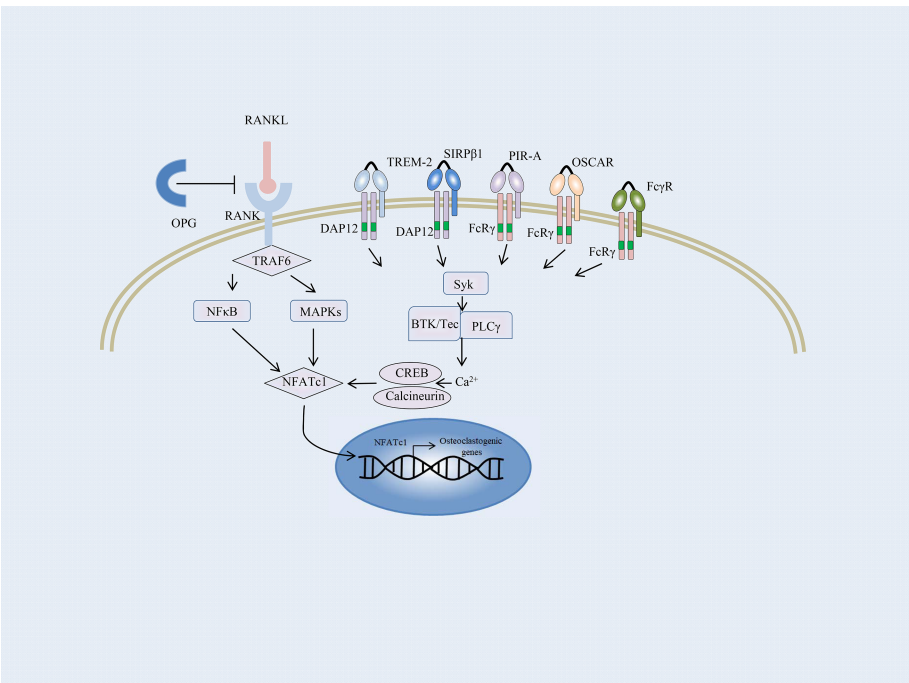


FIGURE 2 | Overview of the osteoclast signaling network. A schematic representation of ITAM-mediated costimulatory signal in the RANKL-induced TRAF6 signaling pathway of osteoclast differentiation. In osteoclast precursors, phosphorylation of ITAM stimulated by immunoreceptors and RANKL–RANK interaction recruits the Syk family kinases, thus activating phospholipase Cγ (PLCγ), Bruton's tyrosine kinase (BTK), as well as Tec kinases. They augment the calcium influx required for the activation of NFATc1. NFATc1 subsequently migrates to the nucleus, where it binds to its gene promoter and triggers an auto-amplifying feedback loop. Calcium signals in osteoclast precursors are provided by the ITAM-bearing proteins, Fc receptor γ subunit, and its functional analog DNAX activation protein of 12 kDa (DAP12). FcRγ-chain is associated with the immunoglobulin (Ig)-like receptors, such as osteoclast-associated receptor (OSCAR) and paired Ig-like receptor-A (PIR-A). DAP12 is associated with its signaling counterpart, triggering receptor expressed on myeloid cell-2 (TREM-2), and signal-regulatory protein β1 (SIRPβ1).

stimulated by IgG complexes on osteoclast precursor cells (25). Although FcγRs are required for osteoclastogenesis and bone resorption in inflammatory disorders, their specific role in bone homeostasis is not completely understood.

FcγRs and Bone Erosion

Costimulatory signals mediated by the ITAM motif cooperate with RANKL for bone homeostasis, suggesting the link between ITAM-harboring adaptors FcγRs and bone erosion (117). As an important link binding the bone system and immune system, FcγRs are not only receptors for the Fc portion of IgG but are also costimulatory molecules for RANKL-induced osteoclastogenesis (119, 121). Bone damage has been reported in seropositive RA patients before clinical disease onset, highlighting that osteoclastogenesis is independent of joint inflammation (128). This finding challenged the concept that inflammation is the primary trigger for bone erosion in inflammatory arthritis and indicated that bone loss might precede inflammation (129).

The expression of Fcγ receptors on osteoclast precursor cells and mature osteoclasts has been measured. FcγRI and FcγRIII are primarily expressed on human preosteoclasts, whereas the inhibitory FcγRIIB is majorly expressed on mature osteoclasts (127). Under physiological conditions, activating FcγRI and FcγRIV in mice does not have a major role in bone characteristics and osteoclast development (22). Bone homeostasis is not significantly different in mice with FcγRI or IV deficiency compared with wild mice (17). In addition, the deficiency of FcγRIIB does not affect osteoclastogenesis (23). Activating FcγRs transmit the positive signal. In contrast, FcγRIII functions as an inhibitory receptor in the differentiation of osteoclast precursor cells under physiological conditions. FcγRIII deprives the FcRγ subunit's availability for other Ig-like receptors activating receptors, such as PIR-A and OSCAR, thus transmitting an ITAM-mediated inhibitory signal for osteoclastogenesis (130). Naive FcγRIII$^{-/-}$ mice have increased osteoclast numbers and an osteoporotic phenotype (22).

The relative importance of various FcγRs in osteoclastogenesis changes in the inflammatory arthritis microenvironment. Studies demonstrated that the stimulation of FcγRI and FcγRIV increases both osteoclast differentiation and function both *in vitro* and *in vivo* (22, 30). FcγRIII levels are increased, and FcγRIIB levels are decreased on bone marrow cells from mice with collagen-induced arthritis (CIA), indicating that FcγRIII induces osteoclastogenesis under inflammatory conditions (22). Furthermore, human RA patients with the *FcγRIIIa-158V* allele endure severe bone erosion compared with patients with the *FcγRIIIa-158F* allele (131, 132). Similarly, artificial crosslinking of FcγRI and FcγRIV leads to increased osteoclast differentiation without affecting their resorbing function *in vitro* (17). Osteoclast numbers and bone erosion were decreased in FcγRIV$^{-/-}$mice compared with wild mice in a serum transfer model (17). FcγRIIB$^{-/-}$mice spontaneously developed osteoporosis, which was reversed by an additional knockout of activating FcγRs (22).

De-sialylated IgGs binding to FcγRs with strong affinity have substantially high stimulatory effects on both murine and human osteoclasts (127, 133). In addition, IgGs were less sialylated during inflammation (22). Harre et al. confirmed that the interactions between immune complexes and osteoclasts were related to the degree of IgG sialylation, and only non-sialylated or low-sialylated immune complexes drive osteoclastogenesis. RA patients with low Fc sialylation levels of IgGs have significantly higher bone loss. The pro-osteoclastogenic effect of non-sialylated immune complexes is a common feature of all IgG antibodies (127). A recent study showed that in induced pluripotent stem cell derived mesenchymal stem cell (iMSCs), the sialylation degree of IgG determines the antibodies directed osteogenic potential by regulating immune responses and osteoclastogenesis (24),but desialylated IgG complexes do not affect arthritis-mediated bone loss (134).

Although the signaling of activating FcγRs mediated by immune complex increases osteoclast differentiation, different results exist for immune complex/FcγR on osteoclastogenesis and osteoclast function (**Table 1**). Previous studies demonstrated the immune complex-induced inhibition of osteoclastogenesis, which possibly acts *via* activating FcγRs (23, 139). This suggests that FcγRs may have dual roles in bone destruction in inflammatory arthritis. High levels of autoantibodies are a characteristic feature of SLE compared with other inflammatory arthritis (140, 141). The deposition of autoantibodies or immune complexes causes lupus nephritis (142), skin damage (143), splenomegaly (144), and damage to other organs. Lupus IgG can promote the differentiation of monocytes into DCs (145). These indicate that lupus autoantibodies may also play a protective role in bone destruction in inflammatory arthritis. Recently, our research results (26) demonstrated that joint-deposited lupus IgG induced arthritis without bone erosion by intraarticular injection of lupus IgG in mice. Monocytes/macrophages and their product TNFα are required for the development of lupus IgG-induced arthritis. To understand the mechanism of lupus IgG-induced arthritis with deficiency of bone erosion, we determined whether lupus IgG inhibited RANKL-induced osteoclastogenesis. We found that lupus IgG directly suppressed RANKL-induced osteoclastogenesis in a dose-dependent manner *in vitro*. The inhibitory effect of lupus IgG on osteoclastogenesis is related to timepoint in lupus IgG and RANKL treated macrophages. Deficiency of FcγRII and FcγRIII did not affect the inhibitory effect of lupus IgG on osteoclastogenesis, indicating that the inhibitory effect of lupus IgG on osteoclastogenesis is dependent on FcγRI. Lupus IgG and RANKL can downregulate the surface expression of FcγRI on bone marrow macrophages (20). Research results suggest that lupus IgG inhibits osteoclastogenesis by competitively occupying FcγRI on monocytes/macrophages and reducing RANKL signaling. The effect of activation or repression of RANKL-induced osteoclastogenesis depends on the extent of FcγRI occupancy by IgG. This protective mechanism explains non-destructive arthritis in SLE. In addition, it implies that FcγRI could be a therapeutic target for bone erosion in inflammatory arthritis.

The deposition of ACPA is important for osteoclastogenesis in RA (146). Different studies have identified that ACPA prevalence is significantly increased in SLE patients with erosive arthritis (16). Recent studies have explored the direct effect of ACPA-mediated bone erosion. ACPA IgG together with their citrullinated antigens forms immune complexes that

TABLE 1 | Different roles of FcγRs in arthritis and bone destruction.

FcγR subtype	Animal model	Function	Mechanism	Reference
FcγRI	CIA; K/BxN arthritis	activation	involving in the early arthritis pathology	(18)
	lupus-like arthritis;	activation OR inhibition	depending on the extent of FcγRI occupancy by IgG and RANKL	(26)
FcγRIIA	CIA; K/BxN arthritis	activation	crosstalking with C5a receptor; driving the osteoclastogenesis independent of RANKL and inflammatory cytokines by binding to IgG-ICs	(107, 135)
FcγRIIB	AIA; CIA; lupus-like disease in FcγRIIB−/− mice	inhibition	inhibition of FcγRI/III; efficient clearance and endocytosis of ICs	(18, 89, 136, 137)
FcγRIII	CIA; K/BxN arthritis	activation	being required for early arthritis onset	(18, 35)
FcγRIV	AIA; K/BxN arthritis	activation	cross-linking with ICs directly; inducing the influx of S100A8/A9-producing neutrophils into the arthritic joint.	(5, 17, 35)
Unclassified	K/BxN arthritis	inhibition	activating FcγRs, but not FcγRIIB mediate IC-induced inhibition of osteoclastogenesis	(23)
	CIA; IC-induced bone destruction	activation OR inhibition	the relative expression of FcγRI/III/IV and FcγRIIB; the availability of ICs	(22)
	CIA	activation OR inhibition	the degree of IgG sialylation determines the effect of FcγRs	(127)
	TNF-induced osteolysis model	inhibition	cross-linking of FcγRs with IVIG suppresses osteoclastogenesis by inducing A20 expression.	(138)

CIA, collagen-induced arthritis; AIA, antigen-induced arthritis; IC, immune complex; IgG, immunoglobulin G; IVIG, Intravenous immunoglobulin; RANKL, receptor activator of nuclear factor kappa-B ligand; TNF, tumor necrosis factor.

stimulate immune cells *via* their interaction with FcγRs (93, 147). By using polyclonal ACPAs purified from ACPA-containing serum of RA patients, Harre et al. provided the first validation that ACPAs can directly promote osteoclast differentiation and activation (7). ACPA IgG might affect osteoclastogenesis by the activation of Fc receptors on osteoclasts directly. IgG Fc sialylation is crucial for immune complex–osteoclast interactions (127). Besides, ACPA IgG is shown less sialylated than random IgG (148). There are other published papers regarding the detailed mechanisms of ACPA's direct regulation, but the exact mechanism of ACPA's direct effect on erosion remains unclear (149–152).

FcγR IMMUNOTHERAPY

The crucial role of FcγRs in both inflammatory arthritis and bone erosion may offer a promising therapeutic target for bone destruction in inflammatory arthritis. One indirect mechanism involves the neutralization of autoimmune IgG Fc by soluble FcγRs, These drugs include the recombinant soluble FcγIIB receptor SM101 (NCT03851341) and monoclonal antibody targeted the receptors. For example, antagonistic monoclonal antibody against the hFcγRIIIA has been shown to be effective in a patient with immune thrombocytopenia (ITP) refractory to all conventional therapies (153). And human recombinant soluble FcγRIIB treatment could ameliorate collagen-induced arthritis by reducing immune complexes-stimulated inflammation and joint swelling (154). Besides, recombinant human soluble FcγRII was evaluated as an effective therapeutic strategy in inhibiting chronic murine lupus pathology (155).

Another mechanism involves the direct blocking of the IgG-binding site on FcγRs. Recombinant multimeric Fc fragments with a high affinity for FcγRs have been shown to be efficacious in animal models of RA, ITP, and graft-versus-host disease (GVHD) (156). These include PF-06755347 (NCT03275740), CSL730 (NCT04446000) and CSL777 (Preclinical) (157). However, nonspecific crosslinking of activating FcγRs could lead to undesired clinical adverse events, and monovalent antibody derivatives, such as Fab, may reduce severe clinical adverse events (158). Up to now, results of above molecules from clinical trials have been promising in autoimmune diseases, but further long-term data are needed (159, 160).

Intravenous immunoglobulin (IVIG) treatment is efficient in several different immune disorders (161, 162). IVIG consists predominantly of IgG and a small fraction of immune complexes. It exerts anti-inflammatory effects in both humans and animal models by its Fc but not Fab fragments (163). Besides, previous studies confirmed that IVIG could directly inhibits human osteoclastogenesis by suppressing the RANK signaling, the suppressive effect is partly mediated by IgG immune complexes contained within IVIG preparations (138). Our study showed that lupus IgG induced synovial inflammation but inhibited RANKL-induced osteoclastogenesis. The suppressive effect is mediated by the competitive occupation of FcγRI on monocytes/macrophages (26).

CONCLUSIONS AND FUTURE PERSPECTIVES

Bone erosions are remarkable features in inflammatory arthritis, such as RA, but not in lupus arthritis. Osteoclasts are major cells for bone erosions. Activating FcγR containing ITAM motifs is required for RANKL-induced osteoclastogenesis. FcγR can effectively regulate inflammatory arthritis and bone erosions. Based on published studies, we conclude that FcγR may have dual roles in osteoclastogenesis. The effect of activating and inhibiting

osteoclastogenesis depends on the extent of FcγRI occupancy by IgG and RANKL, respectively. Specific IgG molecules or Fc fragments with a high affinity for FcγRI designed to occupy FcγRI may exert the inhibitory effect on bone erosion. The sialylation level of IgG Fc binding to FcγRs needs to be taken into account as well. A deeper understanding of FcγRs involved in physiological and pathological osteoclastogenesis will be valuable in identifying new targets and developing potential therapeutic strategies for inflammatory arthritis.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

YZ and G-MD designed the manuscript and figures. YZ and G-MD drafted the manuscript and approved the final version of the manuscript. G-MD revised the final version of the manuscript critically. All authors contributed to the article and approved the submitted version.

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Astragaloside IV Alleviates the Experimental DSS-Induced Colitis by Remodeling Macrophage Polarization Through STAT Signaling

Lianlian Tian[1†], Jun-Long Zhao[2†], Jian-Qin Kang[1†], Shi-bo Guo[1], Nini Zhang[1], Lei Shang[3], Ya-Long Zhang[1], Jian Zhang[4], Xun Jiang[1*] and Yan Lin[1*]*

[1] Department of Pediatrics, Tangdu Hospital, Air Force Medical University, Xi'an, China, [2] State Key Laboratory of Cancer Biology, Department of Medical Genetics and Developmental Biology, Air Force Medical University, Xi'an, China, [3] Department of Health Statistics and Ministry of Education, Key Laboratory of Hazard Assessment and Control in Special Operational Environment, Air Force Medical University, Xi'an, China, [4] State Key Laboratory of Cancer Biology, Department of Biochemistry and Molecular Biology, Air Force Medical University, Xi'an, China

Inflammatory bowel disease (IBD) is characterized by chronic and relapsing intestinal inflammation, which currently lacks safe and effective medicine. Some previous studies indicated that Astragaloside IV (AS-IV), a natural saponin extracted from the traditional Chinese medicine herb *Ligusticum chuanxiong*, alleviates the experimental colitis symptoms *in vitro* and *in vivo*. However, the mechanism of AS-IV on IBD remains unclear. Accumulating evidence suggests that M2-polarized intestinal macrophages play a pivotal role in IBD progression. Here, we found that AS-IV attenuated clinical activity of DSS-induced colitis that mimics human IBD and resulted in the phenotypic transition of macrophages from immature pro-inflammatory macrophages to mature pro-resolving macrophages. *In vitro*, the phenotype changes of macrophages were observed by qRT-PCR after bone marrow-derived macrophages (BMDMs) were induced to M1/M2 and incubated with AS-IV, respectively. In addition, AS-IV was effective in inhibiting pro-inflammatory macrophages and promoting the pro-resolving macrophages to ameliorate experimental colitis *via* the regulation of the STAT signaling pathway. Hence, we propose that AS-IV can ameliorate experimental colitis partially by modulating macrophage phenotype by remodeling the STAT signaling, which seems to have an essential function in the ability of AS-IV to alleviate the pathological progress of IBD.

Keywords: inflammatory bowel disease, astragaloside IV (AS-IV), macrophages polarization, STAT signaling, pro-resolving macrophage

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***Correspondence:**
Yan Lin
yiyilin19800823@126.com
Xun Jiang
jiangx@fmmu.edu.cn
Jian Zhang
biozhangj@fmmu.edu.cn

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

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INTRODUCTION

Inflammatory bowel disease (IBD) includes two distinct disorders, Crohn's disease (CD) and ulcerative colitis (UC), which is a complex disease caused by the interaction of environmental factors, immune disorders, and bacterial imbalances in the genetic background (1–3). The incidence of IBD is rising in the world, especially in Africa and Asia (4, 5). Multiple factors, such as genetic

background, environmental changes, abnormal gut microbiota, and mucosal immune dysregulation, have been suggested to contribute to IBD pathogenesis. The current therapies for IBD mainly include anti-inflammatory drugs and biologics, such as corticosteroids, 5-aminosalicylic acid (5-ASA)-based agents, azathioprine, and TNF-α inhibitors (6, 7). However, the side effects of these medicines were unacceptable to some people, such as nausea, diarrhea, and abdominal pain. More important, even with an aggressive top-down approach to therapy, the majority of patients fail to achieve prolonged, steroid-free remission and are at particular risk of requiring surgery. Therefore, it is crucial to devise novel therapeutic strategies for the treatment of IBD.

The previous studies have demonstrated that mucosal immunity plays a significant role in establishing and maintaining gut homeostasis, especially for intestinal macrophages. During inflammation, the terminal differentiation of monocytes and maturation of intestinal macrophages are disrupted (8, 9). At the onset of intestinal inflammation, accumulated immature macrophages maintain their pro-inflammatory ability by secreting inflammatory cytokines, such as IL-12, IL-23, and IL-1β, which is similar to the classic macrophage activation pattern (also known as M1). With the development of IBD, phagocytosis of apoptotic cells triggers the functional transition of macrophages to the pro-resolving phenotype (10, 11), similar to the alternative macrophage activation pattern (also known as M2). The pro-resolved intestinal macrophages increase anti-inflammatory cytokines production, repress pro-inflammatory factors secretion, enhance phagocytic activity, and acquire the expression of scavenger receptors that are essential for homeostasis (12). It is significant for IBD recovery to remodel mucosal immunity and facilitate pro-resolving macrophages development.

Traditional herbs have been used to treat colitis in China for hundreds of years (13) and have been increasingly recognized worldwide for their low toxicity, low side effects, and is well tolerated. Of note, AS-IV (3-O-β-D-xylopyranosyl-6-O-β-D-glucopyranosyl cycloastragenol), a natural saponin isolated from *Astragali radix*, has been reported to present antioxidant, cardioprotective, anti-inflammatory, antiviral, antibacterial, antifibrosis, anti-diabetes, and immunoregulation effects (14, 15). In addition, AS-IV could alleviate experimental colitis symptoms *in vitro* and *in vivo* (16, 17). However, the definite mechanisms of AS-IV remain unclear. Recent studies have shown that AS-IV enhances diabetic wound healing involving upregulation of alternatively activated macrophages (18). In addition, AS-IV inhibits progression and metastasis of lung cancer by regulating macrophage polarization through the AMPK signal (19). AS-IV also has been demonstrated to ameliorate steroid-induced osteonecrosis of the femoral head by repolarizing the phenotype of pro-inflammatory macrophages (20). However, during IBD progression, whether and how AS-IV exerts pro-resolving effects *via* remodeling intestinal macrophages development remains to be explored. In this study, we reported that AS-IV attenuated clinical activity of DSS-induced colitis and resulted in the phenotypic transition of macrophages. Further mechanism studies revealed that AS-IV ameliorates experimental IBD partially by modulating macrophage phenotype *via* the regulation of the STAT signaling pathway.

MATERIALS AND METHODS

Animals

Male specific-pathogen-free (SPF) C57BL/6 J mice (6-8 weeks old, 21 ± 2 g) were purchased from Experimental Animal Center, Air Force Medical University (Xi'an, China). They acclimated for 7 days with tap water and a pelleted basal diet before the start of the experiments. The temperature was maintained at 23°C ± 2°C, humidity 50%-60%, 12h light/dark cycles. Throughout the experiments, mice were fed with a standard chow diet and tap water. The animal room was cleaned regularly during the holding period.

DSS-Induced Colitis

To induce experimental colitis, mice were induced in the animals *via* oral administration of 2.5% (w/v) DSS (MW 36,000–50,000; MP Biomedicals) in fresh drinking water for 5 consecutive days. For evaluation of colitis progression, mice received normal drinking water for additional 10 days. AS-IV powders ($C_{41}H_{68}O_{14}$, molecular weight: 784.9702, purity >98%) (Dalian Meilun) was dissolved in 0.5% sodium carboxymethyl cellulose (CMC) at a concentration of 10mg/mL or 20mg/mL before experiment. The mice were orally administered 50 or 100 mg/kg AS-IV once daily for 10 days (from day 0 to day 10) and 5-Aminosalicylic acid(5-ASA) 150 mg/kg was used as the positive control (Med Chem Express Co.). The body weight of the mice was monitored every day. The disease activity index (DAI) score was determined as previously reported (21). In the termination of the study, the mice were euthanized using CO_2, and the colon was removed from animals. The length of the colon was measured. All experimental procedures were approved by the Ethical Committee of Air Force Medical University.

Histological Analysis

The distal section of colon tissues was immediately fixed in 4% formalin overnight at room temperature, embedded in paraffin wax, and stained with H&E. The histological score of the colon was determined as previously described (22).

Measurement of MPO

The distribution number of neutrophils in colonic samples was detected by Myeloperoxidase (MPO) activity. Colonic samples were weighed and homogenized using reaction buffer. Then, the MPO activity assay kit (Nanjing Jiancheng Co.) was applied to assess the MPO activity.

Real-Time PCR Assay (RT-PCR)

After obtaining murine lamina propria immune cells or BMDMs, Trizol reagent (Invitrogen) was used to extract total RNA immediately. NanoDrop 2000/2000c UV-Vis (Thermo scientific, USA) was used to measure RNA quantification. We also assessed

the contamination by the A260/280, and the integrity by electrophoresis to confirm RNA quality which including purity and integrity. cDNA was synthesized by using 1μg of total RNA with a reverse transcription kit (Takara, Dalian, China) following the supplier's instructions that means the reaction was run under the conditions of 37°C for 15 minutes and 85°C for 5 seconds. 250 nM of specific primers, a kit (SYBR Premix EX Taq, Takara) and the ABI Prism 7500 Real-Time PCR System were used for quantitative real-time PCR in triplicates under the conditions of step one (95°C for 15 minutes) and 40 cycles of step two (one cycle: 95°C for 10 seconds, 58°C for 20 seconds and 72°C for 20 seconds), with β-actin as an internal control. The gene expression level was normalized by subtracting the expression level of β-actin of the same group, and the different expression levels were calculated using the comparative Ct ($2^{-\triangle\triangle}$Ct) method. Primers are listed in **Table S1** in **Supplementary Material**.

Cell Culture and AS-IV Treatment

Bone marrow cells were harvested from tibias and femurs of C57BL/6J mice and subjected to erythrolysis by using red blood cell lysis buffer. Then, the cells were cultured at a density of 2×10^6/mL in DMEM containing 10% FBS and 25 ng/mL murine macrophage-colony stimulating factor (M-CSF) (PeproTech, Rocky Hill, NJ) for 7 days to obtain BM-derived macrophages (BMDMs). BMDMs were stimulated with IFNγ (20 ng/ml, SinoBiology, China) and LPS (50 ng/mL, Sigma, Saint Louis, MO) or IL-4 (20 ng/mL, SinoBiology) for 24 h before further analyses. Macrophages treated with PBS, LPS + IFN-γ, or IL-4 were named M^{PBS}, M^{LPS}, or M^{IL-4}, according to Epelmann et al. (23).

AS-IV was dissolved in dimethyl sulfoxide (DMSO) for the treatment of macrophages. The final concentration of DMSO was less than 0.1% (v/v). For treatment with AS-IV (100μM), cells were incubated for 24 h after BMDM treated with PBS, LPS + IFN-γ, or IL-4. In some experiments, phosphorylated STAT1 inhibitors (Med Chem Express Co.) were added and treated with AS-IV (100μM) after BMDM treated with PBS, LPS + IFN-γ, or IL-4. To exclude the effects of DMSO, the adherent macrophages were treated with DMSO (0.1%) alone for 24 h.

Isolation of Murine Lamina Propria Immune Cells From Colonic Tissues

Isolation of colonic lamina propria cells was performed according to the protocol. Entire colons from each group were longitudinally cut and washed to remove feces. They were then cut into 1 cm pieces, followed by incubation with a predigestion solution containing 5 mM EDTA and 0.145 mg/ml DTT for 20 min at 37°C on a shaking platform. After removal of EDTA by three washes in PBS and passing through a cell strainer (100 μm), the suspension of epithelial, subepithelial, and villus cells was removed. The remaining colon pieces, including lamina propria cells and muscle layer, were cut by using scissors and then incubated in digestion media containing 0.05 g of collagenase D (Roche), 0.05 g of DNase I (Sigma-Aldrich), and 0.3 g of dispase II (Roche) for 25 min at 37°C on a shaking platform. After digestion, the lamina propria cells were enriched using Percoll density gradient centrifugation. The resulting cells were then used for flow cytometry analysis.

Western Blot Analysis

BMDMs and colonic lamina propria cells were harvested and the whole-cell lysates were extracted on ice with the RIPA buffer containing a protease inhibitor cocktail (Beyotime, Haimen, China). Lysates were centrifuged and the supernatants were collected. Protein concentration was determined using a BCA protein assay kit (Pierce). Aliquots of protein lysates were separated by 10% SDS-PAGE and blotted onto polyvinylidene fluoride membrane. Membranes were blocked with bovine serum albumin solution and then incubated overnight at 4°C with a 1:1000 dilution of STAT1, p-STAT1, STAT3, p-STAT3, and β-Actin antibodies, followed by HRP-conjugated secondary antibodies. Protein bands were visualized with chemiluminescent reagents (Pierce).

Flow Cytometry

Colonic lamina propria cells were stained with antibodies against CD45, CD11b, F4/80, MHC II, or Ly6C for 30 min at 4°C in the dark, then washed twice and resuspended in 500μL of phosphate-buffered saline (PBS). The cells were sorted on a FACS AriaIII flow cytometer (BD Immunocytometry Systems) and analyzed using the FlowJo software (Ashland, OR).

Luciferase Assay

A DNA fragment containing 4 × STAT1 recognized elements were synthesized and inserted into pGL3-promoter to construct a pGL3-STAT1 reporter, which is used to monitor the activation of STAT1 protein. HeLa cells (2×10^4) were transfected with pGL3-basic and pGL3-STAT1 reporters using Lipofectamine 2000™ (Invitrogen), TNF-α, and AS-IV of different concentrations were added simultaneously. The luciferase activity was assessed 48 h later using Luminoskan Ascent (Labsystems, Helsinki, Finland) and a Dual-Luciferase Reporter Assay Kit (Promega) according to the manufacturer's protocol. All luciferase activity was normalized to the Renilla luciferase activity.

Immunostaining and Confocal Microscopy

Colon tissues were removed, rinsed in PBS, embedded in O.C.T. compound (Sakura Finetek), and frozen in an isopentane bath on dry ice. Sections (5 μm) were cut on a microtome and were stained with a mixture of antibodies, including an anti-Rabbit CD206 and anti-Rat F4/80. The slides were counterstained with DAPI (Vector Laboratories, Inc.) to identify nuclei. Images were observed and captured with mages were captured with an FV1200 laser scanning confocal microscope (Olympus, Inc.). The positive area was quantified using Viewer software CaseViewer and analyzing five high power fields per section and per animal.

Homology Modeling and Molecular Docking

STAT1 protein sequences were downloaded from the UniProt database (https://www.uniprot.org/), then submitted sequences

to the Robetta server (http://robetta.bakerlab.org/). Homology modeling of protein structure was performed using the TrRefineRosetta modeling method. Further, the protein structure was submitted to the website (https://saves.mbi.ucla.edu/) for Ramachandran plot analysis. The results showed that the Ramachandran plot score was 92.4%, which indicated that the structure of the protein was more accurate and could be used for the subsequent molecular docking study. The docking of AS-IV with STAT1 was performed with the Autodock 4.1 software. Firstly, the protein structure was set by hydrogenation and charge additional. Then the energy of the small molecule compound ligand (AS-IV) was minimized, and rotatable bonds and the numbers were set. The docking pocket coordinates were x: 25.0, y: 34.0, and z: -32.0, with a radius of 20 Å. Finally, the molecular docking was evaluated according to the docking energy, and the optimal docking conformation was retained to demonstrate a 2-dimensional diagram.

Statistical Analysis

Statistical analysis was performed with the Graph Pad Prism 8.0 software. Student's t-test or one-way ANOVA test was used for statistical analyses. All the experiments were performed at least three times, and the acquired results are shown as mean ± SD. $P<0.05$ was considered statistically significant.

RESULTS

Immature Macrophages Recruitment Facilitated the Inflammatory Progression of IBD

IBD is featured by a series of events comprising an activation phase and gradually followed by a resolution phase (12, 24). To assess the changes of inflammation in experimental IBD mice at different phases, we established the DSS-induced colitis model (**Figure 1A**). The mice treated with DSS presented significant presentations of IBD, including weight loss, bloody stool, and diarrhea. The weight loss observed in the DSS group was significantly aggravated from the 7th day after DSS treatment and began to regain weight from the 10th day (**Figure 1B**). The results of the DAI score showed that the gross colitis of DSS-induced mice appears and gradually worsened from the 3rd day of modeling (**Figure 1C**). Colon length was further measured and statistically processed. Compared with the control group, the results showed that colon length was shortened in the activation phase and increased in the resolution phase (**Figure 1D**). The changes of mice's intestinal mucosa in the acute active phase group were observed using HE staining. The results showed specific pathological changes of IBD, including epithelial cell shedding, crypt structure destruction, infiltration of lymphocytes and neutrophils, connective tissue hyperplasia, and intestinal gland dilatation. However, intestinal inflammation gradually returned to homeostasis in the resolution phase (**Figure 1E**). The histopathological score were significantly higher in the activation phase group than in the control group. Compared with the activation phase group, the histopathological scores of the resolution phase group decreased and were close to the control group (**Figure 1F**).

Most steady-state mouse colonic macrophages are derived from a CC-chemokine receptor 2 (CCR2)-dependent infiltration of lymphocyte antigen 6C-high ($Ly6C^{hi}$) blood-derived monocytes. When $Ly6C^{hi}$ monocytes enter the lamina propria, they undergo a gradual differentiation and acquire expression of major histocompatibility complex II (MHCII) whereas loss expression of Ly6C. This phenotypic differentiation of blood-derived monocytes is paralleled by a progressive acquisition of typical functions of mature pro-resolving macrophages (8, 25). Therefore, we evaluated the development of immune cells by using FACS. The results indicated that the inflammatory macrophages recruited ($CD11b^{+}F4/80^{low}$, Inf-Mφ) from bone marrow were increased significantly at the activation phase (DSS 10 days) and retrieved at the resolution phase (DSS 15 days), while the resident macrophages ($CD11b^{+}F4/80^{hi}$, Res-Mφ) presented no difference. More importantly, the promotion of inf-Mφ was majorly contributed to the immature macrophage population with $MHCII^{-}Ly6C^{+}$ phenotype (**Figures 1J, K**). It has been reported that $MHCII^{-}Ly6C^{+}$ macrophages were identified with M1-like polarization and presented pro-inflammatory functions (12). Therefore, we further determined the relative mRNA levels of inflammatory cytokines, including *Il-1β*, *Tnf-α*, and *Il-6*. The results suggested that the expression of these cytokines was increased at the activation phase and reduced at the resolution phase (**Figures 1G–I**). Taken together, these data demonstrated that the recruitment of $MHCII^{-}Ly6C^{+}$ immature macrophages promoted the inflammatory response and facilitated the damage of IBD.

AS-IV Attenuates Inflammatory Progression of DSS-Induced Colitis

At present, IBD is recurrent and lacks effective medicine (26). Some previous studies demonstrated that AS-IV alleviates the symptoms of multiple inflammatory diseases (15). To characterize the effect of AS-IV on the evolution of IBD, we established the DSS-induced colitis model with AS-IV administration. Meanwhile, the treatment of 5-ASA, which is identified as the routine medicine for IBD, was considered as the positive control. After AS-IV treatment, bodyweight loss in the colitis model was significantly alleviated and similar to the Ctrl group and 5-ASA group (**Figure 2A**). Additionally, the DSS/AS-IV50 group and DSS/AS-IV100 group exhibited a dose-dependently decreased cumulative DAI compared with the DSS+CMC group (**Figure 2B**). Furthermore, the reduction in colon length, which is a marker of intestinal inflammation, was less pronounced in DSS/AS-IV50 group and DSS/AS-IV100 group compared with the DSS group (**Figures 2C, D**). As expected, MPO activity, an indicator of neutrophil aggregation, was significantly reduced in the AS-IV treatment group than in the DSS group (**Figure 2E**). Additionally, H&E histopathology results showed that treatment with AS-IV significantly alleviated the inflammatory response in comparison with treatment with the DSS group (**Figure 2G**). Compared with the DSS group, the blinded histological injury scores in the distal colon of the AS-IV treatment group

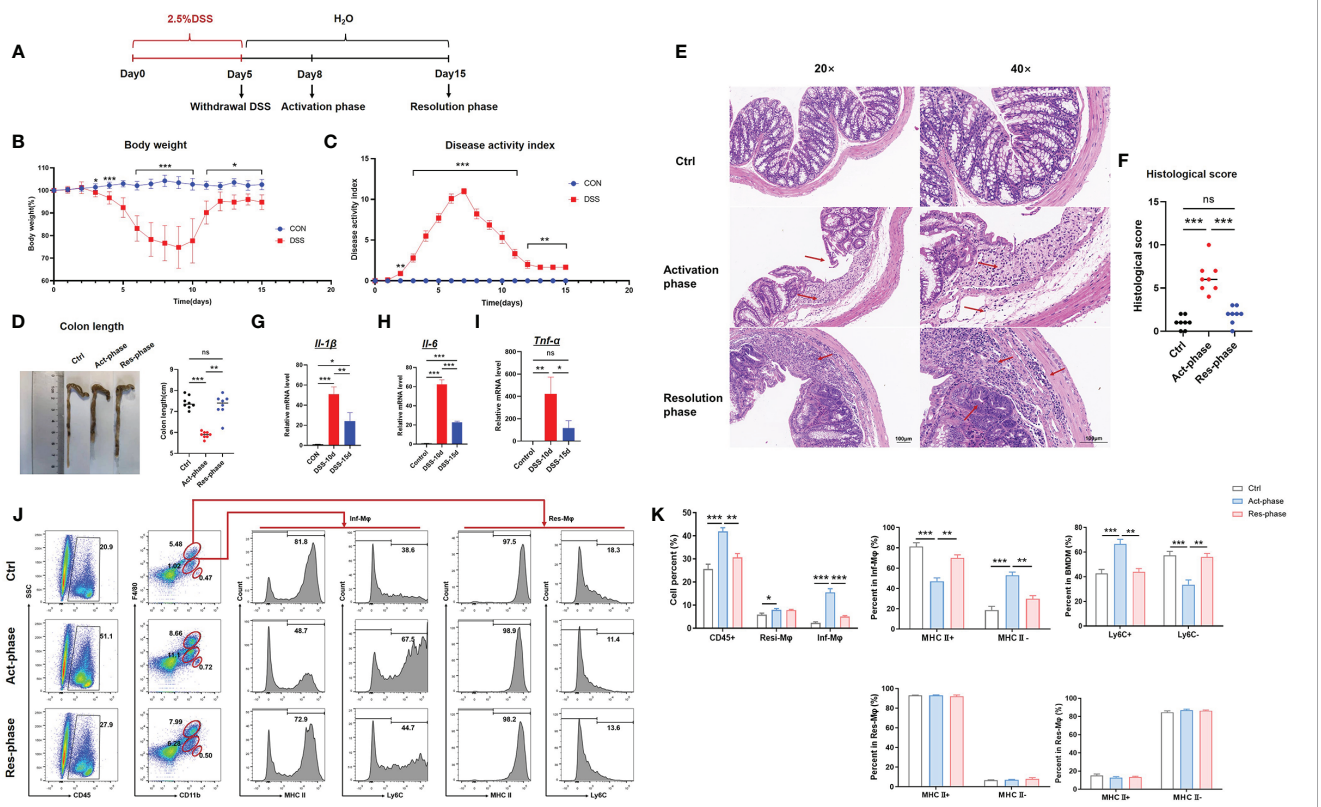

FIGURE 1 | Immature macrophages recruitment facilitated the inflammatory progression of IBD. **(A)** Establishment of DSS-induced colitis mice. **(B)** The daily mean weight change in the activation phase and resolution phase of DSS-induced colitis. **(C)** The changes in DAI in different stage phases scored from diarrhea, bleeding, and body weight loss. **(D)** On the activation phase (Act-phase) and resolution phase (Res-phase), the mice were sacrificed, their colons were removed, and the lengths of their colons were measured and recorded. Macroscopic appearance of the colon, as represented by the colon with the mean colon length. **(E)** The histopathological changes in the colon tissue samples were examined by H&E staining (20×, 40×). **(F)** Histopathological scores were determined for the colon tissue samples. **(G–I)** The relative mRNA expression levels of the inflammatory cytokines *Il-1β*, *Il-6*, and *Il-10* in colon tissue were determined by RT-PCR. **(J)** Intestinal macrophages were analyzed by fluorescence-activated cell sorting (FACS) after staining with anti-F4/80, anti-CD11b, and anti-MHCII (or anti-Ly6C). **(K)** The number of the different subpopulations in **(J)** was determined and quantitatively compared. The student's t-test or One-way ANOVA test was used for statistical analyses. Bars represent means ± SD; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. ns, no significance.

were significantly decreased (**Figure 2F**). Previous studies showed certain cytokines and pro-inflammatory mediators were increased in DSS-induced colitis. Therefore, we further investigated whether the protective effects of AS-IV on DSS-induced colitis in mice were correlated with reductions in pro-inflammatory mediator and cytokine production. The results indicated that the expression of *Il-1β* and *Tnf-α* mRNA was significantly increased in the colon of the DSS group, whereas AS-IV could substantially decrease the expression of *Tnf-α* and *Il-1β* in a dose-dependently manner (**Figures 2H, I**). Meanwhile, AS-IV could dramatically increase the expression of *Il-10* and *Tgf-β* in a dose-dependently way (**Figures 2J, K**). These results indicated that AS-IV attenuates the inflammatory progression in DSS-induced colitis *in vivo*.

AS-IV Remodeled the Development of Intestinal Macrophages

As mentioned above, macrophages are the gatekeepers of intestinal immune homeostasis. Decreasing intestinal macrophage leads to a loss of tolerance to symbiotic bacteria and food antigens, which is thought to be the foundation for the chronic inflammation observed in IBD (27). Therefore, immunofluorescence staining was performed to evaluate the number of infiltrated macrophages. The results suggested that DSS-induced promotion of macrophage was retrieved by AS-IV administration (**Figures 3A, B**). In addition, different subpopulations of intestinal macrophages play different roles during IBD progression. The immature macrophage with inflammatory functions was increased significantly at the activation phase of IBD (**Figure 1J**). We detected the effect of AS-IV on the development of intestinal macrophages by FACS (**Figure 3C**). The results indicated that the increase of Inf-Mφs induced by DSS stimulation was restored by AS-IV administration, although Res-Mφs were not influenced. Moreover, AS-IV significantly promoted $MHCII^{lo}Ly6C^{hi}$ immature macrophages to differentiate into $MHCII^{hi}Ly6C^{lo}$ mature macrophages with resolving functions (**Figures 3D–H**). Furthermore, the number of infiltrated granulocytes ($CD45^{+}CD11b^{+}F4/80^{hi}$) was also reduced by AS-IV treatment (**Figure 3C**). Taken together, these findings indicated that AS-IV improved the immune

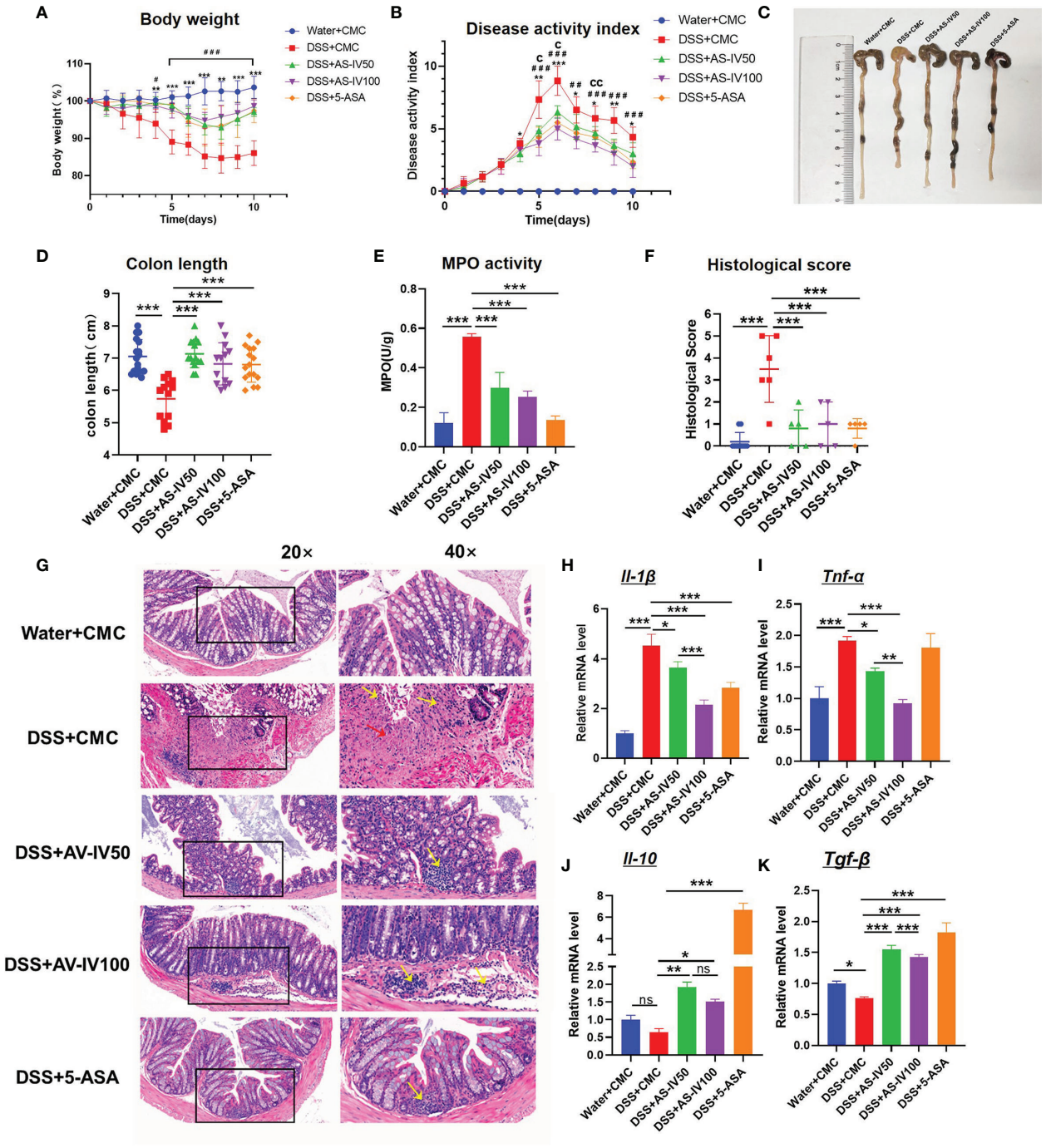

FIGURE 2 | AS-IV treatment attenuates inflammatory progression of DSS-induced colitis mice. The following groups of mice were used in the study: Ctrl (Water+CMC); Model group (DSS+CMC); DSS+AS-IV50mg/kg (DSS+AS-IV50); DSS+AS-IV100mg/kg (DSS+AS-IV100); and 5-ASA 150 mg/kg treatment(5-ASA) (n=8), and each group was used for three independent experiments. The mean values ± SEMs are represented by bars. **(A)** The daily mean weight change in each group (*P < 0.05, **P < 0.01, ***P < 0.001.DSS+AS-IV50 *versus* DSS+CMC). ($^{\#}$P < 0.05, $^{\#\#}$P < 0.01, $^{\#\#\#}$P < 0.001.DSS+AS-IV100 *versus* DSS+CMC). **(B)** The changes in DAI, scored from diarrhea, bleeding, and body weight loss (^{C}P < 0.05, ^{CC}P < 0.05 DSS+AS-IV100 *versus* DSS+AS-IV50). **(C)** On day 10, the mice were sacrificed, their colons were removed, and the lengths of their colons were measured and recorded. **(D)** Macroscopic appearance of the colon, as represented by the colon with the mean colon length. **(E)** Colonic MPO activity. **(F)** Histopathological scores were determined for the colon tissue samples. **(G)** The histopathological changes in the colon tissue samples were examined by H&E staining (20×, 40×). **(H–K)** The relative mRNA expression levels of the inflammatory cytokines *Il-1β*, *Tnf-α*, *Il-10*, and *Tgf-β* in colon tissue were determined by RT-PCR, Student's t-test or One-way ANOVA test was used for statistical analyses. Bars represent means ± SD; *P <0.05, ** P <0.01, *** P <0.001. ns, no significance.

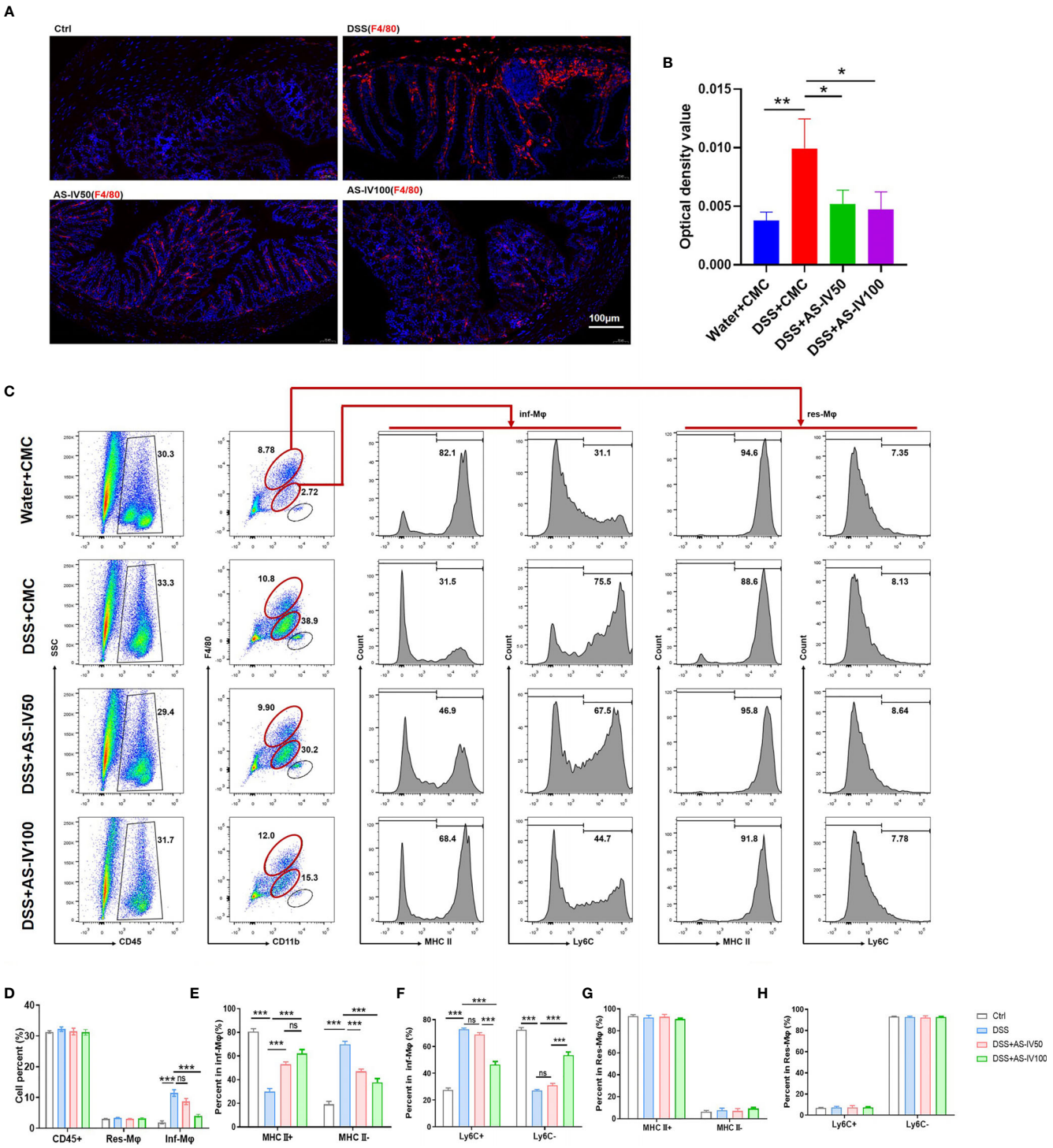

FIGURE 3 | AS-IV remodeled the development of intestinal macrophages. **(A)** F4/80 protein expression in colon tissue was detected by immunofluorescence. Positive immunoreactivity for F4/80 protein expression is indicated by the red color. The slides were counterstained with DAPI (blue). In addition, the sum of OD was analyzed in **(B). (C)** Intestinal macrophages were collected and analyzed by fluorescence-activated cell sorting (FACS) after staining with anti-F4/80, anti-CD11b, and anti-MHCII (or anti-Ly6C). **(D–H)** The number of the different subpopulations in **(C)** was determined and quantitatively compared. One-way ANOVA test was used for statistical analyses. Bars represent means ± SD; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. ns, no significance.

microenvironment *via* remodeling intestinal myeloid cells development and protected from DSS-induced colitis.

AS-IV Administration Modulated the Plasticity of Intestinal Macrophages

Previous studies have shown that macrophages exhibit a distinct phenotype and functional differences in response to different inflammatory stimuli, manifested as M1/M2 polarized macrophages. However, the phenotype of a macrophage is difficult to accurately define as M1 or M2 polarization in many diseases. The plasticity of intestinal macrophages would be altered during the progression of IBD (12, 27). To assess the effect of AS-IV on macrophages polarization, we sorted intestinal macrophages by flow cytometric and examined the mRNA expression levels of polarization markers and cytokines by RT-PCR, including *Il-12*, *Il-1β*, *Il-6*, *Tnf-α*, *CD206*, *Arg1*, *Il-10*, and *Tgf-β*. As presentation in **Figures 4A–D**, DSS-induced increases in the mRNA expression levels of the pro-inflammatory cytokines *Tnf-α* were decreased by AS-IV treatment. In contrast, mRNA expression levels of the pro-resolving cytokines *CD206* and *Tgf-β* were increased by AS-IV treatment compared with the DSS group (**Figures 4E–H**). Additionally, we examined the protein expression levels of the F4/80 and CD206 in colon tissue by immunofluorescence, which represented pro-resolving macrophages (**Figures 4I–J**). Consistent with the above results, immunofluorescence results

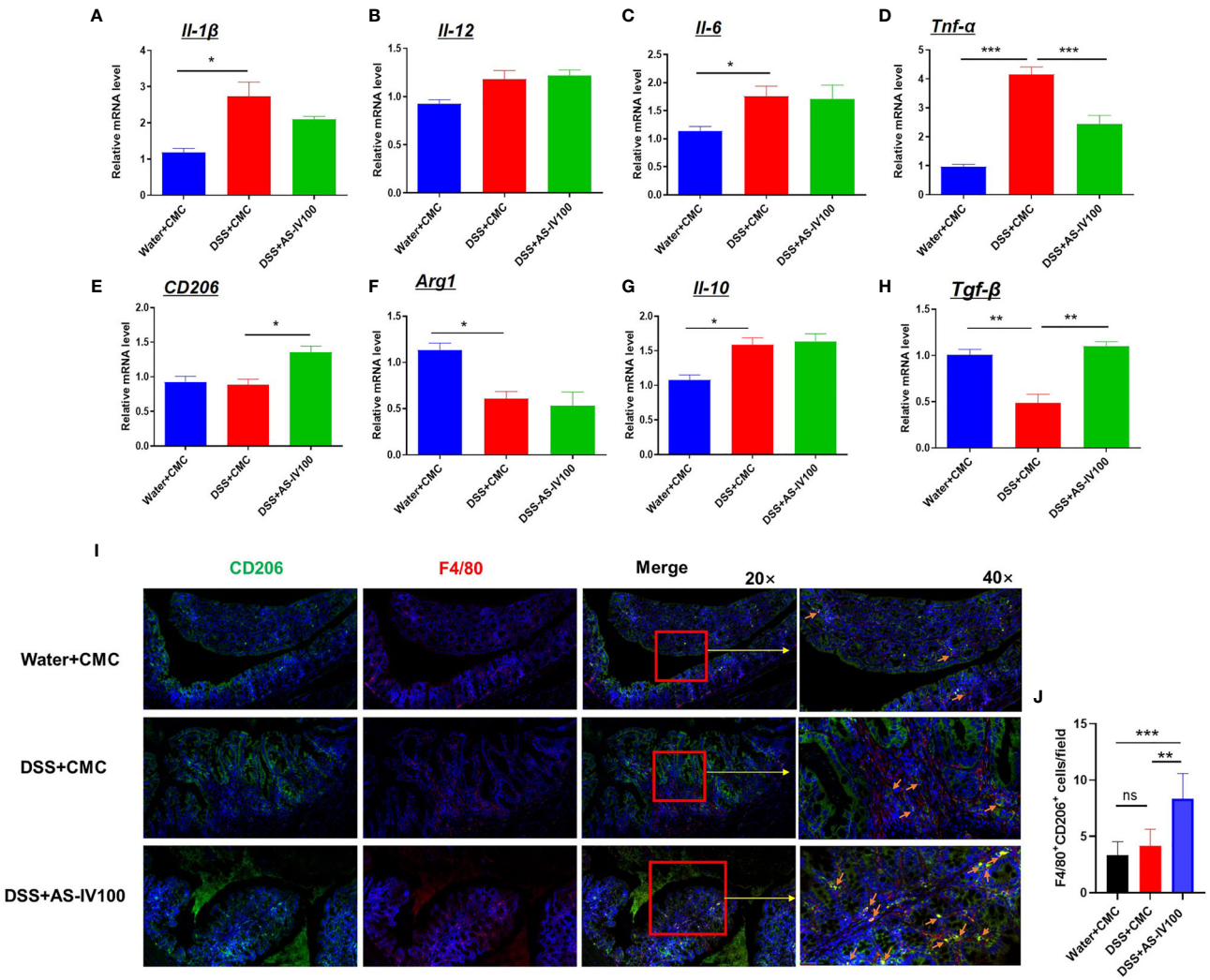

FIGURE 4 | AS-IV administration modulated the plasticity of intestinal macrophages. **(A–H)** Intestinal macrophages were collected with F4/80 maker by flow cytometric sorting. The relative mRNA expression levels of the inflammatory cytokines *Il-1β*, *Il-12*, *Il-6*, *Tnf-α*, *CD206*, *Arg1*, *Il-10*, and *Tgf-β* in F4/80 positive intestinal macrophages were determined by RT-PCR. The mean values ± SEMs are represented by bars. **(I)** The protein expression levels of the F4/80 (red color) and CD206 (green color) in colon tissue by immunofluorescence, and the slides were counterstained with DAPI (blue). **(J)** The number of $F4/80^{+}CD206^{+}$ cells in **(I)** were counted and quantitatively compared. One-way ANOVA test was used for statistical analyses. Bars represent means ± SD; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. ns, no significance.

showed that pro-resolving macrophages were significantly increased by treatment with AS-IV. Taken together, these results indicated that AS-IV had protective roles against DSS-induced colitis mediated by the phenotypic transformation of macrophages from pro-inflammatory macrophages to pro-resolving macrophages.

AS-IV Promoted Transformation of Macrophage Subsets *In Vitro*

To further study whether the therapeutic effect of AS-IV on DSS-induced colitis leads to changes in macrophage phenotypes. Firstly, BMDMs were cultured and then stimulated with PBS (M^{PBS}), LPS + IFN-γ (M^{LPS}), or IL-4 (M^{IL-4}) following treatment with AS-IV, and the expression of polarization markers was detected by qRT-PCR. Then we examined the expression of M1-related genes, including *Tnf-α*, *Il-1β*, *Il-6*, *iNOS*, and the expression of M2-related genes, including *Il-10*, *Tgf-β*, *Ym1*, *CD206* in the colon. The results showed that AS-IV downregulated the expression of M1 markers *Il-1β*, *Il-6*, *iNOS* under LPS+IFN-γ stimulation. Whereas the expressions of M2 marker *Tgf-β*, *Ym1*, *CD206* were upregulated under IL-4 stimulation (**Figures 5A–H**). Taken together, these results indicated that AS-IV significantly suppresses pro-inflammatory macrophage subsets and promotes pro-resolving macrophage subsets *in vitro*.

AS-IV Regulated Macrophages Phenotype Through STAT1 Signaling

There are several signals, including NF-κB, JAK-STATs, and IRFs, which play regulatory roles in the development, functional regulation, and subpopulation transformation of macrophages (28, 29). These pathways form a complex regulatory network through close interaction and crosstalk. Among them, STAT signaling cascades are vital regulators of the differentiation and function of the macrophage (30).

Previous studies showed dysfunction of and genetic variation in JAK-STAT signaling pathways correlated with a spectrum of IBD (31). Therefore, we focused on the STAT signaling pathway and evaluated STAT signaling activity after different stimulation in the presence or absence of AS-IV in DSS-induced colitis in mice. Western blot analysis indicated that p-STAT1 was down-regulated with AS-IV treatment, while p-STAT3 was up-regulated with AS-IV treatment (**Figures 6A–C**). We further evaluated STAT signaling activity in the presence or absence of AS-IV in the different phenotypes of BMDM. The results showed that the expression of p-STAT1 and p-STAT3 in M^{LPS} macrophages was significantly higher than that in M^{PBS} and M^{IL-4} macrophages. Meanwhile, western blot analysis similarly indicated that p-STAT1 was down-regulated with AS-IV treatment and p-STAT3 was up-regulated with AS-IV treatment especially in LPS stimuli (**Figures 6D–F**).

It has been reported that multiple polyhydroxy glycosides regulated the phosphorylation of different proteins (32, 33). Chemically speaking, polyhydroxy substrates induce the transfer of phosphate groups from phosphorylated proteins. However, this phosphorylation transfer requires the spatial proximity of phosphorylated protein to the polyhydroxy molecule. Therefore, to investigate the mechanisms of AS-IV regulating STATs phosphorylation, the molecular docking simulation was carried out. The results indicated that AS-IV could bind with a pocket of STAT1 consisting of 699-701 aa (**Figures 6G–I**). Meanwhile, we constructed the reporter system with the STAT1 recognition motif,

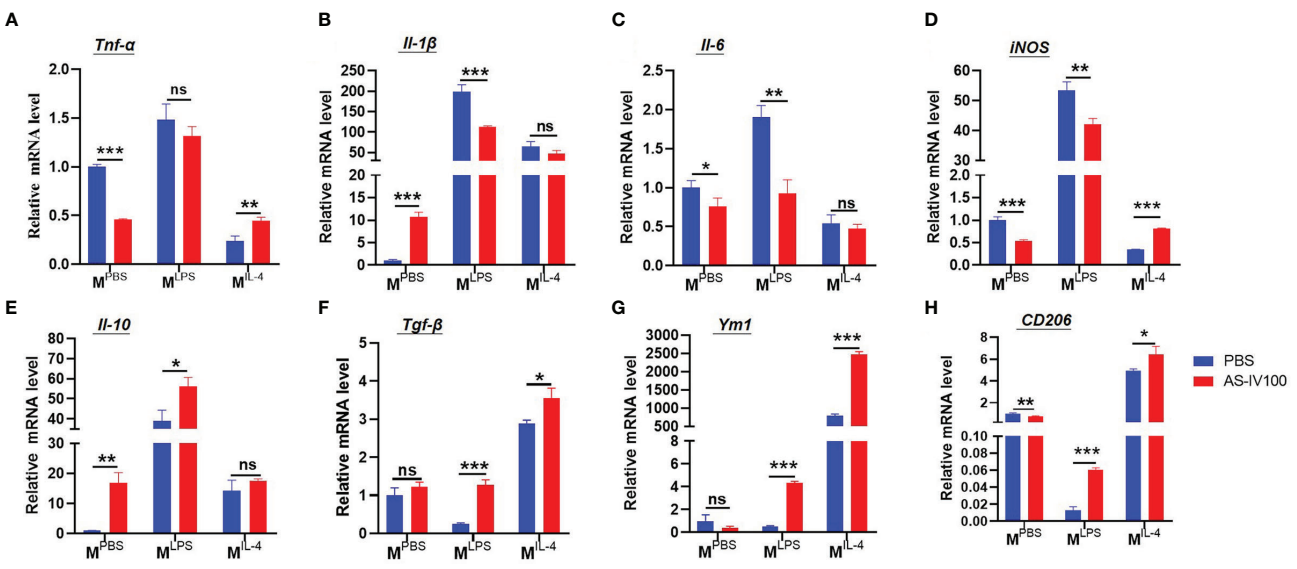

FIGURE 5 | AS-IV promoted transformation of macrophage subsets *in vitro* **(A–H)** Bone marrow-derived macrophages (BMDMs) were stimulated with PBS (M^{PBS}), LPS + IFN-γ (M^{LPS}), or IL-4 (M^{IL-4}) following treatment with AS-IV. The relative mRNA expressions of the M1-related genes *Tnf-α*, *IL-1β*, *Il-6*, *iNOS*, and the expression of M2-related genes, including *Il-10*, *Tgf-β*, *Ym1*, *CD206* were determined by RT-PCR. Student's t-test was used for statistical analyses. Bars represent means ± SD; **P* < 0.05, ***P* < 0.01, ****P* < 0.001. ns, no significance.

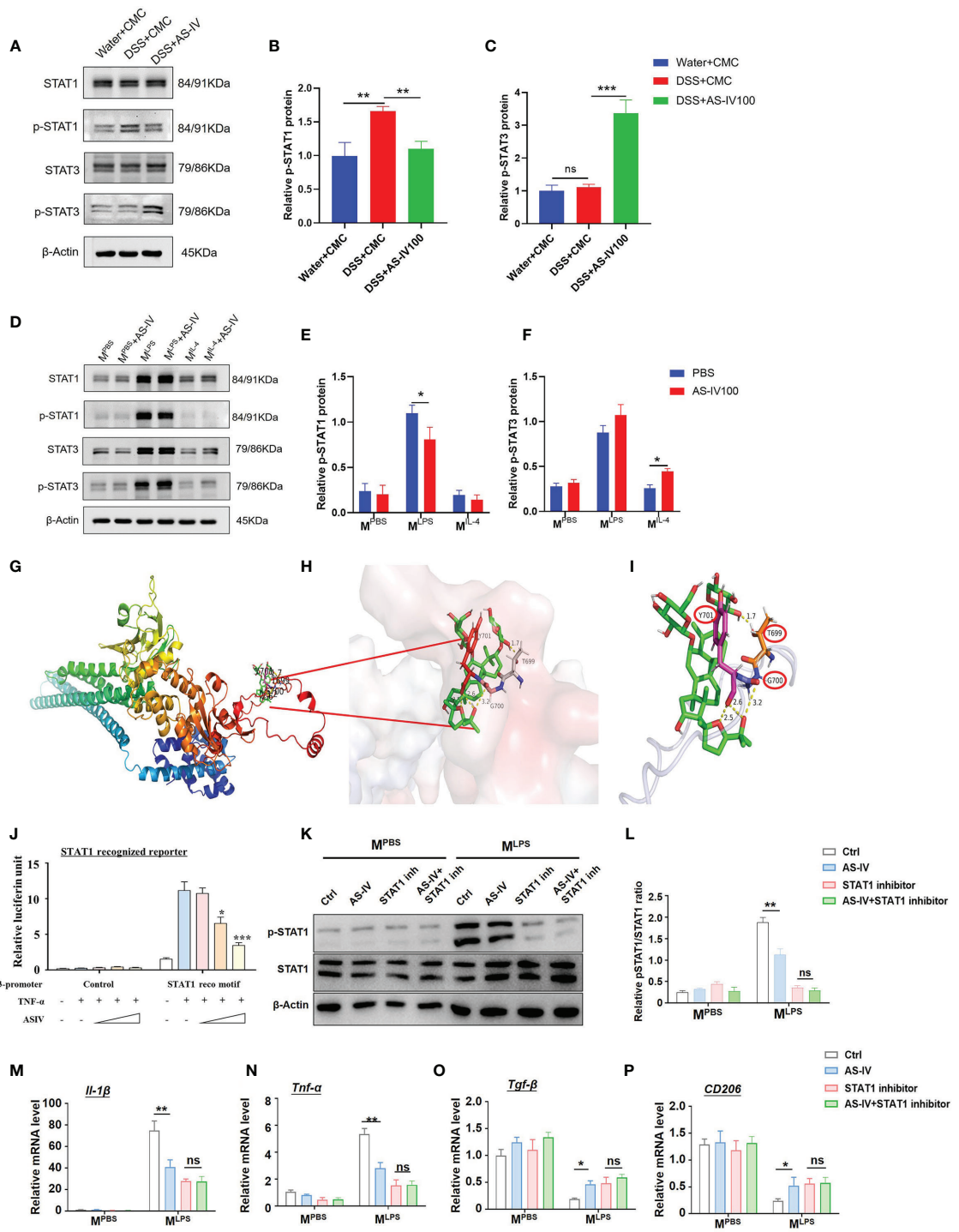

FIGURE 6 | AS-IV regulated macrophages phenotype through STAT1 signaling. **(A–C)** Western blotting analysis of STAT1, p-STAT1, STAT3, and p-STAT3 protein expression in mice colon tissue. **(D–F)** Western blotting analysis of STAT1, p-STAT1, STAT3, and p-STAT3 protein expression after BMDMs were stimulated with PBS (M^{PBS}), LPS + IFN-γ (M^{LPS}), or IL-4 (M^{IL-4}) following treatment with AS-IV. **(G–I)** Homology modeling and molecular docking were used to analyze the interaction between STAT1 and AS-IV. **(J)** HeLa cells were transiently transfected with STAT1 recognized reporters, TNF-α and AS-IV of different concentrations were added simultaneously. The luciferase activity was assessed 48 h later. **(K–L)** STAT1 was inhibited in M^{PBS}/M^{LPS} by STAT1 inhibitor, followed by AS-IV treatment, and then the expression of p-STAT1and STAT1were detected by western blotting analysis. **(M–P)** STAT1 was inhibited in M^{PBS}/M^{LPS} by STAT1 inhibitor, followed by AS-IV treatment, and then the expression of *Il-1β*, *Tnf-α*, *CD206*, and *Tgf-β* was detected by RT-PCR. student's t-test or One-way ANOVA test was used for statistical analyses. Bars represent means ± SD; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. ns, no significance.

which could evaluate the activation of the STAT1 pathway by the intensity of luciferin. The reporter assay indicated that the activity of the STAT1 pathway was activated by TNF-α stimulating immensely, while was repressed by AS-IV administration in a dosage-dependent manner (**Figure 6J**). The results demonstrated that AS-IV interacted with STAT1 and mediated the dephosphorylation and deactivation of STAT1. Meanwhile, western blot analysis further indicated that p-STAT1 was down-regulated with AS-IV treatment in M^{LPS} macrophages, while inhibition of p-STAT1 completely canceled the effect of AS-IV on macrophage in LPS stimuli **(Figures 6K, L)**. In addition, STAT1 was inhibited in M^{PBS}/M^{LPS} by p-STAT inhibitor, followed by AS-IV treatment; and then the expression of macrophage polarization markers was detected. The results suggested that inhibition of p-STAT1 completely canceled the effect of AS-IV on macrophage polarization switch, indicating that the function of AS-IV was dependent on the regulation of STAT1 signaling **(Figures 6M–P)**. Considering that STAT1 signaling could repress the activation of the STAT3 pathway, AS-IV might regulate STAT3 signaling through repressing STAT1 activation. The data above indicated that AS-IV regulates macrophage function *via* repressing the STAT1 signaling pathway.

DISCUSSION

IBD, including UC and CD, is a chronic and relapsing intestinal inflammation with symptoms involving acute abdominal pain, chronic diarrhea, and loss of body weight. The pathogenesis of the disease is still unclear, and the available therapeutic strategies for the disease are not up to the researcher's expectations. Several compounds extracted from Chinese herbs have been shown to have therapeutic effects against UC (13). AS-IV is a lanolin alcohol-shaped tetracyclic triterpenoid saponin as one of the major active substances of *Astragalus membranaceu* (15). Recent studies have found that AS-IV regulates energy metabolism and further improves 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis (16). Additionally, AS-IV has a therapeutic effect on experimental UC *in vitro* and *in vivo via* inhibiting inflammatory molecules and downregulating NF-κ B signaling (17). Our studies also showed that AS-IV attenuated the DAI and MPO activity, downregulate the expression of *Tnf-α* and *Il-1β* and increase the level of *Il-10* and *Tgf-β* in a dose-dependently manner in DSS-induced colitis mice. Although these studies have pointed to that AS-IV alleviates the symptoms of experimental ulcerative colitis, the target cells of AS-IV acting in the intestine have yet to be fully elucidated.

Intestinal macrophages restrain excessive inflammation responded to harmless commensal microorganisms and improve tolerance mainly *via* the production of Il-10 (34). Therefore, intestinal macrophages play pivotal roles in establishing and maintaining gut homeostasis. However, recent genome-wide association studies identified key driver genes of macrophages for inflammatory disorders (35). When intestinal homeostasis is disturbed, the composition of the intestinal macrophage pool will change significantly (36). Therefore, we first observed that the immature Inf-Mφs population recruited from bone marrow were increased significantly at the activation phase and retrieved at the resolution phase. While the number of Inf-Mφs decreased after the administration of AS-IV at the activation phase. Immunofluorescence validated the conclusion that AS-IV decreased macrophage percentages in DSS-induced colitis mice. Previous studies have indicated that macrophages show great phenotypic and functional differences in response to different external inflammatory stimulus signals, which are manifested as M1/M2 macrophages. However, M1/M2 macrophage classification is largely based on *in vitro* differentiation, which does not accurately reflex the complexity of *in vivo* macrophage plasticity and heterogeneity (37). Current evidence suggests that monocytes that have entered tissues differentiate into macrophages displaying varying M1-like or M2-like characteristics (38, 39). Accumulation of M2-like pro-resolving macrophages in the intestinal microenvironment appears to play a vital role in re-establishing gut tissue homeostasis (40, 41). Further, we studied the effect of AS-IV on the phenotype and function of intestinal macrophages. In this study, we observed that AS-IV administration resulted in the phenotypic transformation of macrophages from pro-inflammatory M1 (M^{LPS}) macrophages to pro-resolving M2 ($M^{IL\text{-}4}$) macrophages. Meanwhile, flow cytometry results showed AS-IV treatment could reduce the number of bone marrow-derived proinflammatory macrophages and promote their functional maturation.

The activation of stimulus-specific transcription factors within this macrophage-specific transcriptional landscape is probably to dictate the polarization of macrophages, such as STAT family, nuclear receptor PPARγ, CREB–C/EBP axis, and interferon regulatory factors (42). JAK-STAT signaling is an important signaling pathway associated with a spectrum of hematological malignancies and autoimmune disorders, such as IBD (43). In addition, JAK-STAT signaling mediated essential cytokines (IL-6, IL-10, IL-2 or IL-22) participating in immune and stromal gut cell homeostasis and those well-described mediators (IFN-γ, IL-12, IL-23 or IL-9) involving in pathological processes in IBD (31, 44). Several small-molecule JAK inhibitors have shown efficacy in the treatment of IBD within the past 5 years (45). However, JAK activation leads to structure alteration, which triggers a series of subsequent modifications and ultimately results in the phosphorylation, dimerization, and activation of the STATs family. While a number of studies have indicated that STATs are crucial factors in macrophage polarization and involved in IBD progression (46, 47). Particularly, p-STAT1 has the potent effect on promoting M1 type macrophage activation in the presence of IFN-γ (48). Studies have reported amelioration of experimental colitis has been observed in STAT1-deficient mice (49), suggesting has a pro-inflammatory effect. However, regulatory and/or anti-inflammatory properties have also been described for STAT1 in intestinal epithelial cells or macrophages (50, 51). In this study, we observed that p-STAT1 significantly upregulated in the DSS group, while decreased with AS-IV treatment. But p-STAT3 was up-regulated with AS-IV treatment. STAT3 is a key immunomodulatory transcription factor that has a fundamental role in IBD (43, 52). Previous studies showed STAT3 has an antagonistic effect on

immunostimulatory effects of STAT1 and imbalance of STAT1/3 signals is the pathogenesis of various inflammatory diseases (53, 54). Our results showed AS-IV treatment seems to regulate the balance between p-STAT1/3. The mechanism investigation demonstrated that AS-IV, as a ligand, specifically interacts with STAT1, mediating the dephosphorylation of Tyr701 and deactivation of STAT1. Meanwhile, many studies indicated that STAT1 signaling could repress the activation of the STAT3 pathway. Therefore, we inferred that AS-IV directly repressed STAT1 signaling, and subsequently activated STAT3 signaling. Further, we examined the effects of AS-IV on STAT signaling during macrophage polarization *in vitro*. The protein expression of p-STAT1 was significantly decreased in M^{LPS} macrophages treated with AS-IV, while p-STAT3 was increased in M^{LPS} macrophages treated with AS-IV. Further reporter assay showed that STAT1 pathway was repressed by AS-IV administration with a dosage-dependent manner directly. Finally, we confirmed that the AS-IV treatment canceled the regulation of macrophages phenotype after inhibiting STAT1 signal by salvage experiment. Taken together, these data demonstrated that AS-IV may regulate macrophages phenotype through STAT1 signaling.

In conclusion, we studied the therapeutic effects and mechanism of AS-IV on experimental colitis and provided compelling evidence that AS-IV was effective in IBD treatment. Our findings showed that AS-IV attenuated clinical disease activity, diminished pro-inflammatory cytokine production, up-regulated anti-inflammatory cytokine production, and decreased the percentages of macrophages by blocking the M1 polarization of macrophages partially through the STAT1 signaling pathway in DSS-induced colitis. Taken together, these findings indicate that transition of intestinal macrophage subsets is a likely therapeutic target in IBD therapy and provide new insights regarding the therapeutic potential of AS-IV for IBD treatment.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethical Committee of Air Force Medical University, China.

AUTHOR CONTRIBUTIONS

YL, XJ, JZ, and J-LZ designed the study and wrote the manuscript. LT, S-BG, Y-LZ, and J-LZ performed experiments. YL, LT, N-NZ, LS, and JQ-K analyzed data. YL supervised the work and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.740565/full#supplementary-material

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Anti-TLR7 Antibody Protects Against Lupus Nephritis in NZBWF1 Mice by Targeting B Cells and Patrolling Monocytes

Yusuke Murakami[1,2†], Ryutaro Fukui[1†], Reika Tanaka[1], Yuji Motoi[1], Atsuo Kanno[1], Ryota Sato[1], Kiyoshi Yamaguchi[3], Hirofumi Amano[4], Yoichi Furukawa[3], Hitoshi Suzuki[5], Yusuke Suzuki[5], Naoto Tamura[4], Naomi Yamashita[2] and Kensuke Miyake[1,6]*

[1] Division of Innate Immunity, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan, [2] Faculty of Pharmacy, Department of Pharmaceutical Sciences & Research Institute of Pharmaceutical Sciences, Musashino University, Tokyo, Japan, [3] Division of Clinical Genome Research, Advanced Clinical Research Center, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan, [4] Department of Internal Medicine and Rheumatology, Juntendo University School of Medicine, Tokyo, Japan, [5] Department of Nephrology, Juntendo University Faculty of Medicine, Tokyo, Japan, [6] Laboratory of Innate Immunity, Center for Experimental Medicine and Systems Biology, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by autoantibody production and multiple organ damage. Toll-like receptor 7 (TLR7), an innate immune RNA sensor expressed in monocytes/macrophages, dendritic cells (DCs), and B cells, promotes disease progression. However, little is known about the cellular mechanisms through which TLR7 drives lupus nephritis. Here, we show that the anti-mouse TLR7 mAb, but not anti-TLR9 mAb, protected lupus-prone NZBWF1 mice from nephritis. The anti-TLR7 mAb reduced IgG deposition in glomeruli by inhibiting the production of autoantibodies to the RNA-associated antigens. We found a disease-associated increase in Ly6C^{low} patrolling monocytes that expressed high levels of TLR7 and had upregulated expression of lupus-associated IL-10, CD115, CD31, and TNFSF15 in NZBWF1 mice. Anti-TLR7 mAb abolished this lupus-associated increase in patrolling monocytes in the circulation, spleen, and glomeruli. These results suggested that TLR7 drives autoantibody production and lupus-associated monocytosis in NZBWF1 mice and, that anti-TLR7 mAb is a promising therapeutic tool targeting B cells and monocytes/macrophages.

Keywords: toll-like receptor, lupus nephritis, inhibitory monoclonal antibody, monocytes, autoantibody

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Masayuki Mizui,
Osaka University, Japan
Adriana Karina Chavez-Rueda,
Mexican Social Security Institute (IMSS), Mexico
Hiroki Mitoma,
Kyushu University, Japan

***Correspondence:**
Kensuke Miyake
kmiyake@ims.u-tokyo.ac.jp

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

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INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease of unknown etiology characterized by autoantibody production and clinical manifestations affecting the skin, joints, kidneys, and central nervous system (1). Immunosuppressive agents such as antimalarial drugs, hydroxychloroquine (HCQ), non-steroidal anti-inflammatory drugs, glucocorticoids, and mycophenolate mofetil have been administered to control SLE. However, life-threatening

manifestations, such as lupus nephritis, develop in resistant patients despite such treatments. Furthermore, the use of glucocorticoids is limited due to various adverse effects. Therefore, a novel therapeutic agent with fewer adverse effects is required.

Causative autoimmune responses are driven by autoreactive B cells that produce autoantibodies to nucleic acid (NA)-associated autoantigens, conventional dendritic cells (cDCs) that produce proinflammatory cytokines, and plasmacytoid dendritic cells (pDCs) that produce type I interferons (IFNs) (2, 3). In addition to these cells, monocytes/macrophages infiltrate glomeruli and play pathogenic roles in glomerular damage associated with SLE, independently of immune complex deposition (4–6).

Toll-like receptor 7 (TLR7) is an innate immune RNA sensor that is expressed in B cells, dendritic cells, and monocytes/macrophages. This receptor responds not only to pathogen-derived single-stranded RNA (ssRNA), but also to self-derived ssRNA, and drives autoimmune diseases such as SLE and psoriasis (7–9). A lupus-prone mouse strain, Y-linked autoimmune accelerator (Yaa), has a duplicate copy of the TLR7 gene that results in TLR7 hyperactivation, leading to lupus-like states (10, 11). The TLR7 agonist imiquimod drives lupus nephritis in mice (12, 13), whereas lupus nephritis spontaneously developed in the lupus-prone strain, New Zealand Black/New Zealand White F1 mice (NZBWF1 mice) is ameliorated by a small chemical TLR7 inhibitor (14). The immune complex (IC)-independent glomerular accumulation of $Ly6C^{low}$ patrolling monocytes causes lupus nephritis in lupus-prone mouse strain lacking the human SLE susceptibility gene *Tnip1* (6). Although both TLR7 and TLR9 drive lupus nephritis in this strain (6), TLR7 might play unique pathogenic roles in patrolling monocytes because they express abundant TLR7 (15).

We previously reported that the anti-TLR7 mAb inhibits TLR7 responses in B cells, dendritic cells, and monocyte/macrophages (16). The anti-TLR7 mAb binds to cell surface TLR7, which is internalized into the endosomal compartment. Because TLR7 shuttles between cell surface and the endosomal compartment, endosomal TLR7 comes out of the cell surface and becomes accessible to the anti-TLR7 mAb (16). Therefore, the TLR7-mAb immune complex gradually increases with the mAb treatment. When endosomal TLR7 is mostly complexed with the anti-TLR7 mAb, endosomal TLR7 responses are inhibited. The inhibitory effect of the anti-TLR7 was also observed *in vivo*, rescuing *Unc93b1*$^{D34A/D34A}$ mice from TLR7-dependent autoimmune hepatitis. Here, we investigated the pathogenic role of TLR7 in NZBWF1 mice using an anti-TLR7 inhibitory mAb This mAb ameliorated lupus nephritis in NZBWF1 mice by acting on B cells and monocytes/macrophages, thereby reducing IgG deposition in glomeruli and diminishing autoantibody production. These findings suggested that the activation and differentiation of autoreactive B cells in NZBWF1 mice is TLR7-dependent. Furthermore, the numbers of $Ly6C^{low}$ patrolling monocytes, which are thought to be tissue macrophages in the circulation, TLR7-dependently increased in the spleen, circulation, and kidneys. Transcriptome and FACS analyses revealed increased expression of lupus-associated molecules such as IL-10, which promotes nephritis, in monocytes that accumulated in the spleen (17). These results suggested that TLR7 is a therapeutic target for SLE and that anti-TLR7 mAb is a promising therapeutic tool targeting both B cells and monocytes in SLE.

MATERIAL AND METHODS

Reagents and Antibodies

Standard saline was purchased from Otsuka Pharmaceutical Co., Ltd. (Tokushima, Japan). Anti-mouse TLR7 mAb (mouse IgG1, κ, clone A94B10), anti-mouse TLR9 mAb (mouse IgGa, κ, clone NaR9), and isotype control mAb (IgG1, κ, clone TF904 or IgG2a, κ, clone YN907) were purified by us. FITC-conjugated anti-mouse IgG antibody was purchased from Southern Biotech (1030-02, Birmingham, UK). FITC-conjugated anti-mouse C3 antibody was purchased from MP Biomedicals (SKU:0855500, CA, USA). The anti-mouse CD19 (clone 6D5), GL7 (clone GL7), CD8α (clone 53-6.7), CD11c (clone N418), Ly-6C (clone HK1.4), Ly-6G (clone 1A8), TREML4 (clone 16E5), CD273 (clone TY25), CD31 (clone 390), CD115 (clone AFS98), CD14 (clone Sa14-2), CD41 (clone MWReg30), CD85k (clone H1.1), CD54 (clone YN1/1.7.4), CD132 (clone TUGm2), PD-1H (clone MH5A), CD169 (clone 3D6.112), CD274 (clone 10F.9G2), CD61 (clone 2C9.G2), integrin β7 (clone FIB27), CD63 (clone NVG-2), CD88 (clone 20/70), TER119 (clone TER-119), CD117 (2B8), and NK1.1 (clone PK136) antibodies were purchased from BioLegend (San Diego, CA, USA). Anti-mouse CD138 (clone 281-2), I-A/I-E (clone M5/114), CD3ϵ (clone 145-2C11), CD4 (clone GK1.5), CD44 (clone IM7), CD62L (clone MEL-14), CD49b (clone HMα2), CD11b (clone M1/70), Siglec-H (clone 440c), CD16.2 (clone 9E9), and CD45.2 (clone 104) antibodies were purchased from BD (Franklin Lakes, NJ, USA). Staining buffer (1x PBS, 2.5% FBS, 2 mM EDTA, and 0.1% sodium azide) was prepared by us. The LEGENDScreen Mouse PE Kit was purchased from BioLegend.

Mice

Female C57BL/6NCrSlc and NZBWF1/Slc mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). The mice were housed in a specific-pathogen-free (SPF) environment with free access to food and water, under the approval of the Animal Experiment Committee of The Institute of Medical Science, The University of Tokyo (approval numbers PA-84 and A17-83).

Antibody Treatment

10 mg/kg of anti-mouse TLR7 mAb, isotype control mAb, or the same volume of saline was injected into the peritoneal cavity of NZBWF1 mice once a week. Administration was started at 12-16 weeks of age and ended at 35-40 weeks.

Biochemical Tests

Urine and serum were collected from mice aged 30-40 weeks. Urine albumin, urine creatinine, and blood urea nitrogen (BUN)

levels were measured by ORIENTAL YEAST CO., LTD. (Tokyo, Japan).

Histological Analysis

Mouse kidneys were fixed in 20% formalin neutral buffer solution. Fixed kidneys were then embedded in paraffin wax for sectioning. The sections were stained with hematoxylin and eosin (HE) or periodic acid-Schiff (PAS). The sections were visualized using an EVOS microscope (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Pathological scores for glomerulonephritis were defined as the average of the scores derived from 50 glomeruli. Glomerulonephritis was scored as 0, normal; 1, cell proliferation or infiltration; 2, membranoproliferation, lobulation, or hyaline deposition and 3, crescent formation or global hyalinosis.

Immunohistochemistry (IHC)

Mouse kidneys were embedded in Tissue Tek embedding medium for frozen tissue blocks (Sakura Finetechnical Co., Ltd., Tokyo, Japan). Frozen kidney sections were sectioned and incubated with FITC-conjugated anti-mouse IgG antibody (Southern Biotech Birmingham, UK). Stained samples were mounted with Fluoromount/Plus (Diagnosis Biosystems, CA, USA). All samples were visualized using an FM1000D confocal laser scanning microscope (Olympus, Tokyo, Japan), and the images were analyzed using the FV10-ASW viewer (Olympus) or Image J software (Schneider, Nat. Methods 2012). For the IHC of color development, frozen kidney sections were incubated with anti-mouse CD11b (clone M1/70), anti-mouse CD16.2 (FcγRIV) (clone 9E9), anti-mouse TREML4 (clone 16E5), and anti-mouse Ly-6G (clone 1A8). Samples were mounted with Fluoromount/Plus (Diagnosis Biosystems) and analyzed using an EVOS microscope (Thermo Fisher Scientific) or a BZ-X710 fluorescence microscope (Keyence, Osaka, Japan). The number of glomeruli assessed was 5 to 10 from an individual kidney in B6 WT (n = 4 or 5), saline (n = 5), control IgG1 (n = 6), and anti-TLR7 (n = 5). The dots show the average percentage of the indicated positive staining area. Ratios (%) of the indicated positive staining area to the total area of one glomerulus were calculated using the BZ-X710 fluorescence microscope software.

ELISA

Anti-Sm and anti-SSA/Ro60 antibodies were measured using an ELISA kit (Alpha Diagnostic International Inc., San Antonio, TX, USA). Anti-double-stranded DNA antibodies in serum were measured using an ELISA kit (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). Serum IL-10 was measured using a Duo Set ELISA kit (R&D Systems, Minneapolis, MN, USA). Serum ACP5 levels were measured using an ELISA kit (Novus Biologicals, MN, USA).

Preparation of Cells in Tissues and Peripheral Blood

Spleens were harvested from mice and dissociated into single-cell suspensions with glass slides. Single cell suspensions of kidney were prepared using Multi Tissue Dissociation Kit 2 and Debris Removal Solution (Miltenyi Biotec, Bergisch Gladbach, Germany). Peripheral blood was harvested from the buccal blood vessels and kept in EDTA-treated microtubes. Red blood cells in the prepared samples were lysed using RBC Lysis Buffer (BioLegend).

Flow Cytometry Analysis

Fc receptors on the cells were blocked by non-conjugated anti-CD16/32 (2.4G2, Bio X cell, Lebanon, NH, USA) for 10 min at room temperature. Cells were stained with antibodies for 15 min at 4°C. Stained cells were fixed with BD Cytofix Fixation Buffer (BD) for 20 min at 4°C and washed twice with staining buffer 2 times. For intracellular staining of TLRs, fixed cells were permeabilized using BD Perm/Wash buffer (BD) and incubated with anti-TLR antibody for 30 min at 4°C. Prepared cells were suspended in staining buffer and analyzed by BD FACS Aria III, BD LSR Fortessa X-20, or BD FACSLyric. The obtained data were analyzed using the FlowJo software (BD). Antibodies were diluted with a staining buffer. The reaction of these steps was performed at 1×10^7 cells/mL.

RNA Extraction and cDNA Synthesis

RNA was prepared from cells using the RNeasy Mini or Micro kit (Qiagen, Venlo, Netherlands). To extract RNA from the kidneys, the kidneys were incubated with Sepasol-RNA I Super G solution for RNA isolation (Nacalai Tesque, Osaka, Japan) and homogenized with metal beads in a multi-bead shocker (Yasui Kikai, Osaka, Japan). Chloroform (200 μL/kidney) was added and centrifuged at 15300 × g at 4°C for 15 min. The supernatant was collected, and 500 μL/kidney isopropanol was added. The sample was mixed well, and the mixture was centrifuged at 15300 × g at 4°C for 10 min. The supernatant was discarded, and 70% ethanol was added to the nucleic acid pellets. The pellet was then centrifuged at 15300 g at 4°C for 5 min. The supernatant was removed, and the nucleic acid pellet was dried and dissolved in 200 μL of water. Complementary DNA (cDNA) was synthesized from the extracted RNA using ReverTra Ace qPCR Master Mix (Toyobo, Osaka, Japan).

Real-Time PCR

To measure mRNA levels, cDNA was quantified by real-time PCR using TaqMan probes and primers (Thermo Fisher Scientific) in a total volume of 20 μL. mRNA expression was calculated according to the comparative threshold cycle method, using the hypoxanthine-guanine phosphoribosyltransferase gene (*Hprt*) as an internal control. The accession numbers of TaqMan probes are; *Hprt*: Mm03024075_m1, *Tnfsf15*: Mm00770031_m1, *Il10*: Mm01288386_m1, *Acp5*: Mm00475698_m1, *Il34*: Mm01243248_m1, *Csf1*: Mm00432686_m1, *Tnf*: Mm00443258_m1, *Il6*: Mm00446190_m1, *Il12b*: Mm01288989_m1, *Cxcl1*: Mm04207460_m1, *Ifna4*: Mm00833969_s1, *Ifnb1*: Mm00439552_s1.

Cell Sorting

Single-cell suspensions of splenocytes were prepared with 0.65 WU/ml of Liberase TL and 200 U/ml of DNase I (Sigma-Aldrich, St. Louis, MO, USA). After RBC lysis, Fc receptors on the cells were blocked, as previously described. To exclude T cells, B cells, NK cells, erythroblasts, granulocytes, and cDCs, cells were

incubated with biotin-conjugated anti-mouse CD3ϵ, CD19, NK1.1, TER119, Ly6G, and CD117. Labeled cells were incubated with anti-biotin MACS beads (Miltenyi Biotec), and crude monocytes were collected as the negative fraction using Auto MACS separator (Miltenyi Biotec). Cells were stained with anti-mouse CD11b, CD11c, Ly6C, I-A/I-E, CD49b, and CD16.2 as described. Stained cells were subjected to cell sorting with BD FACS Aria III to collect patrolling monocytes and classical monocytes (**Figure 3E**). Collected cells were suspended in RNA protect cell reagent (QIAGEN) and stored at -80°C.

RNA Sequencing

Total RNA was extracted from the cells as previously described. RNA samples were checked using a Bioanalyzer 2100 with RNA 6000 nano kit (Agilent Technologies, Santa Clara, CA, USA), and RNA integrity number (RIN) was calculated. The high-quality RNA (RIN > 7.2) samples were subjected to RNA sequencing using the Ion Torrent NGS system (Thermo Fisher Scientific). Briefly, RNA libraries were prepared using 10.62 ng of total RNA with an Ion AmpliSeq Transcriptome Mouse Gene Expression kit, and sequenced on Ion Proton using an Ion PI Hi-Q Sequencing 200 kit and Ion PI Chip v3 (Thermo Fisher Scientific). The data were analyzed using AmpliSeqRNA plug-in v5.2.0.3 in the Torrent Suite Software v5.2.2 (Thermo Fisher Scientific), and normalized using RPM (reads per million mapped reads) method. The normalized data were further analyzed and visualized using GeneSpring v14.9.1 software (Agilent Technologies), R Studio (R Foundation for Statistical Computing, Vienna, Austria), and Microsoft Excel (Microsoft, Redmond, WA, USA).

Statistics

Statistical significance was calculated by performing Log-rank test, two-tailed unpaired Student's *t*-test, Welch's *t*-test, one-way ANOVA, or two-way ANOVA. A p-value of < 0.05 was considered statistically significant. If the result of one-way ANOVA was significant ($p < 0.05$), Tukey's multiple comparison test was performed. Prism software (GraphPad Software, San Diego, CA, USA) was used for the statistical analyses.

RESULTS

Anti-TLR7 mAb Protected NZBWF1 Mice From Lupus Nephritis

To understand the role of TLR7 in disease progression in NZBWF1 mice, we intraperitoneally administered anti-TLR7 mAb (10 mg/kg weekly) to 12-16-week-old NZBWF1 mice. By the age of 40 weeks, all mice that were administered the anti-TLR7 mAb remained alive, whereas 60%-75% of the mice administered saline or the isotype control Ab died (**Figure 1A**). The NZBWF1 mice died of kidney failure, indicated by elevated urinary albumin/creatinine (ALB/CRE) ratios and blood urea nitrogen (BUN) (**Figures 1B, C**). These values did not increase in mice administered the anti-TLR7 mAb. Consistent with these findings, histological analyses revealed glomerular changes such as mesangial cell proliferation in mice administered control IgG1 or saline, but significantly less changes in those administered the anti-TLR7 mAb (**Figures 1D, E**). These results suggested that the anti-TLR7 mAb protected the NZBWF1 mice from lupus nephritis.

The single-stranded DNA (ssDNA) sensor, TLR9, also promotes lupus nephritis in lupus-prone *Tnip1*$^{-/-}$ mice (6). To assess the role of TLR9 in lupus nephritis, we administered our inhibitory anti-TLR9 mAb to NZBWF1 mice (18). Although the anti-TLR9 mAb ameliorates TLR9-dependent lethal hepatitis (18), we did not identify any healing effect in NZBWF1 mice (**Figures 1F, G**). These results suggested that TLR9 is dispensable for disease progression in NZBWF1 mice.

Anti-TLR7 mAb Reduced IgG Deposition in Glomeruli and Autoantibody Production

The anti-TLR7 mAb significantly decreased IgG deposition in glomeruli (**Figure 2A**). A previous study found that TLR7 drives autoantibody production in response to RNA-associated autoantigens in MRL/lpr mice (7). In NZBWF1 mice, autoantibodies to Sm and SSA antigens were detectable at 20 wk old and their titer increased with age (**Supplementary Figure 1** and **Figure 2B**). The anti-TLR7 mAb significantly reduced the titers of these se autoantibodies to Sm and SSA antigens at 30-40 wk old. Anti-TLR7 mAb weakly but significantly decreased serum levels of anti-dsDNA autoantibodies (**Figure 2B**). Although production of anti-dsDNA autoantibodies depends on TLR9, not TLR7, in MRL/lpr mice (7), TLR7 partially contributes to production of anti-dsDNA autoantibodies in TLR7 transgenic mice (19). Because TLR7 responds to DNA-derived deoxyguanosine (20), TLR7, in addition to TLR9, would be activated by DNAs in autoreactive B cells. TLR7 activation by DNA would drive anti-dsDNA autoantibody production. The anti-TLR7 mAb would directly act on autoreactive B cells, because it inhibits B cell responses to TLR7 ligands *in vitro* (16).

We analyzed splenic B cell subsets by flow cytometry to assess the effects of the anti-TLR7 mAb on B cells in NZBWF1 mice. The anti-TLR7 mAb decreased the higher ratios (%) of germinal center B cells and plasma cells, in NZBWF1, compared to those in C57BL/6 mice (**Figure 2C**). The mAb did not alter the frequencies of naïve and memory B cells (**Supplementary Figure 2**). These results suggested that the anti-TLR7 mAb inhibits the TLR7-dependent activation and differentiation of autoreactive B cells, leading to reduced levels of serum autoantibodies and decreased IgG deposition in the glomeruli.

The anti-TLR7 mAb also significantly decreased ratios (%) of $CD4^+$ memory T cells, but not those of $CD8^+$ T cells (**Figure 2D**). Because the anti-TLR7 mAb inhibited TLR7 responses in dendritic cells (DCs) *in vitro* (16), its inhibitory effect on DCs might decrease $CD4^+$ memory T cells in NZBWF1 mice.

Increases of Patrolling Monocytes Were Inhibited by the Anti-TLR7 mAb

The anti-TLR7 mAb abolished splenomegaly in NZBWF1 mice manifesting as spleen weight and splenocyte numbers (**Figures 3A–C**). We analyzed TLR7-dependent cellular changes

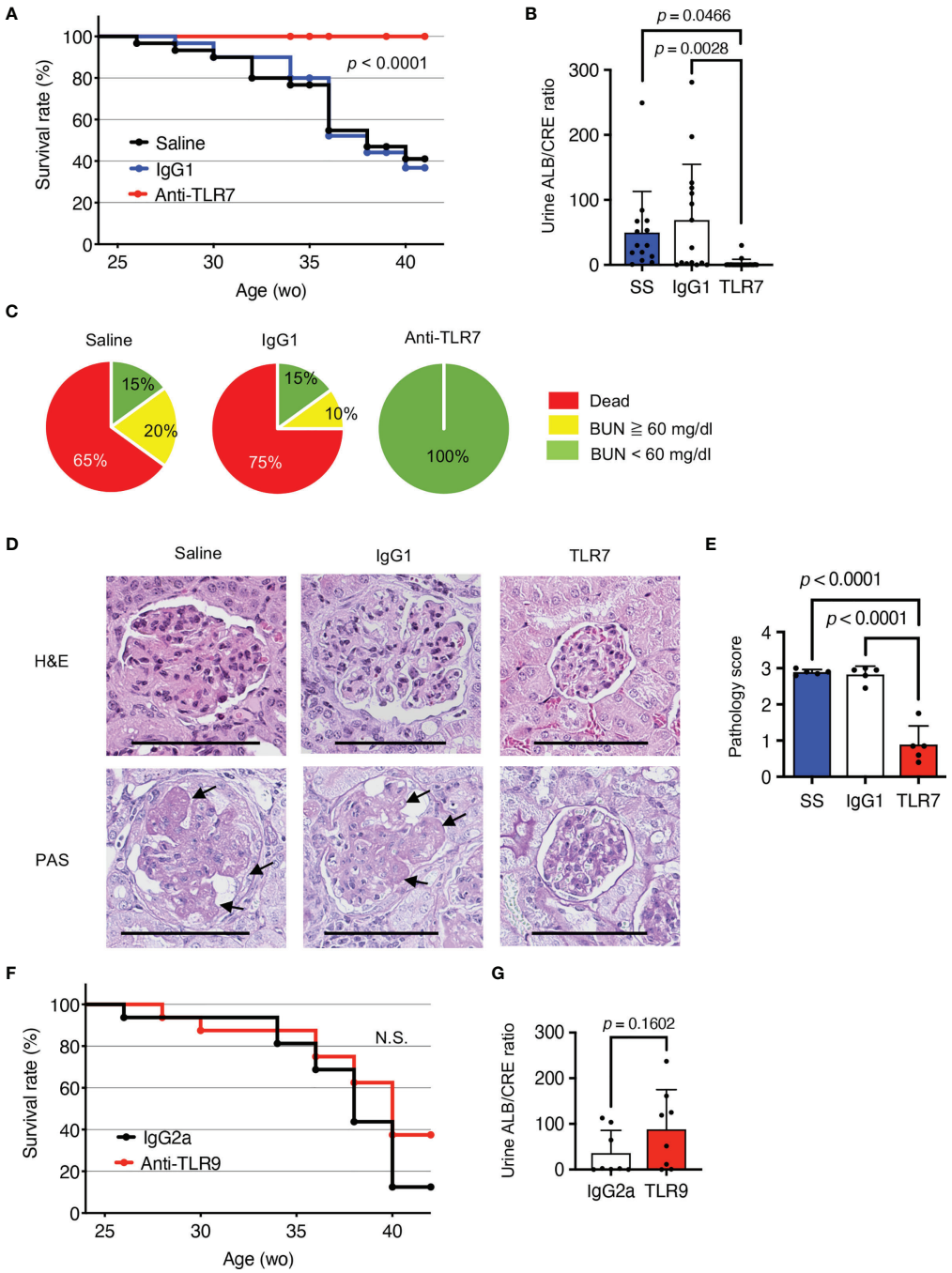

FIGURE 1 | Protective effects of anti-TLR7 mAb against lupus nephritis. **(A-E)** NZBWF1 mice were intraperitoneally administered 10 mg/kg of anti-TLR7 mAb (clone A94B10, IgG1), isotype control (clone TF904, IgG1) mAb or standard saline (control) weekly from age 12-16, until 35-40 weeks. **(A)** Survival of NZBWF1 mice (n = 30 per group). **(B)** Urinary albumin (ALB)/creatinine (CRE) ratio in 30-40-week-old NZBWF1 mice (n = 30). **(C)** Ratios (%) of mice with blood urea nitrogen (BUN) above (yellow) or below (green) 60 mg/dL at age of 40 weeks and those of dead mice (red; n = 20). **(D)** Representative images of glomerular sections visualized by hematoxylin and eosin (HE) or periodic acid-Schiff (PAS) staining. Arrows, increased numbers of mesangial cells. Scale bar, 100 µm. **(E)** Pathology scores from 0 to 3 (n = 5). **(F, G)** NZBWF1 mice were administered anti-TLR9 mAb (clone NaR9, IgG2a, n = 16) or isotype control mAb (clone YN907, IgG2a, n = 16) at 10 mg/kg from age 12-40 weeks. **(F)** Survival curves of mice administered Ab (n = 16). **(G)** Urinary ALB/CREA ratio in mice aged 30-40 weeks (n = 8). Data were statistically analyzed using Log-rank tests **(A, F)**, one-way ANOVA with Tukey's multiple comparison tests **(B, E)**, or Student's t-tests **(G)**. Data are shown as individual points and as means ± SD for each experimental group **(B, E, G)**.

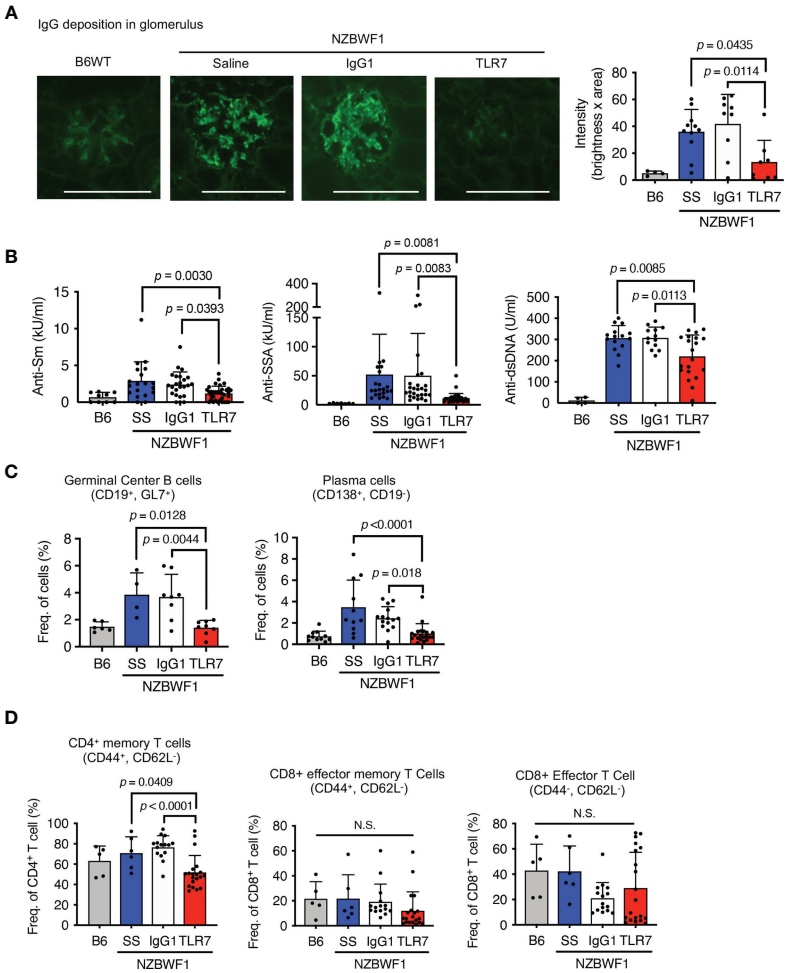

FIGURE 2 | Inhibition of IgG deposition in glomeruli and autoantibody production induced by anti-TLR7 mAb. **(A–D)** NZBWF1 mice were administered saline, IgG1 or anti-TLR7 mAb from ages of 12-16 to 35-40 weeks and compared with age-matched WT C57BL/6 (B6) mice. **(A)** Representative images of immunohistological staining with anti-IgG Ab (left). Scale bar, 100 μm. Quantitation of fluorescence intensity in IgG-positive areas in glomeruli (right). Numbers of glomeruli assessed: B6 (n = 4), saline (n = 9), control IgG1 (n = 9), and anti-TLR7 (n = 7). **(B)** Serum levels of autoantibodies in mice aged 30-40 weeks. B6 ($n \geq 4$), saline ($n \geq 15$), control IgG1 ($n \geq 13$), and anti-TLR7 mAb ($n \geq 20$). **(C, D)** Ratios of B **(C)** and T **(D)** cell subsets in spleen. B6 ($n \geq 4$), saline ($n \geq 4$), control IgG1 ($n \geq 8$), and anti-TLR7 mAb ($n \geq 8$). Data were statistically analyzed using one-way ANOVA with Tukey's multiple comparison tests. N.S., not significant. Data are shown as individual points and as means ± SD for each experimental group.

using flow cytometry. Among the immune cells in the spleen, the anti-TLR7 mAb decreased the frequencies of B cells and monocytes, not those of cDCs, pDCs, granulocytes, NK cells, and T cells (**Figure 3D**). The absolute numbers of B cells, T cells, monocytes and plasma cells were decreased by anti-TLR7 treatment (**Figure 3E**). T cell decrease would be due to impaired activation of B cells and cDCs. Because changes in monocytes were apparent as those in B cells, we focused on monocyte subsets, Ly6C^{hi} classical, and Ly6C^{low} patrolling monocytes (21). These subsets were defined by cell surface markers such as F4/80, CD43, CX3CR1 (**Supplementary Figure 3**). The anti-TLR7 mAb significantly decreased ratios (%) of CD11b^{+} Ly6C^{low} FcγRIV^{+} patrolling monocytes, but did not affect those of Ly6C^{hi} classical monocytes (**Figures 4A–C**) (21, 22).

We explored TLR7 and TLR9 expression of these monocyte subsets in NZBWF1 mice. The expression of TLR7 increased, whereas that of TLR9 decreased with maturation from classical, to patrolling monocytes (21) in NZBWF1 mice (**Figures 4D, E**). Although increased TLR7 expression in patrolling monocytes might be consistent with their pathogenic roles in these mice, such changes in TLR7 and TLR9 expression were also observed in C57BL/6 mice (**Figures 4D, E**).

Anti-TLR7 mAb Inhibited Disease-Associated Increases in Patrolling Monocytes in the Circulation and Glomeruli

Classical monocytes mature in bone marrow and enter the circulation (21), where they mature into patrolling monocytes

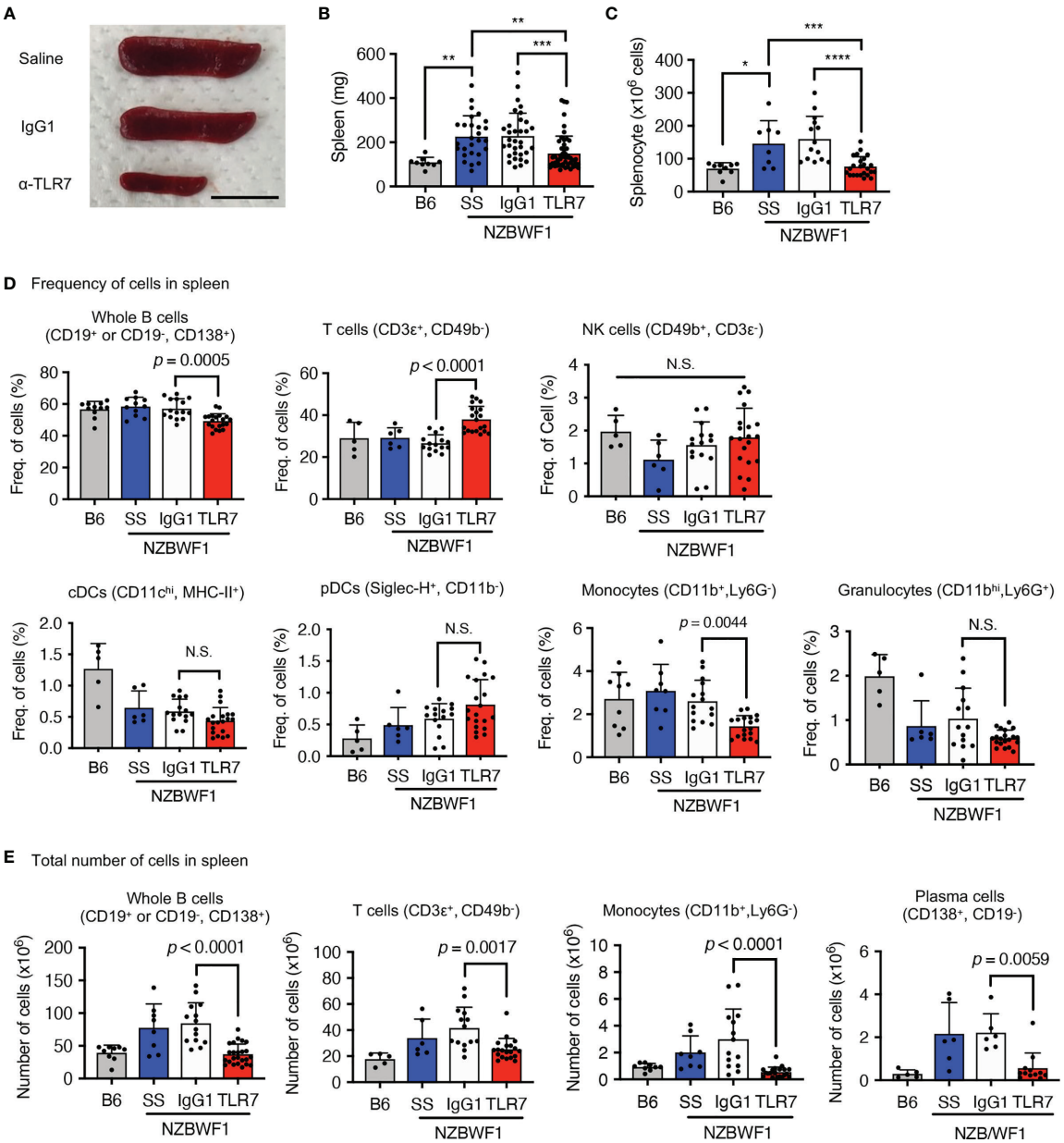

FIGURE 3 | Anti-TLR7 mAb inhibited monocytosis in spleens. **(A-D)** Age-matched B6 and NZBWF1 mice were administered saline, IgG1 or anti-TLR7 mAb from age of 12-16 to 35-40 weeks. **(A-C)** Macroscopic appearance **(A)**, weight **(B)**, and cell numbers **(C)** in spleens. B6 (n = 9), saline (n = 8), control IgG1 ($n \geq 14$), and anti-TLR7 mAb ($n \geq 24$). **(D, E)** Ratios **(D)** and absolute numbers **(E)** of indicated immune cells in spleens: B6 ($n \geq 5$), saline ($n \geq 6$), control IgG1 ($n \geq 15$), and anti-TLR7 ($n \geq 18$). Data were statistically analyzed using one-way ANOVA. The results found significant by ANOVA ($p < 0.05$) were further assessed by Tukey's multiple comparison tests **(B, C)**. For flow cytometry analysis, Tukey's multiple comparison test was performed between control IgG1 and anti-TLR7 groups, and the p-values are shown **(D, E)**. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, **** $p < 0.0001$. N.S., not significant. Data are shown as individual points and as means ± SD for each experimental group.

that are regarded as blood macrophages that TLR7-dependently clear damaged endothelial cells (22). We analyzed the numbers of patrolling monocytes in the peripheral blood of NZBWF1 mice that had been administered control IgG1 or the anti-TLR7 mAb. We found TLR7-dependent increases in the number of circulating patrolling monocytes in NZBWF1 mice at the ages of 30 and 37 weeks (**Figure 5A**), much later than the beginning of autoantibody production. Because the anti-TLR7 mAb did not decrease patrolling monocytes, the effect of the anti-TLR7 mAb is unlikely cytotoxic. TLR7 activation was probably required for the increases in these monocytes in mice with lupus, but not in healthy mice, and the anti-TLR7 mAb inhibited

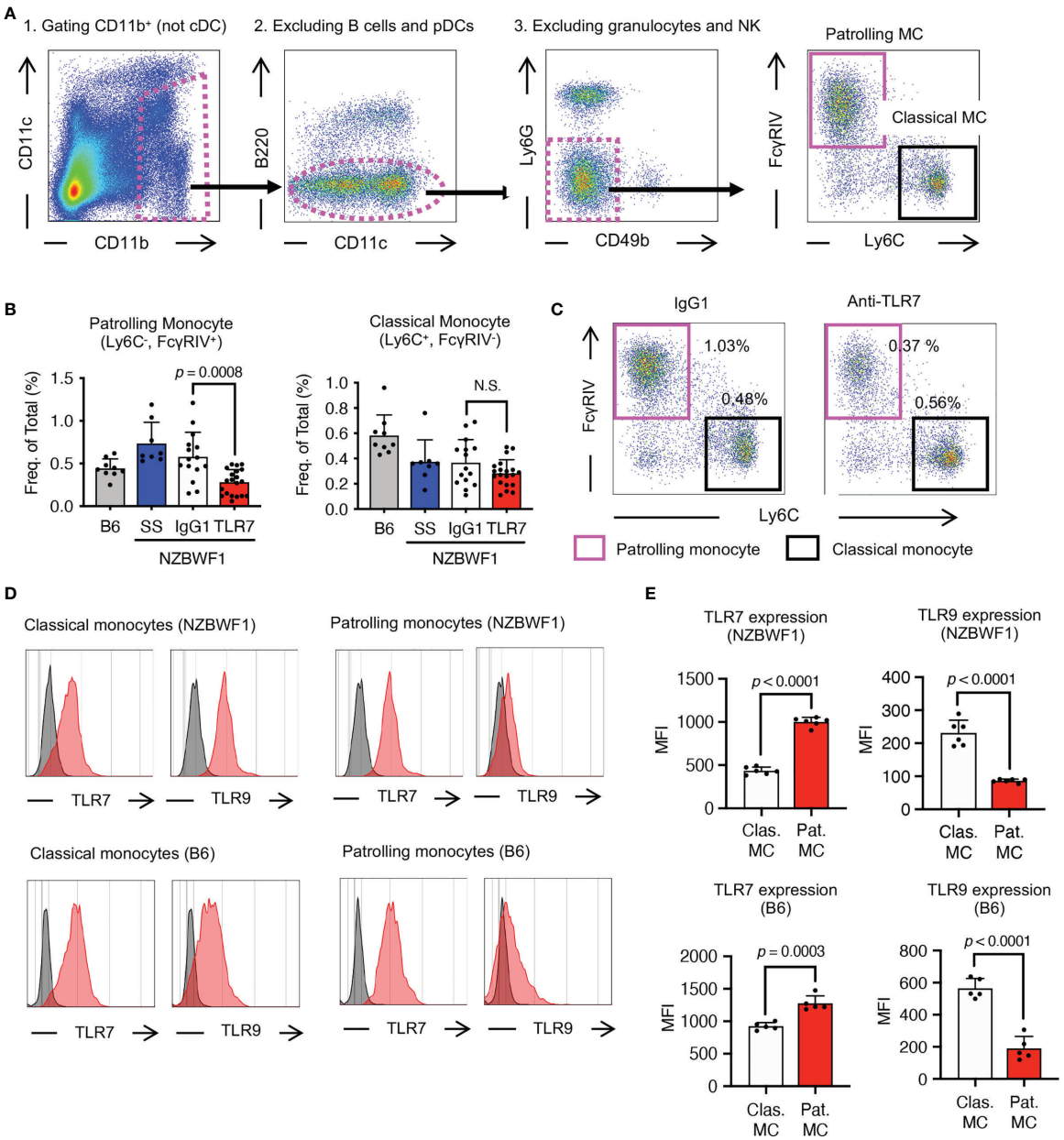

FIGURE 4 | Patrolling monocytes TLR7-dependently increased in NZBWF1 mice. **(A)** Gating strategy and markers for flow cytometry of monocyte subsets. Cells in magenta dotted gates were assessed using drill-down analyses. **(B, C)** NZBWF1 mice were administered saline, IgG1 or the anti-TLR7 mAb from age 12-16 to 35-40 weeks and compared with age-matched B6 mice **(B)** Ratios of monocyte subsets in spleens: B6 ($n \geq 5$), saline ($n \geq 6$), control IgG1 ($n \geq 15$), and anti-TLR7 ($n \geq 18$). **(C)** Representative dot plots show monocyte subsets in NZBWF1 mice administered IgG1 or anti-TLR7 mAb. **(D)** Red histograms show expression of TLR7 and TLR9 in classical and patrolling monocytes in spleens of 14-week-old NZBWF1 mice detected by membrane permeabilized staining. Gray histograms, staining with isotype-matched control antibodies. **(E)** Statistical analysis of mean fluorescence intensity (MFI) of TLR7 and TLR9 staining in monocyte subsets ($n = 6$). Data were statistically analyzed by ordinary one-way ANOVA with Tukey's multiple comparison tests **(B)** or Student's t-test **(E)**, and p-values are shown. Data are shown as individual points and as means ± SD for each experimental group **(B, E)**.

TLR7-dependent increase of monocytes. In contrast, the number of classical monocytes in peripheral blood did not increase even at the age of 37 weeks, and the anti-TLR7 mAb did not affect them (**Figure 5A**). These results suggested that TLR7-activation in NZBWF1 patrolling monocytes occurs after their maturation from classical monocytes.

We compared monocytes in the kidneys of young (pre-onset) NZBWF1 mice aged 12 weeks, with those of elderly mice aged 35-40 weeks that were administered IgG1 or anti-TLR7 mAb. The number of immune CD45.2$^+$ cells, CD11b$^+$ monocytes, and granulocytes were increased in the NZBWF1 mice given IgG1, and anti-TLR7 mAb inhibited this increase (**Figure 5B**). Among

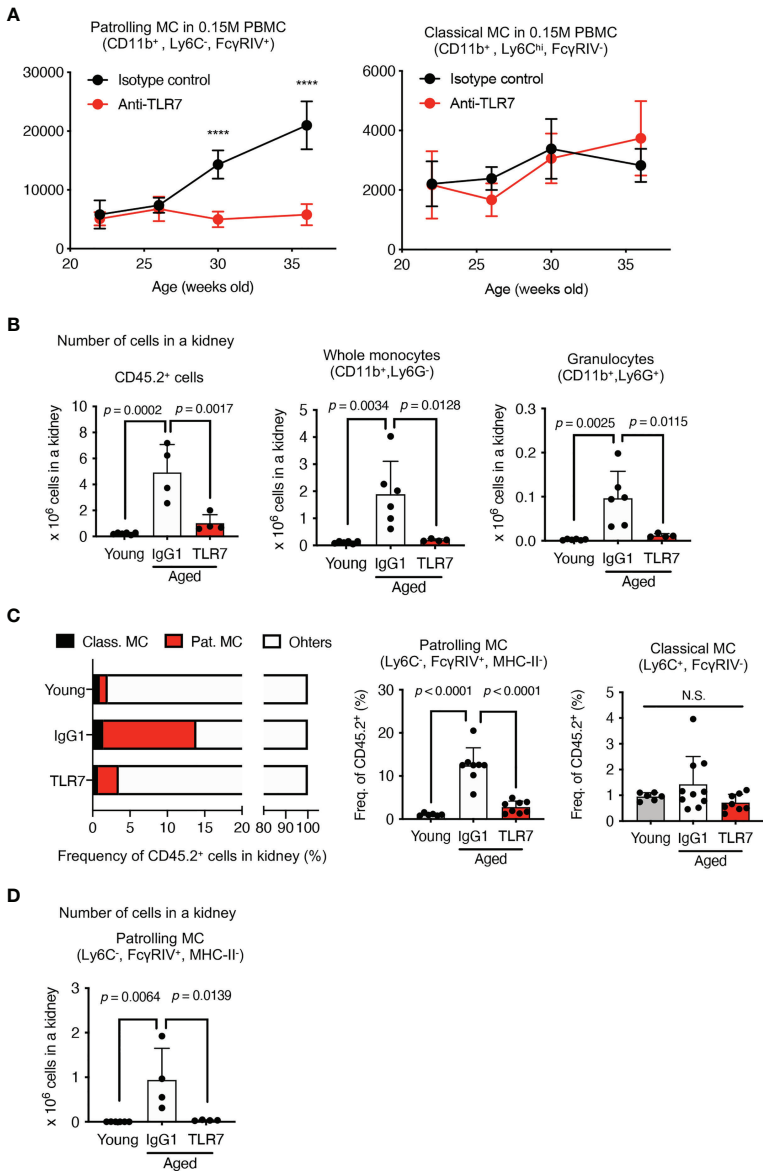

FIGURE 5 | Anti-TLR7 mAb inhibited patrolling monocyte increases in circulation and kidneys. **(A–D)** NZBWF1 mice were administered saline, IgG1 or anti-TLR7 mAb from age 12-16, to 35-40 weeks. **(B–D)** Young (pre-onset) NZBWF1 mice without treatment aged 12 weeks. **(A)** Average numbers of patrolling and classical monocytes in 15 x 10^4 PBMCs of NZBWF1 mice administered control IgG1 (n = 4) or anti-TLR7 mAb (n = 7). **(B)** Numbers of CD45.2$^+$ leukocytes, CD11b$^+$ Ly6G$^-$, and CD11b$^+$ Ly6G$^+$ monocytes in kidneys. Young mice (n = 6), NZBWF1 mice treated with control IgG1 ($n \geq 4$), or anti-TLR7 mAb ($n \geq 4$). **(C)** Ratios (%) of classical monocytes, patrolling monocytes, and other leukocytes in the kidney. **(D)** Numbers of patrolling monocytes in kidneys. Data were statistically analyzed by 2-way ANOVA **(A)** or ordinary one-way ANOVA **(B–D)**. For 2-way ANOVA, Sidak's multiple comparisons test was performed to calculate the row factor at each age. ****$p < 0.0001$; N.S., not significant. For ordinary one-way ANOVA, the results found significant by ANOVA ($p < 0.05$) were further assessed by Tukey's multiple comparison tests. Data are shown as individual points and as means ± SD for each experimental group.

the immune cells in the kidney, the ratios (%) and numbers of patrolling monocytes significantly and TLR7-dependently increased (**Figures 5C, D**). These results showed that the numbers of patrolling monocytes TLR7-dependently increased in the kidney and the circulation.

We used immunohistochemical staining to determine whether abundant patrolling monocytes infiltrated the glomeruli. The ratio (%) of glomeruli containing CD11b$^+$ cells in elderly NZBWF1 mice was ~ 40%, compared with those in age-matched C57BL/6 mice, which were < 10% (**Figures 6A, B**). The anti-TLR7 mAb decreased the number of glomerular CD11b$^+$ cells (**Figures 6A, B**). To confirm that patrolling monocytes infiltrated the glomeruli, we stained glomeruli for FcγRIV and TREML4, which are expressed at high levels in patrolling monocytes (23, 24). We also stained Ly6G to detect infiltrative neutrophils in glomeruli, because neutrophils were

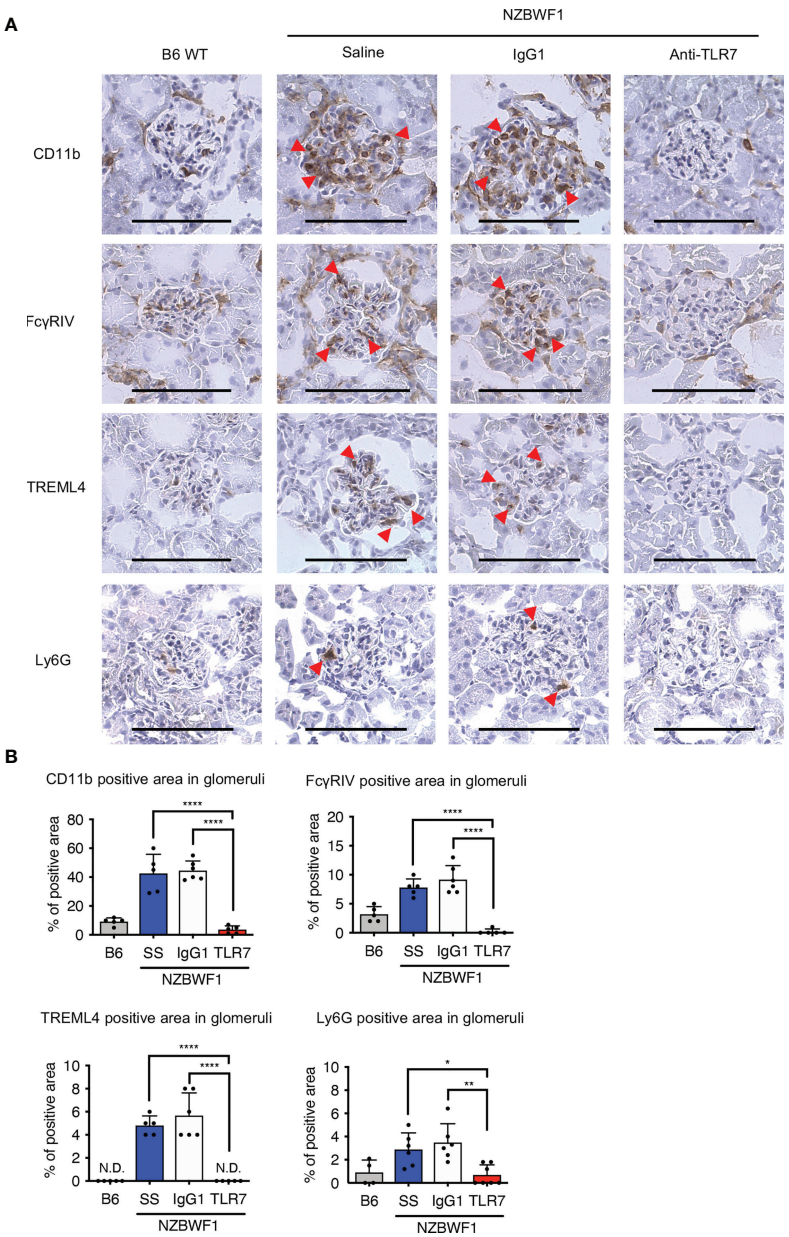

FIGURE 6 | Anti-TLR7 mAb inhibited patrolling monocyte infiltration into glomeruli. **(A, B)** NZBWF1 mice were administered saline, IgG1 or anti-TLR7 mAb from age 12-16, to 35-40 weeks and compared with age-matched B6 mice. **(A)** Representative images show immunohistological findings of glomerular myeloid cells expressing CD11b, FcγRIV, TREML4 or Ly6G. Red arrowheads, positively stained cells. Scale bar, 100 μm. **(B)** Ratios of stained areas in each glomerulus statistically analyzed using fluorescence microscopy. Numbers of glomeruli assessed (5-10 from one kidney in B6 ($n \geq 4$), saline ($n = 5$), control IgG1 ($n = 6$), and anti-TLR7 mAb ($n \geq 5$) **(A, B)**. Data were statistically analyzed using one-way ANOVA with Tukey's multiple comparison tests. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$. N.D., not detected. Data are shown as individual points and as means ± SD for each experimental group.

increased in the kidneys of NZBWF1 mice (**Figure 5B**). The expression of FcγRIV and TREML4 suggested that patrolling monocytes infiltrated the glomeruli of NZBWF1 mice (**Figures 6A, B**). Although Ly6G$^+$ neutrophils were detected in significant numbers of glomeruli, the ratios (%) of Ly6G$^+$ glomeruli were much lower than those of patrolling monocytes (**Figure 6B**). The anti-TLR7 mAb significantly decreased neutrophil recruitment to the glomeruli. These results might be consistent with the finding that patrolling monocytes recruit neutrophils to cause endothelial damage (22). Neutrophil-independent damage might occur in glomeruli with patrolling monocytes but without neutrophils. These results suggested that anti-TLR7 mAb mitigates lupus nephritis by inhibiting the accumulation of patrolling monocytes in glomeruli.

Expression of Lupus-Associated Genes in Patrolling Monocytes in NZBWF1 Mice

We characterized the abundant patrolling monocytes in NZBWF1 mice using transcriptome analysis. We compared $Ly6C^{hi}$ classical and $Ly6C^{low}$ patrolling monocytes because classical monocytes were not increased in NZBWF1 mice (**Figures 4B**, **5A**). The expression of 924 genes significantly differed between patrolling monocytes and classical monocytes. Among them, 112 genes were upregulated > 4-fold in patrolling monocytes (**Figures 7A, B**, and **Supplementary Table 1**). Some of these upregulated genes (*Fabp4, Il10, Pecam1*, *Pparg*, *Vwf*, *Cd36*, *Cd274*, and *Tnfsf15*), are upregulated in SLE as well (25–30). Although loss-of-function mutations of *Acp5* cause spondyloenchon-drodysplasia with immune dysregulation (SPENCDI), which is a skeletal and neurological disorder with lupus-like symptoms and a type I interferon signature (31), the expression of *Acp5* mRNA increased in patrolling monocytes in NZBWF1 mice.

To determine whether these SLE-associated genes are TLR7-dependently upregulated, we measured levels of mRNAs encoding SLE-associated genes in isolated patrolling monocytes using real-time PCR. The anti-TLR7 mAb decreased *Tnfsf15* expression in splenic patrolling monocytes (**Figure 7C**). Although the anti-TLR7 mAb did not significantly change levels of mRNAs encoding the other genes, it returned the elevated serum IL-10 and ACP5 to normal levels (**Figure 7D**). Anti-TLR7 mAb might impact monocytes during transcription and post-transcriptional processes. We examined the expression of mRNAs encoding these upregulated genes in the kidney using real-time PCR. The anti-TLR7 mAb decreased the upregulated mRNAs encoding *Il10, Acp5*, and *Tnfsf15* in NZBWF1 mice to the levels in C57BL/6 kidneys (**Figure 7E**). Expression of mRNAs encoding inflammatory cytokines and type I interferons were not changed by the anti-TLR7 mAb (**Supplementary Figure 4**). These results suggested that patrolling monocytes TLR7-dependently expressing lupus-associated genes such as *Il10, Acp5*, and *Tnfsf15* infiltrated the kidneys of NZBWF1 mice.

Expression of TLR7-Dependent Cell Surface Markers on Patrolling Monocytes

We applied an antibody array system to identify the TLR7-dependent expression of a cell surface marker in patrolling monocytes, because the anti-TLR7 mAb might act on monocytes at the post-transcriptional level. Flow cytometry using 258 antibodies revealed that 106 markers were expressed on patrolling monocytes. The mean fluorescence intensity (MFI) of 60 markers was reduced by the anti-TLR7 mAb, and we focused on 11 among them that were reduced > 50% by the anti-TLR7 mAb (**Figures 8A, B**). Splenic patrolling monocytes were stained with these antibodies for verification, and the MFIs of the markers CD273 (PD-L2), CD31 (platelet endothelial cell adhesion molecule 1; PECAM), CD115 (CSF1-R), CD14, CD41, CD85k (GP49B), CD54 (ICAM1), CD132, and CD274 (PD-L1) were significantly decreased by the anti-TLR7 mAb (**Figure 8C**).

We compared patrolling monocytes in the kidneys of young (pre-onset) and elderly NZBWF1 mice administered IgG1 or the anti-TLR7 mAb, to determine the expression of these markers in patrolling monocytes in the kidney. The expression of CD273, CD31 (PECAM), CD115, CD14, CD85k, and CD54 (ICAM1) was significantly increased by disease progression and was sensitive to the anti-TLR7 mAb (**Figure 8D**). These markers might play pathogenic roles in lupus nephritis. For example, the CD115 inhibitor GW2580 attenuates nephritis and neuropsychiatric diseases in the lupus-prone mouse strain, MRL-lpr/lpr (31). In this context, the fact that mRNAs encoding M-CSF and IL-34, ligands for CD115, were TLR7-dependently increased in the kidneys of NZBWF1 mice is notable (**Figure 8E**). As these cytokines are produced by mesangial cells and podocytes in lupus nephritis (32), glomerular infiltration by $CD115^+$ patrolling monocytes might upregulate the expression of mRNAs encoding CD115 ligands, leading to a vicious circle between patrolling monocytes and glomerulus-intrinsic cells such as mesangial cells and podocytes.

DISCUSSION

Our results showed that TLR7 activation increased the number of patrolling monocytes in the spleen, circulation, and kidneys of NZBWF1 mice. Lupus-associated TLR7-dependent monocytosis has been identified in the *Yaa* mouse model of lupus (33), in which TLR7 is hyperactivated due to its gene duplication (10, 11). Furthermore, the numbers of patrolling monocytes in the kidney are increased in lupus-prone MRL/*lpr*, B6.*Sle yaa*, and *Tnip1*$^{-/-}$ mice (6). Patrolling monocytes infiltrate the glomeruli of patients with SLE (6, 22, 34). These findings suggest that increased numbers of patrolling monocytes directly drive lupus nephritis. Consistent with this, lupus nephritis in B6.*Sleyaa* and *Tnip1*$^{-/-}$ mice is ameliorated by preventing monocyte maturation into patrolling monocytes by deleting their master transcription factor NR4A1 (6, 35). Our anti-TLR7 mAb findings suggested that TLR7 drives lupus-associated increases in patrolling monocytes in NZBWF1 mice. Because the anti-TLR7 mAb did not impair homeostatic monocyte maturation into patrolling monocytes, TLR7 was activated only in a subpopulation of patrolling monocytes that infiltrated the kidney of NZBWF1 mice. Macrophage-specific *Tnip1* deletion is sufficient for nephritis to develop in *Tnip1*$^{-/-}$ mice, and high levels of TLR7 were expressed in patrolling monocytes of NZBWF1 mice. These results suggest that patrolling monocytes are increased by cell-autonomous TLR7 activation in these mice. Endogenous retroelements, such as Alu RNAs, might activate TLR7 in patrolling monocytes (36). A mechanism through which TLR7 promotes monocytosis might be suggested by our finding of increased CD115 expression in patrolling monocytes. Because CD115 delivers a survival signal in patrolling monocytes (37), upregulated CD115 activation might underlie lupus-associated monocytosis in NZBWF1 mice.

Lupus-associated increases in patrolling monocytes were detected not only in the circulation, where classical monocytes mature into patrolling monocytes (21), but also in the spleen and kidney. Circulating patrolling monocytes are likely to infiltrate

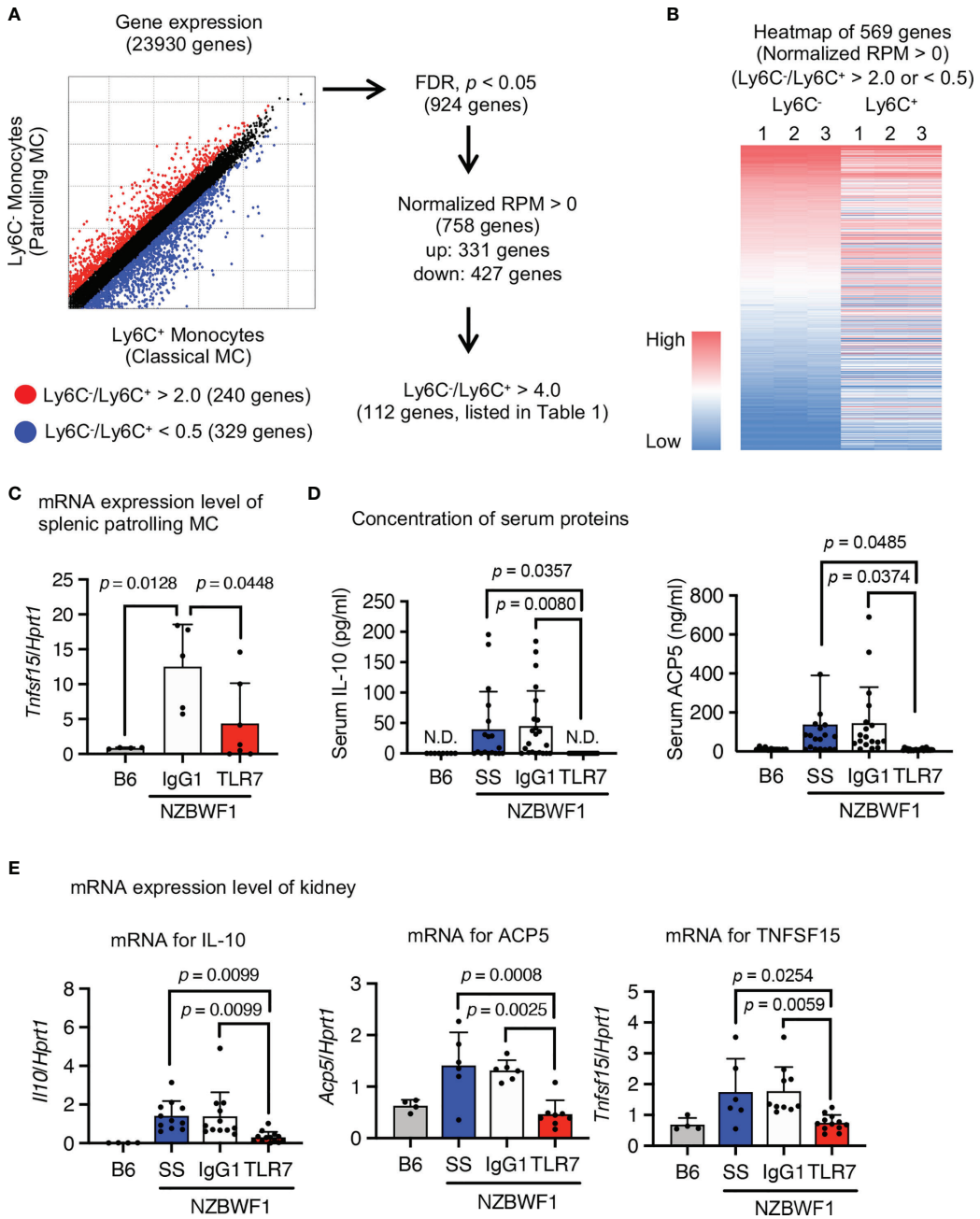

FIGURE 7 | Expression of SLE-associated genes in patrolling monocytes in NZBWF1 mice. Expression of 23,930 analyzed genes in patrolling monocytes and classical monocytes of NZBWF1 mice aged 30-35 weeks. Red and blue dots show genes with expression ratios in patrolling monocytes compared with classical monocytes that were > 2 or < 0.5, respectively. We identified 924 significantly changed genes ($p < 0.05$; Benjamin-Hochberg FDR tests), and 758 with normalized expression (reads per million, RPM) > 0. Among them, the expression of 331 genes was considered as upregulated in patrolling monocytes compared with classical monocytes. Among these 331 genes, 112 with a patrolling/classical expression ratio > 4 are shown in **Supplementary Table 1**. **(B)** Heatmap of RPM of 569 genes (red and blue dots in **(A)** in descending order based on that of patrolling monocytes. Results are from individual samples (1–3). **(C–E)** NZBWF1 mice administered saline, IgG1 or anti-TLR7 mAb from age 12-16 to 35-40 weeks compared with age-matched B6 mice. **(C, E)** Expression of mRNAs encoding indicated genes in splenic patrolling monocytes **(C)** and kidney **(E)** were analyzed by real-time PCR. Results are normalized by *Hprt1* mRNA. **(D)** Concentration of IL-10 and ACP5 in serum measured by ELISA. B6 ($n = 4$), saline ($n \geq 6$), control IgG1 ($n \geq 5$), and anti-TLR7 mAb ($n \geq 6$). Data were statistically analyzed using one-way ANOVA. The results found significant by ANOVA ($p < 0.05$) were further assessed by Tukey's multiple comparison tests and the *p*-values are shown **(C–E)**. Data are shown as individual points and as means ± SD for each experimental group **(C–E)**.

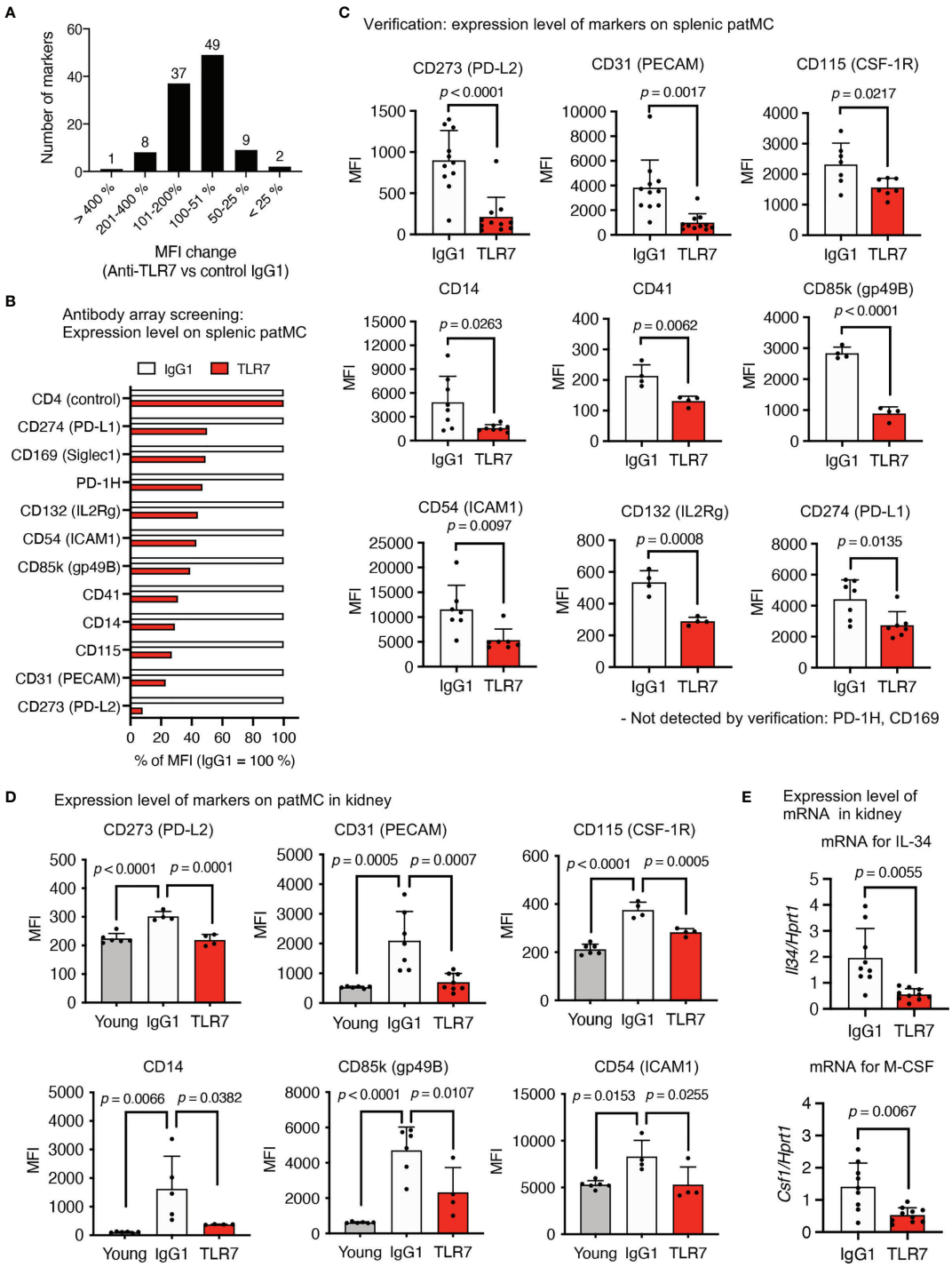

FIGURE 8 | TLR7-dependent expression of the cell surface molecules on patrolling monocytes in NZBWF1 mice. **(A–E)** NZBWF1 mice were administered saline, IgG1 or the anti-TLR7 mAb from age of 12-16 to 35-40 weeks. Young (pre-onset) untreated NZBWF1 mice were analyzed at 12 weeks of age. **(A)** Splenic patrolling monocytes were stained using LEGENDScreen antibody array. Distribution of ratios (%) of anti-TLR7 to IgG1 treatment is shown. **(B)** Mean fluorescent intensity of markers with > 50% less expression caused by anti-TLR7. Results are average values from four mice. **(C)** Expression of markers on patrolling monocytes in spleen. Control IgG1 ($n \geq 4$), and anti-TLR7 mAb ($n \geq 4$). **(D)** Expression of markers on patrolling monocytes in kidneys. Young (pre-onset) 12-week-old NZBWF1 mice were controls. Young mice ($n = 6$), control IgG1 ($n \geq 4$), and anti-TLR7 ($n \geq 4$). **(E)** Expression of mRNA encoding IL-34 and M-CSF in kidneys analyzed by real-time PCR. Results are normalized to *Hprt1* mRNA. Control IgG1 ($n \geq 7$), and anti-TLR7 ($n \geq 6$). Data were statistically analyzed by Student's t-tests, Welch's t-tests **(C, E)**, or one-way ANOVA **(D)**. The results found significant by ANOVA ($p < 0.05$) were further assessed by Tukey's multiple comparison tests. Data are shown as individual points and as means ± SD for each experimental group **(C–E)**.

these organs. Transcriptome analyses and an antibody array showed a TLR7-dependent increase in CD31, which belongs to the immunoglobulin superfamily and is expressed at the intercellular junctions of endothelial cells and leukocytes including monocytes (38). Homophilic interactions between leukocyte CD31 and endothelial CD31 enable trans-endothelial leukocyte migration (39). Thus, patrolling monocytes might use CD31 to migrate into the glomeruli of NZBWF1 mice. The expression of CD31 in the peripheral blood of patients positively correlates with SLE disease activity (29); therefore, CD31 expression might also increase TLR7-dependently in human SLE.

Messenger RNAs encoding M-CSF and IL-34 that are ligands for CD115, were TLR7-dependently increased in the kidneys of NZBWF1 mice. These ligands are produced by mesangial cells and podocytes, respectively, in glomeruli (32), indicating that glomerular patrolling monocytes could change the microenvironment to drive glomerulonephritis. Consistent with this, the CD115 inhibitor GW2580 attenuates nephritis and neuropsychiatric disease in a lupus-prone mouse strain MRL-lpr/lpr (40). The molecular mechanisms through which patrolling monocytes drive glomerular damage, might be *via* neutrophil recruitment, which results in endothelial cell damage (22). We detected TLR7-dependent increases in neutrophils in the kidneys, but considerably fewer neutrophils than patrolling monocytes infiltrated the glomeruli of NZBWF1 mice. Patrolling monocytes are likely to drive nephritis without neutrophil recruitment when glomeruli do not harbor neutrophils. Notably, patrolling monocytes TLR7-dependently produced IL-10. Although large amounts of IL-10 are produced by various types of immune cells such as $CD4^+$ T cells and DCs in NZBWF1 mice (41), the anti-TLR7 mAb significantly decreased serum IL-10 and IL-10 mRNA in the kidneys, suggesting that patrolling monocytes are a major source of IL-10 in NZBWF1 mice. Interleukin-10 is a pleiotropic cytokine that mediates both immunostimulatory and immunoregulatory effects in humans and mice (42). The anti-IL-10 mAb delays disease development in NZBWF1 mice, whereas IL-10 accelerates disease progression (17). Elevated levels of serum IL-10 have also been found in other kidney diseases, such as mesangioproliferative glomerulonephritis, IgA nephropathy, and diabetic nephropathy (40). As IL-10 promotes glomerular damage by increasing mesangial proliferation and the mesangial deposition of immune complexes (43, 44), our results suggested that the TLR7-dependent production of IL-10 in glomerular patrolling monocytes promotes lupus nephritis. Tumor necrosis factor superfamily 15 (TNFSF15), also known as TNF-like ligand 1A (TL1A) or vascular endothelial growth inhibitor (VEGI), is produced by monocytes and macrophages stimulated with immune complexes (ICs) but not by TLR ligands, including the TLR7/8 ligand R848 (45), which inhibits IC-dependent TNFSF15 expression in human peripheral blood monocytes (46). The activation of TLR7 in NZBWF1 mice might be distinct from R848-mediated TLR7/8 activation in human peripheral blood monocytes. The Y RNA binding protein, Ro60, is an autoantigen that binds to endogenous retroelements such as Alu RNAs, which might activate TLR7 (36). Immune complexes containing Alu RNA-Ro60 might act on cell surface FcRs and subsequently on endosomal TLR7 in patrolling monocytes to induce TNFSF15, which acts on death receptor-3 expressed on endothelial cells in kidneys (47). Therefore, TNFSF15 might contribute to glomerular damage in NZBWF1 mice.

Because deleting TLR7 is not sufficient to rescue MRL/*lpr* or *Tnip1*$^{-/-}$ mice from nephritis (6, 7), other TLRs such as TLR9 might also drive the increases in lupus-associated patrolling monocytes in these mice (6). In this context, TLR7-dependent increase in serum ACP5, also known as tartrate-resistant acid phosphatase (TRAP) is notable. High levels of ACP5 are expressed in osteoclasts, and serum ACP5 is considered a marker of osteoclast activation. Our results suggested that serum ACP5 could be a marker of TLR7-dependent monocyte activation in SLE. On the other hand, biallelic loss-of-function mutations cause spondyloenchondrodysplasia with immune dysregulation (SPENCDI), a skeletal and neurological disorder with lupus-like symptoms and a type I interferon signature (31), in which TLR9 hyperactivation is suggested to cause immune dysregulation. If SLE can be subdivided into one type that depends on TLR7 and another that depends on TLR9, they might be distinguished from each other by serum ACP5.

The CD115 inhibitor GW2580 inhibits monocyte accumulation in the kidneys, but not IgG deposition in glomeruli of MRL/*lpr* mice (40). Lack of the FcRγ chain impairs the activation of FcR^+ monocytes, thus protecting NZBWF1 mice from lupus nephritis, despite unaltered immune complex deposition in glomeruli (4, 5). These previous studies suggest that monocytes/macrophages are activated independently of B cell activation. Because autoreactive B cell also plays pathogenic roles in NZBWF1 mice, B cell is a therapeutic target for the control of lupus nephritis. The anti-TLR7 mAb inhibited both monocytosis and IgG deposition, suggesting that the anti-TLR7 mAb acted on both monocytes and B cells. The anti-TLR7 mAb also inhibits TLR7 responses in BM-cDCs and BM-pDCs *in vitro* (16), which might explain the decrease in number of splenic T cells by the anti-TLR7 mAb. We concluded that TLR7 is a promising therapeutic target for controlling SLE, and a mAb against TLR7 is a promising modality with which various immune cells can be targeted.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: National Center for Biotechnology Information (NCBI) BioProject database under accession number PRJDB12445 and in DDBJ under accession number DRA012916.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Experiment Committee of The Institute of Medical Science, The University of Tokyo.

AUTHOR CONTRIBUTIONS

YMu, RF, and KM conceived and designed the experiments. YMu, RF, and KM wrote the manuscript. YMu, RF, RT, YMo, AK, RS, and HA performed the experiments. YMu, RF, YMo, and AK generated the antibodies. KY and YF conducted and performed RNA sequencing analysis. HA, NT, HS, YS, and NY analyzed the data with clinical insights. All the authors reviewed and edited the results and comments on the manuscript. YMu and RF contributed equally as co-first authors. All authors contributed to the article and approved the submitted version.

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RNASE2 Mediates Age-Associated B Cell Expansion Through Monocyte Derived IL-10 in Patients With Systemic Lupus Erythematosus

Yantong Zhu[1], Xiaojun Tang[1], Yang Xu[1], Si Wu[1], Weilin Liu[1], Linyu Geng[1], Xiaolei Ma[1], Betty P. Tsao[2], Xuebing Feng[1] and Lingyun Sun[1]*

[1] Department of Rheumatology and Immunology, The Affiliated Drum Tower Hospital of Nanjing University Medical School, Nanjing, China, [2] Division of Rheumatology and Immunology, Department of Medicine, Medical University of South Carolina, Charleston, SC, United States

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Naoki Iwamoto, Nagasaki University Hospital, Japan

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***Correspondence:**
Xuebing Feng
fengxuebing@hotmail.com

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Systemic lupus erythematosus (SLE) is characterized by the production of pathogenic autoantibodies. Ribonuclease A family member 2 (RNase2) is known to have antiviral activity and immunomodulatory function. Although RNASE2 level has been reported to be elevated in SLE patients based on mRNA microarray detection, its pathologic mechanism remains unclear. Here, we confirmed that RNASE2 was highly expressed in PBMCs from SLE patients and associated with the proportion of $CD11c^{+}T\text{-}bet^{+}$ B cells, a class of autoreactive B cells also known as age-associated B cells (ABCs). We showed that reduction of RNASE2 expression by small interfering RNA led to the decrease of ABCs *in vitro*, accompanied by total IgG and IL-10 reduction. In addition, we demonstrated that both RNASE2 and IL-10 in peripheral blood of lupus patients were mainly derived from monocytes. RNASE2 silencing in monocytes down-regulated IL-10 production and consequently reduced ABCs numbers in monocyte-B cell co-cultures, which could be restored by the addition of recombinant IL-10. Based on above findings, we concluded that RNASE2 might induce the production of ABCs *via* IL-10 secreted from monocytes, thus contributing to the pathogenesis of SLE.

Keywords: systemic lupus erythematosus, age-associated B cells, ribonuclease A family member 2, interleukin 10, monocytes

INTRODUCTION

Systemic lupus erythematosus (SLE) is a prototypic autoimmune disease characterized by the production of various autoantibodies and damage to multiple organ systems (1). It is widely recognized that B cell dysfunction plays a major role in the pathogenesis of lupus (2). Among which, the age-associated B cell subset (ABCs) has been the focus of increasing interest over the last decade (3). ABCs express myeloid markers CD11c and depend on T-box transcription factor (T-bet) for their generation, thus also called $CD11c^{+}T\text{-}bet^{+}$ B cells (4). ABC-like B cells have been detected in human SLE and animal models (5), and were associated with disease activity and specific

autoantibody profiles (6). However, the molecular pathways that promote the expansion of ABC population in SLE patients is still largely unknown.

Recently, it has been recognized that the innate immune system not only initiates the inflammatory cascade in SLE, but also continues to promote adaptive immune response throughout the disease process (7, 8). Ribonuclease A family member 2 (RNase2), commonly known as eosinophil-derived neurotoxin, belongs to RNaseA superfamily and is one of the four major secretory proteins released upon activation of eosinophils (9). Besides eosinophils, human monocyte-derived macrophages can also produce this RNase after the stimulation (10). Our previous study has found that RNASE2 was one of the most prominent up-regulated genes in PBMCs of SLE patients through high-throughput sequencing technique (data not shown). Besides, RNASE2 has been identified as a marker gene that is cross-validated in multiple types of human SLE samples (11), while the mechanism by which this occurs remains obscure. Dependent on its ribonuclease activity, RNase2 has broad antiviral activity against single strand RNA like respiratory syncytial virus and human immunodeficiency virus (12, 13). Treatment of RNase2 could result in maturation of dendritic cells and trigger the production of a variety of soluble mediators, mostly pro-inflammatory cytokines and chemokines (14). More recently, it has been shown to be required in immune sensing of live pathogens by Toll-like receptor 8 (15). Thus, RNase2 may serve as a bridge between innate and adaptive immunity.

In this study, we demonstrated that RNASE2 mRNA was highly expressed in peripheral blood mononuclear cells (PBMCs) from SLE patients and correlated with disease activity, autoantibody levels as well as proportion of ABCs. Silencing RNASE2 reduced the number of ABCs *in vitro*. We also observed that interleukin (IL)-10 served as a major effector of RNASE2. By increasing the expression of IL-10 in monocytes, RNASE2 could promote the production of ABCs. Moreover, our data revealed that IL-10 has a two-way action on ABCs. Altogether, our study provides a novel mechanistic view into the upstream regulation of ABCs.

MATERIALS AND METHODS

Subjects

Study protocol was reviewed and approved by the Ethics Committee of the Affiliated Drum Tower Hospital of Nanjing University Medical School. The enrollment of volunteers was conducted in compliance with the Declaration of Helsinki. Patients excluding tumors and infection are recruited from rheumatology and immunology department. Patients with SLE, rheumatoid arthritis (RA) and primary Sjögren's syndrome (SS) fulfilled the 1997 updated American College of Rheumatology (ACR) classification criteria, the 1987 ACR criteria and the 2002 American-European consensus criteria respectively (16–18). SLE disease activity was assessed and scored by the SLE disease activity index (SLEDAI) and the British Isles Lupus Assessment Group (BILAG) index (19, 20). Written informed consent was obtained from all patients and healthy donors that provided blood samples 10ml or more specifically for the study. When only residual blood was used, written informed consent was waived.

Cell Sorting

To simultaneously isolate multiple cell subsets from the same patient, flow cytometry sorting was applied. PBMCs were isolated from 10 ml peripheral venous blood with Ficoll-Hypaque discontinuous gradient method, donated by 5 HC and 8 SLE patients. PBMCs were stained with FITC-antiCD14 (BioLegend), eflour450-antiCD19 (ebioscience), PE-cyanine7-antiCD4 (ebioscience) antibodies, then flow cytometry sorting technology was applied: $CD14^+$ gate and $CD14^-$ gate were set on BD FACSAria III flow cytometer (BD Biosciences), $CD14^+$ monocytes were separated; for $CD14^-$ cells, $CD19^+$ B cells and $CD4^+$ T cells and the remaining $CD14^-CD19^-CD4^-$ cells were isolated with $CD4^+$ and $CD19^+$ gates.

For B cell solo-cultures, B cell isolation kit (Miltenyi Biotec) was used to separate B cells from PBMCs. CD2, CD14, CD16, CD36, CD43, and CD235a (glycophorin A) positive cells were subsequently magnetically labeled with anti-Biotin microbeads for depletion. B cells obtained by depletion of magnetically labeled non-B cells were stained with percp cy5.5-antiCD19 (Biolegend), and flow cytometry showed the purity of $CD19^+$ cells was over 90% (**Supplementary Figure 1A**).

For monocyte solo-cultures, human CD14 positive selection kit II (Stem cell) was applied to separate monocytes from lupus PBMCs according to the manufactory's instructions. $CD14^+$ monocytes were sorted using magnetic beads with a purity over 90% (**Supplementary Figure 2**).

RNASE2 siRNA Silencing

Four pairs of small interfering RNA (siRNA) sequences targeting RNASE2 and one pair of non-targeting siRNA sequences (**Supplementary Table 1**) were designed and synthesized by personnel at Dharmacon Cells were suspended in siRNA buffer and distributed to 96-well round-bottomed plates (Costar) at 1×10^5 per well, with RNASE2 siRNA pairs or nontargeting siRNA at a final concentration of 1uM. Cells were incubated at 37 °C with 5% CO_2 for 3 days.

Cell Cultures

PBMCs were isolated from peripheral venous blood with Ficoll-Hypaque discontinuous gradient method, donated by SLE patients. Then PBMCs were silenced by RNASE2 siRNA (1 uM, Dharmacon) for 3 days. Magnetic sorted B cells (1×10^5) from SLE donors were stimulated with anti-CD40 (0.1 μg/ml, goat IgG, R&D Systems) and anti-IgM F(ab')2 (5.0 μg/ml, Jackson ImmunoResearch Laboratories), and silenced by RNASE2 siRNA as described above. Magnetic sorted monocytes (Stem cell) were silenced as described above.

To confirm the role of monocytes on B cells and the link to RNASE2 and IL-10, 1×10^5 monocytes sorted by Flow cytometry were treated with RNASE2 siRNA or anti IL-10 antibody (1 ug/ml and 5 ug/ml, Ebioscience) in 96 well plate with a volume of 100 ul for 6 hours, and then co-cultured with $0.5\text{-}1\times10^5$/100 ul B cells from the same individual. For monocytes treated with RNASE2 siRNA, recombinant human IL-10 (50 ng/ml,

Peprotech) was added in some of the co-cultures with B cells to observe the changes of ABCs.

To verify the effect of IL-10 on B cells, recombinant human IL-10 was either added into monocytes and B cells co-cultures at an increasing concentration of 0, 25, 50 or 100 ng/ml, or added into B cells solo-cultures stimulated with anti-CD40 and anti-IgM F(ab')2 at low concentrations of 0, 2, 10 and 40 ng/ml without the presence of monocytes. Three days later, cells were harvested for examining by flow cytometry and culture supernatants were stored at -80 °C for use.

RNA Isolation and Quantitative Real Time PCR

To detect RNASE2 and IL-10 expression, we isolated total RNA from PBMCs, $CD19^+$ B cell, $CD4^+$ T cell, monocytes and $CD14^-CD19^-CD4^-$ cells using Trizol reagent (Vazyme). Contaminating DNA was removed by deoxyribonuclease treatment, and total RNA was quantified using spectrophotometry. The RNA was converted to complementary DNA (cDNA) using transcriptor first-strand cDNA kit (Vazyme), and qPCR was performed on StepOnePlus Real-Time PCR instrument with gene-specific primers and analyzed with StepOne SoftwareV2.3 (Applied Biosystems). The relative gene quantification was done by using the $2^{-\triangle\triangle Ct}$ method following normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). For details of primers used in this study, see **Supplementary Table 2**.

Flow Cytometry

For ABCs detecting, PBMCs/B cells to be detected were incubation with FcR blocking reagent (Miltenyi Biotec) for 10 minutes at 4 °C, then stained with BV421 anti-CD11c (BioLegend) and percp-Cy5.5 anti-CD19 (BioLegend) at 4 °C for 30 minutes. For intracellular staining, cells were surface stained, fixed using eBio fix/perm kit (eBioscience), washed and stained with PE-Cyanine7 anti-T-bet (BioLegend) in 1 × eBio fix/perm buffer for 1 hour at 4 °C. Mouse IgG1 kappa Isotype Control, PE-Cyanine7 (eBioscience) was used as isotype control for T-bet staining.

For RNASE2 detecting, PBMCs to be detected were incubation with FcR blocking reagent (Miltenyi Biotec) for 10 minutes at 4 °C, then stained with APC anti-CD14 and viability dye efour 506 (Invitrogen) at 4 °C for 30 minutes. For intracellular staining, cells were surface stained, fixed with fixation/permeabilization solution (Cytofix/Cytoperm kit, BD), washed and stained with RNASE2 antibody (Invitrogen) in 1× Perm/Wash buffer for 1 hour at 4 °C, then stained with AlexaFlour 488 Goat anti-rabbit IgG (FCMACS). Cells stained with CD14 and AlexaFlour 488 Goat anti-rabbit IgG were used as isotype control for RNASE2 staining.

Then Cells were analyzed on a BD FACSAria III flow cytometer (BD Biosciences) using FACSDiva software. Cells were analyzed on a BD FACSAria III flow cytometer (BD Biosciences) using Flowjo VX software.

ELISA and Luminex

Total IgG and IL-10 levels in supernatants or plasma were detected by Human IgG total ELISA Kit (FMS-ELH102, Fcmacs) and Human IL-10 High Sensitivity ELISA Kit (70-EK110HS-96, MultiSciences). For total IgG level detection, samples and diluted standards were added to pre-embedded plate and incubated for two hours at 37 °C. Then biotinylated anti-human detection antibody was added to each well. The plate was incubated for one hour at 37 °C and developed with the addition of Streptavidin-HRP and tetramethylbenzidine as a substrate. The optical density for each well was documented with a microplate reader (Tecan Sunrise, Männedorf, Switzerland) set to 450 nm and levels of IgG and IL-10 were calculated according to their standard curves. For IL-10 level detection, amplification reagent was added to plate after the first addition of Streptavidin-HRP to amplify the detected signal. Levels lower than the lower limit of detection (LLOD) were regarded as having the minimum detection value (0.05 pg/ml).

B cell related cytokines was evaluated with customized MILLIPLEX MAP Human Th17 Magnetic Bead Panel (HTH17MAG-14K, Merck&Millipore) according to the manufacturers' instructions, a kit used for the simultaneous quantification of the following cytokines: IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-21, IFN-γ, TNF-α and TNF-β.

Western Blot Analysis

Magnetic sorted 5×10^5 $CD14^+$ monocytes from SLE patients were silenced by RNASE2 siRNA (1uM, Dharmacon) for 3 days. Then cells were collected and proteins were extracted in RIPA buffer supplemented with EDTA-free protease inhibitor cocktail (Shanghai Epizyme) and phosphatase inhibitor cocktail (Shanghai Epizyme). Proteins were run on 12.5% gradient gel (BioRad), blotted onto PVDF membrane and detected with RNASE2 (Invitrogen) or GAPDH (Cell Signaling Technology) antibody. Images were captured and analyzed on Tanon−5200 Chemiluminescent Imaging System.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 8.0.1 software. Data were shown as means and Standard Error of Mean (SEM). Unpaired two tailed t-test was applied to compare the difference between two groups, and Welch's correction was applied for those with unequal variance. Paired two tailed t test was used for pairing data. Relations between two variables were evaluated using the Pearson correlation test or Spearman correlation test, dependent on whether the variables were normally distributed. A p value less than 0.05 was considered significant.

RESULTS

Increased RNASE2 mRNA Expression in SLE Patients

Elevated peripheral blood RNASE2 mRNA expression was validated by real-time PCR in 60 SLE patients, compared with 20 patients with rheumatoid arthritis (RA), 20 patients with primary Sjögren's syndrome (SS) or 37 HC (**Figure 1A** and **Supplementary Table 3**). RNASE2 mRNA expression positively correlated with SLEDAI score, the amount of 24hour proteinuria, as well as creatinine (**Figures 1B–D**), but not uric acid (**Figure 1E**). Except

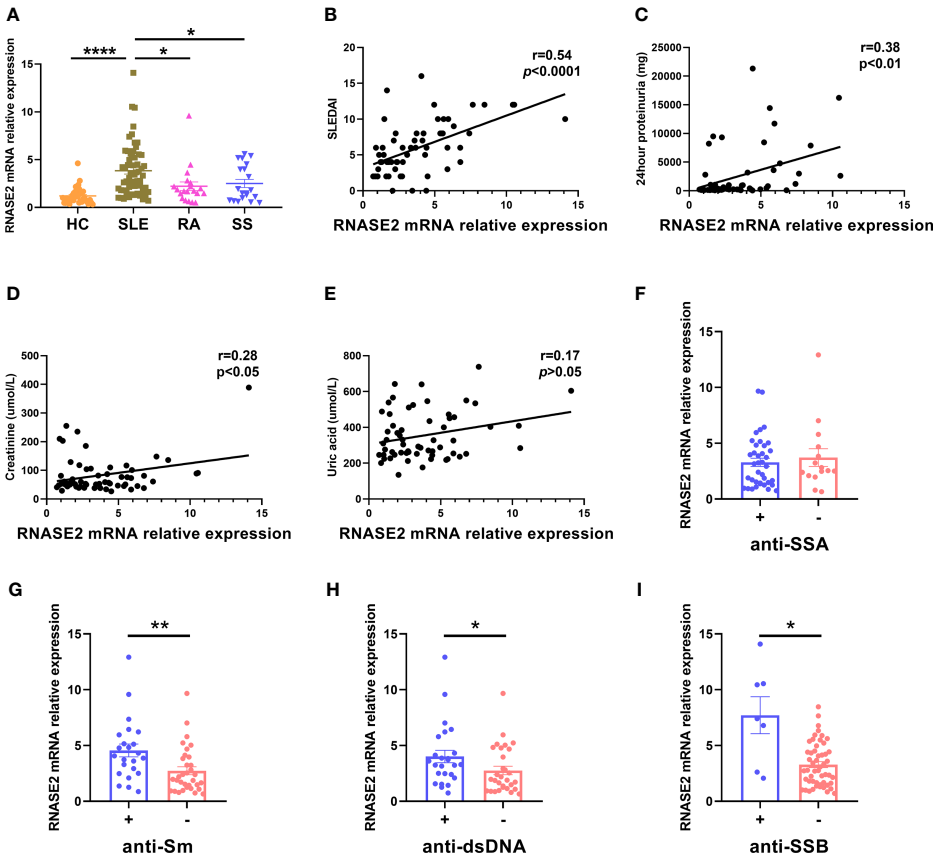

FIGURE 1 | Increased RNASE2 mRNA expression in PBMCs from SLE patients. **(A)** Detection of RNASE2 expression by real-time PCR in PBMCs from 37 healthy controls (HC), 60 SLE patients, 20 patients with rheumatoid arthritis (RA) and 20 patients with primary Sjögren's syndrome (SS). **(B–E)** Associations of RNASE2 mRNA levels with SLE disease activity index (SLEDAI) score, the amount of 24hour proteinuria as well as the levels of serum creatinine and uric acid by the Pearson or Spearman correlation test. **(F–I)** RNASE2 mRNA expression in SLE patients with or without positive anti-SSA, anti-Sm, anti-dsDNA or anti-SSB antibodies. Data are presented as mean ± SEM, *p < 0.05, **p < 0.01, ****p < 0.0001.

for anti-SSA (**Figure 1F**), seropositive SLE patients for anti-Sm, anti-dsDNA or anti-SSB antibodies had increased RNASE2 levels (**Figures 1G–I**), implying a possible link between upregulated RNASE2 and autoantibody production.

Association of ABC Proportion With RNASE2 Level

The role of RNASE2 in SLE disease progression is unclear at present. Our data confirmed elevated proportion of peripheral ABCs in SLE patients (n=26) compared to that in HC (n=24) (**Figure 2A**), which was associated with both SLEDAI score and BILAG score as well as 24hour proteinuria levels (**Figures 2B–D**). Meanwhile, SLE patients with positive anti-Sm antibody also had higher levels of ABCs (**Figure 2E**). Given that RNASE2 and ABCs were both related to disease activities and autoantibodies production in SLE patients, it was speculated that these two factors might be connected. Then we detected peripheral RNASE2 expression by real-time PCR and ABCs proportion by flow cytometry simultaneously in another 14 SLE patients. Interestingly, a positive correlation between these two factors was truly observed (r=0.640, p=0.014) (**Figure 2F**).

RNASE2 Silencing Down-Regulated The Proportion of ABCs

To further prove the link between RNASE2 and ABCs, SLE PBMCs were cultured with RNASE2 small interfering RNA (siRNA) or non-targeting siRNA for 3 days. As showed in **Figure 3A**, there was an over 80% decrease of RNASE2 expression after silencing. Along with the decline of RNASE2, the proportion and absolute number of ABCs in PBMCs were both significantly reduced (**Figure 3B**), while the proportion and absolute number of total B cells in PBMCs showed no significant difference (**Supplementary Figure 3**). The levels of total immunoglobulin G (IgG) were also decreased after RNASE2 siRNA silence (**Figure 3C**), and the change of IgG levels was positively correlated with the range of ABCs reduction (r=0.82, p < 0.05) (**Figure 3D**), supporting that RNASE2 is involved in ABCs regulation and may consequently promotes antibody production.

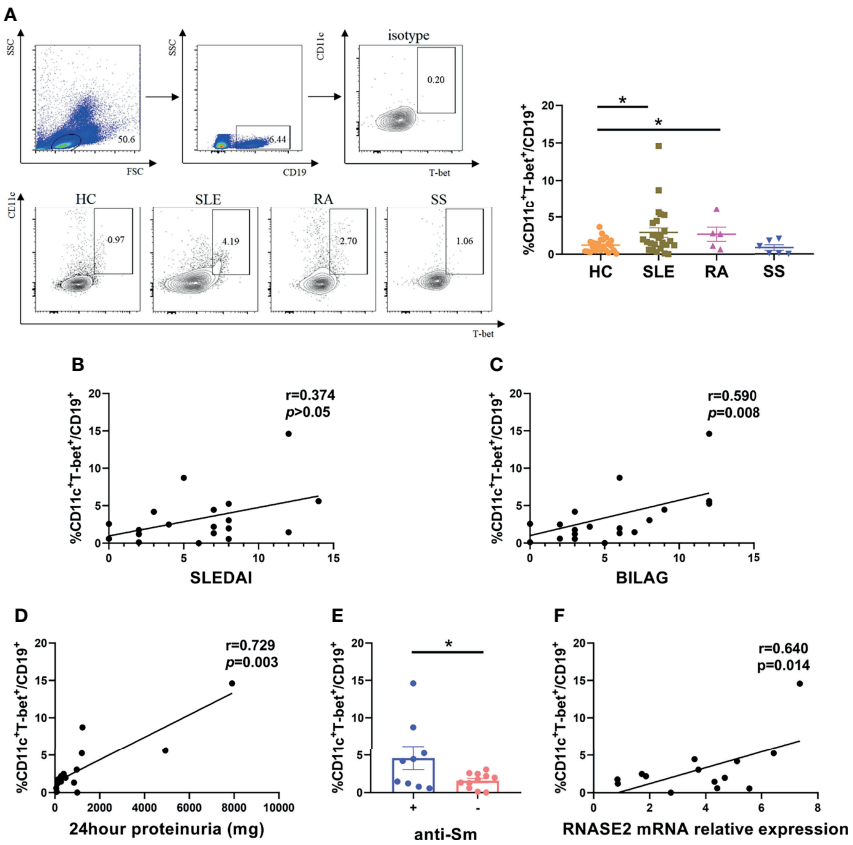

FIGURE 2 | Association of age-associated B cells subset (ABCs) with RNASE2 expression in SLE patients. **(A)** PBMCs from 24 HC, 26 SLE, 5 RA and 6 SS patients was collected and analyzed for the proportion of $CD11c^+T\text{-}bet^+$ cells in $CD19^+$ B cells by flow cytometry. **(B, C)** The percentage of $CD11c^+T\text{-}bet^+$ B cells was related to SLE disease activity score (SLEDAI and BILAG) (by Spearman correlation test). **(D)** The percentage of $CD11c^+T\text{-}bet^+$ B cells was associated with the amount of 24hour proteinuria (by Spearman correlation test). **(E)** The proportion of $CD11c^+T\text{-}bet^+$ B cells was increased in SLE patients with positive anti-Sm antibody. **(F)** The proportion of $CD11c^+T\text{-}bet^+$ B cells was closely related to RNASE2 mRNA levels in SLE patients (n=14). Data are presented as mean ± SEM, $^*p < 0.05$.

Reduced IL-10 Level After RNASE2 Silencing

Next, we asked the question how ABCs were regulated by RNASE2. As RNASE2 was broadly connected to the production of cytokine mediators (14), the levels of B cell related cytokines in cultured supernatants after RNASE2 silencing were measured by using Luminex liquid phase chip technology, including IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-21, interferon (IFN)-γ, tumor necrosis factor (TNF)-α and TNF-β. Our data showed undetectable levels of IFN-γ and TNF-β, and no difference in levels of IL-2, IL-4, IL-6, IL-12p70, IL-21 or TNF-α between RNASE2 silent group and control group (**Figures 4A–C, E–G**). As showed in **Figure 4D**, only IL-10 level was significantly decreased after RNASE2 silencing. In order to confirm this result, an independent sample validation test was applied by using ELISA and a nearly 90% decline of IL-10 was observed after RNASE2 silencing (**Figure 4H**). Consistently, we also found that plasma level of IL-10 in lupus patients were higher than those in normal subjects and positively correlated with peripheral RNASE2 expression (**Figures 4I, J**).

IL-10 Mainly Derived From Lupus Monocytes

To search for the source of IL-10 in peripheral blood of SLE patients, we sorted out $CD14^+$ monocytes, $CD19^+$ B cells, $CD4^+$ T cells and the remaining $CD14^-CD19^-CD4^-$ cells from lupus PBMCs using flow cytometry sorting technology (**Figure 5A**), and measured the mRNA expression of IL-10 in each cell subgroup by real-time PCR. As showed in **Figure 5B**, similar to that in normal subjects, IL-10 expression in monocytes was highest among the groups in SLE patients. Meanwhile, RNASE2, usually highly expressed in eosinophils from normal subjects (**Supplementary Figure 4**), was also significantly elevated in lupus monocytes (**Figure 5C**). There was a trend towards significance between RNASE2 and IL-10 mRNA expression in SLE monocytes (r= 0.59) (**Figure 5D**). To verify the effect of RNASE2 on IL-10 secretion, we isolated $CD14^+$ monocytes from lupus PBMCs by magnetic beads and cultured with RNASE2 siRNA. There was a significant decrease of RNSAE2 protein levels in SLE monocytes after siRNA treatment (**Supplementary Figure 5**). Levels of IL-10 mRNA and protein secretion in culture supernatants were both down-regulated after

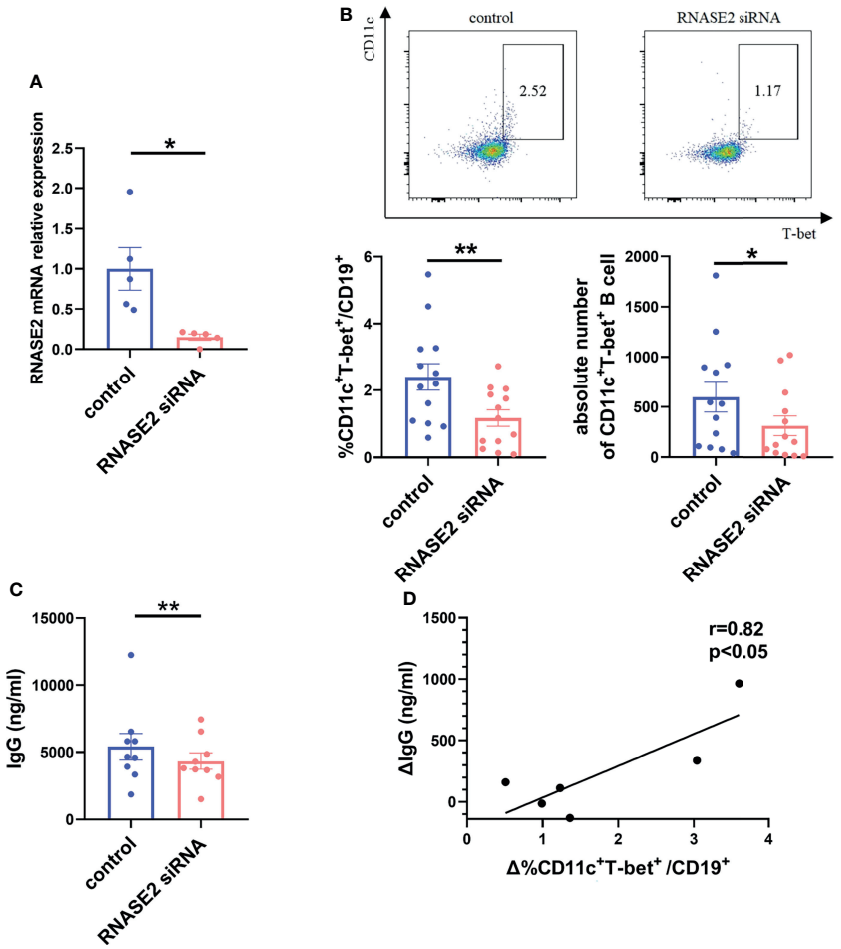

FIGURE 3 | RNASE2 participated in the regulation of ABCs. **(A)** Nearly 80% reduction in the gene expression 3 days after the treatment of RNASE2 siRNA *in vitro* (n=5). **(B)** Decreased percentage and absolute number of CD11c$^+$T-bet$^+$ B cells after RNASE2 silencing by flow cytometry (n=13). **(C)** Down-regulated expression of total immunoglobulin (Ig) G in culture supernatants from RNASE2 silencing group (n=9), as determined by enzyme-linked immunosorbent assay (ELISA). **(D)** The difference of IgG level between silence group and control group was positively correlated to the alteration of ABCs proportion (n= 6). Data are presented as mean ± SEM, *p < 0.05, **p < 0.01.

silencing of RNASE2 silencing (**Figures 5E, F**), suggesting that RNASE2 promotes IL-10 production in lupus monocytes.

RNASE2 Regulated ABCs Through the Modulation of IL-10

To explore the relationship among RNASE2, IL-10 and ABCs, lupus B cells were negatively isolated from PBMCs by magnetic beads and cultured *in vitro* with or without the presence of RNASE2 siRNA, which showed no significant change in ABC percentages after RNASE2 silencing (**Supplementary Figure 1B**). Next, lupus monocytes and B cells were isolated respectively from SLE PBMCs. The monocytes were firstly silenced by RNASE2 siRNA for 6 hours, and then co-cultured with syngeneic B cells with or without human recombinant IL-10. As showed in **Figure 6A**, RNASE2 silencing significantly down-regulated the percentages of ABCs. While adding recombinant IL-10 to the co-culture fully restored ABCs levels after monocyte RNASE2 silencing, only high doses of anti-IL-10 antibody inhibited the production of ABCs in monocyte-B cell co-cultures (**Figure 6B**), suggesting that the presence of just a small amount of IL-10 in the culture system could promote the production of ABCs. Consequently, we identified a two-way action of IL-10: when IL-10 was added into monocyte-B cell co-cultures at relatively high concentration, the proportion of ABCs was actually down-regulated (**Figure 6C**), while only under low concentration (2ng/ml) and without the presence of monocytes, IL-10 promoted the expansion of ABCs (**Figure 6D**).

DISCUSSION

Recently, ABCs have attracted much attention in the pathogenesis of SLE. These CD11c$^+$T-bet$^+$ B cells are the main source of extra-follicular autoantibody production (6) and correlated with lupus

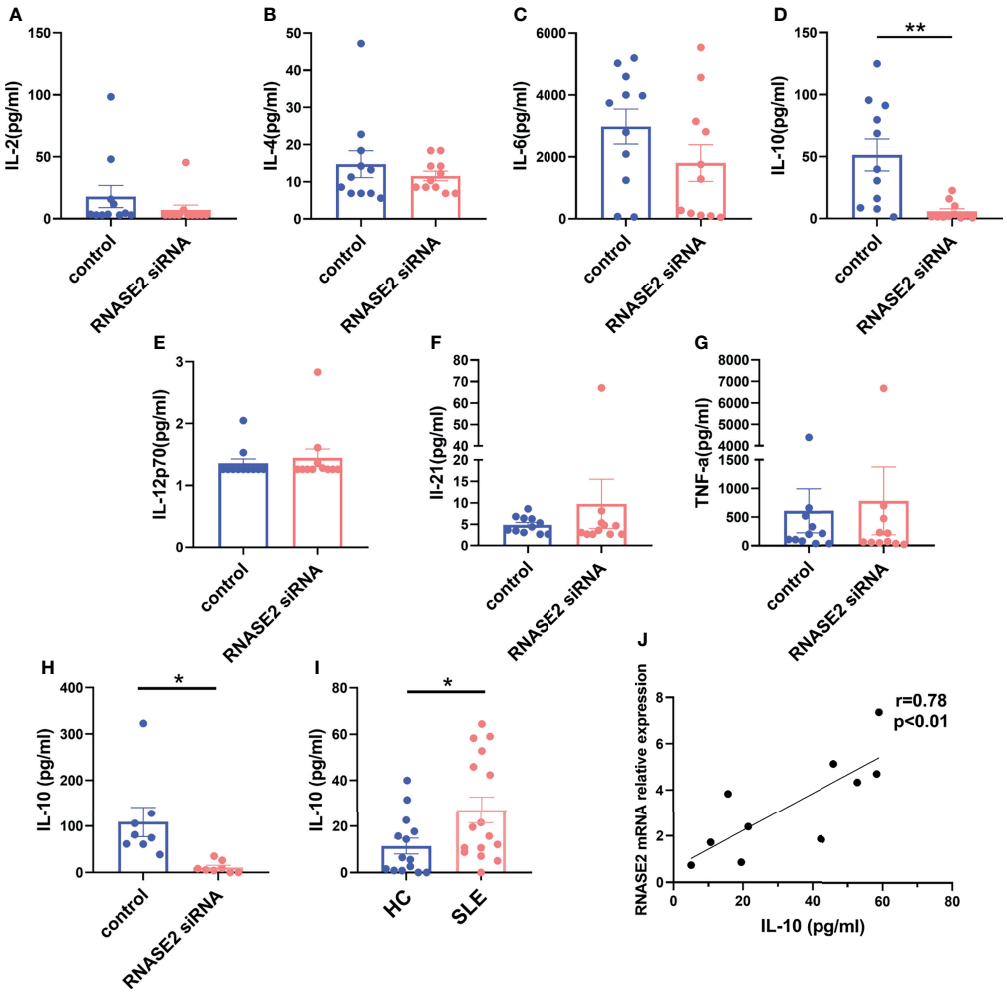

FIGURE 4 | IL-10 was a major effector of RNASE2. **(A–G)** The expression of multiple cytokines (IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-21 and TNF-α) in cultured supernatant was determined by using luminex liquid phase chip, among which only IL-10 level was significantly decreased in RNASE2 silencing group (n=11). The lower limit of detection (LLOD) for each cytokine was 2.96 pg/ml, 5.61 pg/ml, 0.72 pg/ml, 0.74 pg/ml, 1.26 pg/ml, 2.69 pg/ml and 0.98 pg/ml respectively, and levels lower than LLOD were regarded as having the minimum detection value. **(H)** Independent sample validation by ELISA confirmed the reduction of IL-10 level in cultured supernatants after RNASE2 silencing (n=8) (LLOD 0.05pg/ml). **(I)** *In vivo* study showed the level of plasma IL-10 was increased in SLE patients (n=16) compared with healthy controls (n=14). **(J)** Plasma IL-10 levels in SLE patients were closely linked to peripheral RNASE2 mRNA expression (n=10). Data are presented as mean ± SEM, *p < 0.05, **p < 0.01.

manifestations, and their depletion *in vivo* may lead to reduction of autoreactive antibodies (21). In addition, excessive $CD11c^{+}T\text{-}bet^{+}$ B cells could promote activated T cells differentiated into follicular T-helper (Tfh) cells through their potent antigen-presenting function and consequently compromise affinity-based germinal center selection, participating in antibody-affinity maturation (22). However, despite cumulative data showing that ABCs are increased in SLE, current understanding regarding their expansion and regulation is rather limited. As reported, Tfh cells or T peripheral helper cells could provide help to ABCs *via* the secretion of IL-21 (23, 24). IL-21 acts synergistically with TLR7/9 to induce naive B cells differentiation into ABCs and promote T-bet expression, while IL-4 could inhibit the up-regulation of these cells and antagonize T-bet induction (25, 26). Besides, the SWEF proteins, a newly identified risk variant for human SLE, have also been found to be involved in ABCs regulation, which is dependent on cognate interactions with Tfh cells (27).

In this study, we showed for the first time that another upregulated gene in SLE, RNASE2, participated in the expansion of ABCs in the absence of T-cell help. Although RNASE2 has been recognized as a common marker gene associated with SLE (11), this gene does not receive much attention in recent years due to its unclear biological function. Normally, RNase2 is secreted by eosinophils and has broad antiviral activity (28). However, lupus monocytes appeared to be the most prominent cell type to produce this protein (**Figure 5C**). Lupus $CD16^{+}$ monocytes are reported to have enhanced impacts on B cells to differentiate into plasma B cells with more Ig production, and less inhibition on

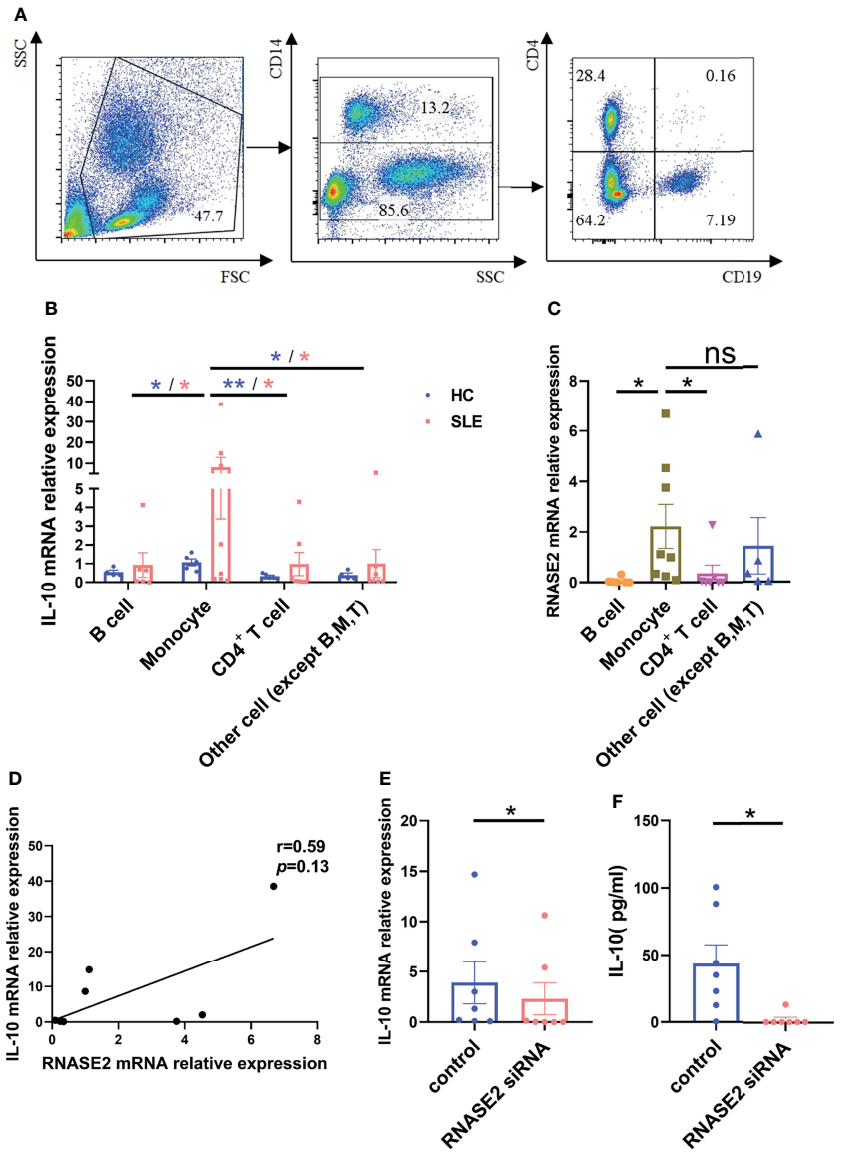

FIGURE 5 | RNASE2 promoted the secretion of IL-10 from lupus monocytes. **(A)** Flow cytometry sorting technology was used to separate lupus $CD14^+$ monocytes, $CD19^+$ B cells, $CD4^+$ T cells and the remaining $CD14^-CD19^-CD4^-$ cells. **(B)** mRNA expression of IL-10 in each cell subgroup was detected by real-time PCR, and the highest expression was seen in monocyte subgroup (n=5), especially those from SLE patients (n=8). **(C, D)** mRNA expression of RNASE2 was also prominent in monocyte subgroup from lupus patients, which was correlated with monocyte IL-10 mRNA level (by Spearman correlation test, n=8). **(E, F)** RNASE2 silencing down-regulated IL-10 mRNA expression in lupus monocytes and IL-10 protein levels in cultured supernatants (n=7). Data are presented as mean ± SEM, *p < 0.05, **p < 0.01, ns, no significant statistical difference.

regulatory B cells (29). The specific mechanism is unclear, which could only be partially attributed to the elevated surface expression of CD80, CD86 and HLA-DR on $CD16^+$ monocytes. High expression of RNASE2 in lupus monocytes might contribute to promote B cell differentiation and Ig production.

Our next question is how RNase2 affected the function of lupus monocytes so as to promote ABCs expansion. Previously, we have tested the plasma levels of RNase2 and found there were no difference between SLE patients and healthy controls (data not shown), suggesting that RNase2 is less likely to act through secretion to distant target tissues in SLE pathogenesis, although it is one of the four major secretory proteins released upon activation of eosinophils under normal circumstances (30, 31). Since RNase2 could serve as a chemoattractant of dendritic cells and promote the secretion of a lot of cytokines and chemokines (14, 32), we focused on the measurement of several major cytokines related to B cells development. Surprisingly, we found that IL-10 but not IL-12 was the most effective cytokine to restore ABC levels after RNASE2 silencing, which was tightly associated with the decline of RNASE2 either in mRNA or in protein level.

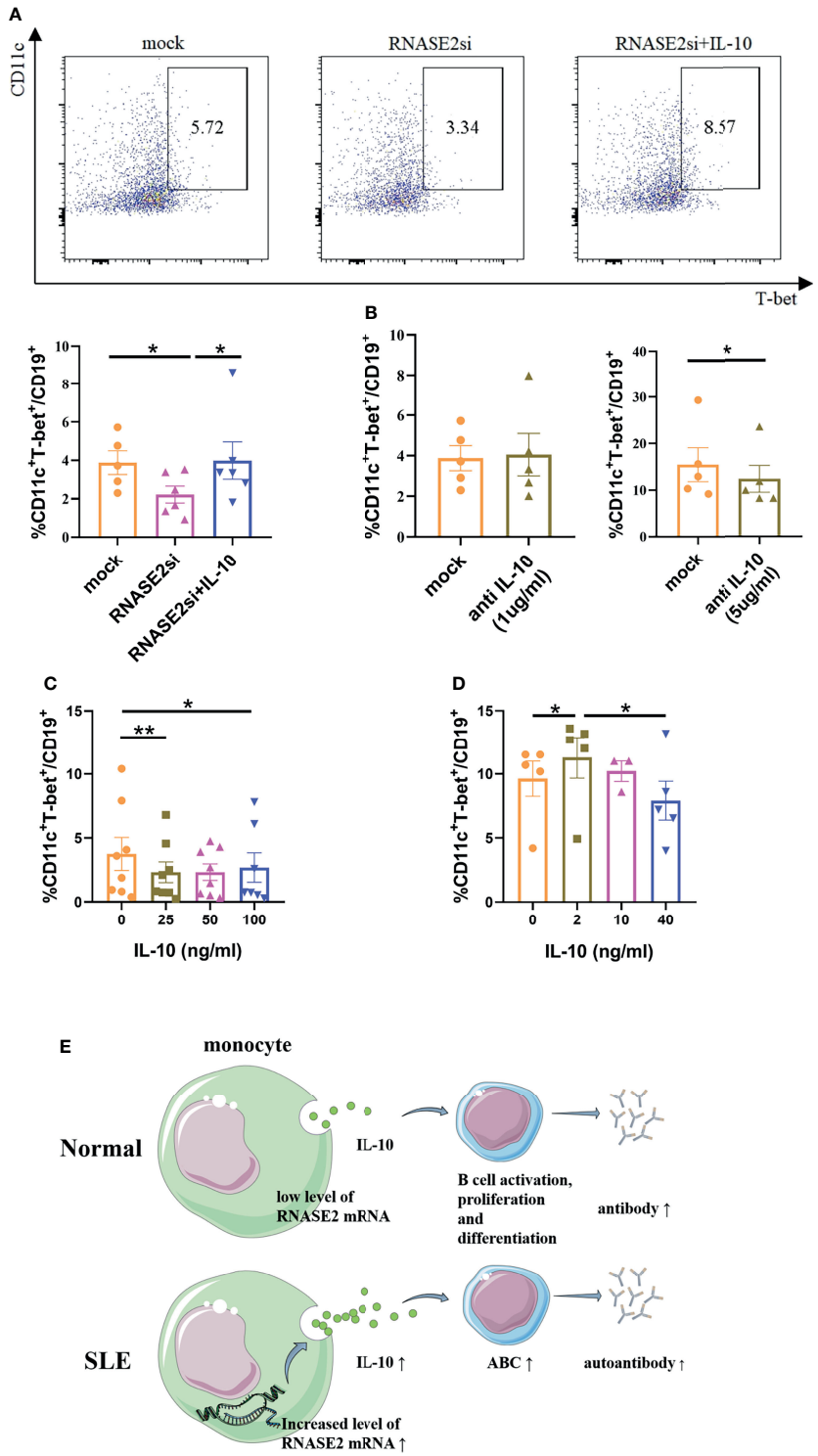

FIGURE 6 | IL-10 regulated the production of ABCs in SLE patients. **(A)** Lupus B cells were co-cultured with monocytes with the presence of either RNASE2 siRNA (RNASE2si) or RNASE2 siRNA plus 50ng/ml human recombinant IL-10 (RNASE2si+IL-10) (n=6). The proportion of $CD11c^+T\text{-}bet^+$ B cells was restored after the replenishment of IL-10 in RNASE2 silencing group. **(B)** High dose anti-IL-10 (5ug/ml) but not low dose anti-IL-10 (1ug/ml) blocked the production of $CD11c^+T\text{-}bet^+$ B cells in B cell and monocyte co-cultures (n=5). **(C)** The effect of recombinant IL-10 on the proportion of $CD11c^+T\text{-}bet^+$ B cells in B-cell plus monocyte co-cultures (n=8). **(D)** The effect of recombinant IL-10 on the proportion of $CD11c^+T\text{-}bet^+$ B cells in $CD19^+$ B cell culture (n=5). **(E)** Hypothesis schema of RNASE2 pathogenetic role involved in SLE patients (picture material was from http://smart.servier.com). Data are presented as mean ± SEM, paired t test, *$p < 0.05$, **$p < 0.01$.

Thus far, the role of IL-10 in SLE remains controversy. IL-10 can promote humoral immune responses, enhancing B cell proliferation, differentiation, and autoantibody production (33). Serum IL-10 levels have been reported to be increased in lupus patients and correlated with disease activity and anti-dsDNA antibodies (34). On the other hand, IL-10 is considered as a potent anti-inflammatory cytokine that can inhibit production of pro-inflammatory cytokines, antigen presentation, and cell proliferation (33, 35, 36). Our data support that IL-10 is generally pathogenic to promote the production of ABCs, yet it has the opposite effect at high concentrations. Also, it seems unrealistic to implement the corresponding antibodies to treat SLE patients, because there will be no efficacy until IL-10 is almost completely blocked.

It remains to be elucidated how RNASE2 regulates IL-10 production in lupus monocytes. Evidence has suggested that RNase2 is an endogenous ligand of TLR2. It appears to be a TLR2-specific as its capacity to enhance immune responses is independently of TLR1 or TLR6 (32). Recently, RNase2 has been found to act synergistically with RNase T2 to release uridine from RNA ligands, and help to process RNA into TLR8 ligands (15). Thus, through the stimulation of TLR, a lot of downstream signaling pathways, especially myeloid differentiation factor 88 (MyD88) (32) and mitogen-activated protein kinase (MAPK) (37), may be activated to promote the production of pro-inflammatory factors, including IL-10.

Based on the above data, a novel hypothesis of lupus pathogenesis has emerged: over-expression of RNASE2 in SLE patients may trigger monocytes to secrete more IL-10, consequently inducing the expansion of ABCs and leading to the production of various autoantibodies (**Figure 6E**). Therefore, RNASE2 may serve as a promising new target for the treatment of SLE.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Affiliated Drum Tower Hospital of Nanjing University Medical School. The patients/participants provided their written informed consent to participate in this study, which was waived when only residual blood was used.

AUTHOR CONTRIBUTIONS

XF designed, coordinated and supervised the study. YZ drafted the manuscript. YZ, XT, and YX carried out most of the experiments, performed data acquisition and analysis. SW, WL, LG, and XM contributed to sample collecting and data interpretation. BT and LS participated in study design and helped revise the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.752189/full#supplementary-material

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Restoration of Default Blood Monocyte-Derived Macrophage Polarization With Adalimumab But Not Etanercept in Rheumatoid Arthritis

Audrey Paoletti[1†], Bineta Ly[1†], Samuel Bitoun[1,2], Gaëtane Nocturne[1,2], Elodie Rivière[1,2], Jessica J. Manson[3], Andrea Matucci[4], Marc Pallardy[5,6], Niek De Vries[7] and Xavier Mariette[1,2]*

[1] Université Paris-Saclay, INSERM UMR1184, Center for Immunology of Viral Infections and Autoimmune Diseases, Le Kremlin Bicêtre, France, [2] Rheumatology Department, Hôpital Bicêtre, Assistance Publique – Hôpitaux de Paris, Le Kremlin Bicêtre, France, [3] Department of Rheumatology, University College Hospital, London, United Kingdom, [4] Department of Rheumatology, Università di Firenze, Firenze, Italy, [5] Université Paris-Saclay, INSERM, Inflammation Microbiome Immunosurveillance, Châtenay-Malabry, France, [6] Anti-Biopharmaceutical Immunization: Prediction and Analysis of Clinical Relevance to Minimize the Risk (ABIRISK), Châtenay-Malabry, France, [7] Amsterdam Rheumatology and Immunology Center, Academic Medical Center of the University of Amsterdam, Amsterdam, Netherlands

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***Correspondence:**
Audrey Paoletti
audrey.paoletti@inserm.fr

[†]These authors have contributed equally to this work

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Introduction: We previously reported a specific defect of rheumatoid arthritis (RA) monocyte polarization to anti-inflammatory M2-like macrophages related to increased miR-155 expression in all RA patients except those receiving adalimumab (ADA). In this longitudinal study, we examined whether different tumor necrosis factor inhibitors were able to restore monocyte polarization to M2-like macrophages and their effect on the transcriptomic signature.

Methods: M2-like polarization induced by human serum AB was studied in 7 healthy donors and 20 RA patients included in the ABIRA cohort before and 3 months after starting ADA or etanercept (ETA). The differential gene expression of M2- and M1-related transcripts was studied in macrophage-derived monocytes after differentiation.

Results: At baseline, RA monocytes showed a defect of polarization to M2-like macrophages as compared with healthy donor monocytes, which was negatively correlated with disease activity. M2-like polarization from circulating monocytes was restored only with ADA and not ETA treatment. The transcriptomic signature demonstrated downregulation of M2-related transcripts and upregulation of M1-related transcripts in active RA. In patients receiving ADA, the transcriptomic signature of M2-related transcripts was restored.

Conclusion: This longitudinal study demonstrates that ADA but not ETA is able to restore the M2-like polarization of monocytes that is defective in RA.

Keywords: rheumatoid arthritis, monocyte-derived macrophages, M2-like macrophages, Adalimumab, Etanercept

Abbreviations: RA, rheumatoid arthritis; ADA, adalimumab; ETA, etanercept; IFX, infliximab; HD, healthy donor; M1-like, classically activated macrophage phenotype; M2-like, alternatively activated macrophage phenotype; SAB, human serum AB; ABIRISK, Anti-Biopharmaceutical Immunization prediction and analysis of clinical relevance to minimize the RISK; ABIRA, Anti-Biopharmaceutical Immunization in Rheumatoid Arthritis; DAS28, Disease Activity Score in 28 joints; IPA, ingenuity pathway analysis; MTX, methotrexate; TNFi, tumor necrosis factor inhibitor.

HIGHLIGHTS

- In patients with rheumatoid arthritis, blood monocyte differentiation in anti-inflammatory macrophages is impaired and associated with disease activity.
- This *in vitro* defective blood monocyte polarization to anti-inflammatory macrophages was restored only in patients receiving adalimumab and not etanercept.
- Different types of tumor necrosis factor inhibitors do not have the same effect on monocytes/macrophages.

INTRODUCTION

Monocytes/macrophages are key players in the pathogenesis of rheumatoid arthritis (RA) (1, 2) by secreting tumor necrosis factor α (TNF-α) among other inflammatory cytokines. Synovium tissue macrophages are the most common resident immune cells in the healthy synovial membrane. In active RA with myeloid and lymphoid synovitis, the synovial membrane is leucocyte-rich, including an increased number of pro-inflammatory macrophages. Indeed, besides resident macrophages, blood monocytes can differentiate into monocyte-derived macrophages (MDMs) that may join the synovium and have different phenotypes and functions (3–5).

The "classically activated M1 macrophage phenotype" is considered to be pro-inflammatory, and the "alternatively activated M2 macrophage phenotype" is considered to be regulatory and anti-inflammatory in tissues. Actually, there is a continuum from pro-inflammatory to anti-inflammatory macrophages, with high plasticity between the different states. Classically activated macrophages contribute to RA pathogenesis by secreting pro-inflammatory cytokines and are the main producers of TNF.

We previously showed that RA patients had defective monocyte polarization toward an M2-like macrophage phenotype ($CD11b^{Lo}$–$CD71^{Lo}$; $CD206^{Lo}$; $CD163^{Lo}$ with decreased interleukin 10 [IL-10] secretion) in favor of an M1-like phenotype (inducible nitric oxide $synthase^{+}$, interferon regulatory factor 5^{+}, and increased levels of pro-inflammatory cytokines such as IL-1β–IL-6–macrophage inflammatory protein 1α) (6). This defect was specific to RA because it was not found in healthy donors (HDs) or those with other inflammatory diseases (such as Sjogren's syndrome (SS) and spondyloarthritis (SpA) patients). Moreover, we have also found this specific defect in M2-like polarization of monocytes in RA patients receiving etanercept (ETA) but not adalimumab (ADA).

The objectives of this study were to longitudinally study the roles of the different TNF inhibitors (TNFis) for modifying monocyte polarization in RA patients and to study in detail the macrophage population before and after initiation of ADA treatment.

MATERIALS AND METHODS

Patients

All RA patients fulfilled the 2010 American College of Rheumatology and European League Against Rheumatism RA criteria (7). The Disease Activity Score in 28 joints (DAS28) was used to assess the disease activity of RA patients. Because of the impact of corticosteroids on macrophages, patients receiving corticosteroid therapy ≥10 mg per day were excluded (8). Cells from two different cohorts of patients were obtained:

- Peripheral blood mononuclear cells (PBMCs) from patients in the ABIRA cohort included in the European consortium ABIRISK. This prospective cohort, set up to look for predictors of immunization to biologics, included patients with failure of methotrexate (MTX) and receiving for the first-time ADA or ETA with at least 3-month follow-up (ClinicalTrials.gov: NCT02116504). MTX resistance was defined by disease activity defined by the DAS28 (>3.2, moderate activity; >5.1, high activity) after at least 3 months of MTX. Patients included in the ABIRA cohort were used for phenotyping of monocytes and macrophages before and after anti-TNF treatment.
- Blood MDMs included for RNA-sequencing (RNA-seq) were from patients referred to the Department of Rheumatology of Hôpitaux Universitaires Paris-Sud between September 2017 and May 2019, who all gave their informed consent for use of cells for clinical research. The study was approved by the ethics committee (CPP Sud Méditerranée V2020-A00509-30).

For PBMCs and blood MDMs from HDs, our institutional review board gave their approval for the collection of blood from healthy people (centralized for all French healthy blood donors at Etablissement Francais du Sang in France) under the control of convention with the INSERM.

To compare patients included in the ABIRA cohort, HD PBMCs were frozen under the same condition. Briefly, PBMCs were frozen at 6 to 10.10^6/ml with autologous plasma–10% dimethyl sulfoxide (DMSO); then cells were placed at −80°C for 48 h and then transferred to −150°C.

Monocyte Selection, Peripheral Blood Mononuclear Cell Thawing, and Differentiation Into Macrophages

For PBMC thawing, cells were placed in a water bath at 37°C and rapidly transferred to complete Roswell Park Memorial Institute (RPMI). Cells were then washed and counted for monocyte staining and polarization to M2 macrophages, we remove samples under 1.10^6 PBMCs and viability under 80% of trypan blue. For M2 differentiation with human serum AB (SAB), fresh blood monocytes were isolated by using a pan-monocyte negative selection according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany) to achieve purity of 90% (for RNA-seq) or thawed PBMCs (from patients included in the ABIRA cohort) and then cultured at 1.10^6 cells/ml for 6 days in hydrophobic Teflon dishes (Lumox; Duthsher) in a macrophage medium (RPMI 1640 medium supplemented with 200 mM of L-glutamine, 100 U of penicillin, 100 µg of streptomycin, 10 mM of HEPES, 10 mM of sodium pyruvate, 50 µM of β-mercaptoethanol, 1% minimum essential medium vitamins, and 1% nonessential amino acids) containing 15% of heat-inactivated human serum AB as a natural source of *M-CSF*, *IL-10*, *IL-4*, and *IL-13*. After 6 days of culture, MDMs were then

harvested and suspended in 100% fetal bovine serum to avoid cell death, scratched, and washed with phosphate-buffered saline (PBS) 1×.

Flow Cytometry

For monocyte subpopulation staining, PBMCs were saturated with FcBlock and incubated for 30 min at 4°C with anti-CD2-CD19-CD56 (PerCP-cy5.5), anti-HLA-DR (FITC), anti-CD16 (APC), and anti-CD14 (Amcyan) antibodies; dead cells were excluded by using fixable live-cells (APC-cy7) (gating strategy in **Supplementary Figure 1A**). For determining macrophage phenotype after monocytes polarization by SAB, cells were harvested in 100% fetal bovine serum and scratched, washed once with phosphate-buffered saline, saturated with FcBlock, and incubated for 30 min at 4°C with anti-CD2-CD19-CD56 (FITC), anti-HLA-DR (AmCyan), anti-CD11b (PerCP-cy5.5), anti-CD71 (PE), anti-CD206 (Pe-cy7), and anti-CD163 (PB) antibodies; dead cells were excluded by using fixable live cells (APC-cy7) (gating strategy in **Supplementary Figure 1B**). The indicated antibodies and isotype-matched antibodies used were obtained from Biolegend (San Diego, Californie). Stained cells were acquired by using a BD FACSCanto II (BD Biosciences, San Jose, CA, USA) and analyzed by using FlowJo V10; gates were defined by using an isotype for each patient.

RNA-Sequencing and Bioinformatics Analysis

For RNA-seq, after blood monocyte isolation and differentiation into M2-like macrophages with SAB, mRNAs from macrophages were isolated by using the GeneJet RNA purification Kit (Life Technologies, Carlsbad, CA, USA) and QIAshredder (Qiagen, Valencia, CA, USA). The quality of the samples (RNA integrity number [RIN]) was assessed on the Agilent 2100 Bioanalyzer following the manufacturer's instructions. To construct the libraries, 250 ng of high-quality total RNA (RIN > 8) was processed by using the TruSeq Stranded mRNA kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. Briefly, after purification of poly-A-containing mRNA molecules, mRNA molecules are fragmented and reverse-transcribed with random primers. Replacement of dTTP by dUTP during the second-strand synthesis achieved strand specificity. The addition of a single A base to the cDNA was followed by ligation of Illumina adapters.

Libraries were quantified by a combination of Qubit concentration values and library average size from profiles obtained with the DNA High Sensitivity LabChip kit on the Bioanalyzer 2100. Libraries were sequenced on an Illumina Nextseq 500 instrument using paired-end 75 base-length reads (chemistry v2.5). The average number of reads per sample was 36.7 ± 4.4 million. After sequencing, a primary analysis based on AOZAN software (automated post-sequencing data-processing pipeline) was used to demultiplex and control the quality of the raw data (based on bcl2fastq v2.20.0.422 and FastQC v0.11.5). Obtained fastq files were then aligned by using the STAR algorithm (v2.7.1a). Reads were counted by using RSEM, and the statistical analyses of the read counts involved using the DESeq2 package v1.22.2 (R v3.5.3) to determine the proportion of differentially expressed genes between two conditions. The ingenuity pathway analysis (IPA) database was used to examine the potential functions and regulatory mechanisms of differentially expressed genes in RA-MTX patients versus HDs or RA-ADA versus RA-MTX patients by "upstream regulators" analysis.

Statistical Analysis

The data were analyzed by using Graph Pad Prism V9.0.1. Data were tested by the Mann–Whitney for two groups and the Kruskal–Wallis test with Dunn's multiple comparisons for multiple groups, expressed as mean ± SD or SEM with plot individual values. Correlation between variables was tested with two-tailed nonparametric Spearman's correlation, with a 95% CI. $p < 0.05$ was considered significant.

RESULTS

Patients

Twenty RA patients with failure of MTX treatment were included and analyzed before and at 3 months after TNFi initiation (n = 10 ADA; n = 10 ETA). We also included 7 HDs. Nine RA patients were recruited at Bicêtre hospital, and 4 HDs were used for transcriptomic analysis. Their characteristics are in **Table 1**.

Defective M2 Polarization of Rheumatoid Arthritis Monocytes at Baseline Correlated With Disease Activity

At baseline, we confirmed that in RA patients, macrophages differentiated from monocytes with human SAB showed significantly fewer cells with the pan-macrophage markers CD11b and CD71 ($p = 0.0005$) as compared with HDs (**Figure 1A**) and fewer cells with expression of M2-like markers such as CD206 ($p = 0.008$) and CD163 ($p = 0.0003$) (**Figures 1B, C**). We found a negative correlation (Spearman = −0.313) between disease activity, measured by the DAS28, and the number of M2-like $CD206^+$ macrophages (**Figure 1D**), Thus, patients with a higher monocyte defect of polarization in M2-like macrophages had higher disease activity.

Defective M2 Polarization of Rheumatoid Arthritis Monocytes Is Restored After 3 Months of Adalimumab But Not Etanercept

Longitudinal follow-up of these patients at 3 months after ADA treatment showed that the M2-like polarization was restored to levels similar to HDs for pan-macrophage markers and CD206 (**Figures 2A, B**). ADA was only partially effective in restoring CD163 levels in macrophages (**Figure 2C**). Conversely, ETA was unable to reverse the M2-like polarization defect (**Figures 2D–F**). Unfortunately, we are not able to demonstrate any correlation between M2-like markers at baseline (such as CD206 or CD163) and clinical or EULAR response at 1, 3, 6, or 12 months after anti-TNF treatment.

TABLE 1 | Demographic and clinical data.

A. Healthy donors (HDs) and ABIRA patients with rheumatoid arthritis (RA)

	HD (n = 7)	RA (n = 20)
Female, n (%)	29	85
Age, mean (SD)	38 (16.5)	55 (23–73)
Disease duration, years, median (range)	–	10 (0–20)
ACPA, n (%)	–	70
RF, n (%)	–	65
DAS28, median (range)	–	4.65 (2.4–5.8)
CRP, mg/ml, median (range)	–	7 (0.09–48)
Co-treatment, n (%)		
MTX		15 (75)

B. HDs and patients included in RNA-seq analysis

	HD (n = 4)	RA (n = 9)
Female, n (%)	3 (75)	8 (89)
Age, mean (SD)	38.2 (11.9)	57.1 (17.9)
Disease duration, years, median (range)	–	11.5 (3–22)
Anti-CCP, n (%)	–	78
DAS28, median (range)	–	4.24 (1.7–7.1)
Treatment groups		
• MTX	–	5
• ADA	–	3
• IFX	–	1
Co-medications in the ADA/IFX group, n (%)		
Methotrexate		3 (75)
Leflunomide		1 (25)

ACPA, anti-citrullinated protein antibodies; RF, rheumatoid factor; DAS28, Disease Activity Score in 28 joints; MTX, methotrexate; ADA, adalimumab; IFX, infliximab.

Monocyte Subpopulations and Activation Status Do Not Account for Adalimumab-Induced Rescue of the Polarization Defect

The polarization defect of monocytes to macrophages might be due to the abnormal distribution of monocyte subpopulations or their degree of activation. However, longitudinal follow-up of monocytes ($CD14^{hi}CD16^{-}$, $CD14^{hi}CD16^{hi}$ and $CD14^{dim}CD16^{high}$) did not significantly differ before and after treatment with ETA or ADA or between ETA and ADA, which suggests that the correction of the defect of M2-like polarization by ADA was not due to a change in the distribution of the monocyte subsets (**Figure 3A**). The expression of HLA-DR was also unchanged after TNFi therapy (**Figure 3B**).

RNA-Sequencing Analysis Demonstrated the Pro-Inflammatory Endotype of Rheumatoid Arthritis Monocyte-Derived Macrophages and Rescue by Adalimumab

To further analyze in detail the M2-like polarization defect and the role of ADA in reversing it, we used transcriptomic profiling

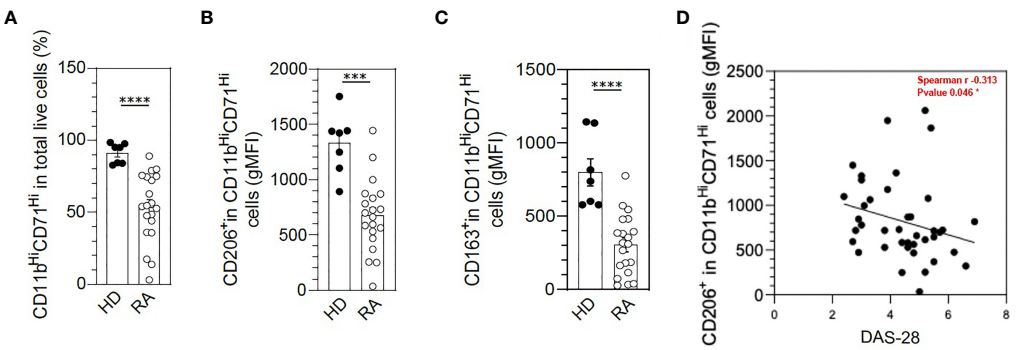

FIGURE 1 | M2-like macrophage polarization defect in rheumatoid arthritis (RA) monocytes under serum AB differentiation. The differentiation of frozen peripheral blood mononuclear cells (PBMCs) to M2-like macrophages was assessed in healthy donors (HDs) (n = 7) and RA patients (n = 20) by flow cytometry with anti-CD11b and anti-CD71 antibodies **(A)**. Specific markers of M2-like macrophage polarization were assessed: CD206 **(B)** and CD163 **(C)**. Spearman's correlation analysis of macrophage CD206 expression in RA patients (geometric mean [gMFI]) and disease activity (Disease Activity Score in 28 joints [DAS28]) **(D)**. Data are shown as symbols and mean ± SEM and were compared by Mann–Whitney t-test. ***$p < 0.001$ and ****$p < 0.0001$.

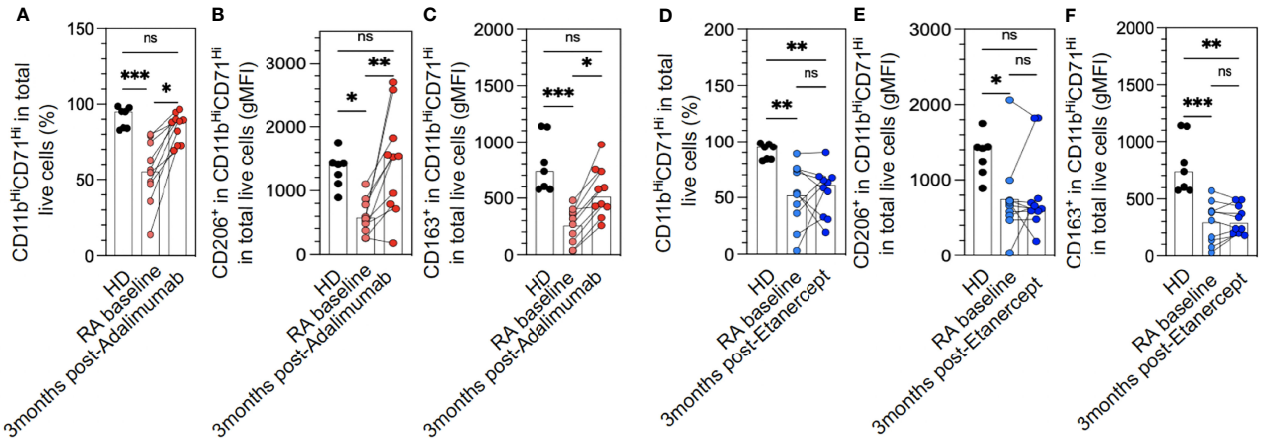

FIGURE 2 | M2-like macrophage polarization defect in rheumatoid arthritis (RA) monocytes is restored with adalimumab (ADA) but not etanercept (ETA) treatment. The differentiation of frozen peripheral blood mononuclear cells (PBMCs) to M2-like macrophages was assessed in healthy donors (HDs) (n = 7) and RA patients at baseline and divided upstream between patients who would receive ADA **(A–C)** or ETA **(D–F)** (n = 20), RA at 3 months after ADA (n = 10, red dots), and RA at 3 months after ETA (n = 10, blue dots) by flow cytometry analysis using anti-CD11b and anti-CD71 antibodies **(A, D)**. Specific markers of M2-like macrophage polarization were assessed: CD206 **(B, E)** and CD163 **(C, F)**. Results are shown as symbols, lines, and mean ± SEM and were compared by Kruskal–Wallis test with Dunn's multiple comparisons. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. ns, not significant.

of a transversal set of MDMs from 4 HDs and 9 RA patients. We separated monocytes from RA patients who received MTX alone (n = 5; 4 of them non-responders) and those who received monoclonal anti-TNF antibodies (ADA or infliximab [IFX]) (n = 4; 2 of them non-responders).

Unsupervised IPA of differentially expressed genes between MTX-treated RA patients versus HDs revealed a significant ($p < 0.05$) enrichment of 69 canonical pathways. The top 10 enriched pathways were related to ERK/MAPK, PI3K/AKT, STAT3, or granulocyte-macrophage colony-stimulating factor (GM-CSF) signaling (**Supplementary Figure 2A**).

This observation was further complemented by the enrichment of several pro-inflammatory cytokines or transcription factors involved in macrophage polarization, such as TNF-IFN-γ/α/β-IL-1β-IRF5-TP53-STAT1-ERK-p38MAPK-NFκB, and inhibition of IL-10-SOCS-1 ($p < 0.05$) (**Figure 4A**), as top upstream regulators by IPA. Interestingly, miR-155 was the most upregulated miR in MTX-treated RA patients versus HDs (**Figure 4A**). ADA/IFX-treated RA patients showed inhibition of a pro-inflammatory macrophage response and activation of an anti-inflammatory response, such as the factors SOCS-1, IL-10, CEBPβ, and c-MYC (9) (**Figure 4A**). There was no statistical

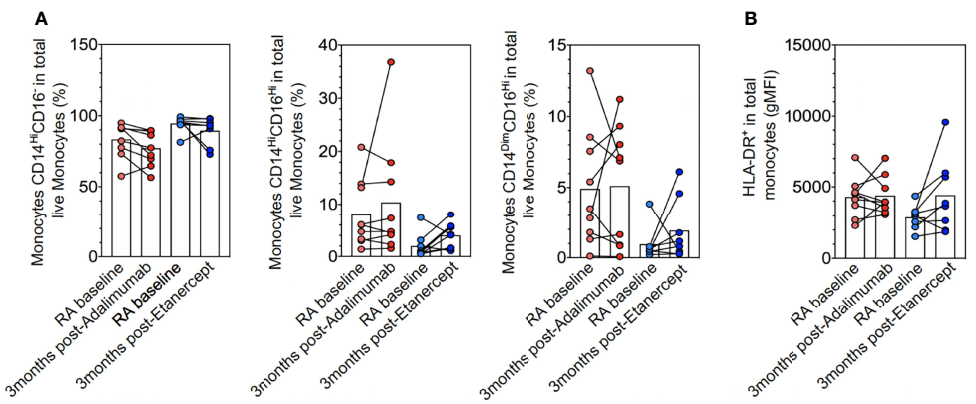

FIGURE 3 | Monocyte subpopulations and activation. *Ex vivo* CD14 and/or CD16 monocytes **(A)** and HLA-DR **(B)** expression in rheumatoid arthritis (RA) at baseline (n = 17), RA at 3 months after adalimumab (ADA) (n = 9), and RA at 3 months after ETA (n = 8) determined by flow cytometry analyses of frozen peripheral blood mononuclear cells (PBMCs with anti-HLA-DR, anti-CD14, anti-CD16, anti-CD2, anti-CD19, and anti-CD56 antibodies. Results are shown as symbols, lines, and mean ± SEM and were compared by Kruskal–Wallis test with Dunn's multiple comparisons and the two-tailed nonparametric test.

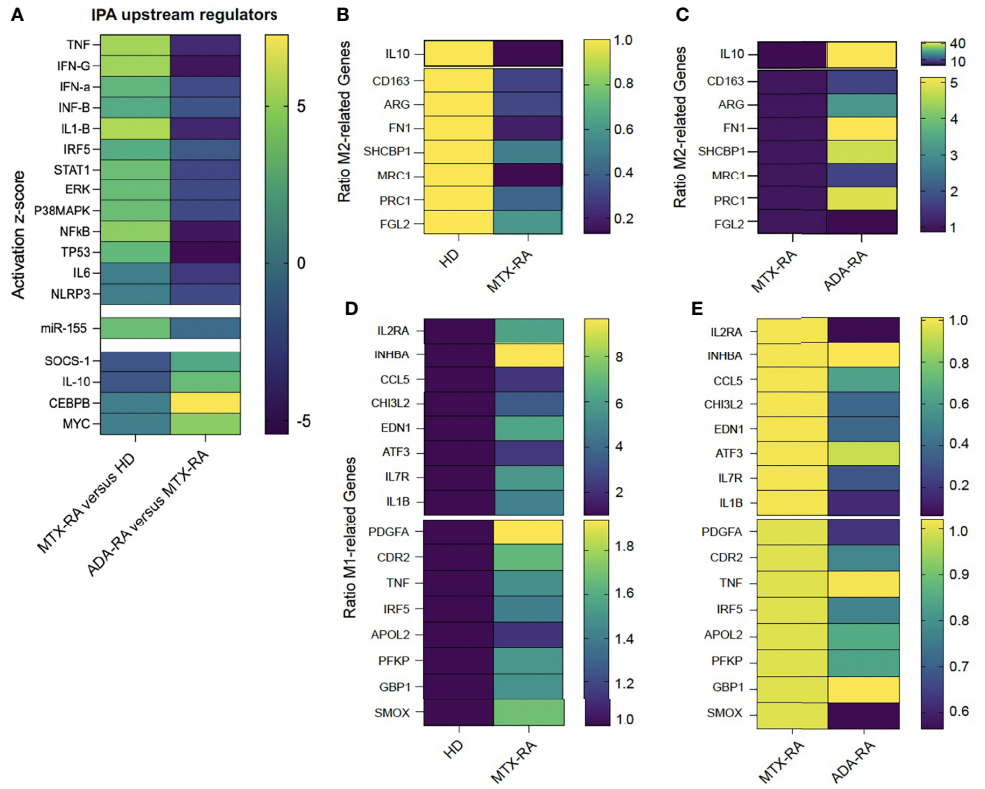

FIGURE 4 | The defect in M2-like polarization leads to an M1-like macrophage phenotype. Heatmap of a predicted upstream regulator effect on M2-like macrophages after monocyte polarization with serum AB (SAB) differentiation in rheumatoid arthritis-methotrexate (RA-MTX) patients versus healthy donors (HDs) or RA-adalimumab (RA-ADA) versus RA-MTX patients. The color of each square represents the activation z-score: activated = yellow (+5), inhibited = dark blue (–5) **(A)**. Heatmap of the ratio of the normalized count of M2-related genes in RA-MTX patients versus HDs (HD = 1 yellow) **(B)** or RA-ADA patients versus RA-MTX patients (RA = 1 dark blue) **(C)**. Ratio of normalized counts of M1-related genes in RA patients versus HDs (HD = 1 dark blue) **(D)** or RA-ADA patients versus RA patients (RA = 1 yellow) **(E)**. MRC-1 gene = CD206 receptor.

difference in miR-155 expression between MTX-treated and ADA/IFX-treated RA patients (**Figure 4A**).

Then, we performed a supervised analysis looking at genes associated with macrophage polarization. The terminology and markers to describe macrophage activation are heterogeneous; to address obstacles in describing macrophage activation, we selected the papers of Murray et al., Locati et al., and Sica et al. to choose M2 and M1 markers (10–12).

We defined a set of 8 M2- and 16 M1-associated genes (10–12). The 8 M2-related genes were downregulated in MTX-treated RA as compared with HDs (**Figure 4B**), and the 16 M1-related genes were upregulated (**Figure 4D**). Conversely, as compared with MTX-treated RA patients, in ADA/IFX-treated RA patients, 7 M2-related genes were upregulated (**Figure 4C**) and 12 M1-related genes were downregulated (**Figure 4E**).

Finally, IPA of RA patients receiving ADA/IFX and those receiving no TNFi confirmed, according to the top diseases and functions, a decrease in rheumatic disease (activation score −2.2, 89 molecules related) and inflammation of joints (activation score −2.3, 69 molecules related) (**Supplementary Figure 2B**).

DISCUSSION

In RA patients with failure of MTX who were enrolled in the ABIRA study, we confirmed specific abnormalities of monocyte polarization in anti-inflammatory macrophages. The proportion of anti-inflammatory macrophages was negatively correlated with disease activity. This defective monocyte polarization in anti-inflammatory macrophages was reversed by ADA but not ETA treatment. The transcriptomic analysis confirmed that stimulation in HD orientates to an anti-inflammatory macrophage phenotype, which leads to RA to a few anti-inflammatory macrophages and a majority of pro-inflammatory macrophages, which was reversed by ADA treatment.

The link between the proportion of blood monocyte-derived anti-inflammatory macrophages and disease activity supports the importance of this regulatory population even outside the joint. Previous studies suggested that synovial macrophages (SMs) have two major origins, namely, tissue-resident and monocyte-derived SMs, where the pro-inflammatory-M1 or anti-inflammatory-M2 concept does not reflect the phenotype of SMs (13, 14). The

complexity of synovial tissue macrophages (STMs) leads to several clusters of macrophages (13–15), and Alivernini et al. demonstrated an increase in the population of $CD163^{pos}CD206^{pos}$ STMs in patients in remission who had received TNFi and MTX. Moreover, the amount of $CD163^{pos}CD206^{pos}$ STMs is higher in sustained RA remission than in patients with flare (15).

This study has some limitations. The number of patients followed up longitudinally was low (10 with each drug), the duration of disease was heterogeneous, and age and sex differ between HDs and RA patients. The transcriptomic analysis was not performed in patients who received ETA. The choice of the M1- and M2-related genes may be the subject of discussion, we used only CD206 and not CD163 for defining M2-like macrophages and we did not provide functional experiments.

Despite these limitations, this study sheds some light on a possible different mechanism of action of the different types of TNFi. With a longitudinal 3-month follow-up, we showed a restoration of M2-like anti-inflammatory macrophages with ADA but not ETA treatment. Monoclonal anti-TNF antibodies (such as ADA/IFX) and the TNF receptor 2 (TNFR2)-immunoglobulin (ETA) can bind soluble TNF and membrane TNF (mTNF), but the stability of the interaction with mTNF is higher, with monoclonal anti-TNF antibody revealing a possible differential mechanism of action of these molecules (16). Our study shows that monocyte polarization could be used as a biomarker to help to choose the best anti-TNF in RA patients. In case of a defect of polarization of monocytes into anti-inflammatory M2-like macrophages, ADA could be preferred over ETA. In addition, the assessment of polarization could be used as a biomarker for predicting and following the efficacy of anti-GM-CSF treatment, a new and very promising drug in RA. Indeed, anti-GM-GSF is able to inhibit the differentiation of monocytes into pro-inflammatory M1-like macrophages and thus can reverse the defect of polarization into anti-inflammatory M2-like macrophages.

Thus, if TNFi in RA acts mainly by inhibiting soluble TNF and if this action is the same between both types of drugs, there might be a supplementary action of monoclonal anti-TNF antibody by binding mTNF, which is mainly expressed by monocytes/macrophages. Some examples of a differential action of TNFi have been published regarding the activation of T regulatory cells specific to the monoclonal anti-TNF antibody *via* an increase in mTNF level and direct stimulation of T regulatory cells *via* TNFR2 (17). Recently, Diallo et al. demonstrated in mice transgenic for TFNR1−/−, TNFR2−/−, and tmTNFKI/KI (3TG mice), with canonical TNF signaling abolished and soluble TNF not secreted by monocytes/macrophages, that M1 macrophages polarized from monocytes of bone marrow and treated by an anti-murine-TNF antibody (MP6-XT22) or ETA inhibited the expression of pro-inflammatory cytokines and inducible nitric oxide synthase mainly by upregulating arginase 1 (18).

The mechanism of the restoration of M2-like macrophage differentiation by monoclonal anti-TNF antibody remains to be explored. Our transcriptomic study clearly showed that monocytes could not differentiate from anti-inflammatory macrophages. The link between the binding to mTNF and this defect is still unknown. In our previous study, we found that miR-155 was overexpressed in RA monocytes and M2 macrophages except in ADA-treated patients and could lead to this defect when miR-155 was introduced in healthy monocytes. In this study, we confirm that miR-155 is increased in M2-like macrophages from MTX-treated RA but also in M2-like macrophages from ADA/IFX-treated patients.

In conclusion, our study demonstrated that ADA but not ETA could restore the M2-like polarization of monocytes that is defective in RA. This is another example of the differential action of the two types of TNFi. The pathway involved in this restoration should be further studied to identify novel therapeutic targets.

DATA AVAILABILITY STATEMENT

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (19) and are accessible through GEO Series accession number GSE196743 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE196743).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by CPP SudMéditerranée V2020-A00509-30 for patients included in the RNA-Seq Analysis and ClinicalTrials.gov:NCT02116504 for patients included in the ABIRA cohort. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AP and BL performed all experiments. AP, SB, GN, and XM contributed to the conception and design of the study and wrote the manuscript. NDV organized the ABIRA sample storage. All authors contributed to the manuscript revision and read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.832117/full#supplementary-material

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Abnormal Changes of Monocyte Subsets in Patients With Sjögren's Syndrome

Yan He [1,2†], Rongjuan Chen [1,2†], Mengqin Zhang [1,2], Bin Wang [1,2], Zhangdi Liao [1,2], Guixiu Shi [1,2] and Yan Li [1,2*]*

[1] Department of Rheumatology and Clinical Immunology, The First Affiliated Hospital of Xiamen University, School of Medicine, Xiamen University, Xiamen, China, [2] Xiamen Key Laboratory of Rheumatology and Clinical Immunology, Xiamen Science and Technology Bureau, Xiamen, China

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***Correspondence:**
Guixiu Shi
gshi@xmu.edu.cn
Yan Li
Liy010203@163.com

[†]These authors have contributed equally to this work

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Background: Recent studies have proven the existence of distinct monocyte subsets, which play a significant role in the development of some rheumatic diseases such as systemic lupus erythematosus (SLE). This study was performed to define the changes of monocyte subsets in patients with Sjögren's Syndrome (SjS).

Methods: Single cell RNA-sequencing (scRNA-seq) data of monocytes from SjS patients and controls were analyzed. The transcriptomic changes in monocyte subsets between SjS and controls were identified and potential key functional pathways involved in SjS development were also explored.

Results: A total of 11 monocyte subsets were identified in the scRNA-seq analyses of monocytes. A new monocyte subset characterized by higher expression of VNN2 (GPI-80) and S100A12 (Monocyte cluster 3) was identified, and it was increased in SjS patients. Compared with controls, almost all monocyte subsets from SjS patients had increased expression of TNFSF10 (TRAIL). Moreover, interferon (IFN)-related and neutrophil activation-associated pathways were main up-regulated pathways in the monocytes of SjS patients.

Conclusion: This study uncovered the abnormal changes in monocyte subsets and their transcriptomic changes in SjS patients, and identified TNFSF10 $^{high/+}$ monocytes as a potential key player in SjS pathogenesis and a promising target for SjS treatment.

Keywords: Sjögren's syndrome, monocyte subsets, single cell RNA-sequencing, pathogenesis, transcriptomic analyses

INTRODUCTION

SjS is a complex rheumatic disease characterized by the infiltration of immune cells into exocrine glands such as salivary glands, and effective targeted therapies for SjS are still lacking (1, 2). Current studies suggest that some factors such as disease susceptibility genes, immune abnormalities, and viral infections are synergistically involved in its pathogenesis of SjS (3–5). Among those factors, abnormal immune factors such as B cell hyperactivity have been considered as key players in SjS pathogenesis and potential targets for SjS treatment (6, 7). Nevertheless, the immune mechanisms involved in SjS pathogenesis and progression have not been fully clarified. To reveal potential targets of immunotherapy, it is necessary to further study the immune cell subsets that play a critical pathogenic role in SjS.

Mononuclear phagocytes (MNPs) are the most common innate immune cells with key roles in both immunity and autoimmunity (8–11). MNPs in blood are mainly composed of monocytes and dendritic cells (DCs), both of which have heterogenerous subsets with distinct phenotypes (12, 13). Recent studies using scRNA-seq have demonstrated the existence of distinct monocyte subsets and they have crucial roles in the development of some rheumatic diseases such as SLE (14, 15). For instance, monocytes can participate in the pathogenesis of SLE by immune mechanisms such as secreting pro-inflammatory cytokines and assisting in the activation of B cells and T cells (16–19). In recent years, the roles of monocytes in the pathogenesis of SjS have also gained increased attentions, and some studies have proved possible key roles of monocytes in the development and progression of SjS (20–24). However, the mechanisms of monocytes in SjS have not been fully clarified, and further research is required. At present, there is a lack of relevant research exploring the changes of monocyte subsets in SjS patients *via* scRNA-seq. This study aimed to analyze the abnormal changes of monocyte subsets in peripheral blood of SjS patients by scRNA-seq data, and further explore the key transcriptomic changes in monocytes of SjS patients.

MATERIALS AND METHODS

Transcriptomic Data of Monocytes of SjS Patients

scRNA-seq data of monocytes from SjS patients and controls in GSE157278 from Gene Expression Omnibus (GEO) were used in our study. In GSE157278, peripheral blood mononuclear cells (PBMCs) from 5 SjS patients and 5 controls were analyzed by scRNA-seq, but this study did not analyze the abnormal changes of monocyte subsets in peripheral blood of SjS patients by scRNA-seq analyses. In addition, a sample with low quality of sequencing was further excluded. Therefore, we analyzed scRNA-seq data of monocytes from 5 SjS patients and 4 controls. This study was conducted in accordance with the Declaration of Helsinski and was approved by the Ethics Committee of our hospital.

To further explore the transcriptomic changes in monocytes of SjS patients, we further analyzed bulk RNA-sequencing (RNA-seq) data of monocytes of SjS patients in GSE173670. In GSE173670, RNA-sequencing of $CD14^+$ monocytes from SjS patients and controls was carried out. We analyzed the transcriptomic changes in monocytes of 12 SjS patients and 11 healthy controls.

scRNA-Seq Analyses

scRNA-seq analyses were performing using Seurat (Version 3.0) and SingleR (25, 26). Quality control was performed mainly by the amount of feature genes and the percentage of mitochondrial genes expression. To characterize the subsets of monocytes precisely, scRNA-seq data with high quality were analyzed. Cells with detected genes above 1000 and the percentage of mitochondrial genes less than 10% were regarded as cells with high quality. Cells were omitted if they were more than 10% in the percentage of mitochondrial genes expression. Monocytes in each sample were identified by SingleR and dentritic cells were filtered (25), in which up to 15 principal components (PCs) were used in the clustering of cells. Gene counts were normalized with SCTransform function of Seurat. Intergraded data from 5 SjS patients and 4 controls were clustered with 9 PCs in combination with the dimensional reduction method of uniform manifold approximation and projection (UMAP) or t-Distributed Stochastic Neighbor embedding (t-SNE). Cell type annotation was performed with SingleR and those cells annotated to be monocytes were extracted for subsequent analyses. Feature genes of monocyte subsets were calculated through the differential expression analyses in Seurat.

Differential Expression Analyses

In the analyses of RNA-seq transcriptome datasets, gene expression analyses with raw count were first used if available, and differential expression analyses were performed with DESeq2 (27). For RNA-seq transcriptome datasets in other data forms such as FPKM (Fragments Per Kilobase Million) or TPM (Transcripts Per Million), differential expression analyses were performed with limma package (28). In the differential gene expression analyses above, outcome lists of differentially expressed genes (DEGs) were obtained for subsequent analyses. Those genes with the log2 value of fold changes (log2FC) no less than 1 and adjusted P values less than 0.05 were deemed to be DEGs. In scRNA-seq analyses, DEGs of monocyte subsets between SjS patients and controls were calculated through the differential expression analyses in Seurat.

Enrichment Analyses of DEGs

Functional annotation of the DEGs was performed with clusterProfiler (29), and gene sets of gene ontology (GO) terms and hallmark gene sets were mainly analyzed in the functional enrichment analyses. Genes sets with an adjusted P values less than 0.05 were considered as significantly enriched pathways.

Expression of Key Genes in SjS Patients

The aberrant expression of potential key genes in the monocytes of patients was validated with the transcriptomic data from 12

SjS patients and 11 healthy controls in GSE173670. The expression levels of potential key genes were extracted, and difference between SjS patients and controls was then analyzed.

Statistical Analyses

Results were shown as mean or median with 95% confidence intervals (95%CI). The difference in the expression levels of potential key genes between SjS cases and controls was analyzed with the unpaired t test. R software (Version 3.6.1) and GraphPad Prism (Version 8) were used in data analyses and $P<0.05$ suggested statistically significant difference. An online software (http://www.openepi.com/) was used in the power calculation. As the expression values in the transcriptomic data of GSE173670 had been standardized and inter-sample variability was small, the pre-defined standard deviation was 0.85. For a gene with a difference of 1.0 in the expression level, a sufficient power over 80% needed a combined set of 12 cases and 12 controls. In the *post-hoc* power analyses, the power of detecting a statistical significant difference in TNFSF10 (TRAIL) expression level between cases and controls was 95.5%.

RESULTS

scRNA-Seq Analyses Revealed Changes in Monocyte Subsets Among SjS Patients

In the scRNA-seq analyses of monocytes of SjS patients, a total of 11 monocyte subsets were identified (Cluster 0 to Cluster 10; shown as C0 to C10 in **Figure 1**). A new monocyte subset characterized by higher expression of VNN2 (GPI-80) and S100A12 (Monocyte cluster 3, C3) was identified, and it was increased in SjS patients (**Figures 1B, C**). However, owing to the limited samples in current study, the feature genes of monocyte subsets were not highly specific, and were also expressed in other subsets (**Figure 1D**). The changes in monocyte subsets among SjS patients still need to be explored by scRNA-seq analyses of larger number of samples.

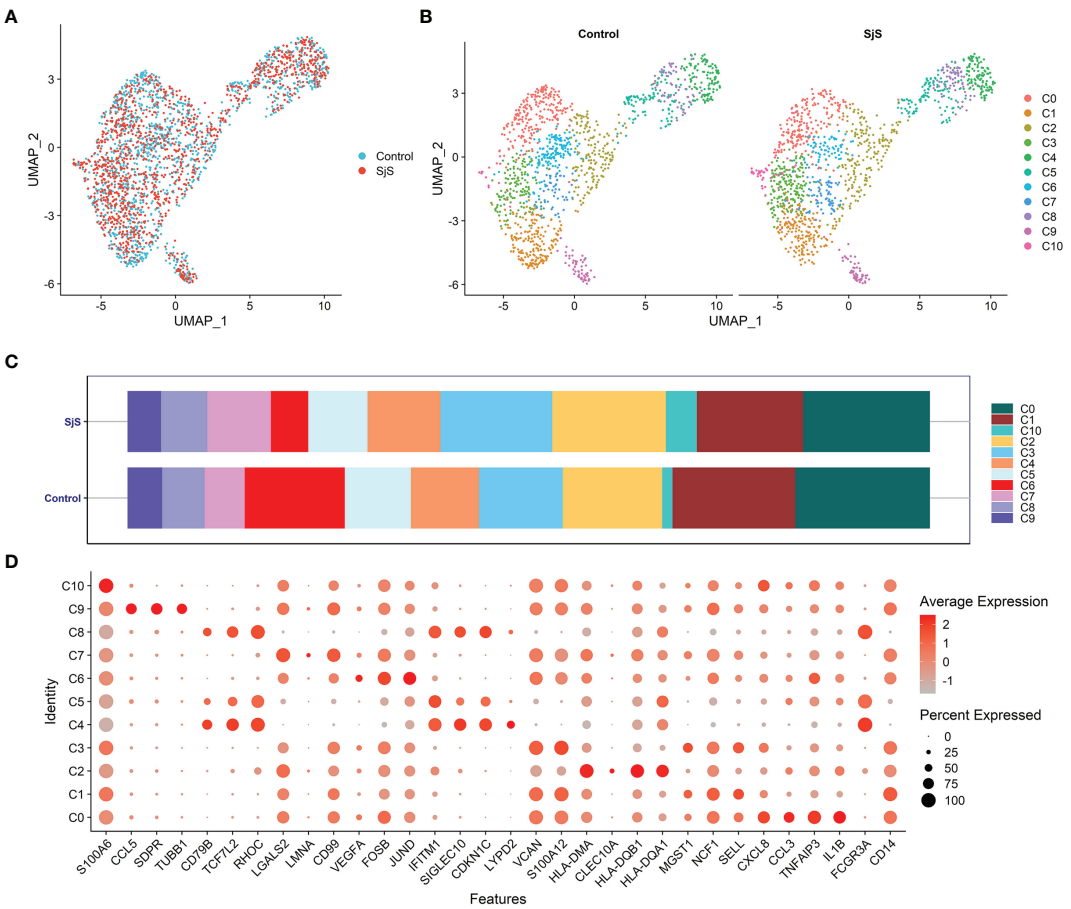

FIGURE 1 | Identification of monocyte subsets among SjS patients *via* scRNA-seq transcriptome analyses. **(A)**, Visualization of the distribution of monocytes between SjS patients and controls *via* the UMAP dimension reduction method. **(B)**, Visualization of clusters of monocytes between SjS patients and controls *via* the UMAP dimension reduction method. **(C)**, Comparison of the percentages of monocyte clusters between SjS patients and controls. **(D)**, Dot plot shows the expression percentages and the expression levels of feature genes of different clusters of monocytes.

Transcriptomic Changes in Monocytes of SjS Patients

Through scRNA-seq transcriptome analyses, a number of significant DEGs in monocyte subsets of SjS patients were identified, such as TMEM176B, TMEM176A, HLA-DRB5, FOS, TXNIP, ARPC1B, GRN, FGL2, SAMHD1, CEBPD, CTSZ, HLA-DQB1, SNX17, TNFSF10, WASF2, ATP5A1, ZFP36L2 and CORO1A (**Figures 2A, B**). Some of those significant DEGs above such as HLA-DRB5 and TNFSF10 have been proved to be key players in the pathogeneses of many autoimmune or rheumatic diseases. Enrichment analyses of those significant DEGs identified neutrophil activation-associated pathways and IFN-related pathways as the main up-regulated pathways in the monocytes of SjS patients, (**Figures 2C, D**) suggesting that those pathways had the vital roles in SjS pathogenesis.

Bulk transcriptome analyses of monocytes identified a number of significant genes aberrantly expressed in the monocytes of SjS patients such as TRIM22, MX2, MS4A4A, IFI44, IFIT2, STAT2, SAMD9L, STAT1, EPSTI1, IFI44L, SIGLEC1, TNFSF10, CX3CR1 and ISG15 (**Figure 3A**). Enrichment analyses of those significant DEGs suggested that

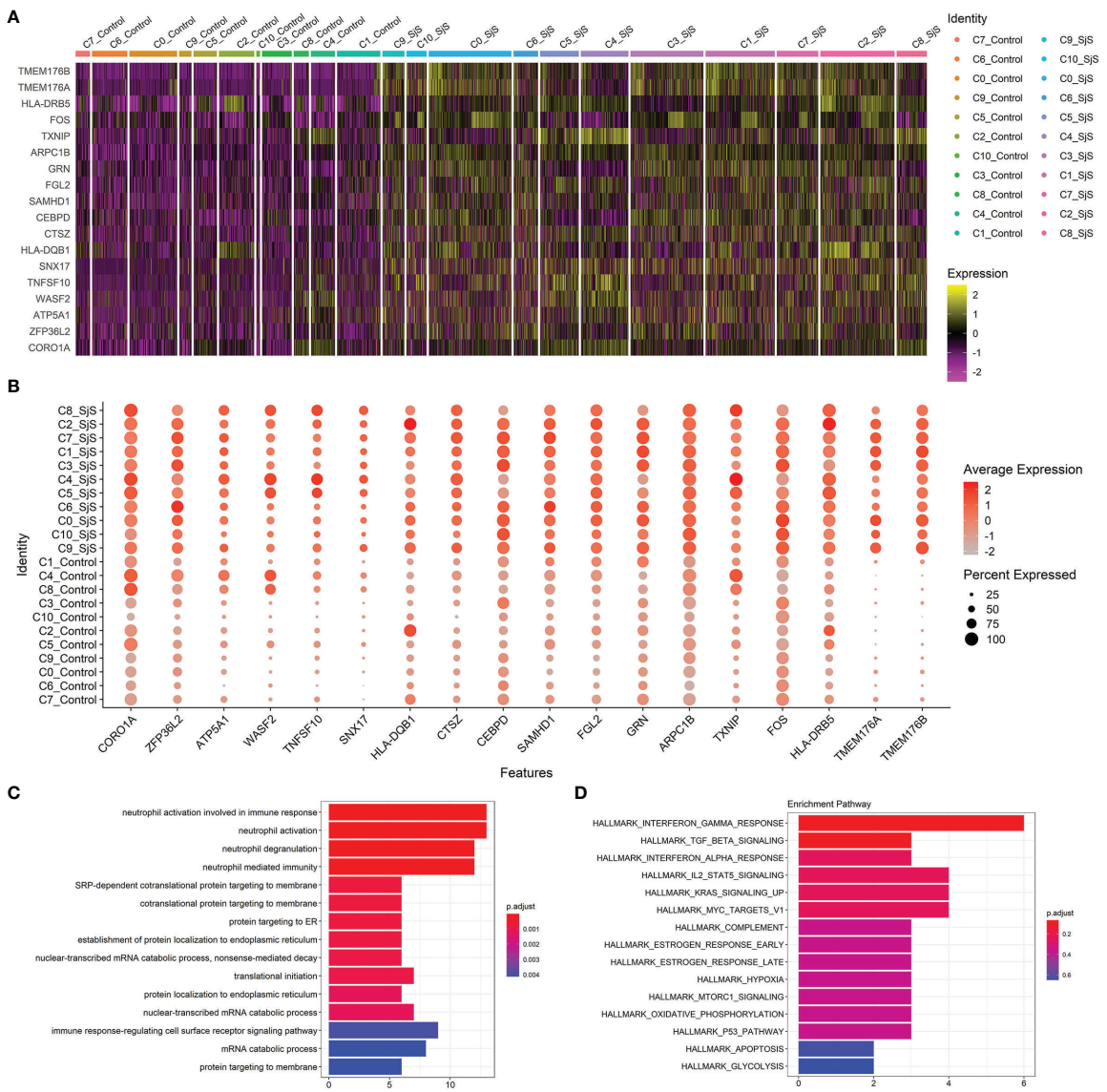

FIGURE 2 | Identification of transcriptomic changes in monocyte subsets of SjS patients *via* scRNA-seq transcriptome analyses. **(A)**, Heatmap shows the expression changes of key genes in those monocyte subsets between SjS patients and controls. **(B)**, Dot plot shows the expression percentages and the expression levels of key DEGs of monocyte subsets between SjS patients and controls. **(C)**, Main enriched GO pathways of those significant DEGs identified in scRNA-seq transcriptome analyses *via* clusterProfiler. **(D)**, Main enriched Hallmark pathways of those significant DEGs identified in scRNA-seq transcriptome analyses *via* clusterProfiler.

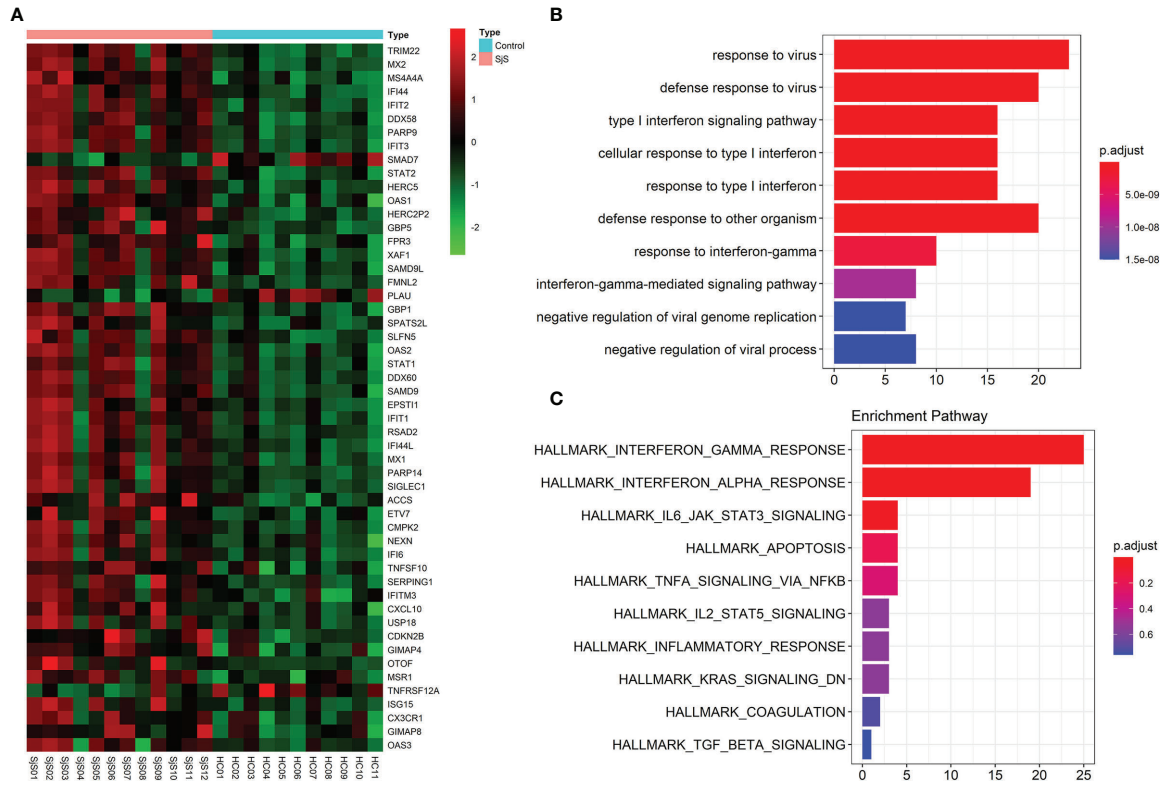

FIGURE 3 | Bulk transcriptome analyses of monocytes identify key genes and functional pathways involved in SjS. **(A)**, Top DEGs in the bulk transcriptome analyses of monocytes from SjS patients. **(B)**, Main enriched GO pathways of those significant DEGs *via* clusterProfiler. **(C)**, Main enriched Hallmark pathways of those significant DEGs *via* clusterProfiler.

those genes were enriched in the pathways such as virus infection-associated and IFN-related pathways (**Figures 3B, C**), indicating that those pathways played a key role in the pathogenesis of SjS.

Increased Expression of TNFSF10 (TRAIL) in the Monocytes of SjS Patients

Among those significant DEGs, the increased expression of TNFSF10 (TRAIL) in the monocytes of SjS patients was identified by both scRNA-seq transcriptome analyses (**Figure 2B**) and bulk transcriptome analyses of monocytes (**Figure 3A**). As shown in **Figures 4A, B**, the increased expression of TNFSF10 (TRAIL) was found in most monocyte subsets of SjS patients. In addition, validation study also confirmed the increased expression of TNFSF10 (TRAIL) in monocytes of SjS patients (**Figure 4C**). The outcomes above suggested TNFSF10 $^{high/+}$ monocytes as a potential key player in SjS pathogenesis and a promising target for SjS treatment.

DISCUSSION

The roles and underlying mechanisms of monocytes in SjS development have not been fully clarified. This study analyzed the abnormal changes of monocyte subsets in SjS patients by scRNA-seq data, and further explored the key transcriptomic changes of monocytes in SjS patients. We found a new monocyte subset characterized by higher expressions of VNN2 (GPI-80) and S100A12 (Monocyte cluster 3), which was increased in SjS patients. Moreover, virus infection-associated pathways, IFN-related pathways and neutrophil activation-associated pathways were the major up-regulated pathways in the monocytes of SjS patients. Finally, compared with controls, almost all monocyte subsets from SjS patients had increased expression of TNFSF10 (TRAIL). Therefore, this study uncovered the abnormal changes in monocyte subsets and their transcriptomic changes in SjS patients, and identified TNFSF10 $^{high/+}$ monocytes as a potential key player in SjS pathogenesis and a promising target for SjS treatment.

Mononuclear phagocytes including monocytes are the main antigen presenting cells (APCs) and can initiate protective immune processes against pathogens, but they can also initiate autoimmune processes in autoimmune diseases (30–32). Recent studies have proven the existence of distinct monocyte subsets and they have critical roles in the development of some rheumatic diseases such as SLE and rheumatoid arthritis (RA), and targeting monocytes is a potential treatment for those diseases (17, 33). There are also some studies focusing on the

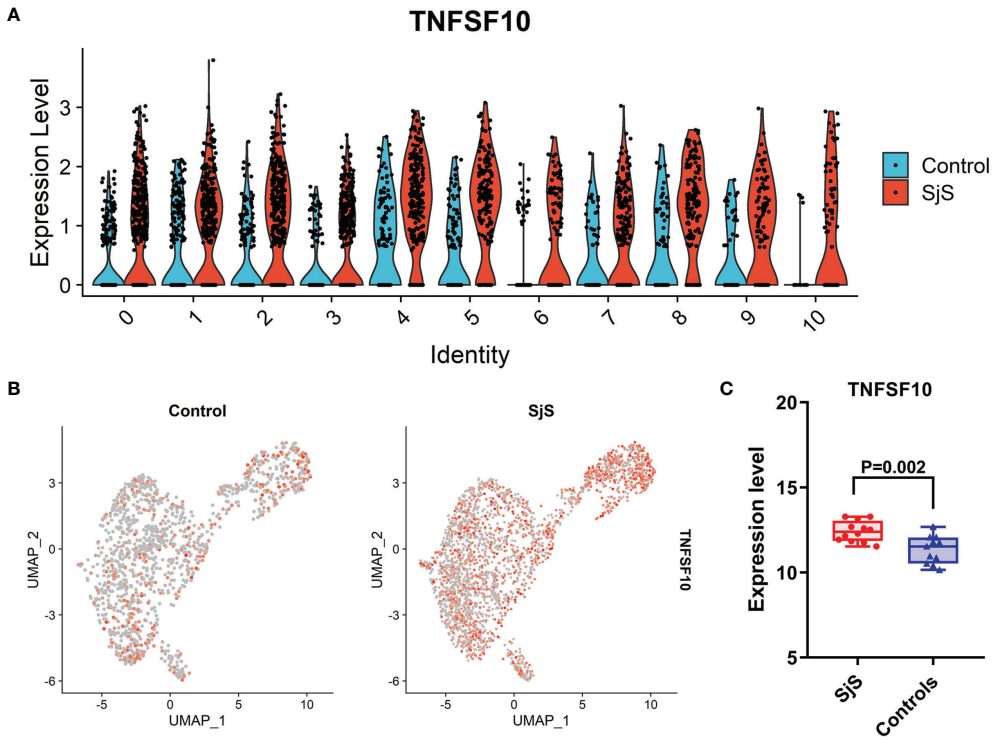

FIGURE 4 | Increased expression of TNFSF10 (TRAIL) in monocytes of SjS patients. **(A)**, Violin plot shows the increased expression of TNFSF10 (TRAIL) in most monocyte sunsets of SjS patients in the scRNA-seq transcriptome analyses. **(B)**, Feature plot shows the increased expression of TNFSF10 (TRAIL) in monocytes of SjS patients in the scRNA-seq transcriptome analyses. **(C)**, Validation study confirms the increased expression of TNFSF10 (TRAIL) in monocytes of SjS patients.).

roles of monocytes in SjS, and some have shown that aberrant changes in immunophenotypes, intracellular functional pathways and epigenetics exist in SjS patients (20–24). Those outcomes suggest the key roles of monocytes in the development and progression of SjS, though the underlying mechanisms are largely elusive.

Monocytes have several subsets with functionally distinct phenotypes, and peripheral monocytes have been calcified as proinflammatory or classic monocytes ($CD14^{++}CD16^{-}$), intermediate monocytes ($CD14^{++}CD16^{+}$) and nonclassic monocytes ($CD14^{+}CD16^{++}$) (34). Though many studies have explored the roles of monocytes in human diseases, findings are inconsistent in both the immunophenotypes of monocyte subsets and their functions. The development of scRNA-seq has provided new opportunities in uncovering monocyte subsets and defining their disturbances in those diseases (14, 35–37). In the present study, we tried to identify changes in monocyte subsets and transcriptome in SjS patients *via* scRNA-seq analyses. A total of 11 monocyte subsets were identified in the scRNA-seq analyses of monocytes, and a new monocyte subset characterized by higher expressions of VNN2 (GPI-80) and S100A12 (Monocyte cluster 3) was found to increase in SjS patients. However, owing to the limited samples in current study and the minimal heterogeneity among those mononuclear phagocyte subsets, the features genes of those monocyte subsets identified in our study were not highly specific. scRNA-seq transcriptome analyses with limited number of cells or samples could undoubtedly result in high difficulty in defining unique subpopulations with specific features genes. Therefore, the changes in monocyte subsets among SjS patients still need to be explored by further scRNA-seq analyses of larger number of samples. These studies may provide new perspectives in the landscape of mononuclear phagocytes and uncover the potential key pathogenic subset in SjS.

This study suggested that IFN-related signaling and virus infection-associated pathways were key up-regulated pathways in the monocytes of SjS patients and they were involved in SjS pathogenesis. Currently, the role of IFN-α pathway in SjS pathogenesis has long been clearly defined, and therapies targeting IFN-α may be a candidate treatment strategy for SjS (38–40). Besides, there are some published literatures which could confirm the up-regulation of IFN-related signaling in the monocytes of SjS patients. A study by Brkic et al. reported that type I IFN inducible genes such as IFI44L, IFI44, IFIT3, LY6E and MX1 were systematically up-regulated in monocytes of SjS patients and were associated with high disease activity (41). Wildenberg et al. also reported that there was an upregulation of IFN-related genes such as IFI27, IFITM1, IFIT4, and IFI44 in monocytes of SiS patients (42). Sialic acid binding Ig like lectin 1 (Siglec-1), a biomarker of the activation of type I IFN pathway,

was highly expressed in monocytes of SjS patients and was positively correlated with the EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI) score (43). Pertovaara et al. found increased cytokine-induced STAT1 activation in monocytes of SjS patients *via* flow cytometry (44). Data from another 2 studies also supported the up-regulation of molecules related to the activation of IFN-related signaling in the monocytes of SjS patients (45, 46). Therefore, there is good evidence supporting the up-regulation of IFN-related signaling in the monocytes of SjS patients.

Virus infection such as Epstein-Barr virus (EBV) infection has long been studied as an important environmental risk factor of SjS, but definite conclusion on its pathogenic role in SjS is still lacking (47–50). The findings from this study support virus infection as an important player in SjS pathogenesis. However, the molecular mechanisms underlying the pathogenic roles of virus infection in SjS are still not clear, and need to be elucidated in future studies. Apart from IFN-related pathways and virus infection-associated pathways, neutrophil activation-associated pathways were also identified to be up-regulated pathways in the monocytes of SjS patients. There is accumulating evidence implicating those neutrophils as key players in the pathogeneses of autoimmune or rheumatic diseases (51–53). The hyperactivation of neutrophils have been implicated in the pathogeneses of rheumatic diseases such as systemic lupus erythematosus (SLE) (54–56). An early research reported that neutrophil adhesion was enhanced in SjS patients, which indicated an increased activation of neutrophils in SjS patients (57). The up-regulation of neutrophil activation-associated pathways in the monocytes of SjS patients suggests that a possible role of monocytes-neutrophils cross-talk in the pathogenesis of SjS. Previous studies have revealed that monocytes can mediate neutrophil activation *via* multiple distinct mechanisms and is involved in diseases such as SLE (58–60). However, studies focusing on the roles of neutrophil activation or monocytes-neutrophils cross-talk in SjS are still limited, and further studies are needed.

Tumor Necrosis Factor-Related Apoptosis Inducing Ligand (TRAIL/TNFSF10) is a key cytokine of the TNF superfamily, and has significant roles in regulating immunity. Previous studies have identified abnormal changes in TNFSF10 (TRAIL) among patients with distinct autoimmune and rheumatic diseases such as SLE (61–63). Another study found that $TRAIL^{+}$ monocytes played critical roles in lung damage (64). However, the roles of TNFSF10 $^{high/+}$ monocytes in common autoimmune and rheumatic diseases have not been clearly defined. In present study, the increased expression of TNFSF10 (TRAIL) in the monocytes of SjS patients was identified by both scRNA-seq transcriptome analyses (**Figure 2B**) and bulk transcriptome analyses of monocytes (**Figure 3A**). The outcomes above suggested TNFSF10 $^{high/+}$ monocytes as a potential key player in SjS pathogenesis and a promising target for SjS treatment. Besides, more studies exploring the roles and potential mechanisms of TNFSF10 $^{high/+}$ monocytes in SjS development are needed.

A limitation of this study was the small size of samples of SjS patients and controls especially in the scRNA-seq transcriptome analyses. In the scRNA-seq analyses, there were only 5 SjS cases and 4 controls, which could undoubtedly cause impaired statistical power in detecting differences across distinct monocyte subsets. The total number of monocytes in the scRNA-seq analyses was also limited, which could result in suboptimal analyses of monocyte subsets and their functions. Therefore, further scRNA-seq analyses with larger sample size are recommended in future studies, which may provide much deeper insights into the pathogenesis of SjS.

In summary, this study uncovered the abnormal changes in monocyte subsets and their transcriptomic changes in SjS patients. Both scRNA-seq and bulk RNA-seq transcriptomic analyses identified increased expression of TNFSF10 (TRAIL) in monocytes among SjS patients, suggesting TNFSF10 $^{high/+}$ monocytes as a potential key player in SjS pathogenesis and a promising target for SjS treatment. Further work is needed to explore the roles and underlying mechanisms of monocytes in SjS development.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE157278 https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE173670.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the First Affiliated Hospital of Xiamen University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YH, YL, and GS contributed to conception and design of the study. YH, RC, MZ, and BW analyzed the data. YH, BW, and ZL wrote the manuscript. RC, YL, and GS reviewed and edited the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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The Roles of Monocytes and Macrophages in Behçet's Disease With Focus on M1 and M2 Polarization

Lisa Hirahara†, Kaoru Takase-Minegishi†, Yohei Kirino, Yuki Iizuka-Iribe, Yutaro Soejima, Ryusuke Yoshimi and Hideaki Nakajima*

Department of Stem Cell and Immune Regulation, Yokohama City University Graduate School of Medicine, Yokohama, Japan

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Atsushi Kawakami, Nagasaki University, Japan

Reviewed by:
Yuko Kaneko, Keio University School of Medicine, Japan
Yudong Liu, Chinese Academy of Medical Sciences, China
Kei Ikeda, Chiba University Hospital, Japan

***Correspondence:**
Yohei Kirino
kirino@yokohama-cu.ac.jp

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

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Behçet's disease (BD) is a systemic inflammatory disease characterized by recurrent oral ulcers, genital ulcers, cutaneous inflammation, and uveitis. In addition, other potentially life-threatening lesions may occur in the intestinal tract, blood vessels, and central nervous system. This heterogeneity of the BD phenotype hampers development of a targeted treatment strategy. The pathogenesis of BD is not fully elucidated, but it is likely that genetically susceptible people develop BD in response to environmental factors, such as microbiome factors. Genetic analyses have identified various BD susceptibility loci that function in HLA-antigen presentation pathways, Th1 and Th17 cells, and autoinflammation related to monocytes/macrophages, or that increase levels of pro-inflammatory cytokines, reduce levels of anti-inflammatory cytokines, or act in dysfunctional mucous barriers. Our functional analyses have revealed that impairment of M2 monocyte/macrophage-mediated anti-inflammatory function through IL-10 is crucial to BD pathogenesis. We, therefore, propose that BD is an M1-dominant disease. In this review, we describe the roles of monocytes and macrophages in BD and consider the potential of these cells as therapeutic targets.

Keywords: polarization, genetics, innate immunity, macrophages, monocytes, Behcet's disease

INTRODUCTION

Behçet's disease (BD) is a systemic inflammatory disease initially reported in 1937 by Hulusi Behçet, a prominent Turkish dermatologist. The disease is epidemiologically characterized by a high incidence along the ancient Silk Route, and Professor Shigeaki Ohno has named the disease "Silk Road Disease" (1). The typical manifestations of BD are recurrent oral ulcers, genital ulcers, cutaneous inflammation, and uveitis. However, other potentially life-threatening lesions may occur in the intestinal tract, blood vessels, and central nervous system and this heterogeneity results in the disease being considered a syndrome (2). There are currently no disease-specific antibodies or biomarkers for BD; therefore, diagnosis is made solely on clinical symptoms. The most important symptom is recurrent oral ulcers, which is seen in more than 95% of Japanese patients and is mandatory for diagnosis according to the International Study Group criteria (3, 4). The

pathogenesis of BD is not fully determined, but it is likely that people who are genetically susceptible to the disease may develop BD as a response to environmental factors, such as microbiome factors. HLA-B*51 is the most widely known BD susceptibility gene, with an odds ratio of 5.9 for the development of BD, but its allele frequency is about 20% in the Japanese population and is, therefore, not a disease-causative locus (5). Genetic analyses have identified various susceptibility loci involved in HLA-antigen presentation pathways, Th1 and Th17 cells, pro-inflammatory cytokine regulation, dysfunctional mucous barriers, and autoinflammation related to monocytes/macrophages, the main focus of this review. Our research goal is to categorize the complexity of BD and to establish treatment strategies that are tailored to individual patients. In this respect, macrophages and monocytes could be important targets. In this review, we discuss the roles of monocytes and macrophages based on the results of a recent literature survey and how monocyte-macrophages may be considered as therapeutic targets of BD.

ELUCIDATION OF MONOCYTE FUNCTION

Neutrophil Hyperchemotaxis and Monocytes in BD

Early studies of the involvement of monocytes and macrophages in BD focused on their function as part of the pathogenesis of enhanced neutrophil chemotaxis in response to environmental factors. Aberrant neutrophil chemotaxis in BD was reported in 1975 and colchicine was found to exert a therapeutic effect by inhibiting neutrophil migration (6). Monocyte involvement in the mechanism of neutrophil chemotaxis was then investigated. In a report from 1993, monocytes from patients with active BD displayed increased secretion of pro-inflammatory cytokines, including TNF-α, IL-6, and IL-8 (7). Then, in 1995, increased levels of the soluble monocyte-activation marker, CD14, were reported in the sera of BD patients, and monocyte culture supernatants from BD patients were shown to significantly enhance neutrophil adhesion to endothelial cells (8). These results indicated that monocytes in BD patients are highly activated and involved in chronic inflammation by continuous production of pro-inflammatory cytokines. Histopathological examination then revealed that the cells infiltrating oral ulcer sites in BD patients were lymphocytes and monocytes/macrophages (9). Furthermore, mononuclear cells consisting of $CD4^+$ T cells and monocytes were demonstrated to infiltrate the periphery of small blood vessels at the site of the pathergy reaction, suggesting that monocytes are the essential driver of inflammation in BD (10).

Activation of the Innate Immune System *via* Toll-Like Receptors

Involvement of monocytes in the pathogenesis of BD was further strengthened by the discovery of the pattern recognition mechanism of Toll-like receptors (TLRs). TLRs are a defense mechanism against pathogens-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). Microbes have long been assumed to be an external factor triggering BD inflammation. In fact, Hulusi Behçet reported that BD is induced by herpes virus infection, and herpes simplex-induced animal models elicit BD-like symptoms (11). Herpes viruses replicate in monocytes and can be detected by TLR2 and TLR9, which are highly expressed in monocytes.

Oral commensal bacteria are another candidate risk factor for BD. It is hypothesized that streptococci are particularly prevalent in BD because oral ulcers often worsen after dental treatment (12). Heat shock protein (HSP) 65 produced by these bacteria is homologous to human HSP60, and hyper-reactivity of lymphocytes to HSP has been reported in BD patients. Ten human TLRs have been identified and peptidoglycan (PTG) is a ligand for TLR2 and lipopolysaccharide (LPS) is a ligand for TLR4. Furthermore, HSP60 can bind to several PAMPs and induces cytokine production *via* TLR2 and TLR4 signaling, indicating that TLR2 and TLR4 are involved in the pathogenesis of BD (13).

In 2008, we reported the overexpression of TLR4 in peripheral blood mononuclear cells (PBMCs) of BD patients (14). Subsequently, it was reported that TLR2/TLR4 expression was also increased in monocytes from BD patients (15). In 2013, upregulated expression of TLR2/TLR4 was found in macrophages isolated from BD patients, and that TLR2/TLR4-mediated IL-1β was upregulated in patients with active uveitis when stimulated with peptidoglycan/LPS (16). These results indicate that monocytes are involved in BD pathogenesis in part by activating the innate immune system against external stimuli *via* the TLR pathway. Concordant with these observations, a targeted resequencing of innate-immune genes in Japanese and Turkish populations identified low-frequency *TLR4* variants associated with BD, supporting the hypothesis of innate immune system activation through TLRs in BD (17).

FUNCTIONS OF MONOCYTES AND MACROPHAGES REVEALED BY GENETIC STUDIES

Since 2010, genome-wide association studies (GWAS) and other large-scale genetics studies have been conducted to explore BD. These investigations confirmed that the HLA region has the highest association with BD development. *HLA-B*51, A*26, B*15, B*27, and B*57* were identified as disease-susceptibility alleles, and *A*03* and *B*49* were identified as disease-protective alleles (18). In addition to HLA genes, nearly 20 disease-susceptibility loci were identified, including *IL10*, *IL23R*, and *ERAP1*, which contributed to the proposal of the disease concept of "MHC class-I-opathy", similar to spondyloarthritis (**Table 1**) (19–21, 23). BD-related loci that may affect monocyte and macrophage function include *IL10*, *CCR1–CCR3*, *MEFV*, *IL1B*, *IRF8*, and most recently, *IFNGR1* (27, 28). The pathways associated with BD are summarized in **Figure 1**.

Elucidation and Interpretation of CCR1 Function

An important finding in understanding the function of monocytes and macrophages in the pathogenesis of BD was

TABLE 1 | Genome-wide significant disease susceptibility loci for BD.

Year	Author	Population	Gene
2009	Meguro et al. (19)	Japanese	*HLA-A26*
2010	Mizuki et al. (20)	Japanese	***IL10***, *IL23R*
2010	Remmers et al. (21)	Turkish, mixed populations	***IL10***, *IL23R*
2012	Hou et al. (22)	Chinese Han	*STAT4*
2013	Kirino et al. (23)	Turkish, Japanese	***CCR1-CCR3***, *STAT4, KLRC4, ERAP1*
2013	Kirino et al. (17)	Turkish	***MEFV M694V***
2013	Lee et al. (24)	Korean, Japanese	*GIMAP*
2014	Ombrello et al. (18)	Turkish	*HLA-A03*
2014	Xavier et al. (25)	Iranian	*FUT2*
2015	Kappen JH et al. (26)	Turkish and mixed populations	*IL12A*
2017	Takeuchi et al. (27)	Turkish, Japanese, Iranian	***IL1B***, ***IRF8***, *RIPK2, EGR2, LACC1, PTPN1*
2020	Ortiz Fernández et al. (28)	Turkish, Japanese etc.	***IFNGR1***, *DKK1*

The genes highlighted in bold are the ones that we focused on in this paper as being related to macrophage and monocyte function.

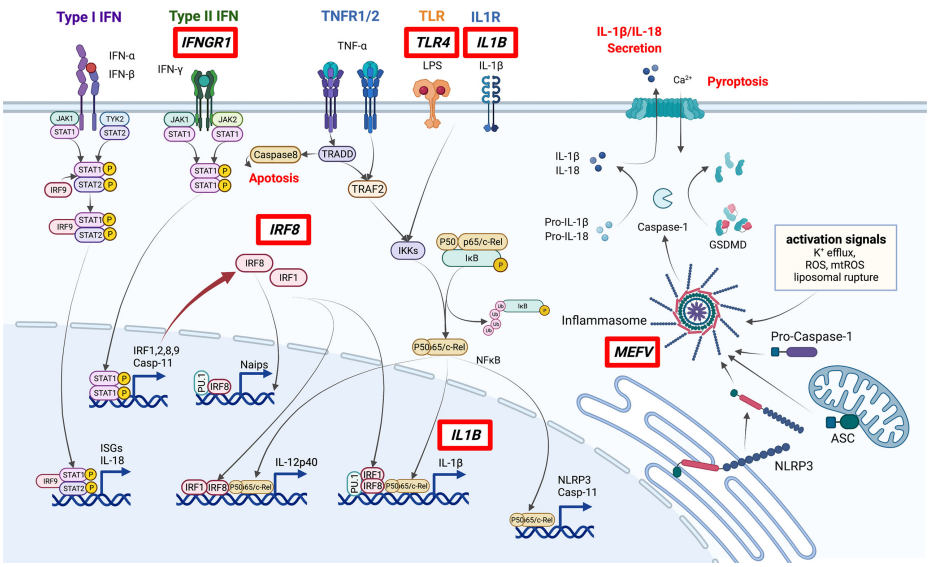

FIGURE 1 | Pathways involved in the pathogenesis of BD. Genes discovered by GWAS to be associated with BD (red boxes) include several involved in macrophage inflammation. In particular, IRF8 and MEFV are important in the regulation of STAT signaling and inflammasome activation. ISGs: Interferon-stimulated genes. This figure was created with BioRender.com.

identification of the *CCR1–CCR3* locus. *CCR1* encodes CC-motif chemokine receptor1(CCR1), which is highly expressed on monocytes/macrophages, whereas CCR3 is highly expressed on eosinophils, basophils, and T cells. The ligands for CCR1 are macrophage inflammatory protein-1 alpha (MIP-1α), regulated on activation normal T expressed and secreted protein (RANTES), and monocyte chemoattractant protein 3 (MCP3). Upon detection of these chemokines, monocytes migrate to the site of high chemokine production (29). GWAS identified a locus tagged with SNP rs7616215 located in the 3′ non-cording region of *CCR1*, and functional analysis revealed that risk allele T was associated with reduced expression of *CCR1*. Furthermore, the migratory ability of monocytes in response to MIP-1α was lower in individuals with risk allele T (23). These results indicate that monocyte chemotaxis is reduced in patients with BD, in contrast to increased monocyte infiltration of lesions. Inconsistent with our GWAS findings, systematic expression quantitative trait analysis with various cell subsets indicated that rs7616215 affects *CCR3* more than *CCR1*, resulting in higher *CCR3* expression (30). However, a recent large GWAS on canker sores, a refractory form of which is considered a "Behçet-spectrum disorder" (31), confirmed association between *CCR1-CCR3*, and the Genotype-Tissue expression of *CCR1* and *CCR3* were down-regulated and up-regulated, respectively, indicating that the locus has a binary effect on *CCR1* and *CCR3* expression (32). In addition, a recent single-cell whole-genome expression quantitative trait analysis (33) showed consistent results that the risk allele T is associated with decreased expression of CCR1 and

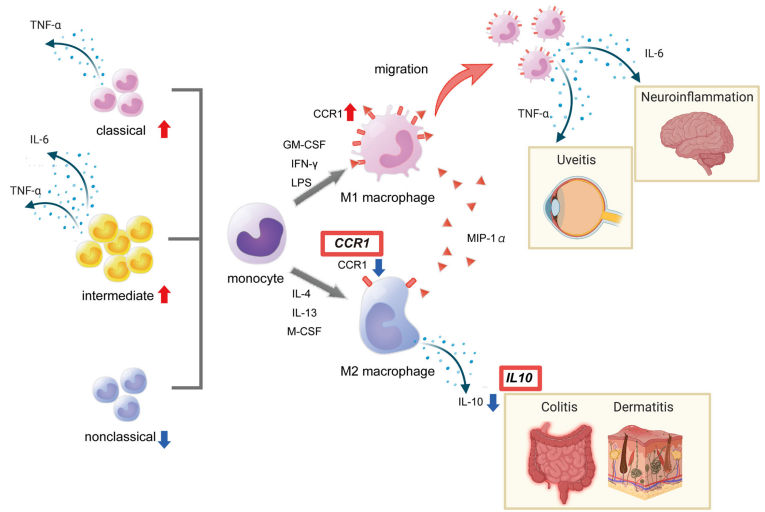

FIGURE 2 | Red boxes show disease susceptibility genes for BD associated with this pathway. This figure was created with BioRender.com.

increased expression of CCR3 in classical monocytes as well as neutrophils and plasmacytoid dendritic cells (**Supplementary Figure 2**).

Abnormal Function of Polarized Macrophages in the Context of IL10

Macrophage polarization has attracted much attention in recent years. M1 macrophages secrete pro-inflammatory cytokines, such as IL-1, IL-6, and TNF-α, which are therapeutic targets of BD, while M2 macrophages secrete anti-inflammatory cytokine represented by IL-10 (34). M2 can be further classified into four subtypes, M2a, M2b, M2c and M2d. Each subtype is characterized by cell surface markers, secreted cytokines and function, but all subtypes secrete IL-10 (35). IL-10 receptor deficiency is known to cause severe inflammation in skin and intestines, symptoms that partially overlap with BD (36). GWAS showed association of BD with *IL10* intronic variant, rs1518111, in both Japanese and Turkish populations (20, 21). *IL10* mRNA and protein levels were reduced in monocytes of individuals with risk alleles compared with those in individuals without risk alleles (21). As mentioned earlier, *CCR1–CCR3* SNPs are associated with BD, and reduced expression of *CCR1* is associated with disease risk.

We addressed how decreased monocyte migration ability revealed by GWAS is related to the pathogenesis of BD by analyzing the polarization of M1 and M2 macrophages (37). Expression of *IL10* and *CCR1* was generally higher in M2 than M1 macrophages, and *CCR1* expression in M1 macrophages was higher in BD patients than in healthy controls. We also found significant infiltration of M1 macrophages in erythema nodosum lesions of BD patients. *CCR1* SNP, rs7616215, is a BD-risk allele associated with reduced M2 migration in response to MIP-1α. These results suggest that M2 macrophage infiltration capacity is lower in BD than in healthy subjects, and that fewer M2 cells and BD-associated SNPs may result in lower levels of IL-10, resulting in M1-predominant inflammation in BD. As mentioned above, recent quantitative trait analysis of single cell expression revealed that CCR1 has a role in the migration of various immune cells, and that not only monocytes but also neutrophils and acquired immune responses are affected by this CCR1-CCR3 locus (**Supplementary Figure 2**). The crosstalk between polarized macrophages and neutrophils and acquired immune cells is not fully understood, but the interaction may be important for inflammation in BD.

In line with our findings, macrophages collected from the peritoneal fluid of herpes simplex virus-induced BD model mice were predominantly M1 (38). Macrophages collected from healthy individuals treated with serum from BD patients promoted differentiation into M1 macrophages, while differentiation into M2 macrophages was suppressed (39). We also reported that the production of heme oxygenase 1, an anti-inflammatory heme degrading enzyme, is high in M2 macrophages but reduced in BD patients in response to TLR4 stimulation (14). Together these results support the hypothesis of impaired M2 function in BD (**Figure 2**).

Importantly, anti-inflammatory M2 macrophages may not be consistently anti-inflammatory. We previously tested whether established M1 macrophages could be converted to M2 macrophages in human primary cells (37). For this purpose, M1 and M2 macrophages that underwent 9 days of *in vitro* polarization in response to GM-CSF or M-CSF were cultured for another 9 days in the presence of the same or other cytokines. Treatment with M-CSF restored the expression of *IL10* (**Figure 3**). Conversely, the expression of *IL10* mRNA was decreased by GM-CSF in M2 macrophages. *IL6* mRNA also showed changes in this experiment. These results suggest that the macrophage phenotype is partially interchangeable between M1 and M2, depending on situational factors, including cytokines.

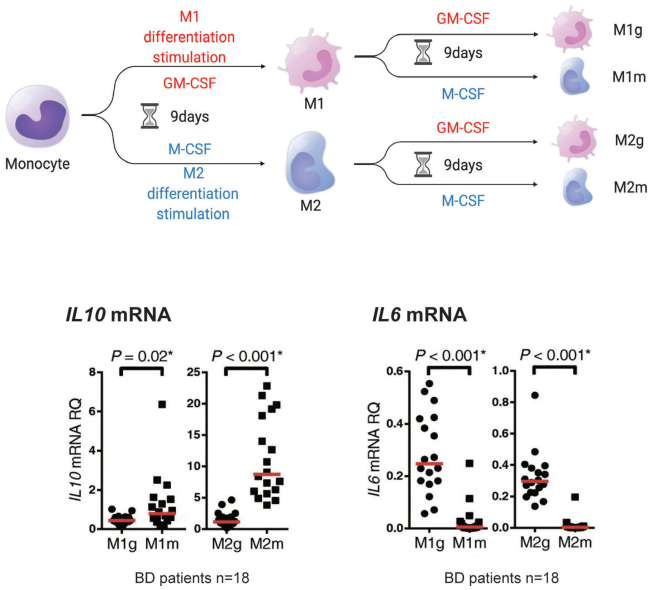

FIGURE 3 | M1 and M2 macrophages may be interchangeable. Monocytes (mono) treated *in vitro* with GM-CSF (M1g and M2g) or M-CSF (M1m and M2m) for 9 days developed an M1 or M2 macrophage-like phenotype (defined here as M1 or M2 stimulation). However, the M2 macrophages stimulated with "M2" or "M1" stimulation for another 9 days (the first M1 stimulus followed by M2 stimulus is defined as "M2g" and the second M1 stimulus followed by M2 stimulus is defined as "M1m") maintained or decreased *IL10* mRNA production. *IL6* mRNA production was reduced by M2 stimulation of M1 macrophages. This figure is a modified version of Figure 4 a-e published in Nakano et al. (37). The figure is modified and distributed under the Creative Commons Attribution 4.0 International License (CCBY4.0, http://creativecommons.org/licenses/by/4.0/). This figure was partially created by BioRender.com.

ABERRANT MONOCYTE SUBSETS IN BD PATIENTS

Interestingly, several recent reports have shown that not only macrophages but also monocyte subsets are imbalanced in BD. Based on the expression of molecules on the cell surface, monocytes can be classified into three categories: classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$), and non-classical ($CD14^{+}CD16^{++}$) (40). These three subtypes are also considered to be functionally distinct. Classical monocyte represents the majority of circulating monocytes. In general, classical monocytes have a pro-inflammatory and phagocytic phenotype. They express high level of CCR2, migrate in response to CCL2(MCP-1) stimulation and are involved in initiation of inflammation. In contrast, non-classical monocytes that patrol the vascular endothelium to survey for damage. Particularly in the area of vascular diseases, they have been focused on as intravascular scavenger that remove cellular debris from blood vessels (41, 42). They produce less reactive oxygen species (ROS) and cytokines in response to stimulation with cell surface TLRs, but produce pro-inflammatory cytokines in response to stimulation with viruses and immune complexes containing nucleic acids (43). Although the functions of intermediate monocytes are not fully understood, they are involved in ROS production and highly express genes related to antigen presentation, cytokine production, apoptosis control, T cell proliferation and cell differentiation (44, 45).

Increased numbers of classical monocytes and decreased numbers of nonclassical monocytes have been reported in BD patients with uveitis (46). In addition, monocytes from BD patients and healthy controls differ in TLR expression by subset, with classical monocytes expressing TLR2, TLR4, and TLR5, nonclassical monocytes expressing TLR2 and TLR5, and intermediate monocytes expressing TLR2 (47). BD patients have increased numbers of intermediate monocytes and decreased numbers of nonclassical monocytes compared with healthy controls. The results showed that classical and intermediate monocytes overproduce TNF-α and intermediate monocytes overproduce IL-6 (48). At present, little is known about monocyte subsets in BD patients. However, there is also a theory that non-classical monocytes can differentiate into M2 macrophages; hopefully future research will answer these questions.

IL1A/IL1B and MEFV

Immunochip analysis in Turkish, Japanese, and Iranian subjects revealed association of *IL1A–IL1B* and *IRF8* loci with BD. Functional analysis revealed that risk SNPs in the *IL1A–IL1B* region were associated with high levels of *IL1β* expression (27). Targeted-resequencing of innate-immune genes in a Turkish population identified association of BD with *MEFV M694V*, a known disease-causing variant of familial Mediterranean fever. *MEFV* encodes a pyrin, which forms pyrin-inflammasomes in response to toxins produced by *Clostridium difficile* (17, 49). A

pyrin ligand β2-microglobulin (β2MG) induces pyrin inflammasome formation, while the caspase-1p20 subunit produced by the pyrin inflammasome inhibits the pyrin-β2MG interaction in neutrophils (50). The M694V mutation weakens the inhibitory effect of caspase-1p20 on the pyrin-β2MG interaction (50). Inflammasomes are composed of pattern recognition receptors, such as NOD-like receptors, and an adaptor protein, pro-caspase 1 (51). When the inflammasome is formed, pro-caspase 1 becomes activated caspase 1, which cleaves pro-IL-1β into active IL-1β (52). Caspase 1 also cleaves gasdermin D, the N-terminus of which forms membrane pores, leading to pyroptosis (53). This pathway is called the canonical pathway. In addition to this process, there is the noncanonical pathway that releases IL-1β/IL-18 and causes pyroptosis *via* caspase 11 (54). Together, these findings indicate that excessive production of IL-1β by activated inflammasomes is likely to be involved in the pathogenesis of BD.

Conclusions from studies using cells from BD patients have been controversial. In monocyte-derived macrophages from BD patients, TLR2 and TLR4 expression and IL-1β/ROS production were upregulated and IL-1β production was suppressed by inhibition of the NLRP3 inflammasome (55). In PBMCs from BD patients, production of NLRP3 inflammasomes and IL-1β in response to LPS stimulation was increased compared with that in healthy controls (56). The expression of NLRP3, caspase 1 and gasdermin D was also significantly higher in the intestinal tissues of BD patients (57). Meanwhile, no enhancement of the caspase 1 pathway was observed in dendritic cells of BD patients (58). The serum level of gasdermin D was lower in BD patients used as disease controls for adult-onset Still's disease (59). The differences in these conclusions might be affected by different cell types and patients' backgrounds. Focusing on the relationship between risk alleles and monocyte/macrophage polarity may provide additional autoinflammatory insights into BD.

IRF8

IRF8 is one of the nine members of the interferon regulatory factor (IRF) family (60). In IRF8-deficient mice, monocytic phagocyte differentiation is inhibited and abnormal differentiation into neutrophils occurs, indicating that IRF8 regulates the differentiation of progenitor cells into monocytic phagocytes (61, 62). IRF8 is also involved in the formation of M1 macrophages (63).

IRFs also function in signaling by TLRs and IFN receptors. IRF8 is expressed in macrophages and is responsible for induction of interferon production; IRF8 regulates transcription by forming a complex with IRF1 and transcription factors such as AP1 and PU.1 (64–67). Stimulation of myeloid cells with LPS results in increased expression of IRF8, which then binds to sites on the genome that are different from its steady-state binding site (68). The activity of IRF8/PU.1 is required for the regulation of *IL18* expression in macrophages in mice (69). Furthermore, IRF8-PU.1 forms a complex with IRF1 and increases the promoter activity of *IL1β* (70). IRF8 is also required to activate the NLRC inflammasome (71).

IRF8 is involved in polarization of T-cells. In antigen-presenting cells (APCs), IFN-γ binding to IFNGR1/2 transactivates *IRF8* expression through a STAT1-mediated pathway (72). IRF8 binds to the promoter region of *IL12p40* and induces the production of IL-12 p40 from APCs, which promotes differentiation into Th1 cells (73, 74). IL-12p40 is a subunit of IL-12, but it is also a subunit of IL-23, which induces Th17 cell differentiation. In addition, IRF8 regulates *IL27* and *TGFβ* expression in APCs. IRF8 expression decreases IL-27 production and activates TGFβ, thereby enhancing the induction of Th17 cell differentiation (75).

The association between IRF8 and BD has been reported in Chinese populations in addition to that described in the aforementioned Immunochip analysis (76). Functional analysis showed increased *IRF8* mRNA expression and IFN-γ production and decreased production of IL-10 in the risk allele group, indicating higher macrophage differentiation ability in BD (76).

IFNGR1

A large genetic association study involving 9,444 individuals from seven diverse populations was recently performed and *IFNGR1* and *LNCAROD/DKK1* were identified as new BD susceptibility loci (28). *IFNGR1* expression was increased in $CD14^+$ monocytes 2 hours after LPS stimulation in the presence of the risk allele (28). IFNGR1 is a receptor for IFN-γ, and when IFN-γ binds to IFNGR1, it regulates the transcription of genes that contain gamma-activating sequences (GAS) in the promoter region *via* the STAT1 pathway (77). In addition to activating macrophages, IFN-γ has many other functions, including controlling Th cell polarity, enhancing antigen presentation, leukocyte homing and cell adhesion (78). Of note, *IFNGR1* polymorphisms affect the immune response to mycobacterium and *Helicobacter pylori*, which are assumed to be pathogens associated with BD (79, 80). Patients with complete loss of IFNGR1 are repeatedly infected with mycobacterium and develop disseminated Bacille Calmette-Guerin (BCG) and die from BCG inoculation (81, 82). Furthermore, IFNGR1-mediated transactivation of caspase 11 has been reported in mice, indicating that IFNGR1 may be involved in the noncanonical pathway of inflammasome formation (83). These results strengthen the hypothesis of an abnormal innate immune response to external stimuli (including pathogens) in BD pathogenesis. The discovery of the involvement of IGNGR1 and STAT1 pathways may support the use of JAK inhibitors for BD.

SERUM CYTOKINE LEVELS IN BD

To clarify the role of cytokines, especially those produced by monocytes and macrophages, in BD pathogenesis, we systematically searched PubMed, the Cochrane Central Register of Controlled Trials, and the Web of Science Core Collection (up to November 30, 2021) for literature comparing cytokine levels in healthy controls and BD patients. Search formulae are presented in **Supplementary Text 1**. Differences in cytokine levels between BD and control subjects were calculated as the standardized mean difference (SMD) with confidence intervals (CIs). Pooled analyses were performed using the generic inverse variance method with a random

effects model. Heterogeneity was indicated by I^2, where 0% meant no heterogeneity and 100% meant the strongest heterogeneity. Review Manager version 5.4 software (Cochrane, London, UK) was used to draw paired forest plots.

Among 2,429 candidate articles, we identified 26 eligible studies (84–95). The quality of the original studies was assessed using the Newcastle-Ottawa Quality Assessment Scale for case-control study design (96). Characteristics of the included studies are summarized in **Supplementary Table 1**. The Newcastle-Ottawa Scale score of the included studies was good (**Supplementary Table 2**).

Proinflammatory cytokines play crucial roles in the initiation and perpetuation of disease. Levels of IL-6 and TNF-α are associated with disease activity according to several studies (91, 94). Serum IL-6 levels were measured in 15 cohorts involving 594 BD patients and 479 controls. The meta-analysis showed that IL-6 levels were significantly higher in BD patients than in controls (SMD = 3.20, 95% CI: 2.14–4.26, I^2 = 97%, p < 0.001) (**Figure 4A**). Similarly, serum TNF-α levels were measured in 14 cohorts involving 552 BD patients and 413 controls, and were significantly increased in BD patients compared with controls (SMD = 3.19, 95% CI: 2.22–4.16, I^2 = 97%, p < 0.001) (**Figure 4B**). Subgroup analysis revealed that IL-6 and TNF-α levels were increased more in active BD than in inactive BD (**Supplementary Figure 1**). Serum IL-1β levels were studied in only four cohorts involving 191 BD patients and 128 controls. IL-1β levels were increased in BD patients compared with controls (SMD = 1.67, 95% CI: 0.18–3.16, I^2 = 97%, p < 0.001).

As noted above, IL-10 confers an anti-inflammatory effect involved in the pathogenesis of various autoimmune diseases. Serum IL-10 levels were measured in six cohorts involving 266 BD patients and 260 controls. Some studies have shown that IL-10 is increased in inflamed tissues of BD patients (92, 93). However, serum levels have been reported to be both increased and decreased in BD, and the meta-analysis showed that IL-10 levels were not higher in BD patients compared with controls (SMD = -2.69, 95% CI: -6.14–0.76, I^2 = 99%, p < 0.001) (**Figure 4C**). Although GWAS have shown decreased *IL10*

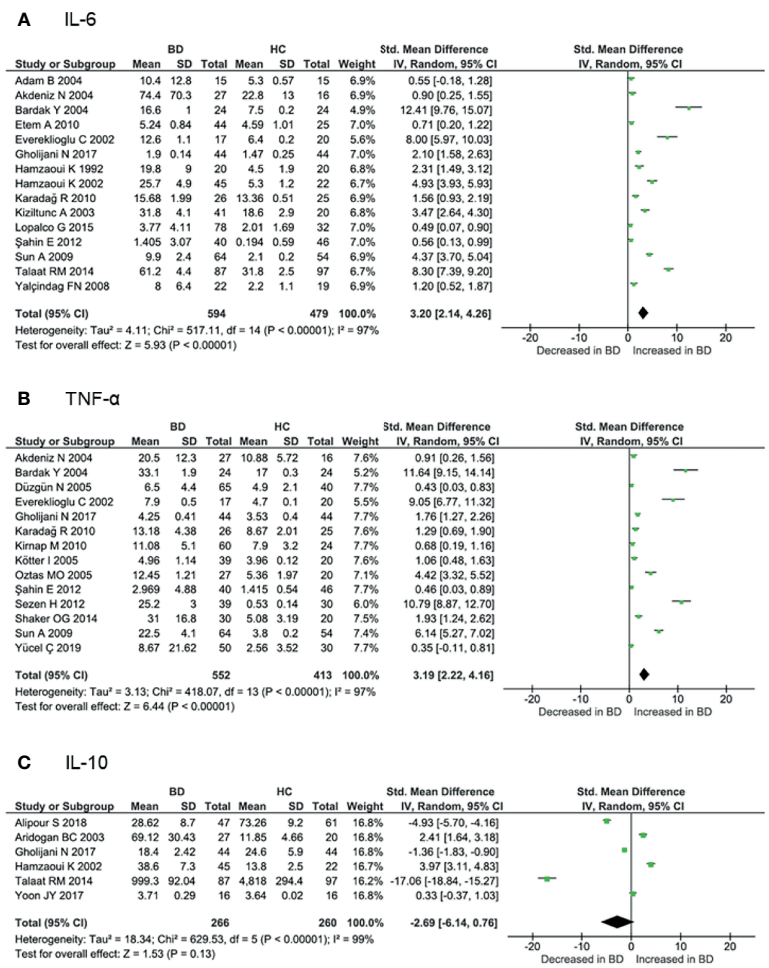

FIGURE 4 | Forest plot of the standardized mean difference of serum cytokine levels in BD patients compared with controls. **(A)** IL-6, **(B)** TNF-α, **(C)** IL-10.

expression associated with BD (20, 21), various disease activities included in the analyses may have influenced the results (**Supplementary Table 1**). Subgroup analysis revealed that IL-10 levels were higher in active BD than in inactive BD (92, 93).

INVOLVEMENT OF MONOCYTES WITH CURRENT TREATMENT STRATEGIES

Colchicine inhibits the polymerization of microtubules and is an established treatment for BD. Inhibition of microtubule polymerization prevents NLRP3 from approaching the adopter molecule apoptosis-associated speck-like protein containing a CARD (ASC) and suppresses activation of the NLRP3 inflammasome (97). Colchicine is also associated with increased numbers of nonclassical monocytes, indicating that it may affect monocyte polarization (98). GWAS indicate abnormal inflammasome activation in monocytes, supporting the use of colchicine as an anchor drug for BD.

Anti-TNF-α antibodies have been used to treat uveitis, and intestinal, neurological, and vascular lesions of BD (99). TNF-α receptors include TNFR1 and TNFR2. TNFR1 has the death domain, Tumor necrosis factor receptor type 1-associated DEATH domain (TRADD), and TRADD-mediated aggregation of induced Fas-associated protein with death domain (FADD)and receptor-interacting protein (RIP) activates caspase-8, leading to apoptosis (100). In contrast, TNFR2 does not have a death domain and activation of the TRAF2-mediated pathway leads to the activation of NFκB (101). Haploinsufficiency of A20, encoded by the *TNFAIP3* gene, causes prolonged activation of the NFκB pathway and BD like symptoms (102–105). Anti-TNF antibodies and monocytes have been studied in response to rheumatoid arthritis treatment. Serum monocyte counts are decreased in patients who are responsive to anti-TNF therapy (106) and an early decrease in circulating monocyte count is a predictor of maintenance of remission (107). Anti-TNF antibodies also affect the polarity of macrophages and monocytes. A psoriasis study showed that anti-TNF therapy inhibited the polarity of M1 macrophages in an IRF1- and STAT1-independent manner (108). Another report of inflammatory bowel disease showed that infliximab infusion caused a decrease in monocyte count, which was more pronounced in classical and intermediate monocytes (109).

In 2019, Apremilast produced successful results in a phase 3 trial for refractory oral ulcers in BD (110). Apremilast is an inhibitor of phosphodiesterase (PDE)4, which regulates signal transduction of the intracellular second messengers, cyclic AMP (cAMP) and cyclic GMP (cGMP). The 11 gene families that comprise the PDE superfamily differ in function, primary structure, affinity for cAMP and cGMP, and regulatory mechanisms. Most cells express more than one PDE family member, but the degree of expression varies among tissues and cells (111). PED4 is cAMP-specific and is distributed throughout the body, but is expressed predominantly on T cells, monocytes, macrophages, neutrophils, dendritic cells, and eosinophils (112). *In vivo* stimulation of $CD14^+$ monocytes with LPS and apremilast increased the expression of the *SOCS3* gene, which then up-regulated the enhanced IL-10 and IL-6 expression, and decreased IFN-γ expression (113), thus apremilast may correct the genetically driven IL-10 impairment in M2 macrophages of BD patients. Similarly, sub-analysis of a phase 3 trial showed serum IFN-γ levels in the apremilast group were significantly lower than in the placebo group at 12 weeks (114).

FUTURE PERSPECTIVES

Elucidation and interpretation of the function of disease susceptibility loci discovered by GWAS have led to significant progress in understanding the pathogenesis of BD. The pathogenesis of BD cannot be described by an abnormality in a single immune process but is undoubtedly a complex interplay of multiple immune processes. Monocytes/macrophages are responsible for initiating the inflammatory response and directing the subsequent activation of the acquired immune system. Considering that current therapies target monocytes, monocytes are promising targets for new agents, especially those that can alter the polarity of macrophages and monocytes.

The current unmet medical needs of BD are residual disease activity in some patients using current therapies, lack of treatment goals, and personalization of treatment regimens. As seen in the cytokine meta-analysis, the heterogeneity of BD symptoms makes the study of BD difficult. The classification of disease type is necessary to inform therapeutic strategies. We and others have analyzed the phenotypic subtypes of BD and have described five subtypes (115, 116). A disadvantage of categorizing the disease into clinical subtypes is the reduction in patient number for each subtype, especially as BD is a rare disease with a regional bias. A large international cohort to increase the number of participants is, therefore, necessary. In addition to symptom subtypes, there may also be genetic and immunological subsets. IL-17 inhibitors and JAK inhibitors were recently shown to be effective for BD (117, 118). By identifying subsets with predominant monocyte/macrophage activity, subsets with predominant Th17 activity, etc., it may be possible to identify patient groups for which inhibition of specific cells is predicted to be useful. We suggest that further subtype analysis and elucidation of immunological pathogenesis will contribute to personalized medicine in BD.

AUTHOR CONTRIBUTIONS

LH, KT-M, and YK designed the research. LH, KT-M, YK, YI-I, YS, and RY conducted the research, statistical analysis, and interpretation of the data. LH, KT-M, and YK drafted the manuscript. YI-I, YS, RY, and HN were involved in writing the article or revising it critically for intellectual content. LH, KT-M, and YK had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the

data analysis. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.852297/full#supplementary-material

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Different Spatial and Temporal Roles of Monocytes and Monocyte-Derived Cells in the Pathogenesis of an Imiquimod Induced Lupus Model

Atsushi Nomura[1], *Miho Mizuno*[1], *Daisuke Noto*[1], *Aki Aoyama*[1], *Taiga Kuga*[1,2], *Goh Murayama*[1,2], *Asako Chiba*[1] *and Sachiko Miyake*[1*]

[1] Department of Immunology, Juntendo University Graduate School of Medicine, Tokyo, Japan, [2] Department of Internal Medicine and Rheumatology, Juntendo University School of Medicine, Tokyo, Japan

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Carmelo Carmona-Rivera, National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIH), United States

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Shruti Sharma, Tufts University School of Medicine, United States
Klaus Tenbrock, RWTH Aachen University, Germany

***Correspondence:**
Sachiko Miyake
s-miyake@juntendo.ac.jp

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Mounting evidence indicates the importance of aberrant Toll-like receptor 7 (TLR7) signaling in the pathogenesis of systemic lupus erythematosus (SLE). However, the mechanism of disease progression remains unclear. An imiquimod (IMQ)-induced lupus model was used to analyze the lupus mechanism related to the aberrant TLR7 signals. C57BL/6 mice and NZB/NZW mice were treated with topical IMQ, and peripheral blood, draining lymph nodes, and kidneys were analyzed focusing on monocytes and monocyte-related cells. Monocytes expressed intermediate to high levels of TLR7, and the long-term application of IMQ increased Ly6C^{lo} monocytes in the peripheral blood and Ly6C^{lo} monocyte-like cells in the lymph nodes and kidneys, whereas Ly6C^{hi} monocyte-like cell numbers were increased in lymph nodes. Ly6C^{lo} monocyte-like cells in the kidneys of IMQ-induced lupus mice were supplied by bone marrow-derived cells as demonstrated using a bone marrow chimera. Ly6C^{lo} monocytes obtained from IMQ-induced lupus mice had upregulated adhesion molecule-related genes, and after adoptive transfer, they showed greater infiltration into the kidneys compared with controls. RNA-seq and *post hoc* PCR analyses revealed Ly6C^{lo} monocyte-like cells in the kidneys of IMQ-induced lupus mice had upregulated macrophage-related genes compared with peripheral blood Ly6C^{lo} monocytes and downregulated genes compared with kidney macrophages (MF). Ly6C^{lo} monocyte-like cells in the kidneys upregulated *Il6* and chemoattracting genes including *Ccl5* and *Cxcl13*. The higher expression of *Il6* in Ly6C^{lo} monocyte-like cells compared with MF suggested these cells were more inflammatory than MF. However, MF in IMQ-induced lupus mice were characterized by their high expression of *Cxcl13*. Genes of proinflammatory cytokines in Ly6C^{hi} and Ly6C^{lo} monocytes were upregulated by stimulation with IMQ but only Ly6C^{hi} monocytes upregulated IFN-α genes upon stimulation with 2′3′-cyclic-GMP-AMP, an agonist of stimulator of interferon genes. Ly6C^{hi} and Ly6C^{lo} monocytes in IMQ-induced lupus mice had different features. Ly6C^{hi} monocytes responded in the lymph nodes of locally stimulated sites and had a higher

expression of IFN-α upon stimulation, whereas Ly6C^{lo} monocytes were induced slowly and tended to infiltrate into the kidneys. Infiltrated monocytes in the kidneys likely followed a trajectory through inflammatory monocyte-like cells to MF, which were then involved in the development of nephritis.

Keywords: classical monocytes, non-classical monocytes, imiquimod, toll-like receptor 7, resident macrophages, systemic lupus erythematosus, lupus nephritis

INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the production of many types of autoantibodies, especially anti-nuclear antibodies (1). Despite the postulated central role of B cells and autoantibodies in SLE, many interventions that target this compartment have not succeeded (2). Mounting evidence indicates the importance of aberrant innate immune signals in the pathogenesis of SLE (3), and symptoms such as neuropsychiatric disorders or nephritis are thought to be partly caused by innate immune mechanisms independent of acquired immunity (4, 5). Among the innate immune signaling molecules, polymorphisms of Toll-like receptor 7 (TLR7) were reported to have a significant association with the development of SLE (6), and the epicutaneous application of TLR7 induced lupus-like disease in mice (7). In that model, plasmacytoid dendritic cells (pDC) were accumulated in the TLR7 agonist-treated skin. pDC have critical roles in the pathogenesis of SLE through the production of IFN-α following TLR7 activation (8). Other than pDC, a study of SLE reported a link between TLR7 and immune cells including B cells (9), monocytes, and monocyte-derived cells (10, 11). Currently, a potential relationship between TLR7 stimulation and lupus-like disease development in the imiquimod (IMQ)-induced lupus model is unclear, other than the mechanism involving pDC.

Monocytes are versatile cells recruited from the circulation to the sites of inflammation, which then differentiate into dendritic cells (DC) or macrophages (MF) (12). Thus, their differentiation and role in the pathogenesis of lupus have gained increased attention (13). Conventionally, monocytes are classified into two types: "classical" Ly6C^{hi} cells in mice and CD16^{-} cells in humans, and "non-classical" Ly6C^{lo} cells in mice and CD16^{+} cells in humans (14). Classical and non-classical monocytes have a lineage relationship in which classical monocytes continuously give rise to non-classical monocytes although phenotypically different intermediate monocytes were recently reported (15, 16). Classical monocytes are involved in acute inflammatory responses. They are recruited to sites of inflammation where they extravasate and give rise to monocyte-derived DCs and MF. In contrast, non-classical monocytes patrol the endothelial surface and coordinate its repair by recruiting neutrophils as required (12). In terms of TLR7 in monocyte-related cells and their relation to SLE, monocyte-derived DCs, especially CD14^{+} cells, and their stimulation *via* TLR7 were reported to be involved in the induction of follicular helper T cells (Tfh), which are important for the differentiation of autoantibody-producing B cells (11). However, CD16^{+} monocytes accumulated in the glomerulus of kidneys in lupus nephritis patients (17) and the stimulation of TLR7 in non-classical monocytes led to inflammation that was related to the pathogenesis of lupus nephritis (18).

In this report, we investigated the involvement of monocytes and monocyte-derived cells in the development of a lupus-like disease in the IMQ-induced lupus model. By analyzing cells in the peripheral blood, lymph nodes, and kidneys at the same timepoints, we showed that numbers of Ly6C^{hi} and Ly6C^{lo} cells increased differently in terms of the developmental stage and organ location. We found that classical monocytes, non-classical monocytes, and monocyte-derived cells were involved differently in the development of SLE.

MATERIALS AND METHODS

Mice and the Induction of Lupus-Like Disease

Female C57BL/6 mice were purchased from CLEA Japan (Tokyo, Japan), NZB mice and NZW mice were purchased from Japan SLC (Hamamatsu, Japan), and female F1 offspring were used as NZB/NZW mice. B6-EGFP mice were originally obtained from Jackson Laboratory (Bar Harbor, ME, USA) and they were bred and maintained in the animal facility at the Juntendo University School of Medicine. Mice were housed under specific pathogen-free conditions. Mice were sacrificed at 12 to 15 weeks of age unless otherwise described. All animal experiments were performed in accordance with the guidelines of laboratory animal experimentation at Juntendo University School of Medicine.

To induce lupus-like disease, mice were treated topically with 5% IMQ cream (Mochida Pharmaceutical, Tokyo, Japan). IMQ cream was applied to the ear skin but if the amount of one dose was greater than 20 mg, it was applied to the ear skin and forelegs. The dosage and duration were dependent on the experiments. Details of the treatments are described in the figure legends.

Proteinuria was analyzed using a DCA Microalbumin/Creatinine Urine Test (Siemens, Erlangen, Germany) according to the manufacturer's instructions.

Immunofluorescent Staining

For fluorescent staining, mouse lymph nodes and kidneys were removed after transcardial perfusion with PBS and then frozen with liquid nitrogen after being embedded in OCT compound (Sakura Finetek Japan, Tokyo, Japan). Four-micrometer cryostat sections were fixed in acetone and stained with anti-mouse IgG

FITC (Southern Biotech, Birmingham, USA), anti-mouse complement C3 (MP Biomedicals, Santa Ana, CA, USA), rat anti-CD11b (Tonbo Biosciences, San Diego, CA, USA), rabbit-anti-CD3 (Abcam, Cambridge, UK) or biotin-conjugated rat anti-CD45R/B220 (BioLegend, San Diego, CA, USA). For multiple staining, primary antibodies were followed by AF 488-conjugated anti-rat IgG (Jackson ImmunoResearch, West Grove, PA, USA), rhodamine (TRITC)-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch) and Cy5-streptavidin (Thermo Fisher Scientific, Waltham, MA, USA). Staining of sections was visualized with a fluorescence microscope (BZ-X700; Keyence, Osaka, Japan).

Immune Cell Isolation

Mice were deeply anesthetized and lymph nodes, spleens, and kidneys were removed after transcardial perfusion with PBS. Blood was drawn from the right ventricle with or without transcardial perfusion depending on the experiment. For the enzymatical digestion of lymph nodes and kidneys, collagenase (Sigma-Aldrich, St. Louis, MO, USA), and DNase (Roche, Basel, Switzerland) dissolved in RPMI 1640 medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin (all from Thermo Fisher Scientific) were used. Kidney tissues were dissociated with GentleMacs Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany), whereas lymph nodes and spleens were ground through a cell strainer. To isolate mononuclear cells, dissociated kidney tissue was suspended in 33% Percoll (GE Healthcare, Chicago, IL, USA) in PBS and overlaid on a 60% Percoll layer. After centrifugation, cells in the intermediate layer were collected and washed. For the isolation of mononuclear cells from the blood, collected blood was overlaid on a 60% Percoll layer and mononuclear cells were isolated using the same method as for kidney cells. For the isolation of splenocytes, spleens were ground through a cell strainer and erythrocytes were removed by ACK lysis. These cells were then used for flow cytometry analysis or cell sorting.

Flow Cytometry

For flow cytometry analysis, isolated cells were pre-incubated for 10 minutes with purified anti-mouse CD16/32 (BioLegend) to block the Fc-mediated non-specific binding of antibodies. Then, cells were stained with the following antibodies: anti-CD64-PE/Dazzle 594, anti-CD115-PE/Dazzle 594, anti-CD3-PerCP/Cy5.5, anti-NK1.1-PerCP/Cy5.5, anti-Ly6G-PerCP/Cy5.5, anti-CD24-PE/Cy7, anti-CX3CR1-APC, anti-CCR5-APC, anti-Ly6C-APC/Cy7, anti-CD45-BV421, anti-I-A/I-E-BV605 (all from BioLegend), anti-CD19-PerCP/Cy5.5, anti-CD11b-AF700 (BD Biosciences, Franklin Lakes, NJ, USA), anti-CCR2-APC (R&D Systems, Minneapolis, MN, USA), and anti-F4/80-PE (Thermo Fisher Scientific) for 20 minutes on ice. After surface staining, dead cells were stained with Zombie aqua™ Fixable Viability Kit (BioLegend). For the intracellular staining of TLR7, cells were fixed and permeabilized with the BD Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD Biosciences) according to the manufacturer's instructions. Then, cells were stained with anti-TLR7-PE (BD Biosciences). Data were acquired on a FACS LSR Fortessa (BD Biosciences) and the percentage of each cell population and mean fluorescence intensity were analyzed using FlowJo software (TreeStar Inc, Ashland, OR, USA).

Cell Sorting

CD11b positive cells were enriched using CD11b microbeads (Miltenyi Biotec) after the isolation of single cells from the blood, spleens, and kidneys of mice as described above. Cells were stained with the fluorochrome-conjugated antibody described above and sorted using a FACSAria Fusion cell sorter (BD Biosciences). Sorted cells were used for RNA-seq, quantitative real-time polymerase chain reaction (qRT-PCR), or cell transfer.

RNA-Seq Analysis

Total RNA was isolated from sorted cells using an RNeasy Micro Kit (Qiagen, Hilden, Germany). RNA-seq libraries were generated with the Ovation SoLo RNA-Seq System, Mouse kit (NuGEN, Redwood City, CA, USA) using 5 ng of total RNA. The cDNA libraries were sequenced by 50-base single-read sequencing on an Illumina HiSeq 2500 sequencer (Illumina, San Diego, CA, USA). The sequencing run and base call analysis were performed according to the HiSeq 2500 System Guide with TruSeq SBS kit v3-HS. After sequencing, raw sequence data were generated with processing by CASAVA-1.8.4 version RTA 1.17.20.0. Reads were mapped to the mm10 genome with tophat2. Normalized FPKM values and differential gene expression analyses were generated with Cuffdiff and CummeRbund. Q-values (Benjamini-Hochberg correction) lower than 0.05 were considered significant. Gene ontology enrichment analysis was performed using metascape (http://metascape.org). Heatmaps were generated using Heatmapper (http://www.heatmapper.ca/).

Stimulation of Monocytes and RNA Preparation for qRT-PCR

Sorted monocytes from the peripheral blood of NZB/NZW mice were placed in a round bottom 96-well plate at 3×10^4 cells per well in RPMI medium supplemented with fetal bovine serum, L-glutamine, penicillin, and streptomycin as described above. Then, cells were stimulated with IMQ (R837) (5 μg/ml; *In vivo*Gen, San Diego, CA, USA) or 2′3′-cGAMP (25 μg/ml; *In vivo*Gen) for 2 h at 37°C in a 5% CO_2 incubator. After incubation, RNA was obtained using an RNeasy Micro Kit.

qRT-PCR Analysis

cDNA was prepared from total RNA by reverse transcription with ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan). Fast SYBR Green Master Mix (Thermo Fisher Scientific) was used for the amplification of *Itga9, Itgad, Mertk, Vcam1, Cxcl13, Ccl5, Il6*, and *Il10* according to the manufacturer's instructions. Results were normalized to *Gapdh*. The following primers were used: *Itga9* forward: 5′-TGTTTTGGCCTGTGCCCATC-3′; *Itta9* reverse: 5′-GGGAATCAGCACCTTGCCTT-3′; *Itgad* forward: 5′-TCCAGAAAGTGGTAGACAGCAA-3′; *Ittad* reverse: 5′-GAGTGTTGTAGTGGCAACCTG-3′; *Mertk* forward: 5′-AGCTGGCATTTCATGGTGGA-3′; *Mertk* reverse: 5′-CTGCACACTGGCTATGCTGA-3′; *Vcam1* forward: 5′-AGAACTAC

AAGTCTACATCTCTCCC-3′; *Vcam1* reverse: 5′-GTCACAGCA CCACCCTCTT-3′; *Cxcl13* forward: 5′-CCACCTCCAGGCAGA ATGAG-3′; *Cxcl13* reverse: 5′-TGGGCTTCCAGAATACCGTG-3′; *Ccl5* forward: 5′-CAGTCGTGTTTGTCACTCGAA-3′; *Ccl5* reverse: 5′-AGAGCAAGCAATGACAGGGA-3′; *Il6* forward: 5′-CACTTCACAAGTCGGAGGCT-3′; *Il6* reverse: 5′-CTG CAAGTGCATCATCGTTGT-3′; *Il10* forward: 5′-CTTTAA GGGTTACTTGGGTTGCC-3′; *Il10* reverse: 5′-TTCTGGG CCATGCTTCTCTG-3′; *Gapdh* forward: 5′-GCAAGGACA CTGAGCAAGAGA-3′; and *Gapdh* reverse: 5′-AGGCCCCT CCTGTTATTATG-3′. TaqMan™ Gene Expression Assay was used for *Ifna4* (Mm00833969_s1), *Ifna5* (Mm00833976_s1), and *Ifnb1* (Mm00439546_s1). Results were normalized to *Gapdh* (Mm99999915_g1). THUNDERBIRD® Probe qPCR Mix (Toyobo) was used for amplification according to the manufacturer's instructions. All qRT-PCR were performed with a 7500 Fast Real-Time PCR System (Thermo Fisher Scientific). Fold-changes in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method.

Monocyte Transfer

Single cells were obtained from the peripheral blood and spleens of IMQ induced or control NZB/NZW mice. Cell sorting was conducted as described above and monocytes were isolated. Monocytes were labeled with CellTrace™ Cell Proliferation Kits (Thermo Fisher Scientific) according to the manufacturer's instructions. Cells were suspended in PBS and 3–5 $\times 10^5$ cells were transferred into recipient mice through the tail vein.

Bone Marrow Transplantation

Using an MBR-1505R2 system (Hitachi Power Solutions, Hitachi, Japan), $EGFP^{-/-}$ mice were irradiated twice with 600 rad 4 h apart. Before irradiation, mice kidneys were protected by covering the abdominal part with a self-made protector consisting of a 0.6 mm thick lead band. This was used to prevent tissue injury and keep resident cells in the tissues (19). A day after irradiation, 1×10^6 total bone marrow cells harvested from the femurs of $EGFP^{+/-}$ mice were suspended in PBS and transferred into irradiated recipient mice through the tail vein. Three weeks after BM reconstruction, chimerism was confirmed by the FACS analysis of peripheral blood.

Statistical Analysis

Statistical analyses (except for RNA-seq) were performed using Prism software (Graphpad, La Jolla, CA, USA). Significance was determined by Student's *t*-test or one-way ANOVA followed by the post-tests described in the figure legends.

RESULTS

Different Spatial and Temporal Increase of Monocyte-Like Cells Induced by IMQ Is Accompanied by the Development of Nephritis

Monocytes can be identified as $CD11b^+CD115^+CX3CR1^+$cells and roughly classified into two types: $Ly6C^{hi}$ and $ly6C^{lo}$ monocytes (12, 15). In the steady state, peripheral blood $CD11b^+$ cells among lineage marker (Lin: CD3, CD19, Ly6G, NK1.1) negative cells were mostly composed of monocytes that expressed CD115 and CX3CR1 (**Supplementary Figures 1A, B**). Considering the importance of TLR7 signals in monocytes and monocyte-related cells in the pathogenesis of SLE (11, 18), we focused on the expression of TLR7 in relation to monocytes. $Ly6C^{hi}$ monocytes expressed TLR7 at a low to intermediate level and $Ly6C^{lo}$ monocytes expressed intermediate levels of TLR7 (**Figure 1A**). There was no significant change in the number of peripheral blood monocytes in mice treated with short-term (3 days) topical IMQ. In contrast, long-term (35 days) IMQ treatment increased the number of peripheral blood $Ly6C^{lo}$ monocytes (**Figure 1B**). At the same time, we investigated the cervical lymph nodes, the draining lymph nodes of IMQ-treated ears. In the steady state, most $CD11b^+Lin^-$ cells in the lymph nodes were $Ly6C^{lo}TLR7^-$ (**Figure 1C** and **Supplementary Figure 1C**), whereas cells with expressions of Ly6C and TLR7 similar to that of peripheral blood monocytes (shown as $Ly6C^{hi}$ mono-like and $Ly6C^{lo}$ mono-like) were scarce. $Ly6C^{hi}$ monocyte-like cell numbers tended to increase with short-term IMQ treatment and were significantly increased with long-term IMQ treatment (**Figure 1D**). $Ly6C^{lo}$ monocyte-like cell numbers also increased with the long-term treatment.

For the kidneys, we had to exclude MF from $CD11b^+Lin^-$ cells before the analysis of monocytes because there were high numbers of TLR7-expressing MF in the FACS gate. In the steady state, $CD11b^+Lin^-$ cells in kidneys were roughly classified into three subsets by the expression of F4/80 and MHC class II (**Figure 1E** and **Supplementary Figure 1D**). In the kidneys, MHC II^{hi} cells were divided into $F4/80^+CD64^+$ MF and $F4/80^-CD64^-$ DC (20). MHC II^{lo} cells were considered to include monocyte-derived cells (21). Because F4/80 is a marker of MF, MHC II^{lo} $F4/80^-$ cells are considered monocyte-like cells that comprise $Ly6C^{hi}$ and $Ly6C^{lo}$ cells based on the expressions of Ly6C and TLR7, which were observed in blood monocytes (**Figure 1E**). $Ly6C^{hi}$ monocyte-like cell numbers did not increase with short-term nor long-term treatment, although long-term treatment increased $Ly6C^{lo}$ monocyte-like cell numbers (**Figure 1F**).

These observed changes of monocytes and monocyte-like cells by long-term treatment were accompanied by the development of proteinuria, which indicates the development of nephritis (**Supplementary Figure 2A**). Immunofluorescent staining of cervical lymph nodes revealed monocyte-like round shaped $CD11b^+$ cells mainly in the T cell zones and high numbers of $CD3^+$ cells in the follicles of long-term treated mice indicating germinal center formation (**Figure 2A**). Deposition of immune-complex in the glomerulus shown by the positive staining for complement C3 and IgG indicated a lupus-like pathogenesis in the nephritis (**Figure 2B**). Increased $CD11b^+$ and $CD3^+$ cell numbers were observed in the glomerulus and interstitium (**Figures 2B, C**). Increased $CD11b^+$ cells can be explained by the increase of $Ly6C^{lo}$ monocytes (round cells) and the upregulated expression of CD11b in MF in the interstitium. The CD11b expression of MF was higher in IMQ-induced lupus mice than in controls (**Supplementary Figure 2B**).

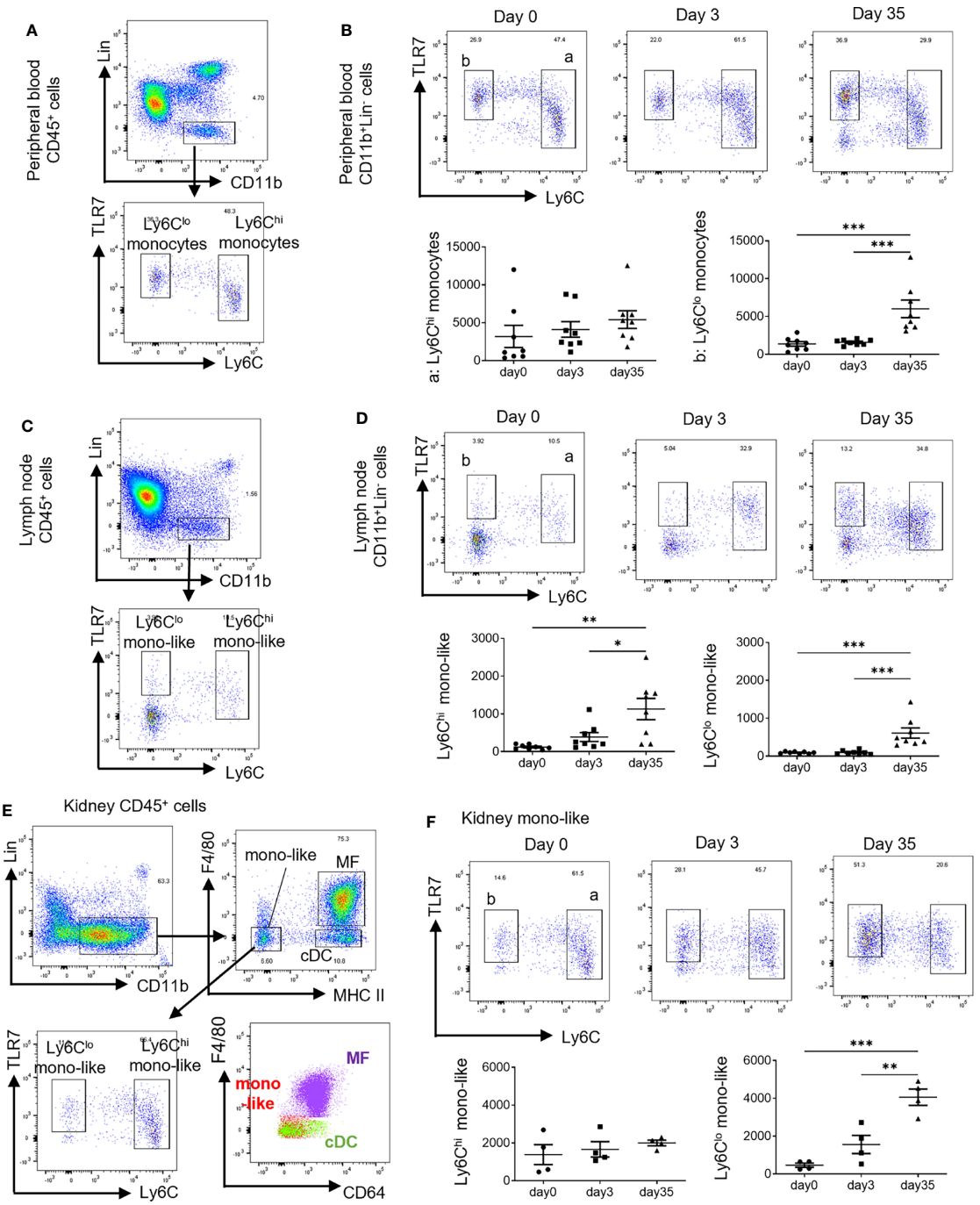

FIGURE 1 | Different spatial and temporal increases in monocyte-like cells by treatment with IMQ. In the short-term analysis, C57BL/6 mice were treated with 40 mg of IMQ on days 0 and 2, then sacrificed on day 3. In the long-term analysis, mice were treated with 40 mg of IMQ three times a week for 35 days. **(A)** Monocytes were identified as $CD11b^+Lin^-$ cells in the peripheral blood. $Ly6C^{hi}$ monocytes and $Ly6C^{lo}$ monocytes are shown relative to the expression of TLR7. **(B)** The change of peripheral $Ly6C^{hi}$ and $Ly6C^{lo}$ monocytes at 3 and 35 days after the application of IMQ. **(C)** Among $CD11b^+Lin^-$ cells in the lymph nodes, $Ly6C^{hi}$ monocyte-like cells and $Ly6C^{lo}$ monocyte-like cells were identified based on the expressions of Ly6C and TLR7. **(D)** The change of cervical lymph node monocyte-like cells 3 and 35 days after the application of IMQ. **(E)** Kidney monocyte-like cells were identified as $CD11b^+Lin^-F4/80^-MHCII^{lo}$ cells. **(F)** Changes in kidney monocyte-like cells at 3 and 35 days after the application of IMQ. **(B, D, F)** show the combined data of two experiments. Numbers in the graph represent the number of cells per 100,000 $CD45^+$ cells. Symbols represent individuals and horizontal lines indicate the mean and SEM. *P < 0.05, **P < 0.01, and ***P < 0.001 by one way ANOVA and *post hoc* Tukey's multiple comparison test. IMQ, imiquimod; Lin, lineage marker (CD3, CD19, Ly6G, NK1.1); mono-like, monocyte-like cells; MF, macrophages; cDC, conventional dendritic cells.

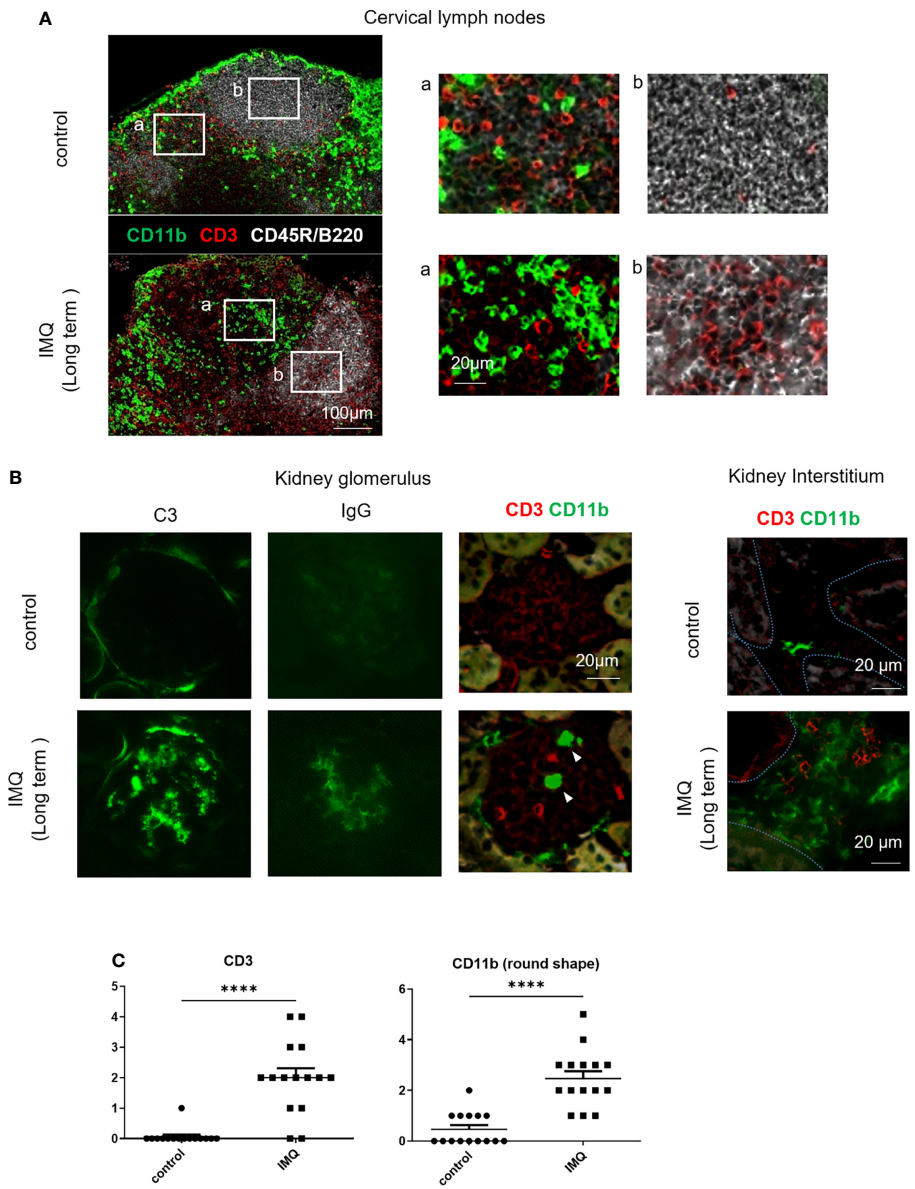

FIGURE 2 | Immunofluorescent staining of cervical lymph nodes and kidneys of IMQ-induced lupus mice. Mice were treated with 40 mg of IMQ three times a week for 5 weeks. Frozen sections were stained with the indicated antibodies. **(A)** Immunofluorescent staining of cervical lymph nodes from control and IMQ-induced lupus mice. **(B)** Immunofluorescent staining of kidneys from control and IMQ-induced lupus mice. Arrowheads indicate round cells in the glomeruli. Dashed lines in the right panel outline the tubular epithelium. **(C)** $CD3^+$ and $CD11b^+$ round cells in five representative glomeruli were counted twice from three mice. Cell number per glomerulus is shown. **(A, B)** are representative images of two experiments (n=3). ****P < 0.0001 by Student's *t*-test. T cell zones (a) and follicles (b) were magnified.

Monocyte-Like Cells and MF in the Kidneys Are Replaced by Peripheral Blood Cells After the Long-Term Application of IMQ

Analysis of monocyte-like cells and MF in kidneys under the treatment of IMQ, showed short-term IMQ treatment decreased MF numbers. In contrast, $F4/80^+$ cells expressing a low level of MHC II (MHC II^{low}MF) were increased at the same time. The long-term application of IMQ decreased MHC II^{low}MF and increased MF. The number of monocyte-like cells, especially $Ly6C^{lo}$ mono-like cells, was increased in the long-term treated mice (**Figures 1F**, **3A, B**). Although MF in the kidneys are usually not replenished by circulating monocytes under steady state conditions, they are replaced by monocyte-derived cells if there is inflammation in the kidneys (21). To investigate whether $CD11b^+Lin^-$ cells in the kidneys of IMQ treated mice were replaced by circulating monocytes, we analyzed the dynamics of $CD11b^+$ cells in the kidneys using a bone marrow chimera

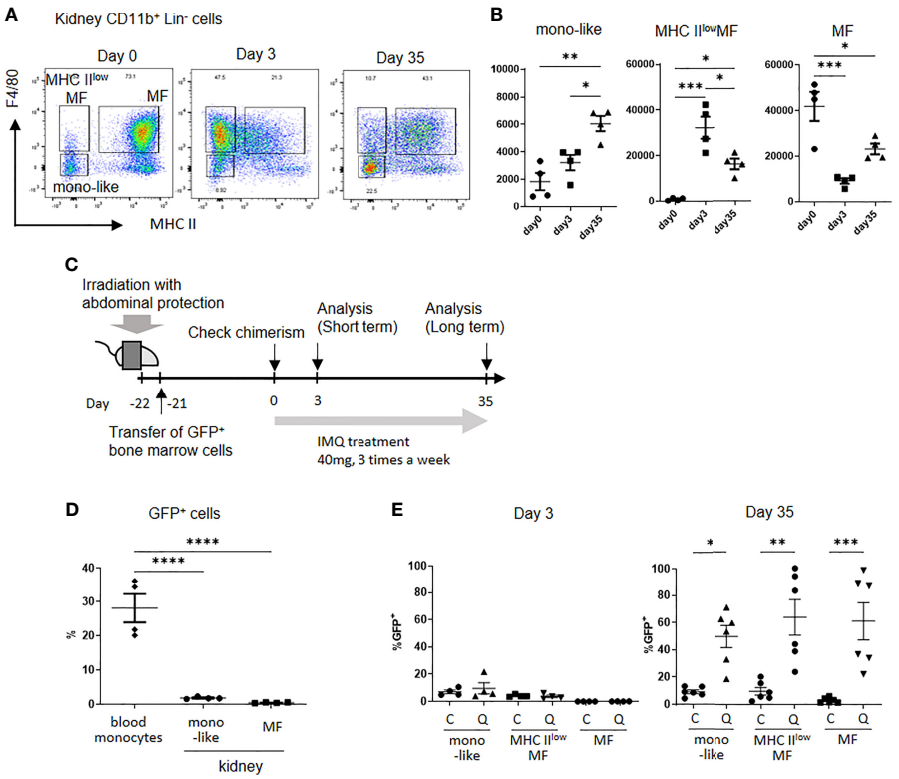

FIGURE 3 | Kidney monocyte-like cells and MF are replaced by peripheral blood cells after the long-term application of IMQ. **(A)** Representative plot of kidney CD11b$^+$Lin$^-$ cells from control (left), short-term IMQ treatment (middle), and long-term IMQ treatment (right) mice. In the short-term analysis, mice were treated with 40 mg of IMQ on days 0 and 2 then sacrificed on day 3. In the long-term analysis, mice were treated with 40 mg of IMQ three times a week for 35 days. **(B)** The change in kidney monocyte-like, MHC II^{low}MF (MHC II^{lo}F4/80$^+$), and MF (MHC II^{hi}F4/80$^+$) cells at 3 and 35 days after the application of IMQ. **(C)** Kidney-protected GFP$^-$ mice were irradiated and GFP$^+$ cells were transferred. Three weeks after transplantation, treatment with topical IMQ was started. **(D)** Percentages of GFP positive cells 21 days after bone marrow transplantation. Partial bone marrow chimera and protection of kidney resident cells were confirmed. **(E)** Percentages of relative GFP$^+$ cells in control **(C)** and IMQ-treated mice (Q) at days 3 and day 35 after treatment. The percentages were adjusted according to the blood GFP$^+$ cell ratio. **(B, D, E)** show the combined data of two experiments. Symbols represent individuals and horizontal lines indicate the mean and SEM. In **(B)**, the number in the graph represents the number of cells per 100,000 CD45$^+$ cells. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 by one way ANOVA and *post hoc* Tukey's multiple comparison test. mono-like: monocyte-like cells, MHC II^{low}MF: MHC II^{lo} macrophage-like cells, MF: macrophages.

generated by the transplantation of B6-EGFP$^{+/-}$ bone marrow cells into B6-EGFP$^{-/-}$ mice (**Figure 3C**). Mice were irradiated with a lead band that covered the abdomen to protect kidney resident cells. Approximately 30% of circulating monocytes became GFP positive, whereas 1%–2% of monocyte-like cells and 0.5% of MF in the kidneys were GFP positive 3 weeks after bone marrow transplantation (**Figure 3D**). When IMQ was applied over the short-term, there was no increase in GFP positive cells in kidney monocyte-like cells (control 7% vs IMQ 9%), MHC II^{low}MF (control 2% vs IMQ 4%), or MF (control 0% vs IMQ 0%) (**Figure 3E**). However, with the long-term application, approximately 50%–60% of monocyte-like cells, MHC II^{low}MF, and MF were replaced by circulating monocytes when normalized by the percentages of GFP positive cells in the circulating monocytes (monocyte-like cells: control 9% vs IMQ 50%; MHC II^{low}MF: control 10% vs IMQ 66%; MF: control 3% vs IMQ 61%) (**Figure 3E**). These results indicate the short-term increase of MHC II^{low}MF did not result from replenishment by circulating monocytes but probably as a result of a phenotypical change of MF in the kidneys. Monocyte-like cells, MHC II^{low}MF, and MF observed in long-term treated mice originated, at least in part, from infiltrated circulating hematopoietic cells.

RNA-Seq Analysis Reveals the Upregulation of Adhesion-Related Genes in Ly6C^{lo} Monocytes and Inflammatory Features of Kidney Monocyte-Like Cells

These findings indicated that long-term IMQ treatment increased Ly6C^{lo} monocytes in the circulation and Ly6C^{lo} monocyte-like cells in affected organs such as kidneys. The monocyte-like cells in the kidneys partially originated from the infiltration of circulating hematopoietic cells. To characterize these monocyte-like cells, we conducted RNA-seq analysis of peripheral blood monocytes and monocyte-like cells in the kidneys. Monocytes and monocyte-like cells were identified as

$CD11b^+Lin^-MHC\ II^{lo}CD64^-F4/80^-CX3CR1^+$ cells (**Supplementary Figure 3**). CD115 was not a suitable marker because of its internalization during the cell isolation process (22). Thus, we used CX3CR1 as a substitute marker for monocytes and monocyte-like cells after the exclusion of MF and DC by excluding $CD64^+$, $F4/80^+$, and MHC II^{hi} cells. Isolated cells were indicated using the following abbreviations: B-Ly6C^{hi}: blood Ly6C^{hi} monocytes; B-Ly6C^{lo}: blood Ly6C^{lo} monocytes; K-Ly6C^{lo}: kidney Ly6C^{lo} monocyte-like cells. RNA was obtained from these cells and analyzed. The number of reads per sample ranged from 16 to 22 million, and 96%–97% were successfully mapped onto the mm10 genome. Among them, we found 8542 differentially expressed genes (DEG).

Using the k-means clustering method, DEG were classified into 20 clusters (**Supplementary Figure 4**). Clusters 16, 17, and 18 were characterized by the genes that were most upregulated in IMQ-induced K-Ly6C^{lo} (**Supplementary Figure 4**). Among them, cluster 17 was characterized by genes also upregulated in IMQ-induced B-Ly6C^{lo}. In this cluster, an enrichment of genes related to cell adhesion was observed (**Figure 4A**). Among the 64 genes in cluster 17, 13 genes had the gene ontology name "cell adhesion" and when compared among peripheral blood monocytes, these genes were the most upregulated in IMQ-induced B-Ly6C^{lo} (**Figure 4B**). The upregulated genes included genes of integrins such as *Itga9* and *Itgad*, which were also confirmed by qRT-PCR analysis (**Figure 4C**). Among the genes in clusters 16, 17, and 18, were those characteristic of MF in the kidneys including *Adam33* (Cluster 16), *Vcam1*, *Mertk*, *C1qa*, *Itga9* (Cluster 17), *CD72*, *C1qc*, and *Itga8*, (Cluster18) (23). IMQ-induced K-Ly6C^{lo} showed the upregulation of MF-related genes compared with monocytes and monocyte-like cells (**Figure 5A**). In terms of chemokines and cytokines, the expression of *CCl5* was generally higher in IMQ-treated mice than controls and it was noteworthy that *Ccr5*, the CCL5 receptor gene, was upregulated in IMQ-induced K-Ly6C^{lo}. The upregulation of several genes encoding cytokines and chemokines including *Il10*, *Il6*, and *Cxcl13* were also observed in IMQ-induced K-Ly6C^{lo}. Furthermore, *Cxcr3* was upregulated in monocytes and monocyte-like cells from IMQ-treated mice by RNA-seq analysis, which was in accordance with the recently reported importance of the IP-10/CXCR3 axis in human lupus nephritis (24).

To assess whether IMQ-induced K-Ly6C^{lo} were macrophage-like and inflammatory, we analyzed kidney monocyte-related

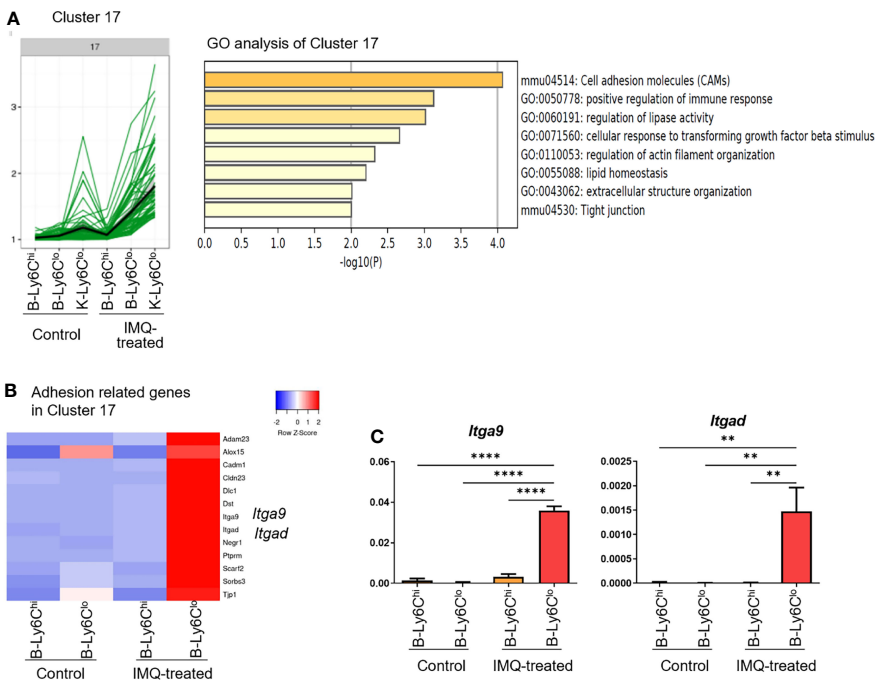

FIGURE 4 | RNA-seq analysis reveals the upregulation of adhesion-related genes in peripheral Ly6C^{lo} monocytes of IMQ-induced lupus mice. **(A)** In the RNA-seq analysis, cluster 17 among 20 clusters was characterized by genes highly expressed in Ly6C^{lo} monocyte-like cells in the kidneys and Ly6C^{lo} blood monocytes in IMQ-treated mice. Gene ontology analysis revealed adhesion-related genes were enriched in this cluster. **(B)** Thirteen genes with the gene ontology term "cell adhesion" in cluster 17. These genes were upregulated in blood Ly6C^{lo} monocytes from IMQ-treated mice. **(C)** qRT-PCR analysis of *Itga9* and *Itgad* in blood monocytes. RNA-seq and q-RT PCR results are of pooled samples from four groups of mice each analyzed individually (16 control or 5 IMQ-treated mice were used for each pooled sample in the RNA-seq analysis. Ten control or 3 IMQ-treated mice were used for each pooled sample in the q-RT PCR analysis). In the qRT-PCR analysis, the y-axis shows the fold change expression in comparison with the expression of *Gapdh*. Bar and horizontal lines indicate the mean and SEM. $^{**}P < 0.01$ and $^{****}P < 0.0001$ by one way ANOVA and *post hoc* Dunnett's multiple comparison test. B-Ly6C^{hi}; blood Ly6C^{hi} monocytes, B-Ly6C^{lo}; blood Ly6C^{lo} monocytes, K-Ly6C^{lo}; kidney Ly6C^{lo} monocyte-like cells.

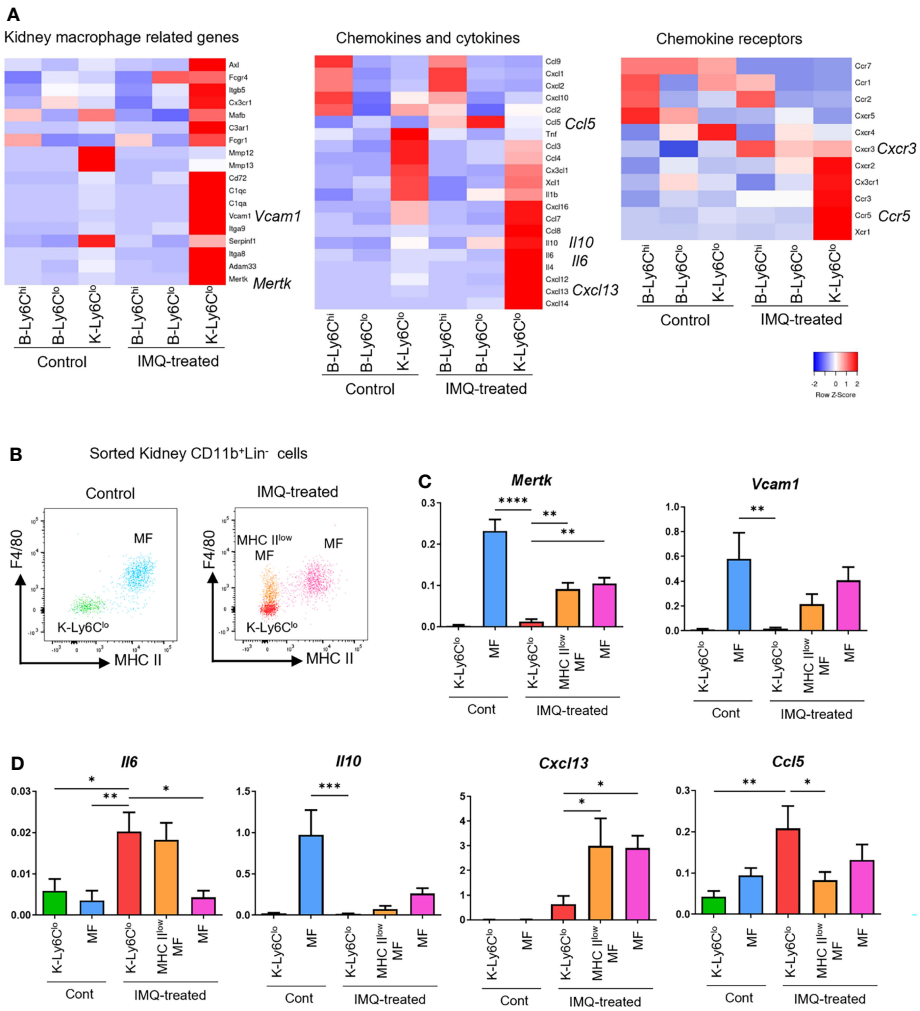

FIGURE 5 | Gene expression features of monocytes, monocyte-like cells, and MF in IMQ-induced lupus mice. **(A)** RNA-seq analysis of blood monocytes and kidney monocyte-like cells in the controls and IMQ-treated mice. **(B)** Plot of sorted monocyte-like cells and MF from control and IMQ-treated mice. Each cell type was sorted from kidney CD11b^{+}Lin^{-} cells as follows. K-Ly6C^{lo} = F4/80^{-}MHCII^{lo}Ly6C^{lo}CX3CR1^{+} cells; MHC II^{low}MF = MHC II^{lo}F4/80^{+}cells; MF = MHC II^{hi}F4/80^{+}cells. **(C, D)** qRT-PCR analysis of sorted kidney cells from control and IMQ-treated mice. RNA-seq and qRT-PCR results are of pooled samples from four groups of mice each analyzed individually (16 control or 5 IMQ-treated mice were used for each pooled sample in the RNA-seq analysis. Ten control or 3 IMQ-treated mice were used for each pooled sample in the qRT-PCR analysis). In the qRT-PCR analysis, the y-axis shows the fold change expression in comparison with the expression of *Gapdh*. Bar and horizontal lines indicate the mean and SEM. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 by one way ANOVA and *post hoc* Dunnett's multiple comparison test. B-Ly6C^{hi}, blood Ly6C^{hi} monocytes; B-Ly6C^{lo}, blood Ly6C^{lo} monocytes; K-Ly6C^{lo}, kidney Ly6C^{lo} monocyte-like cells. MHC II^{low}MF, MHC II^{lo} macrophage-like cells; MF, macrophages.

cells and assessed macrophage-related genes and characteristic cytokine and chemokine genes that were upregulated in IMQ-induced K-Ly6C^{lo} by RNA-seq analysis. MHCII^{low}MF, MF, and K-Ly6C^{lo} were sorted from kidney CD11b^{+}Lin^{-} cells of control and IMQ-treated mice (**Figure 5B**) and gene expressions were analyzed by qRT-PCR. *Mertk* and *Vcam1*, characteristic macrophage-specific genes were upregulated in IMQ-induced K-Ly6C^{lo} by RNA-seq analysis but at a lower level than that in MF (**Figure 5C**). Although *Il6* and *Il10* appeared upregulated in IMQ-induced K-Ly6C^{lo} by RNA-seq analysis, PCR analysis revealed that the expression of *Il10* in K-Ly6C^{lo} was minimal compared with that in control MF (**Figure 5D**). *Il6* was upregulated in IMQ-induced K-Ly6C^{lo} compared with MF. RNA-seq analysis showed the upregulation of *Cxcl13* in IMQ-induced K-Ly6C^{lo} from IMQ-treated mice and PCR analysis revealed its expression was even higher in MHCII^{low}MF and MF from IMQ-treated mice. The CCL5/CCR5 axis was previously reported to be involved in the recruitment of Ly6C^{lo} monocytes to tissues (25). *Ccl5* was upregulated in IMQ-induced K-Ly6C^{lo} and lower in MF (**Figure 5D**).

These results indicated that the upregulated adhesion-related genes in IMQ-induced B-Ly6C^{lo} were likely related to the infiltration of monocytes to the kidneys. Upregulated *Ccl5* in monocytes and monocyte-like cells in IMQ-treated mice and

upregulated *Ccr5* in kidney monocyte-like cells by RNA-seq data suggested the involvement of the CCL5/CCR5 axis in the infiltration of monocytes into the kidneys. Upregulated *Il6* in IMQ-induced K-Ly6C^{lo} indicated the inflammatory features of these cells. The expressions of *Il6* and *Il10* indicated that inflammatory features were decreased related to the differentiation of monocytes to MF. Although MF in IMQ-treated mice had similar gene expression patterns to the control MF, *Cxcl13* expression in MF was limited to IMQ-treated mice, suggesting its importance in the disease pathogenesis.

IMQ-Induced Conditions Are Characterized by the Infiltration of Ly6C^{lo} Monocytes Into Tissues

The upregulated expressions of adhesion-related genes in Ly6C^{lo} monocytes of IMQ-treated mice demonstrated by RNA-seq analysis suggested they were more prone to infiltrate into tissues. To clarify the infiltrating ability of monocytes into tissues, we performed an adoptive transfer experiment using sorted monocytes from IMQ-induced lupus mice.

NZB/NZW mice are a well-known model of the spontaneous development of lupus-like disease. In this model, along with the development of disease, Ly6C^{lo} monocytes and Ly6C^{lo} monocyte-like cells were increased spontaneously in the blood and kidneys of aged mice (**Supplementary Figure 5A**). Similar to IMQ-induced lupus mice, Ly6C^{hi} monocytes tended to be increased in the lymph nodes whereas Ly6C^{lo} monocytes and monocyte-like cells were dominant in the peripheral blood and kidneys. This increase of monocytes and monocyte-like cells was promoted by the application of IMQ to the ears of NZB/NZW mice, and the early development of nephritis was indicated by the development of proteinuria (**Supplementary Figures 2A** and **5A–D**). Thus, the advantage of a shorter time course of IMQ-induced disease development in NZB/NZW mice led us to use this model to analyze the function of monocytes in further experiments.

In the adoptive transfer experiment, monocytes were isolated from the peripheral blood and spleens because these cells are identical (26). Ly6C^{hi} and Ly6C^{lo} monocytes (CD11b^{+}Lin^{-}F4/80^{-} MHC II^{lo}CD115^{+}) from control NZB/NZW mice and IMQ-treated NZB/NZW mice were labeled by CFSE and transferred to recipient mice. Two days after transfer, the cervical lymph nodes, spleens, and kidneys of recipient mice were analyzed (**Figure 6A**). We recovered higher numbers of CFSE^{+} transferred Ly6C^{lo} monocytes from the spleens and kidneys of IMQ-treated mice compared with control mice although there was a negligible infiltration of these cells to the lymph nodes (**Figures 6B, C**). Transferred Ly6C^{lo} monocytes in spleens upregulated MHC class II although monocytes recovered in the kidneys maintained their low expression of MHC II. Ly6C^{hi} monocytes in spleens showed a similar tendency for the high expression of MHC class II compared with those in the kidneys, although the difference was not statistically significant (**Figure 6D**). Ly6C^{lo} monocytes recovered in the kidneys maintained their low expression of F4/80. Thus, they maintained F4/80^{-}MHC II^{lo} monocyte-like surface markers in the short-term after infiltrating into the kidneys (**Figure 6E**). These findings suggest that Ly6C^{lo} monocytes in IMQ-treated mice were more prone to infiltrate into tissues and that they underwent different phenotypical changes dependent on the tissues they infiltrated.

Ly6C^{hi} Monocytes and Ly6C^{lo} Monocytes Have Different Inflammatory Features Upon Stimulation

To further analyze the different features of Ly6C^{hi} and Ly6C^{lo} monocytes in the lupus-like inflammatory environment, peripheral blood monocytes from control and IMQ-treated NZB/NZW mice were sorted and stimulated *in vitro*. Type I interferons are important for the pathogenesis of SLE and serum from SLE patients induced type I interferon-stimulated genes dependent on the agonist of stimulator of interferon genes (STING) activity (27). A study of human SLE reported IFN-α production by monocytes stimulated by 2′3′-cyclic-GMP-AMP (cGAMP), an agonist of STING, positively correlated with SLE disease activity (28). Therefore, we used IMQ and 2′3′-cGAMP to stimulate monocytes and analyzed the gene expressions of proinflammatory cytokines and type I interferons. Upon IMQ stimulation, Ly6C^{hi} and Ly6C^{lo} monocytes tended to upregulate *Il1b*, *Il6*, and *Tnf* compared with 2′3′-cGAMP. Ly6C^{hi} monocytes showed a higher upregulation of *Il6* than Ly6C^{lo} monocytes and Ly6C^{lo} monocytes showed a higher upregulation of *Tnf* in accordance with a previous report of human classical CD14^{+} and non-classical CD14^{lo} non-classical monocytes (10). These features were lost and responses tended to be reduced in monocytes from IMQ-treated mice (**Figure 7A**). However, type 1 IFN genes were upregulated in monocytes when stimulated with 2′3′-cGAMP but not IMQ. Although *Ifnb1* was upregulated similarly in Ly6C^{hi} and Ly6C^{lo} monocytes, IFN-α genes (*Ifna4* and *Ifna5*) were upregulated only in Ly6C^{hi} monocytes when stimulated with 2′3′-cGAMP (**Figure 7B**). This higher response of IFN-α genes in Ly6C^{hi} monocytes to 2′3′-cGAMP correlated with the higher expression of *Tmem173*, which encodes STING protein (mean FPKM of *Tmem173* was 59.78 in Ly6C^{hi} monocytes and 22.62 in Ly6C^{lo} monocytes by RNA-seq analysis).

Taken together, monocytes and monocyte-related cells are likely to be important for the development of lupus pathogenesis. They possess different spatial and temporal roles. The application of IMQ indicated Ly6C^{hi} monocytes responded in the early phase and highly expressed IFN-α genes after the stimulation of STING, a DNA sensor. In contrast, Ly6C^{lo} cells were markedly increased in the late phase. They showed a greater infiltration into tissues, which might be related to the upregulation of adhesion-related molecules when they are in circulation. Furthermore, the gene expressions of proinflammatory cytokines such as *Il6* and chemokines such as *Cxcl13* and *Ccl5* in Ly6C^{lo} monocyte-like cells in the kidneys indicate they are likely to be involved in the inflammatory response.

DISCUSSION

In the present study, we investigated the dynamic change of immune cells in the development of lupus-like disease in an

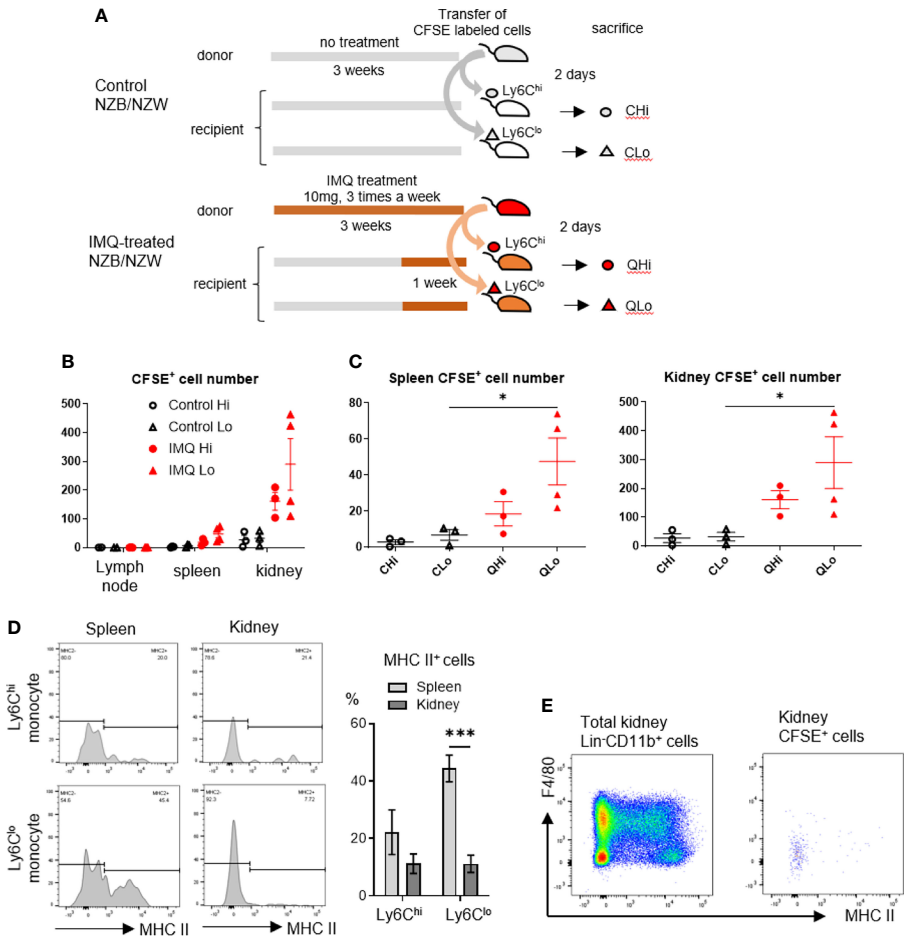

FIGURE 6 | Ly6C^{lo} monocytes from IMQ-treated mice are more prone to infiltrate into tissues. **(A)** Sorted monocytes from control or IMQ-treated (10 mg, three times a week for 3 weeks) NZB/NZW mice were transferred to recipient control or IMQ-treated mice and tissues were analyzed 2 days after transfer. **(B)** Transferred CFSE labeled monocytes recovered from tissues were analyzed. **(C)** Transferred CFSE labeled monocytes recovered from spleens and kidneys were analyzed. **(D)**: CFSE^{+}Ly6C^{lo} monocytes recovered from spleens showed upregulated MHC class II whereas most transfused Ly6C^{lo} monocytes in kidneys remained MHC class II negative. **(E)** Transferred Ly6C^{lo} monocytes in kidneys maintained F4/80^{-}MHC II^{lo} monocyte-like surface markers. In A and B, numbers of CFSE positive cells per 1×10^{5} CD45^{+} cells are shown. Numbers were adjusted according to the transferred cell number that was normalized to 1×10^{5}. **(B–D)** show the combined data of four experiments. Symbols represent individuals and horizontal lines indicate the mean and SEM. *P < 0.05 and ***P < 0.001 by one way ANOVA and *post hoc* Bonferroni's Multiple Comparison Test.

IMQ-induced lupus model focusing on monocytes and monocyte-derived cells.

Ly6C^{hi} monocyte-like cell numbers were increased in the cervical draining lymph nodes starting from the initial phase, which was in accordance with an acute inflammatory response. In the late phase, Ly6C^{lo} monocytes and monocyte-like cell numbers were increased throughout the body. Increased atypical monocytes were previously reported in aged NZB/NZW mice and other lupus models (29, 30). Thus, an increase in Ly6C^{lo} monocytes is a common feature shared among lupus models. Interestingly, Ly6C^{hi} cells continued to increase in the draining lymph nodes whereas an increase in Ly6C^{hi} cells was not observed in the affected kidneys throughout the entire disease course. From these results, we hypothesized that Ly6C^{hi} and Ly6C^{lo} cells have different temporal and spatial roles in the pathogenesis of SLE. Increased numbers of Ly6C^{hi} cells in lymphoid organs are likely to be related to the induction of autoimmunity as previously reported for monocyte-derived DC (moDC) during Tfh induction, a mechanism mediated by the stimulation of TLR7 expressed by moDC (11).

Another indication of the role of Ly6C^{hi} cells in the development of autoimmunity is the production of IFN-α. In the pathogenesis of SLE, type I IFN signals are considered to have an important role by inducing the expression of MHC II on antigen-presenting cells, expanding autoreactive T cells, and increasing the production of antibodies from B cells (31, 32). The increased expression of IFN-α genes by stimulation of the cGAS/STING pathway, which was not observed in Ly6C^{lo} cells, indicated that Ly6C^{hi} monocytes are a potential source of IFN-α. This is similar to the pristane-induced lupus model, in which the

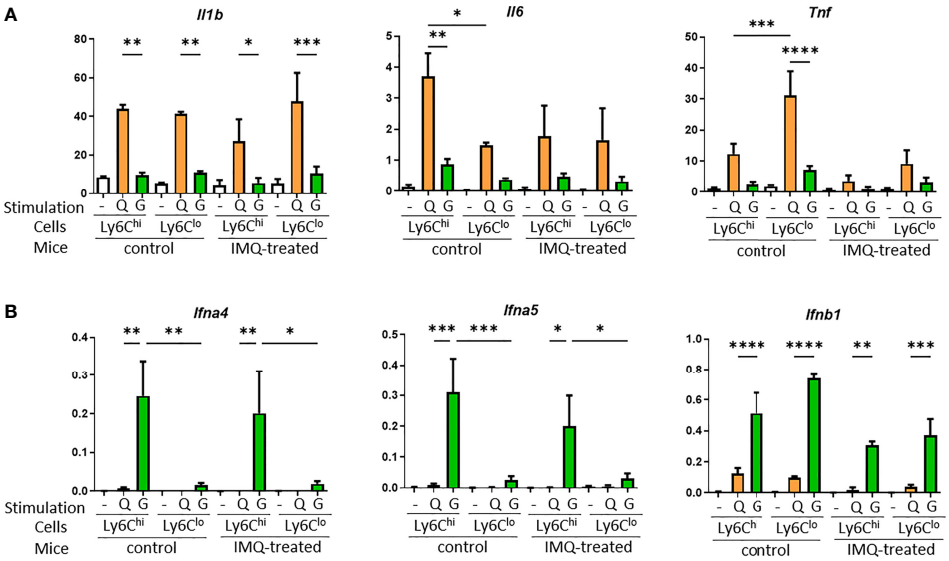

FIGURE 7 | Proinflammatory cytokine and type I interferon expression in monocytes upon stimulation with TLR7 or STING. Sorted monocytes from control or IMQ-treated NZB/NZW mice were stimulated by IMQ or the STING agonist, 2′3′-cGAMP. **(A)** Proinflammatory cytokines were upregulated by IMQ stimulation compared with 2′3′-cGAMP. **(B)** Type I interferon genes were upregulated by stimulation with 2′3′-cGAMP. Expression of IFN–α genes were mostly limited to Ly6C^{hi} monocytes. -, no stimulation; Q, IMQ stimulation; G, 2′3′-cGAMP stimulation. Bar and horizontal lines indicate the mean and SEM. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 by one way ANOVA and *post hoc* Bonferroni's Multiple Comparison Test.

source of type I interferon is thought to be immature Ly6C^{hi} monocytes specifically induced by pristane administration (33). In humans, IFN-α was produced by monocytes *via* the cGAS/STING pathway, and its activation was correlated with disease activity in SLE (28). These findings suggest a role for Ly6C^{hi} monocytes in the induction of Tfh and autoantibody production *via* the stimulation of TLR7 and the cGAS/STING pathway. Therefore, increased Ly6C^{hi} monocyte-derived cells in lymph nodes in the induction phase through to the late phase might be involved in the development of autoimmunity.

Ly6C^{lo} monocytes, the mouse counterparts of human CD16^{+} non-classical monocytes, accumulate in inflamed kidneys during lupus nephritis and are thought to be pathogenic in kidneys (17). In a lupus model of ABIN1 (Tnip1)-deficient mice, which have a dysfunction in the regulation of NF-κB, nephritis occurred with the accumulation of Ly6C^{lo} monocytes in the kidneys. Tnip1$^{-/-}$ Rag1$^{-/-}$ mice, which lack mature T cells and B cells, still developed glomerulonephritis, indicating Ly6C^{lo} monocytes are pathogenic and that adaptive immune system responses were not necessary for kidney pathology (4). In our study, Ly6C^{lo} monocyte-like cells were increased in long-term IMQ-treated kidneys and they originated, at least in part, from monocytes and appeared to have an inflammatory phenotype based on their upregulated inflammatory and chemokine genes including *Il6*, *Cxcl13*, and *Ccl5*.

Our monocyte transfer experiments demonstrated Ly6C^{lo} monocytes from IMQ-treated mice were more likely to infiltrate into organs, which might explain the increased number of Ly6C^{lo} monocyte-like cells in the kidneys of IMQ-treated mice. Gene expression analysis revealed peripheral Ly6C^{lo} monocytes in IMQ-treated mice had upregulated expressions of adhesion-related genes. Furthermore, the upregulation of *Ccl5* in the kidneys of Ly6C^{lo} monocyte-like cells was in accordance with the suggested involvement of the CCL5/CCR5 axis in the recruitment of Ly6C^{lo} monocytes in the CNS of another SLE model in FcγRIIB$^{-/-}$*Yaa* mice (25). Thus, IMQ-induced Ly6C^{lo} monocytes were likely to be primed to infiltrate into tissues and once they reached the kidneys, they had obtained an inflammatory and chemoattractive phenotype. Preventing monocytes from infiltrating the kidneys is a potential strategy for the treatment of nephritis although targeting chemokines and their receptors is still far from practical use (34, 35). Another possibility is targeting adhesion molecules such as integrins as Ly6C^{lo} monocytes that have upregulated *Itga9* and *Itgad*, as demonstrated in our study. Of note, the blockade of integrins might be a therapeutic target for rheumatoid arthritis and multiple sclerosis (36).

The roles of monocytes and MF in lupus nephritis require clarification. The classification of murine kidney mononuclear phagocytes, especially MF and DC, has long been confused, but recently CD64^{+} F4/80^{+} MHC II^{+} spindly processed cells in the interstitium were identified as MF (37), and resident MF were not replaced by bone marrow-derived cells in the steady state (21). Although resident MF were reported to be involved in the initiation of nephritis (38), it was not clear whether resident MF were involved in the long-term pathogenesis. In our study, short-term IMQ treatment caused the disappearance of MHC II^{hi} MF and the emergence of MHC II^{lo} F4/80^{+} cells, which did not accompany the influx of bone marrow-derived cells in the short-term. This was probably caused by the phenotypic change of

resident MF in the kidneys, which may be related to the initiation of nephritis. In contrast, mono-like cells and MF in the kidneys were replaced by bone marrow-derived cells in the long-term. Ly6C^{lo} monocytes were more likely to infiltrate into the kidneys and differentiated to inflammatory mono-like cells or MF. In terms of the pathogenicity and fate of infiltrated monocytes, murine kidney CD11c^{+} myeloid cells, which were differentiated from Ly6C^{lo} monocytes, were reported to promote lupus nephritis by interacting with CD4^{+} T cells (39). A study of human lupus nephritis reported that CD11c^{+} myeloid cells in the urine and kidneys were induced by peripheral monocytes and were proinflammatory. Furthermore, these cells produced proinflammatory cytokines, including IL-6 and IL-1β, when stimulated by nucleic acid, and were recruited to the kidneys *via* the IP10/CXCR3 axis (24), in accordance with our study in which *Cxcr3* was upregulated in the monocytes of IMQ-treated mice. Another study of lupus nephritis by the single cell RNA-seq analysis of kidneys suggested infiltrating non-classical monocytes started as inflammatory blood monocytes and differentiated into phagocytes and then an alternatively activated phenotype (40). Our study supports this idea of a differentiation trajectory based on differences in the expressions of genes encoding cytokines, chemokines, and resident macrophage-specific genes among Ly6C^{lo} monocyte-like cells and MF in the kidneys of controls and IMQ-treated mice. Furthermore, MF in control and IMQ-treated mice appeared phenotypically different in terms of the expression of *Cxcl13*. The upregulation of *Cxcl13* in IMQ-treated mice was noteworthy because this was not observed in control mice. SLE patients with lupus nephritis were reported to have significantly higher levels of serum CXCL13 than controls (41). CXCL13 is also related to the formation of tertiary lymphoid tissues (42) and the local production of autoantibodies (43). Podocytes produce proinflammatory mediators upon stimulation through CXCL13, indicating it might be another pathogenic factor of lupus nephritis (44). This evidence underscores the pathogenic importance of monocyte-derived MF and their expression of *Cxcl13* in the development of lupus nephritis. In summary, resident MF initially responded to stimulation in the kidneys and were probably involved in the initiation of inflammation. Then gradually, Ly6C^{lo} monocytes infiltrated into the kidneys. These monocytes were likely to have more inflammatory features upon arrival, and would differentiate to less inflammatory MF although these monocyte-derived MF were different from resident MF and appeared to have inflammatory features on the basis of their expression of *Cxcl13*.

This study demonstrated the different temporal and spatial roles of Ly6C^{hi} and Ly6C^{lo} monocytes in an IMQ-induced lupus model. We found that Ly6C^{hi} cells were increased in the lymph nodes and upregulated IFN-α genes upon stimulation of the cGAS/STING pathway. Ly6C^{lo} cells were increased in the late phase, and were more likely to infiltrate into tissues and become inflammatory cells in the kidneys. These differences in the functions of monocytes in terms of disease phase and organ involvement should be taken into account when considering monocytes involved in the pathophysiology of SLE.

DATA AVAILABILITY STATEMENT

The RNA-seq datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number can be found below The name of repository: DNA Data Bank of Japan (DDBJ) Accession number: DRA013503.

ETHICS STATEMENT

The animal study was reviewed and approved by laboratory animal experimentation at Juntendo University School of Medicine.

AUTHOR CONTRIBUTIONS

SM and AN designed the experiments. AN, MM, and AA performed the experiments and analyzed the data. TK and GM especially contributed to the experiment of monocyte stimulation and analysis. AN drafted the manuscript. DN and AC critically revised the manuscript. All the authors approved and reviewed the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.764557/full#supplementary-material

Supplementary Figure 1 | Identification of monocytes and monocyte-like cells in the peripheral blood and kidneys. **(A)** Classification of cells in the peripheral blood.

Cells in panel e are monocytes. Monocytes were further classified into Ly6C^{hi} monocytes and Ly6C^{lo} monocytes. **(B)** Most peripheral blood CD45$^+$CD11b$^+$Lin$^-$ cells expressed CD115 and CX3CR1. **(C)** Classification of cells in the cervical lymph nodes. Lymph node monocyte-like cells were identified based on Lin$^-$CD11b$^+$ and the expressions of Ly6C and TLR7. **(D)** Classification of cells in the kidneys. CD11b$^+$Lin$^-$ cells were classified roughly into three subsets of cells according to the expressions of F4/80 and MHC class II. F4/80$^+$ MHC II^{hi} cells and F4/80$^-$MHC II^{hi} cells were considered macrophages and cDC, respectively. F4/80$^-$MHC II^{lo} cells were identified as monocyte-like cells based on Lin$^-$CD11b$^+$ and the expressions of Ly6C and TLR7. **(A–D)** show representative plots of several experiments. mono-like: monocyte-like cells, MF, macrophages; cDC, conventional dendritic cells.

Supplementary Figure 2 | Proteinuria in IMQ-induced lupus mice and CD11b expression in kidney monocyte-like cells and macrophages. **(A)** Nephritis was induced by the long-term application of IMQ, which was confirmed by proteinuria in C57BL/6 mice (left) and NZB/NZW mice (right). C57BL/6 mice were treated with 40 mg topical IMQ three times a week for 5 weeks. NZB/NZW mice were treated with 10 mg topical IMQ three times for 3 weeks. Symbols represent individuals and horizontal lines indicate the mean and SEM. *P < 0.05 by Student's *t*-test. **(B)** Flow cytometry analysis of CD11b expression in Ly6C^{lo} monocyte-like cells and MF in the kidneys of control and IMQ-induced lupus mice. Representative histogram of fluorescence intensity in each subset is shown.

Supplementary Figure 3 | Gating strategy to sort blood monocytes and kidney monocyte-like cells for RNA-seq analysis. Blood monocytes (Ly6C^{hi} and Ly6C^{lo}) and kidney monocyte-like cells were sorted from control and IMQ-induced lupus mice. Lupus like-disease was induced by the application of 20 mg IMQ three times a week for 7 weeks. Representative plots of samples from control mice are shown.

Supplementary Figure 4 | RNA-seq analysis of peripheral monocytes and kidney Ly6C^{lo} monocyte-like cells. Cluster analysis of differentially-expressed genes (DEG) classified genes into 20 clusters. B-Ly6C^{hi}: blood Ly6C^{hi} monocytes, B-Ly6C^{lo}: blood Ly6C^{lo} monocytes, K-Ly6C^{lo}: kidney Ly6C^{lo} monocyte-like cells.

Supplementary Figure 5 | Ly6C^{lo} monocytes and monocyte-like cells are increased in lupus NZB/NZW mice. **(A)** Ly6C^{lo} monocytes are increased in the blood of aged NZB/NZW mice. Monocyte-like cells also tended to be increased in the lymph nodes. **(B)** Ly6C^{lo} monocyte-like cells are increased in the kidneys of aged NZB/NZW mice. **(C)** Application of topical IMQ (10 mg, three times a week for 4 weeks) to NZB/NZW mice increased Ly6C^{lo} monocyte and monocyte-like cells in the peripheral blood and lymph nodes. **(D)** Application of topical IMQ increased Ly6C^{lo} monocyte-like cells in the kidneys of NZB/NZW mice. The numbers in the graph indicate the cell number in 100,000 live CD45$^+$ cells. Symbols represent individuals and horizontal lines indicate the mean and SEM. *P < 0.05, **P < 0.01, and ***P < 0.01 by Student's *t*-test.

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REVIEW
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Pathological Osteoclasts and Precursor Macrophages in Inflammatory Arthritis

Tetsuo Hasegawa[1,2] *and Masaru Ishii*[1,3,4*]

[1] Department of Immunology and Cell Biology, Graduate School of Medicine and Frontier Biosciences, Osaka University, Osaka, Japan, [2] Division of Rheumatology, Department of Internal Medicine, Keio University School of Medicine, Tokyo, Japan, [3] World Premier International Research Center Initiative (WPI)-Immunology Frontier Research Center, Osaka University, Osaka, Japan, [4] Laboratory of Bioimaging and Drug Discovery, National Institutes of Biomedical Innovation, Health and Nutrition, Osaka, Japan

Macrophages comprise a variety of subsets with diverse biological functions, including inflammation, tissue repair, regeneration, and fibrosis. In the bone marrow, macrophages differentiate into multinucleated osteoclasts, which have a unique bone-destroying capacity and play key roles in physiological bone remodelling. In contrast, osteoclasts are also involved in inflammatory bone erosion in arthritis and it has been unclear whether the osteoclasts in different tissue settings arise from similar monocytoid precursors and share similar phenotypes. Rapid progresses in the sequencing technologies have provided many important insights regarding the heterogeneity of different types of osteoclasts. The application of single-cell RNA sequencing (scRNA-seq) to the osteoclast precursor-containing macrophages enabled to identify the specific subpopulation differentiating into pathological mature osteoclasts in joints. Furthermore, an intravital imaging technology using two-photon microscopy has succeeded in visualizing the real-time dynamics of immune cells in the synovial microenvironment. These technologies together contributed to characterize the unique macrophages in the inflamed synovium, termed "arthritis-associated osteoclastogenic macrophages (AtoMs)", causing the pathological bone destruction in inflammatory arthritis. Here, we review and discuss how novel technologies help to better understand the role of macrophages in inflammatory arthritis, especially focusing of osteoclastogenesis at the pannus-bone interface.

Keywords: macrophage, osteoclast, rheumatoid arthritis, single-cell RNA sequencing, intravital imaging

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****Correspondence:***
Masaru Ishii
mishii@icb.med.osaka-u.ac.jp

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INTRODUCTION

Macrophages are distributed throughout the body and possess a variety of biological activities, contributing to tissue homeostasis and a broad spectrum of pathogenesis in autoimmune/autoinflammatory diseases. One of the most unique features of macrophages is their potential to fuse with each other to differentiate into multinucleated osteoclasts in the bone marrow (BM) cavity. Under physiological conditions, they support steady-state bone remodeling together with bone-forming cells, osteoblasts. On the other hand, osteoclasts are also involved in pathological joint

destruction at the synovium-bone interface, called "bare area", in patients with rheumatoid arthritis (RA). Although extensive studies of osteoclast precursor (OP)-containing population in the BM and osteoclast-like cells derived from BM macrophages have been done (1, 2), the microenvironment of inflamed synovium is highly different from the BM in terms of surrounding cell populations, cytokine milieu, and tissue structures. Therefore, a number of fundamental questions regarding pathological bone destruction remained unanswered, such as the phenotype of *in situ* OP populations in the synovium and the origin of pathological osteoclasts in arthritis.

Recent advances in dissecting the inflamed synovium from arthritic mice gave us great insights into macrophages and OPs in the pannus of synovial tissues, the actual site of bone destruction in arthritis (3). In addition, scRNA-seq analysis has succeeded in identifying small subsets in heterogeneous immune cell populations within the joint tissue. Furthermore, the development of intravital imaging system using two-photon microscopy enabled us to to directly analyze the dynamics of osteoclasts and immune cells *in vivo* (4–7). These three skills in combination contributed immensely to better understand of the pathogenesis of arthritic bone erosion and this review introduces recent advances in understanding the role of monocytes/macrophages in inflammatory arthritis.

DIFFERENTIATION TRAJECTORY OF PATHOLOGICAL OSTEOCLASTS IN JOINTS

A healthy synovial membrane consists of scattered macrophage-like cells within a fibroblast stromal tissue and is relatively acellular. However, in RA, the synovial membrane becomes hypertrophic with a variety of immune cells and bone erosion is the central hallmark of the disease, which takes place predominantly at the pannus-bone interface called "bare area". The precise protocol to purely isolate the pannus tissue from the inflamed synovium in an arthritic mouse model has been documented in the previous study (3). *Ex vivo* culture system of the pannus tissue revealed that OPs are within the CX_3CR1^+ cells and $CX_3CR1^{hi}Ly6C^{int}F4/80^+I\text{-}A/I\text{-}E^+$ macrophages had the highest capacity to differentiate into osteoclasts among all the monocytoid cells in the pannus tissue. To elucidate the origin of this specific cell subset, which was designated as arthritis-associated osteoclastogenic macrophages (AtoM) (3), BM chimeric models and a parabiosis model of CX_3CR1-EGFP/TRAP-tdTomato double transgenic mice were used. The results showed that AtoMs and pathological osteoclasts are derived from blood monocytes and not from synovium-resident macrophages (**Figure 1**).

A detailed analysis of the differentiation trajectory of osteoclasts under arthritic conditions showed that $CX_3CR1^{lo}Ly6C^{hi}$ cells in the blood (R1) ingress into the synovium (R2) and differentiate into $CX_3CR1^{hi}Ly6C^{int}$ cells (R3). Global transcriptomic analysis showed that R2 cells predominantly expressed transcripts encoding chemokines, inflammatory cytokines (*Il1*, *Il6*, and *Tnf*), and *Vegfa*, while R3 cells highly expressed osteoclast marker genes, such as *Mmp9*, *Acp5*, *Ctsk*, *Atp6v0d2*, and *Ppargc1b*. Together, the study showed that monocytes acquire a highly inflammatory phenotype when they ingress into the synovium, and abundant M-CSF in the synovial microenvironment induces maturation of some of these cells into AtoMs, leading to osteoclast formation at the pannus-bone interface (**Figure 1**) (3).

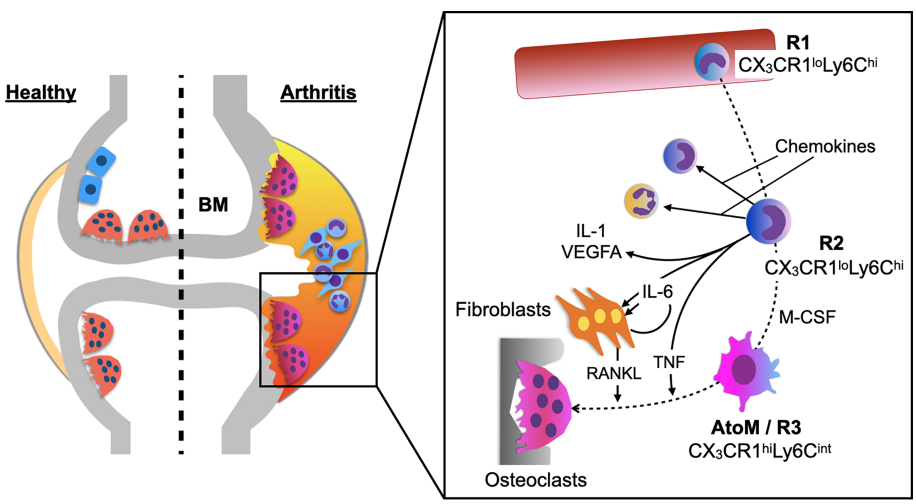

FIGURE 1 | Differentiation trajectory of pathological osteoclasts in arthritis. BM-derived $CX_3CR1^{lo}Ly6C^{hi}$ cells (R1) ingress into the synovium (R2) and produce inflammatory cytokines, chemokines, and VEGFA. Abundant M-CSF in the inflamed synovium induces maturation of some R2 cells into osteoclast precursors, arthritis-associated osteoclastogenic macrophages (AtoMs/R3 cells). When AtoMs localize adjacent to the bone surface, simultaneous stimulation with RANKL and TNF promotes osteoclast formation, leading to joint destruction in arthritis.

CYTOKINES INVOLVED IN PATHOLOGICAL OSTEOCLAST FORMATION

Receptor activator of nuclear factor kappa-B ligand (RANKL) plays a critical role in the articular bone destruction in arthritic mouse models (8), and in RA (9–11). Nevertheless, the primary source of RANKL in the synovial microenvironment has been controversial. A study analyzing conditional knockout mice of RANKL in fibroblasts (Col6a1-Cre) and T cells (Lck-Cre) showed that RANKL expression in fibroblasts contributes mainly to the bone erosion in arthritis (12). Moreover, the analysis of each cell types in the inflamed synovium of arthritic mice showed that RANKL expression in fibroblasts is almost 400 times higher than those in leukocytes (3). Together, these results indicate that fibroblasts are the major source of RANKL in the synovial microenvironment (**Figure 1**).

Various inflammatory cytokines are known to contribute to the bone erosion in arthritis, including IL-1, IL-6, and tumor necrosis factor (TNF). TNF is mainly expressed on macrophages and T cells in the inflamed synovium, which activates TNF receptors type 1 and 2. TNF contributes to osteoclast formation by inducing paired Ig-like receptor-A, a costimulatory receptor for RANK, on OPs (13). Since TNF did not induce osteoclastogenesis when it was administered *in vivo* to RANK knockout mice (14), the permissive levels of RANKL is indispensable for TNF to function against OPs. RANKL priming several days prior to TNF stimulation induces maximal osteoclast formation effect in BM macrophages, while TNF does not promote osteoclast formation when added simultaneously with RANKL (13, 15). In contrast, simultaneous stimulation of RANKL and TNF significantly promotes osteoclastogenesis of OPs in the inflamed synovium, AtoMs (3), indicating that AtoM is distinct from conventional OP populations in the BM and TNF has dual functions in triggering inflammatory osteolysis by inducing inflammation and directly affecting OPs to differentiate into mature osteoclasts in the synovium.

Although IL-1β alone does not directly induce osteoclast formation (16), it can induce osteoclastogenesis from TNF pre-activated OPs by the process independent of NF-κB p50 and p52 (17). In addition, IL-1β enhances stromal cell expression of RANKL, playing an indirect role in osteoclast formation (18). IL-6/sIL-6R directly induce RANKL expression in fibroblasts in RA and this is mediated by the Janus kinase/STAT signalling pathway (19).

SINGLE CELL RNA-SEQ ANALYSIS OF OSTEOCLAST PRECURSOR MACROPHAGES IN THE JOINT TISSUE

Single cell RNA-seq (scRNA-seq) analysis has been applied to synovial macrophages both in mice (20) and human patients (21), revealing heterogeneous subsets that participate in joint inflammation. When this technique was applied specifically to the OP population within the inflamed synovium, the precise number and small subpopulation of macrophages differentiating into pathological osteoclasts *in situ* was estimated. ScRNA-seq analysis of the synovial OP population, AtoMs, showed that about 10% of AtoMs (approximately 1,000 cells per mouse) differentiate into mature osteoclasts in the inflamed synovium (3). A statistical method called pseudotime trajectory analysis, which shows developmental processes from a cell population at asynchronous stages, revealed that AtoMs consist of cells that are under continuous developmental processes into mature osteoclasts, while a minority of cells differentiate into another cell type, which expresses *Nrp1, CD36*, and *C5ar1* (7). The small subpopulation of OPs within AtoMs specifically expressed *FoxM1*, which is a multifaceted transcription factor that plays a prominent role in carcinogenesis by rendering tumour cells to be more aggressive and invasive, leading to metastasis (22). There is a correlation between tumour and pannus tissues that both invade the surrounding tissue and can erode the bone surface. FoxM1 inhibitor and depletion of *Foxm1* in arthritic mice suppressed the articular bone erosion *in vivo*, suggesting that FoxM1 constitutes a potential target for RA treatment. The master regulator of osteoclastogenesis, *Nfatc1*, was also highly expressed in the small subpopulation of OPs in AtoMs, indicating its role in pathological bone destruction. However, expression levels of *Nfatc1* were comparable in bulk RNA-seq analysis between AtoMs and R2 cells, and further investigation is required to analyse the functional contribution of *Nfatc1* in pathological osteoclastogenesis of AtoMs (3).

INTRAVITAL IMAGING SYSTEM FOR THE SYNOVIAL TISSUE

Although conventional methods, such as flow cytometry, micro-computed tomography, and histomorphological analyses, can give us information on the bone structures and molecular expression patterns, intravital information on dynamic cell movements and cellular interactions is not available. An intravital imaging system using two-photon microscopy provided valuable insights into the intravital behaviour and function of immune cells in a variety of organs (23–26). Two-photon microscopy uses two near-infrared photons for exciting the fluorescent molecule to observe the cellular dynamics of deep tissues (100–1,000 μm), to minimize photobleaching and phototoxicity, and to use a nonlinear optical process called second-harmonic generation (SHG) to visualize collagen fibers (**Figure 2A**). The advantages and disadvantages of different modalities used for bone and joint researches are listed in **Table 1**.

While the intravital imaging of macrophages (27) and cancer cells (28) in the BM cavity has been well performed, visualization of deep areas of synovial tissue has been difficult for several reasons. First, the hypertrophied synovial tissue is composed of multiple layers with different refraction indexes, including lining and sublining layers, which limits the depth of observation.

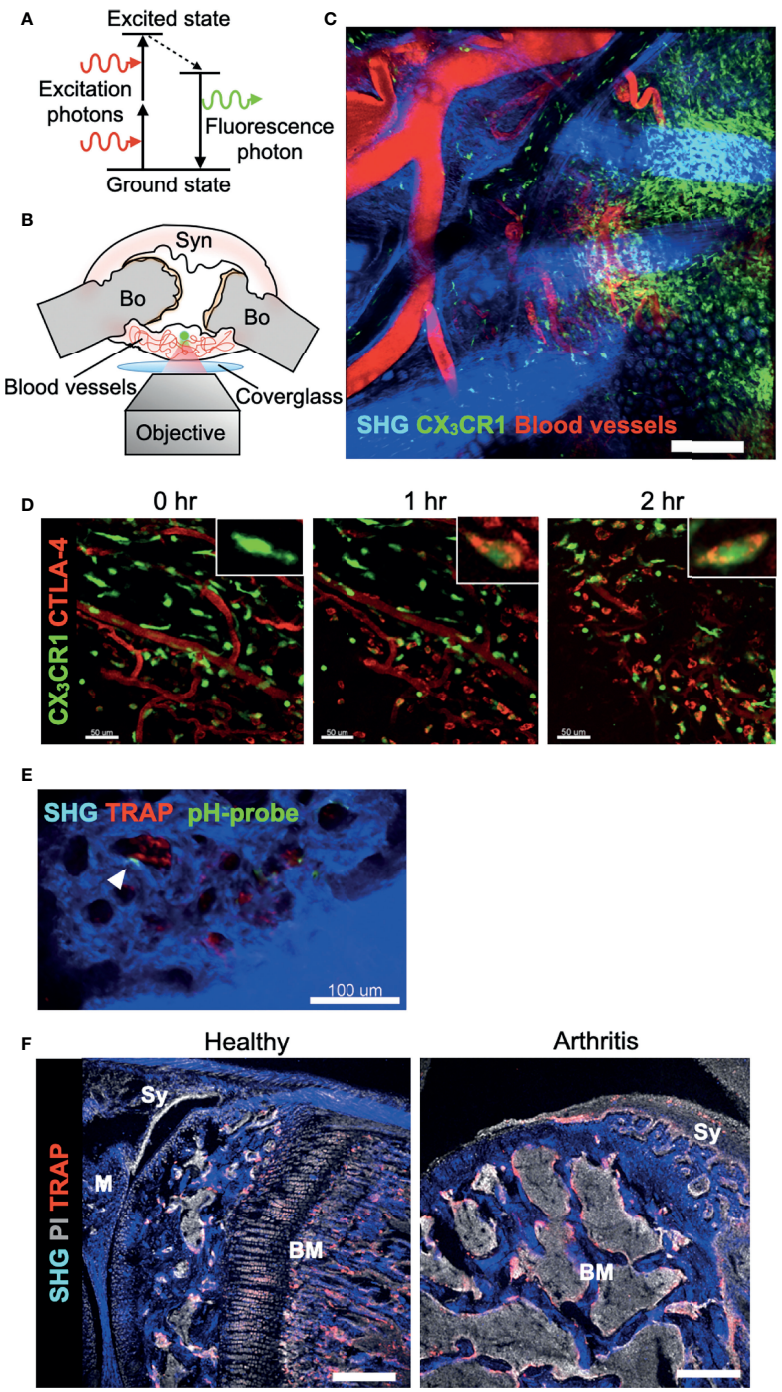

FIGURE 2 | Real-time imaging of the synovium *in vivo* using multi-photon microscopy. **(A)** Basic mechanism of two-photon excitation. Two-photon excitation occurs when a fluorophore absorbs two photons simultaneously. Each photon possesses about half of the energy required to excite the fluorophore. **(B)** Schematic of preparation of exposed synovium, with a coverglass and microscope objective positioning. The green dot shows the area of two-photon excitation. Bo, Bone; Syn, synovium. **(C)** Tile scan image of the inflamed synovium of CX_3CR1-EGFP transgenic mice taken by two-photon microscopy. Blood vessels are visualized *via* intravenous injection of CTLA-4 Ig labeled with AF647 (red). Collagen fibers are visualized by second harmonic fluorescence generated from two-photon excitation (blue). Scale bar: 200 μm. **(D)** Time-lapse imaging of the inflamed synovium of CX_3CR1-EGFP transgenic mice. Intravenously injected CTLA-4 Ig (red) extravasates and binds to CX_3CR1^+ macrophages (green) after one hour. Scale bars: 50 μm. The MIPs of two-dimensional image stacks of vertical synovial slices are shown. **(E)** Intravital images of the third meta phalangeal joint of the CIA TRAP-tdTomato transgenic mice (red) after pHocas-3 (green) injection. Bar, 100 μm. **(F)** Propidium iodide (PI) and TRAP staining fluorescence were visualized by single-photon excitation, while second harmonic generation was produced by two-photon excitation to visualize the bone tissue of the knee joint section. Scale bar: 300 μm. BM, bone marrow; M, meniscus; Sy, synovium.

TABLE 1 | Comparison of different modalities used for bone and joint researches.

Method	Advantages	Disadvantages
Confocal microscopy (CM)	≻Ideal for moderate tissue penetration with simultaneous, multicolor imaging ≻High spatial and temporal resolutions ≻Faster acquisition times compared to MPM	≻Photobleaching and phototoxicity ≻Limitation in thickness of tissues because of light scattering
Multi-photon microscopy (MPM)	≻Ideal for deep tissue penetration ≻Efficient light detection ≻Excitation occurs only at the focal plane, reducing phototoxicity and photobleaching ≻Detection of bone and fibrous tissue by second harmonic generation (SHG) ≻Intravital cellular dynamics and interactions can be observed	≻Higher costs in microscopy purchase/maintenance ≻Artifacts caused by autofluorescence ≻Longer acquisition times compared to CM ≻Limitation in imaging more than four colors due to lack of laser availability ≻Requires skills in handling living animals
MicroCT	≻Three-dimensional visualization of whole bone architecture ≻Quantitative measurements of tissue density ≻Rapid compared to thin sectioning	≻No information on the cellular level ≻No information on the molecular level
Histochemistry	≻Inexpensive ≻Highly specific for individual molecules ≻Many different markers can be combined ≻Can be used for light, confocal, or electronic microscopy	≻The quality of immunolabelling depends on the specificity of the antibody ≻Time and labor consuming ≻Limited to one plane of the section

Second, the visual field of peripheral joints can easily drift in accordance with respiratory movement. Therefore, we decided to expose small joints of the arthritic mice, including wrists and metacarpophalangeal joints, to overcome the first obstacle. Then, we fixed the region of interest to a cover glass and observed the area with inverted microscopy to overcome the second obstacle (**Figure 2B**). A wide field of view can be obtained by tile scan imaging, and monocytes/macrophages are directly visualized *in vivo* in the synovium of a CIA mouse model (**Figure 2C**) (29). When a biological agent, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) Ig, is fluorescently labelled, we could track the intravital distribution of CTLA-4 Ig under arthritic conditions and observe the binding capacity of CTLA-4 Ig to CX_3CR1^+ macrophages in the inflamed synovium (**Figure 2D**). This state-of-the-art technique allows us to observe how the binding of an agent affects the behaviour of macrophages *in situ*.

When the depth of the observation area reaches around 50–100 μm from the synovial surface, mature osteoclasts that resorb the bone matrix can be observed at the pannus–bone interface (29). Osteoclasts are fluorescently labelled in TRAP-tdTomato mice (5) and bone tissue was visualized by SHG. CX_3CR1-$EGFP^+$ cells as well as CX_3CR1-EGFP/TRAP-tdTomato double positive cells were detected at the pannus-bone interface and pathological osteoclasts were making 50-μm-diameter resorption pits (29) (**Figure 2E**). Furthermore, the acidic region caused by functional osteoclasts could be detected by using a pH-sensing chemical fluorescent probe (30). A bisphosphonate group of this probe attaches to the bone surface and boron-dipyrromethene dye emits fluorescence, which has high environmental stability *in vivo*. Combining these techniques, intravital imaging showed that mature osteoclasts were actively resorbing the articular bone without migrating on the bone surface (**Figure 2E**) (29). These results contrast with the osteoclasts under homeostatic conditions in the BM cavity, which are in close contact with osteoblasts and migrate on the bone surface (31). Together, this intravital imaging protocol for the synovium can serve as a platform for exploring the dynamics of immune cells and osteoclasts in the synovial microenvironment. Further studies are required to elucidate which cell subsets and cytokine milieu in the synovium and BM are responsible for the distinctive bone-resorbing behaviour of these osteoclasts.

Another application of two-photon microscopy in the research of bone and joint diseases is the usage of SHG in immunohistochemistry. By combining the images taken by single- and two-photon lasers, we can simultaneously obtain a wide variety of fluorescence signals in combination with SHG signals that detects bone and collagenous synovial layer (**Figure 2F**) (3).

CLINICAL IMPLICATIONS

Identification of osteoclastogenic macrophages in the joint, AtoMs, gives us several clues for the new treatment strategy for inflammatory osteolytic diseases. Since AtoMs originate from blood monocytes, chemokines involved in their ingress into the synovial tissue are potential targets, such as CCR2 and CX_3CL1 (fractalkine). M-CSF and FoxM1 are also shown to be essential for OP formation both *in vitro* and *in vivo*, thereby constituting potential targets for bone destruction in arthritis. Simultaneous stimulation with RANKL and TNF most efficiently induced osteoclastogenesis of AtoMs, implying that TNF-inhibitors possess a direct effect in inhibiting pathological osteoclast formation. In accordance with this finding, TNF blockade can prevent progressive joint damage in patients with RA who have a clinical response as well as in those who do (32, 33), and combination therapy of anti-RANKL monoclonal antibody and biological agents showed the efficacy on radiographic progression in RA (10). Although there's no study that specifically analysed the effect of combination therapy of anti-RANKL monoclonal antibody and TNF-inhibitor, this may be one of the options for patients with progressive bone destruction despite of the use of single biological agent. Although IL-6 didn't have a direct effect on osteoclastogenesis of AtoMs, IL-6/sIL-6R

induce RANKL expression in fibroblasts in RA (19). Therefore, IL-6 may function indirectly in osteoclastogenesis by promoting secretion of cytokines essential for osteoclast formation from stromal cells. Further investigation of IL-6 involvement in osteoclastogenesis with human synovial samples is indispensable to make a final conclusion on this issue.

An important question still remains elusive why synovitis in some diseases, such as RA, leads to bone destruction and others do not. Is it just a matter of the duration and intensity of synovitis? Or are they totally different in terms of their pathogenesis? Although we do not have any clear answer to this question, the differences in the cytokine milieu of synovial microenvironment in each disorder may give us a clue. In diseases that lead to bone destruction, such as RA and septic arthritis, TNF is abundantly expressed in the synovium and causes not only inflammation but also osteoclastogenesis of AtoMs. In addition, M-CSF is present in great quantity to support synovial OP formation at the pannus-bone interface. On the other hand, TNF is not involved in the pathogenesis of SLE, as demonstrated by the possible negative effect of TNF-inhibitors in this disorder, and SLE seldom causes devastating bone erosion. Localization of the inflammation can also play a key role in inflammatory osteolysis. Although TNF is highly involved in the pathogenesis of psoriatic arthritis, enthesis is the representative location of inflammation and it lacks enough capillary networks and the cytokine milieu required for monocytic cells to differentiate into OPs and mature osteoclasts.

AtoMs are distinctive from physiological OPs in the BM in that they express cell surface markers for antigen presentation, such as CD80/86 and MHC class II, and CD11c. Since several studies showed that immature dendritic cells can differentiate into osteoclasts (34,35), these synovial macrophages may be playing a role in antigen presentation in the tertiary lymphoid tissue in the synovium. In fact, fluorescently labelled CTLA4-Ig binds to AtoMs after systemic injection in arthritic mice (29), implying that CTLA4-Ig targets these macrophages in the tertiary lymphoid structure to inhibit auto-antigen presentation in the inflamed synovium. Antigen presenting capacity as well as the expression of CD83, an activation marker for antigen presenting cells, of AtoMs should be assessed in the future.

CONCLUSION

Advances in synovial dissecting procedures, sequencing technologies, and intravital imaging system using multi-photon microscopy have contributed immensely to elucidate the phenotype of macrophages specifically involved in the arthritic bone destruction. Compared to other tissues involved in autoinflammatory/autoimmune diseases, the most characteristic feature of arthritis is bone erosion caused by the final effector cells, osteoclasts. Single cell RNA-seq analysis of the OP-containing macrophages in the joint tissue succeeded in specifying the small subpopulation differentiating into mature osteoclasts at the pannus-bone interface and intravital imaging technique revealed their real-time dynamics. While personalized medicine for autoimmune diseases, including RA, remains a goal out of our reach, novel findings with regard to the differentiation trajectory and distinct phenotype of osteoclastogenic macrophages in the synovium will facilitate us to achieve optimization on treatment for individual patient, and support us to achieve a state of remission as quickly as possible.

AUTHOR CONTRIBUTIONS

TH wrote and MI reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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Trained Immunity Contribution to Autoimmune and Inflammatory Disorders

Samanta C. Funes[1], *Mariana Rios*[2], *Ayleen Fernández-Fierro*[2], *María S. Di Genaro*[1] *and Alexis M. Kalergis*[2,3]*

[1] *Instituto Multidisciplinario de Investigaciones Biológicas-San Luis (IMIBIO-SL), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Universidad Nacional de San Luis (UNSL), San Luis, Argentina,* [2] *Millennium Institute on Immunology and Immunotherapy, Departamento de Genética Molecular y Microbiología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile,* [3] *Departamento de Endocrinología, Escuela de Medicina, Facultad de Medicina, Pontificia Universidad Católica de Chile, Santiago, Chile*

A dysregulated immune response toward self-antigens characterizes autoimmune and autoinflammatory (AIF) disorders. Autoantibodies or autoreactive T cells contribute to autoimmune diseases, while autoinflammation results from a hyper-functional innate immune system. Aside from their differences, many studies suggest that monocytes and macrophages (Mo/Ma) significantly contribute to the development of both types of disease. Mo/Ma are innate immune cells that promote an immune-modulatory, pro-inflammatory, or repair response depending on the microenvironment. However, understanding the contribution of these cells to different immune disorders has been difficult due to their high functional and phenotypic plasticity. Several factors can influence the function of Mo/Ma under the landscape of autoimmune/autoinflammatory diseases, such as genetic predisposition, epigenetic changes, or infections. For instance, some vaccines and microorganisms can induce epigenetic changes in Mo/Ma, modifying their functional responses. This phenomenon is known as trained immunity. Trained immunity can be mediated by Mo/Ma and NK cells independently of T and B cell function. It is defined as the altered innate immune response to the same or different microorganisms during a second encounter. The improvement in cell function is related to epigenetic and metabolic changes that modify gene expression. Although the benefits of immune training have been highlighted in a vaccination context, the effects of this type of immune response on autoimmunity and chronic inflammation still remain controversial. Induction of trained immunity reprograms cellular metabolism in hematopoietic stem cells (HSCs), transmitting a memory-like phenotype to the cells. Thus, trained Mo/Ma derived from HSCs typically present a metabolic shift toward glycolysis, which leads to the modification of the chromatin architecture. During trained immunity, the epigenetic changes facilitate the specific gene expression after secondary challenge with other stimuli. Consequently, the enhanced pro-inflammatory response could contribute to developing or maintaining autoimmune/autoinflammatory diseases. However, the prediction of the outcome is not simple, and other studies propose that trained immunity can induce a beneficial response

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***Correspondence:**
Alexis M. Kalergis
akalergis@bio.puc.cl

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both in AIF and autoimmune conditions by inducing anti-inflammatory responses. This article describes the metabolic and epigenetic mechanisms involved in trained immunity that affect Mo/Ma, contraposing the controversial evidence on how it may impact autoimmune/autoinflammation conditions.

Keywords: trained immunity, autoimmunity, autoinflammation, vaccines, trained immune cells, BCG (Bacille Calmette-Guérin)

INTRODUCTION

Classically the immune response in vertebrates has been classified as innate and adaptive. The latter requires the presence of B and T lymphocytes that, when faced with a pathogen, mount a specific response and establish a memory. Although this process requires time (days), a faster and more effective specific response takes place after subsequent antigen encounters. On the other hand, innate responses have historically been characterized by constitutive systems, such as complement and phagocyte activity, which are non-specific, run rapidly (hours), and do not establish a memory (1). However, a large body of evidence supports that exposure to pathogens can induce a memory-like response in the innate system (2, 3), as we will discuss below in this review.

Trained immunity was first proposed in 2011 as the enhanced innate immune response during a second encounter with the same or different microorganisms (cross-protection) (4). It should be noted that this innate memory differs from adaptive memory since it is not antigen-specific and is based on the strengthening of the innate response to the subsequent encounter with pathogens. This type of immune response cannot be classified as either innate (only observed in the second encounter) or as adaptive (no specific memory itself), so it is defined as another mechanism occurring after a second encounter (4). Trained immunity is mainly mediated by Mo/Ma and NK cells independently of T and B cells. The improvement in cell function in these cell populations is related to epigenetic and metabolic changes that modify gene expression and the phenotype of these cells (**Figure 1**). Although the benefits of immune training have been extensively highlighted in a vaccination context, its effects on autoimmunity and chronic inflammation are still controversial.

BRIEF DESCRIPTION OF TRAINING MECHANISMS

A type of innate memory against previous inflammatory events has been described in plants (5) and various invertebrates as an adaptation to the lack of an adaptive immune system (6). In the case of vertebrates, the stimulation of innate cells through different pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), nucleotide-binding oligomerization domain-

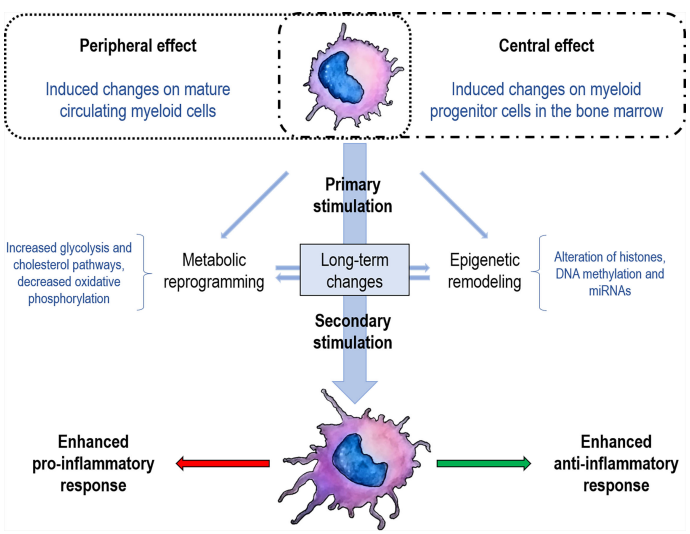

FIGURE 1 | Schematization of trained immunity concepts. The first encounter with a specific stimulus (vaccine, glucan, pathogen) determines metabolic changes and establishes an epigenetic scar either in mature cells (peripheral training) or in stem cells (central training). These marks enable trained immunity to a strengthened response when facing a second stimulus, either to an increase of the pro-inflammatory or anti-inflammatory response. Although the pro-inflammatory response has been the most documented for trained immunity, the anti-inflammatory response has recently been described.

like receptors or NOD-like receptors (NLRs), or C-type lectin receptors (CLRs) promotes long-term modifications that modulate cell metabolism and epigenetic reprogramming (7, 8). PRRs interact with pathogen-associated molecular patterns (PAMPs) and damage/danger-associated molecular patterns (DAMPs). However, the specificity of these receptors can also recognize non-harmful elements generating sterile inflammation (9). For example, macrophages express dectin-1 and TLR-4, recognizing the DAMPs vimentin and high-mobility group box protein-1 (HMGB-1), respectively. These molecules are secreted under injury situations and can train macrophages, making them prone to produce IL-6 and TNF under a second stimulus (10). Therefore, a strict balance between pro- and anti-inflammatory responses is required to avoid chronic inflammation or immune paralysis (11).

Metabolically, cells in a quiescent state have low biosynthetic demand and mainly metabolize glucose *via* glycolysis coupled with oxidative phosphorylation (12). Thus, circulating monocytes in the resting state mostly use the Krebs cycle to synthesize essential molecules or oxidative phosphorylation (13). However, once the cells are activated, they produce biosynthetic precursors by increasing glucose consumption through aerobic glycolysis and oxidative phosphorylation (14). Thus, it has been observed that β-glucans (cell wall components of fungi that are prototype agonists that induce trained immunity) produce a shift in cellular metabolism from oxidative phosphorylation to aerobic glycolysis in monocytes (15). This increased metabolic activity raises the synthesis of metabolites that modulate long-term innate immunity (15, 16).

Metabolic processes, such as glycolysis and fatty acid metabolism can influence immune cell function rather than simply generating energy or modulating general biosynthesis (17). In fact, metabolic reprogramming joins other key immunoregulatory events that influence the immune response (18). Metabolic flexibility in cells is essential to respond to critical changes in the environment and functional demands. In other words, cells can reprogram their metabolism due not only to changes in the availability of nutrients but also in response to the signaling by PRRs and other receptors (cytokine and antigen receptors) (18). Thus, the shift toward aerobics glycolysis and fatty acid synthesis away from the Krebs cycle and fatty acid oxidation is a feature of activated macrophages and DCs (19). Thus, in various immune cells, the increment in glycolysis leads to immune activation contrary to the induction of fatty acid oxidation, oxidative phosphorylation (OXPHOS), and lipid uptake that contribute to immune suppression (20).

Moreover, macrophages are highly plastic cells that can adopt a pro-inflammatory (classical or M1) or anti-inflammatory (alternative or M2) profile, and in each case, their metabolic commitment is adapted accordingly (21). For example, in M1 macrophages aerobic glycolysis predominates while the M2 macrophages engage with OXPHOS and the Krebs cycle (22). As macrophages, DCs undergo cellular changes (morphology, synthesized cytokines, antigenic presentation, increased glycolysis) that define their activated state after stimulation by PRRs (23). Furthermore, the formation of neutrophil extracellular traps (NETs) by neutrophils is dependent on glycolysis, and their activation with PMA increases glucose uptake (24). Similarly, NK cells are activated in the periphery, increasing glucose uptake, glycolysis, and lipid synthesis (25).

On the other hand, despite most of the metabolic studies focusing on glucose pathway shifts, there have also been reports of increased cholesterol synthesis in trained immunity. Consistently with this notion, it was observed that the induction of this pathway is crucial for the establishment of innate memory (26). Furthermore, inhibition of cholesterol synthesis pathways block the trained immunity seen from β-glucan exposure (26), and a deficiency in mevalonate kinase (MVK) associates with a constitutive phenotype of trained immunity and greater susceptibility to sterile inflammation (26).

The above-mentioned metabolic changes are not isolated events within the cellular networks because these changes are closely related to epigenetic alterations capable of regulating innate immune memory (**Figure 2**). This is partly because

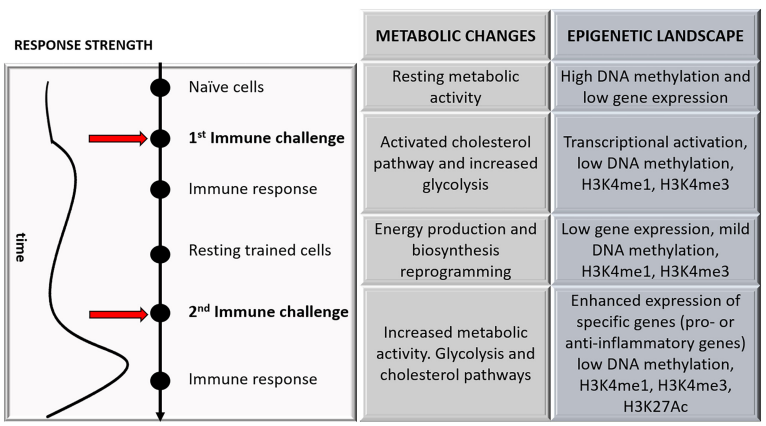

FIGURE 2 | A schematic representation of the changes that occurred during trained immunity over time is shown, focusing on the major metabolic and epigenetic changes.

many epigenetics events are closely associated with metabolic pathways by producing substrates and cofactors required for enzymatic activities (27). Accordingly, it has been observed that epigenetic modifications depend on cellular metabolism changes (16), and these modifications are blocked when metabolic changes are avoided (15, 28). Along these lines, the activation of the cell produces variations in the levels of various intracellular metabolites that lead to changes in the activity of specific enzymes responsible for modifying or reading the modifications in histones or DNA (29). For example, although the mechanisms are not yet fully understood, it is thought that there is a relationship between acetyl-CoA levels and histone acetylation (30). Besides, the accumulation of metabolic intermediates of the Krebs cycle, such as fumarate, inhibits demethylases increasing epigenetic changes in histones and trained immunity in monocytes (31).

On the contrary, the increase in itaconate, a product of the Krebs cycle, reduces epigenetic marks leading to immune tolerance after stimulation with PAMPs (32). Epigenetic regulation refers to phenotype changes without genotype alterations and includes both transient and stable structural alterations of the chromatin that impact gene expression (33). The mechanisms include various post-transcriptional modifications on histones (methylation, acetylation, among others), DNA chemical modifications, and regulation of non-coding RNAs (34). During primary stimulation of innate cells, active gene transcription is made possible by chromatin decondensation that facilitates access of the transcription machinery to DNA. The challenge of monocytes with stimuli such as β-glucans produces a long-lasting enrichment with marks such as the methylation of lysine (K) 4 or the K27 acetylation on histone H3 (H3K4me and H3K27ac, respectively) in the promoters of pro-inflammatory genes, increasing their expression (**Figure 2**) (35).

The effect induced on myeloid cells depends on the nature of the stimulus (the receptor involved) and on the concentration at which the exposure occurs. In this way, the same component can induce an attenuated or strengthened response when used in different concentrations (31). The major TLRs and NLR microbial ligands have been evaluated and their ability to attenuate or enhance the immune response in monocytes to a second encounter (36). These data show that muramyl dipeptide (MDP) and flagellin can induce trained immunity, and the latter is of particular interest because it has been assigned relevance to the pathogenesis of inflammatory bowel diseases (36). Thus, repeated exposure to LPS can induce selective and transient alterations in histones that repress the expression of pro-inflammatory factors in murine macrophages, favoring tolerance and reducing tissue damage by excessive inflammation (37).

On the other hand, exposure to *Candida albicans* or β-glucans induces stable epigenetic changes based on H3K4me, producing expression of inducible genes (35). Thus, β-glucan can at least partially reverse LPS-induced tolerance in Mo/Ma through changes in histones and reactivation of non-responding genes (38). Besides, stimulation with β-glucans in human monocytes produces both H3K4me3 and H3K27ac after seven days, and these changes were associated with induction of the glycolysis pathway (15).

Considering that trained immune response has been described several months after the first encounter (39), and due to the short life span of circulating Mo/Ma and NK cells, the question initially arose about how long-term reprogramming is established in these cells (4). In turn, this programming can be carried out at various levels of cellular function and locations, as will be mentioned in the following sections.

Importantly, trained immunity can be established peripherally in circulating mature cells or centrally in bone marrow progenitor cells, thus maintaining immune training for long periods (**Figure 1**). Different stimuli were shown to induce systemic changes, affecting hematopoiesis and reprogramming progenitor cells in the bone marrow (40, 41). Induction of trained immunity reprograms cellular metabolism in hematopoietic stem cells (HSCs), transmitting a memory-like phenotype to the cells (40, 42, 43). Thus, trained Mo/Ma derived from HSCs typically present a metabolic shift toward glycolysis, which leads to the modification of the chromatin architecture by methylases and acetylases (40). Stem cells express receptors for many inflammatory elements, allowing them to sense and adjust to changes in the environment (44). For example, acute stimulation with LPS induces persistent alterations in specific myeloid lineage enhancers, improving innate immunity against *P. aeruginosa* by a C/EBPβ dependent mechanism (45).

Similar to some infections, vaccines have also been reported to induce trained immunity, conferring non-specific protective effects against other non-related infections. For example, using the Bacillus Calmette-Guérin (BCG) vaccine, metabolic and epigenetic alterations were observed in monocytes both *in vivo* and *in vitro* (16, 46, 47). The result of the exposure of monocytes to the BCG vaccine or β-glucan is an increased cross-response (higher cytokine production) to subsequent exposure to another unrelated pathogen seven days later (35, 48, 49). Below we will detail the most important elements that induce trained immunity and the mechanisms that have been described for each of them.

Vaccines

Vaccines have been developed to induce a specific immune response against a wide variety of pathogens for which they were designed. However, some vaccines can also protect against other pathogens with no specific vaccine by eliciting immune responses related to the concept of trained immunity (46, 47, 50). Furthermore, trained innate cells can boost vaccine strategies by increasing antigen uptake, presentation, migration, and cytokine production (51).

BCG, the vaccine for tuberculosis, has reduced mortality by decreasing morbidities other than tuberculosis in Africa (52). Interestingly, in the current pandemic against SARS-CoV-2, those countries where BCG vaccination is given at birth, it has been shown to have fewer COVID-19-related deaths and a lower contagion rate (46, 47, 53). Accordingly, BCG-vaccinated mice also increase their immune response against *C. albicans* or *Schistosoma mansoni*, at least in part through a T-independent

mechanism (54). Moreover, BCG can improve vaccine performance against viral infections, such as influenza and hepatitis B, by enhancing cytokine production in humans and mice (55). It was shown that three months after BCG vaccination, the production of pro-inflammatory cytokines increased following *ex vivo* stimulation of NK cells with mycobacteria and other unrelated pathogens (48). Furthermore, in response to unrelated bacterial and fungal pathogens, through epigenetic reprogramming of innate immune cells, BCG increased not only the production of IFN-γ but also augmented the release of monocyte-derived cytokines, such as TNF and IL-1β (56). On the other hand, the induction of glycolysis and glutamine metabolism, regulated by epigenetic mechanisms at the chromatin organization level, has been demonstrated to be essential underlying BCG-induced trained immunity in monocytes both in an *in vitro* model and after vaccination of mice and humans (16).

The occurrence of trained immunity has also been observed in live-attenuated vaccines other than BCG, such as vaccines against smallpox (vaccinia virus), measles, polio (live oral vaccine), yellow fever, and the new live-attenuated *M. tuberculosis* candidate vaccine *MTB*VAC (57–62).

The stimulus involved in the induction of trained immunity by vaccines is unclear. However, it is assumed that the immunogen from the vaccine can reach the bone marrow, where the hematopoietic stem and progenitor cells are stimulated, detecting PAMP, or could be indirectly stimulated by detecting systemic inflammatory signals like growth factors and cytokines such as GM-CSG, M-CSG, G-CSF, IL-1β, IL-6 (51). For instance, the bioactive peptidoglycan motif common to all bacterial vaccines is MDP (63), which activates innate cells through PRRs and leads to inflammatory cytokine release. Besides, BCG employs the mechanistic Target of Rapamycin (mTOR) pathway to activate specific downstream metabolic reprogramming and epigenetic changes (16). To date, the only intracellular PRR identified to be involved in the induction of trained immunity is NOD2/Rip2 in response to BCG (**Figure 3**). Moreover, activation of NOD2 (63) stimulates epigenetic changes in macrophages and induces trained immunity (56).

Products Derived From Lipid Metabolism

The endotoxin LPS is the main outer membrane component of Gram-negative bacteria (64) and can regulate innate immune memory by promoting either inflammation or tolerance depending on acute or chronic stimulation and doses (37, 65). For instance, repeated low doses of LPS can increase the inflammatory response in mice after a stroke through changes in H3K4me1 in microglia (65). Moreover, acute stimulation with LPS induced persistent alterations in specific myeloid lineage, improving innate inflammatory immunity against *Pseudomonas aeruginosa* by a C/EBPβ dependent mechanism (45).

Other studies have shown that low doses of LPS reduced the expression of costimulatory molecules and increased expression of iCOS-ligand and DC-SIGN, promoting a mixed M1/M2 phenotype (66). Macrophages with a tolerogenic profile alleviated fibrosis and inflammation in a mouse model of systemic sclerosis (SSc), even by adoptive transfer (66). The gene response after LPS treatment is dynamically regulated to confer the tolerance phenotype (67). Thus, acetylation and methylation of histones are reconfigured to diminish transcription of pro-inflammatory cytokines, lipid metabolism, and phagocytic pathways (38, 67).

On the other hand, non-microbial molecules have been studied as trained immunity inducers, such as endogenous atherogenic particles (68–70). Compared to PAMPs, DAMPs have been much less studied as inducers of trained immunity.

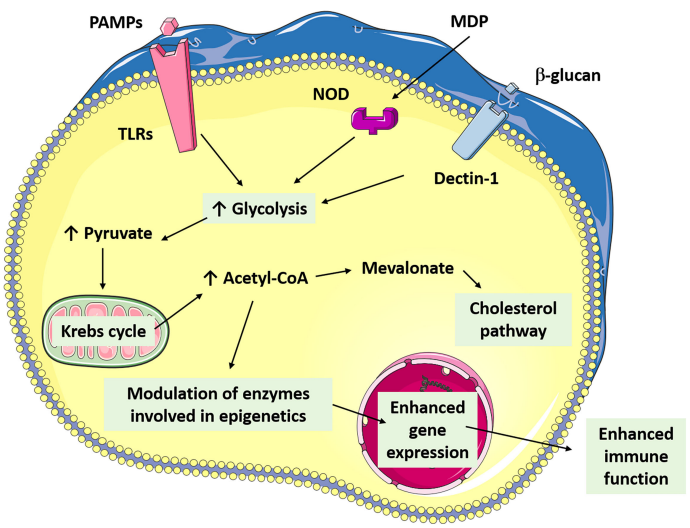

FIGURE 3 | The figure shows in a simplified way the connection of the main metabolic pathways involved in the establishment of trained immunity through the commitment to some PAMPs (β-glucans, BCG, LPS).

DAMPs are host-derived molecules capable of inducing an innate immune response. For example, oxidized low-density lipoprotein (oxLDL) is recognized by receptors as lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), CD36, and scavenger receptor class B type I (SR-BI) and, after a brief exposure, enhances the long-term pro-inflammatory response in Mo/Ma (69). Thus, oxLDL-trained macrophages present an epigenetic reprogramming associated with mTOR signaling showing high histone methylation and a pro-inflammatory profile (68). In addition, large containers of mitochondria have been found after exposure to ox-LDL, which correlates with an increased oxidative phosphorylation activity (69).

Hyperlipidemia has recently been studied as a factor to modulate or induce the inflammatory trained immune cell response (71), but the specific mechanisms regarding this matter remain unknown. A positive correlation between lipid concentration and the induction of metabolic genes has been observed, contributing significantly to trained immunity (70). Hyperlipidemia is linked with the increase of lysoPC and oxLDL as a stimulus for trained immunity (70); however, it has also been linked with increased aldosterone and liver X Receptor (LXR) activation, both proposed as part of a mechanism promoting trained immunity (72, 73).

β-Glucan

The β-glucans are iconic inductors of trained immunity described throughout the literature (35, 74–77). β-glucans are glucose polymers found in the cell wall of fungi, rich in D-glucose units with β-1,3 links and β-1,6 branching, and recognized as PAMPs by dectin-1 in macrophages (**Figure 3**) (78). It has been reported that β-glucans from different sources (algae, yeast, bacteria, oat, and mushroom) can induce a strengthened response *in vitro* in peripheral blood mononuclear cells (PBMCs) (74). In murine models lacking functional T and B lymphocytes, trained immunity can be observed using *C. albicans* and fungal cell wall β-glucan. In that model, β-glucans induce functional reprogramming of monocytes, leading to augmented cytokine production and lower mortality under reinfection (35). Accordingly, the β-glucan treatment produces subsequent protection against *Staphylococcus aureus* infections in mice (75). Similarly, macrophages trained with β-glucan can protect mice against a *P. aeruginosa* infection (76).

The early inflammatory response induces epigenetic and metabolic changes, but interestingly, in this report, the induced immunity was independent of dectin-1 and TLR2 (76). Notably, β-glucan-mediated induction of training in macrophages requires cAMP production and activation of the mTOR-HIF1α pathway and aerobic glycolysis, similar to BCG trained immunity mechanisms (15, 79). Consistently, when glycolysis and glutaminolysis were inhibited, a reduction in histone marks was observed at the promoters of IL-6 and TNF (26). Besides, DCs also have shown enhanced response after fungal exposure. Mice exposed to *Cryptococcus neoformans* have DCs with strong IFN-γ production on a challenge and epigenetic changes (77).

β-Glucan-trained human monocytes undergo chromatin restructuring, identified by increased levels of H3K4me3, H3K27ac, and H3K4me1, as well as DNA demethylation, and increased accessibility of specific transcription factors at gene promoters corresponding to inflammation mediators (38, 79). Importantly, it has been observed that β-glucans also influence the myeloid progenitors of the bone marrow by producing epigenetic remodeling (80). Moreover, β-glucan promotes the expansion of myeloid-biased CD41+ HSCs in mice (41) and induces changes in HSCs in an IL-1β dependent manner (41).

BENEFICIAL AND DETRIMENTAL EFFECTS OF THE INDUCTION OF TRAINED IMMUNITY FOR AUTOIMMUNE DISORDERS

Based on the above, trained immunity could play a pivotal role in defense against pathogens and even cancer cells, which is why it has been proposed as possible new immunotherapy (81). However, it is still debated whether this enhanced phenotype of innate cells could also contribute to establishing or maintaining chronic inflammatory conditions (82). Although the contribution of the genetic profile in the development of autoimmunity and the critical role of the adaptive immune system is well known, the innate response also plays essential functions in these conditions (83, 84). Autoimmune diseases are pathologies in which the immune response is unbalanced, characterized by autoreactive T and B cells, and complex pathogenesis with multifactorial etiology. Thus, immune cells recognize and attack the healthy tissues in a systemic or organ-specific manner, generating even more chronic inflammation (85). Along these lines, alterations in innate response characterized by a pro-inflammatory profile have also been described in innate cells from patients with autoimmune diseases (83, 86).

Two main scenarios can be identified with regard to trained immunity and autoimmune diseases. First, multiple factors, such as epigenetic alterations could be related to the observed inflammatory profile in autoimmune diseases (33, 87) and suggest a "training" state in the innate system under established autoimmunity. These findings have led to proposing new therapeutic strategies based on reversing metabolic or epigenetic changes to reduce or reverse the enhanced inflammatory state of the immune system (88). Second, the promotion of trained immunity could be harmful to individuals prone to developing autoimmune diseases. Therefore, a trained immunity signature in lupus mice and patients has been suggested by the reported reprogramming of HSCs towards the myeloid lineage that could contribute to exacerbated immune responses and flares in systemic lupus erythematosus (SLE) patients (87). Thus, SLE inflammatory milieu could promote immune training memory on bone marrow progenitor cells, similar to the observed β-glucan signature of HSCs after training (41). Besides, exposure to *Candida* β-glucans in two lupus-prone mouse models (*FcGRIIB*$^{-/-}$ and pristane) increased the production of NETs and exacerbated disease activity (**Table 1**) (89). Accordingly, the administration of β-glucans in a lupus mouse model

TABLE 1 | Selection of reports that describe the effects of stimuli that produce trained immunity in patients or *in vivo* models of autoimmunity and the key molecules involved. The administration route is described as well.

Condition	Model	Stimuli	Route of administration	Immune training effect and key molecules involved	References
SLE	FcGRIIB-/- and pristane female mice	*Candida albicans*	Oral	Increased production of NETs and exacerbated disease activity. Induction of prominent NETs formation by Syk and NFκB expression in neutrophilic.	Saithong et al., (89)
	Female NZBxNZW F1 mice	β-glucans from *Saccharomyces cerevisiae*	Oral	More aggressive disease. The involvement of TLRs is suggested.	Fagone et al., (90)
RA	Female SKG mice	β-glucans (Zymosan)	Intraperitoneal	Trigger severe chronic arthritis with a higher incidence. β-glucans stimulate BM-DCs to mature and produce pro-inflammatory cytokines in a Dectin-1- but not TLRs dependent way.	Yoshitomi et al., (91)
	Male CIA model in DBA/1, DBA/2, BALB/c, C3H/HeN and C57BL/10 mice	Particles containing β-glucan prepared from *Candida albicans* by oxidation	Subcutaneous or intraperitoneal	Exacerbate autoimmune arthritis. Genetic background (MHC and complement system) influences the ability of β-glucans as adjuvants.	Hida et al., (92)
	Male CIA model in DBA/1J mice	β-glucans derived from *Aureobasidium pullulans*	Intradermal	Inhibition of histopathological changes in CIA. Molecular mechanisms are unknown.	Kim et al., (93)
T1D	Female NOD/Mrk/TacfBR mice and new-onset diabetic patients	CFA or BCG	Intracutaneous	Inhibited the development of clinical diabetes in mice and clinical remission was observed in BCG-treated patients. Molecular mechanisms are not mentioned.	Shehadeh et al., (94)
	Healthy and diabetic subjects	BCG	Intradermal	Insulin-autoreactive T cell expansion and transient restoration of C-peptide. Mechanism related to TNF-induced death of insulin-autoreactive T cells.	Faustman et al., (95)
	Female NOD mice	CFA	Intradermal	CFA induces TNF-α production, a consequent elimination of TNF-α–sensitive cells and reverses the early stages of disease.	Ryu et al., (96)
SSc	Female HOCl-induced SSc mice	LPS and BCG	Intraperitoneal	Low-dose LPS alleviates fibrosis and inflammation, but BCG-training exacerbates disease. BCG-macrophages enhance the expression of pattern recognition receptors (TLR4, CD206, and CD14), chemokine receptors (CCR2 and CXCR4), costimulatory and/or signalling molecules (CD43, CD14, CD40, CD80, CD68, and Ly6C) and pro-inflammatory cytokines release (IL-6, TNF, and IL-1β). LPS^{low}-macrophages express less costimulatory receptors and pro-inflammatory cytokines but upregulate IL-10, iCOS-ligand and DC-SIGN.	Jeljeli et al., (97)
MS	EAE in C57BL/6 mice	*Fasciola hepatica* total extract	Subcutaneous or intraperitoneal	FHTE increased the expression of *arg1*, *retlna*, *chi3l3*, CD206 and PD-L2 and the secretion of IL-1RA and IL-10 by macrophages while inhibiting TNF and IL-12p40 production in response to a TLRs restimulation. Besides, FHTE trained macrophages suppressed IL-17 production by T cells.	Quinn et al., (98)
	EAE in female C57BL/6 mice	*F. hepatica* excretory-secretory products (FHES)	Subcutaneous	Delay in the induction of murine EAE. FHES activates metabolic pathways (including mTOR) in HSCs, and the BMDM from FHES-treated mice reduces the production of pro-inflammatory cytokines and MHC-II expression but enhances IL-1RA. Besides had reduced costimulatory molecules expression and enhanced TGF-b, IL-10, IL-1R, and IL-6 production.	Cunningham et al., (99)
	EAE in female CD45.2 C57Bl/6J mice	CpG	Intravenous	Protection against EAE development by migration of pre-pDCs to the spine. BM cells stimulated by the TLR-9 agonist CpG generates plasmacytoid dendritic cell (pDC) with enhanced TGF-β and IL-27 production and PD-L1 expression.	Letscher et al., (100)
	EAE in C57BL/6 females	BCG inactivated by extended freeze-drying	Subcutaneous	Attenuates the inflammation systemically and at the CNS level, alleviating EAE. EFD BCG treated mice reduce pro-inflammatory cytokines production (IL-6, IL-1β, TNF-α and IP-10).	Lippens et al., (101)

(NZBxNZW F1) has been reported to produce a more aggressive disease (90) and raises the re-evaluation of these components as immunomodulatory therapy in human lupus patients.

On the other hand, β-glucans derived from *C. albicans* have been employed as an adjuvant for collagen, resulting in mice arthritis and suggesting that fungal metabolites can contribute exacerbating to autoimmune diseases such as rheumatoid arthritis (RA) (92). Accordingly, SKG mice (prone to autoimmune arthritis) failed to develop the disease under a specific pathogen-free (SPF) environment (91, 102). However,

a single administration of fungal β-glucan triggered severe chronic arthritis in SKG mice and transient arthritis in normal mice (91, 102). Moreover, the administration of *C. albicans* β-glucans acts as an adjuvant in the collagen-induced arthritis (CIA) model and induces more severe arthritis (92). In contrast, administering a β-glucan derived from *Aureobasidium pullulans* on the CIA DBA mice model for four weeks markedly reduced arthritis signs in a dose-dependent manner (93). These results suggest that the effect of β-glucan in the case of RA could vary depending on the source of β-glucan and the dose applied. For example, it was shown that β-glucan derived from *Aureobasidium pullulans* can effectively preserve bone mass by an inhibitory effect on osteoclast differentiation and by attenuating the production of pro-inflammatory cytokines (TNF and IL-1β) (103, 104).

Consequently, the encounter with components capable of inducing trained immunity is not always detrimental for autoimmunity (**Table 1**). Training induction in the context of autoimmunity turns out to be complex, and in some of these conditions, it has even been reported to be beneficial by reducing the severity of the symptoms or delaying disease onset. For example, while autoimmunity induces spontaneous IL-17 production and tissue damage, BCG vaccination only induces a primed status of the cells with enhanced secondary pathogen stimulation (105). Indeed, no higher production of these cytokines was seen without second stimulation (105). Consistently with this notion, some studies even reported a beneficial effect of BCG vaccination on autoimmunity (94, 95). As an example, the inoculation with complete Freund's adjuvant (CFA, which is composed in part by *M. tuberculosis*) into young non-obese diabetic (NOD) mice not only prevented the development of type 1 diabetes (T1D) but can also reverse the early stages of disease (94, 96, 106). This impact was associated with the production of TNF, which selectively killed only disease-causing cells (autoreactive T cells) and allowed pancreas regeneration (96). It was shown that CFA or BCG did not inhibit the development of autoimmunity in mice but redirected the disease from a destructive to a non-destructive process (94). Accordingly, a study in humans using a single dose BCG vaccination reported remission by 4-6 weeks with stabilization of blood sugars in 65% of pre-diabetic patients (94). Another trial concluded that BCG treatment or Epstein-Barr virus (EBV) infection could transiently modify T1D severity in humans by stimulating the innate immune response and suggested that BCG or other stimulators of host innate immunity may contribute to the treatment of long-term diabetes (95). Also, the study proposed that more frequent or higher dosing of BCG will likely be helpful for therapeutic and sustained amelioration of the autoimmunity, based on permanent elimination of autoreactive T cells (95, 107). In addition to the above-mentioned effects, the metabolic changes generated by vaccination with BCG could favor a closer control of blood sugar levels, promoting hypoglycemia (108).

In the case of SSc, it has been shown that macrophages treated with BCG adopt a pro-inflammatory profile, and BCG vaccination in an SSc model exacerbated inflammation (66). Conversely, macrophages from the SSc model exposed to low doses of LPS adopted a profile with lower costimulatory molecules and higher expression of iCOS-ligand and DC-SIGN (mixed M1-M2 phenotype) (66).

Trained immunity has been defined as the altered innate immune response (increased or decreased) to a second encounter, and the mechanisms involved are based on epigenetic and metabolic changes (109). Some components from pathogens, such as helminths, can induce lasting epigenetic changes in stem cells, favoring an enhanced anti-inflammatory response rather than a pro-inflammatory one in the face of a second encounter (98, 99). These reports demonstrate a trained immunity characterized by the enhanced anti-inflammatory response, which poses an exciting therapy that could induce long-term tolerance in the context of autoimmunity. Thus, it has been described that other elements such as helminth-derived compounds (*Fasciola hepatica* total extract or FHTE) are capable of inducing an attenuated form of trained immunity (anti-inflammatory profile) that protects against the induction of the experimental autoimmune encephalomyelitis (EAE), which is the animal model for multiple sclerosis (MS) disease (98). An increased production of IL-10 and IL-1RA by macrophages was observed after FHTE exposure (98). Furthermore, it was recently reported that *F. hepatica* excretory-secretory products (FHES) induce an anti-inflammatory profile in HSCs by increasing the differentiation and proliferation of Lys6Clow monocytes (99). These mice also showed an increased proportion of M2 macrophages and all of these events attenuated and delayed the induction of murine EAE for at least eight months. Even more interesting is the fact that the transfer of HSCs from FHES-treated mice to naive mice transferred this resistance to developing EAE, showing that the effect was both peripheral and central (99). Besides, *in vivo* and *in vitro* stimulation of the TLR-9 in bone marrow induced the migration of precursors of plasmacytoid dendritic cells (pDCs) to the spinal cord and induced the production of TGF-β and IL-27, protecting against the development of EAE (100). On the other hand, at the peripheral level, BCG administration also reduced the severity of EAE in mice by promoting pDCs to induce IL-10-producing Treg cells (101). Although it is unknown how training occurs during autoimmunity, knowing the mechanisms that promote inflammation could lead to new strategies based on metabolic and epigenetic modifications (82).

BENEFICIAL AND DETRIMENTAL EFFECTS OF TRAINED IMMUNITY INDUCTION FOR AUTOINFLAMMATION

In contrast with the autoimmune diseases triggered by an aberrant adaptive immune response, in autoinflammatory (AIF) diseases, the innate immune response directly induces tissue inflammation in the absence of autoreactive T cells and high autoantibody titers (110, 111). Autoimmune and AIF diseases share some features despite the different key players,

such as the prefix "auto" indicating a pathological response against self and the chronic inflammation that develops in genetically predisposed individuals (111). Indeed, AIF diseases are monogenic and multifactorial (polygenic) inflammatory conditions whose heterogeneous symptoms are recurrently associated with fever, as in the periodic fever disorders and episodes of acute inexplicable inflammation (85). In the spectrum of AIF, a subset includes hereditary conditions associated with monogenetic mutations affecting the innate immune system, such as the autosomal dominant TNF receptor-associated periodic syndrome (TRAPS), in which mutations in the genes encoding for the tumor necrosis factor receptor (TNFR1) have been identified (112). In the Hyper IgD syndrome (HIDS), the affecting gene encoding MVK is responsible for increased mevalonic acid, IgD, and IL-1β in the serum (113). Remarkably, mevalonate accumulation is one of the contributors to the induction of trained immunity (26). Another subset of AIF is associated with polygenic mutations and involves several environmental factors, such as Crohn's disease, Behcet's disease, and ulcerative colitis (85). In addition, some rheumatic diseases are intermediate autoimmune and AIF settings since they are major histocompatibility complex (MHC)-I associated but mainly autoantibody negative disorders, such as spondyloarthritis (SpA) and other related diseases such uveitis (114).

A critical pathogenetic mechanism in AIF diseases is the dysregulation of the inflammasomes, multiprotein cytoplasmic complexes relevant to innate immunity and inflammatory responses. The main components of inflammasomes are members of the NLR family that detect PAMPs or DAMPs and initiate inflammasome assembly. Thus is induced the proteolytic activation of caspase 1 or 11 and the cleavage and subsequent release of bioactive IL-1β, a key molecule of inflammation and innate immunity (115). Notably, mutations of genes encoding for the components of the proteins involved in the inflammasome (NLRP3) are implicated in the AIF called Cryopyrin-associated periodic syndromes (CAPS) (116).

On the other hand, some AIF diseases are caused by abnormalities of the ubiquitin-proteasome system (UPS), which regulates multiple cellular processes (117). Mutations that cause loss of UPS function in humans lead to a typical type I IFN gene signature and proteasome-associated autoinflammatory syndromes (PRAASs) (118). Although the causes of the induction of sterile inflammation in subjects with PRAAS are still unknown, it is believed that it could be associated with the propagation of endoplasmic reticulum (ER) stress (119). Proteasome defects are known to lead to the retention of misfolded proteins in the ER, leading to inflammation in a pathogen-free setting (120). Another recently described AIF disease related to UPS malfunction is VEXAS (vacuoles, E1 enzyme, X-linked, autoinflammatory, somatic) syndrome (121). Myeloid lineage-restricted somatic mutations of UBA1 (a gene encoding the ubiquitin-activating enzyme 1) characterize VEXAS, leading to inflammation (121). Although the impact of trained immunity in these conditions is unknown to date, it could be assumed that stimuli that establish the capacity of a strengthened response by the innate system could be harmful in these systemic inflammatory diseases.

Aicardi–Goutières syndrome (AGS) is an inherited disease characterized by mutations that produce the accumulation of nucleic acids and ultimately lead to an abnormal IFN response (chronic overproduction of type I IFN) (122). IFN stimulation could induce trained immunity, but chronic exposure to IFN I or IL-1β could cause the HSC pool to become exhausted, in part because of DNA damage caused by replication stress (123). Even though it is still unknown how HSC exhaustion impacts the trained immunity process, it is believed that DNA damage could be influencing renewal capacity and memory in HSCs (124). Remarkably, similar events are described in AGS patients, and it has been reported that they develop AGS during early childhood and many of them after vaccinations or infections (122). Although an association between AGS and trained immunity has not yet been established, it would be a novel approach to study new therapies (124).

Since AIF diseases are characterized by hyperreactivity of the innate immune system, several recent studies have investigated trained immunity in these diseases. Therefore, trained immunity-related signatures such as increased cytokine production, changes in cellular metabolism (mainly increased glycolysis and lactate production in an mTOR/HIF-1α-dependent manner), and epigenetic reprogramming have been analyzed in AIF conditions. Indeed, genetic studies by microarray demonstrated overexpression of IL-1β and IL-1 receptor 1 (IL-1R1) under basal conditions and following LPS stimulation of monocytes of TRAPS compared with controls (125). Another transcriptomic study demonstrated that the treatment with a human anti-IL-1β monoclonal antibody (Canakinumab) reversed the overexpression of inflammatory response genes including IL-1β, suggesting the central role of IL-1β in the TRAPS pathogenesis (126). Also, *in vitro* experiments demonstrated that mTOR contributes to inflammation in TRAPS patients (127), indicating metabolic changes in this AIF disease. In addition, LPS-stimulated peripheral blood monocytes from Behçet's disease patients produced more TNF than healthy volunteers (128). Enhanced spontaneous and MDP-induced cytokine secretion by monocytes suggested an *in vivo* pre-activation of monocytes in SpA patients under conventional therapy, which was reverted under TNF inhibitor treatment (129). In patients with HIDS, circulating monocytes with a trained immunity phenotype have been detected since accumulated mevalonate amplifies the AKT-mTOR pathway, which in turn induces HIF-1α activation and a shift from oxidative phosphorylation to glycolysis (26).

The Mo/Ma activation depends on epigenetically controlled functional reprogramming to coordinate a proper response. Thus, demethylation of several inflammasome-related molecules has been described in stimulated monocytes and macrophages. Also, the epigenetic changes characterize trained immunity phenotype, and they have been reported in Mo/Ma of patients with several monogenic or complex AIF diseases (130, 131). During macrophage differentiation and monocyte activation, DNA methylation levels of inflammasome-related

genes were analyzed in patients with CAPS, an archetypical monogenic AIF syndrome. Monocytes from untreated patients with CAPS undergo more efficient DNA demethylation than those of healthy subjects. Interestingly, patients with CAPS treated with anti-IL-1 drugs display methylation levels similar to those of healthy control subjects (131). Also, when genome-wide DNA methylation patterns were analyzed in monocytes from 16 male patients with Behçet's disease and matched healthy controls, 383 CpG sites were differentially methylated between patients and control (only 125 sites in $CD4^{+}$T cells) (132). Furthermore, mevalonate accumulation induces epigenetic changes in HIDS (26).

Trained immunity could be a likely contributor to AIF diseases. Indeed, heat-inactivated *M. tuberculosis* immunization increased spondylitis and arthritis incidence and accelerated the synchronized onset of spondylitis and arthritis in males and females HLA-B27/Huβ2m transgenic rats (133). On the other hand, etanercept (a TNF inhibitor) treatment delayed the appearance of spondylitis and arthritis and suppressed arthritis severity, evidencing a role of TNF and innate immune activation in the induction phase in this SpA animal model (133).

As aforementioned, BCG vaccination aids in inducting trained immunity. Thus, it has been shown that BCG vaccination enhances the antimicrobial response of innate immune cells (assessed by cytokine production capacity), but at the same time downregulates the systemic inflammation as measured by decreased concentrations of pro-inflammatory proteins in the circulation of a large cohort of healthy volunteers (134). This modulatory effect on systemic inflammation may explain some of the beneficial effects of BCG vaccination in inflammatory diseases (134). Inline, BCG vaccination in mice reduces inflammation in murine models of colitis by stimulating IL-10 and TGF-β production and expansion of Tregs (135). Furthermore, BCG decreased mice's circulating pro-inflammatory cytokines, cholesterol levels, and atherosclerotic lesions [114]. Also, BCG vaccination had a beneficial effect on Alzheimer's disease, downregulating inflammatory processes (136). In addition, a recent study showed that LPS low-tolerized human macrophages elicit a suppressor effect and mitigate the fibro-inflammatory phenotype of endometriotic cells in an IL-10-dependent manner (97). Although much more needs to be learned, these studies show that the manipulation of trained immunity has therapeutic potential for treating a wide range of hyper-inflammatory conditions.

THERAPIES TARGETING TRAINED IMMUNITY IN AN AUTOIMMUNE/ AUTOINFLAMMATORY CONTEXT

As we have discussed throughout this review, understanding trained immunity and its detrimental effect on some autoimmune diseases such as SLE or RA (**Table 1**) or AIF have made a call to consider new factors that could increase the severity of these diseases. In this way, PBMC from RA patients compared to healthy individuals shows a different *in vitro* response against BCG extract exposure. Healthy controls produce higher TGF-β and IL-10 levels and lower IFN-γ by BCG stimulation than RA patients, suggesting a tighter regulation in healthy individuals (101). However, there has not been evidence of established trained immunity in monocytes from RA patients, at least concerning the epigenetic alterations in pro-inflammatory genes TNF and IL-6 (137). On the other hand, the relapses in RA and SpA patients are frequent despite bone marrow transplantation. Hence, it has been suggested that transient infections of the bone marrow close to the synovium and entheses (in RA and SpA, respectively) could have induced lasting epigenetic changes in some bone marrow-derived mesenchymal stem cells (BM-MSCs) (138). Furthermore, a trained immunity signature was detected in HSCs in mice with lupus, and in patients, both showed more significant cell proliferation and differentiation, as well as transcriptional activation of cytokines that lead to myelopoiesis (87).

Hence, some therapies propose different strategies to restore or erase the mark of trained immunity to reduce chronic inflammation and tissue destruction. In this sense, we could find drugs that prevent the activation of NOD2 or dectin-1 (GSK669, GSK717, or laminarin) (139) or those that affect the metabolic pathways associated with trained immunity, such as mTOR inhibitors, such as rapamycin (140). Furthermore, trained immunity induced by β-glucan can be inhibited by blocking the rate-limiting enzyme HMG-CoA reductase (reduction of cholesterol synthesis) with fluvastatin *in vitro* (26). Otherwise, another strategy is to modulate epigenetic changes using inhibitors of enzymes that methylate histones or DNA, including DNA methyltransferases, lysine methyltransferases, and histone deacetylases (141). In this sense, the use of nanocarriers that lead the mentioned compounds to a particular cell type (or its progenitors) emerges as a promising alternative to avoid the damaging effects of blocking the indicated pathways (81).

On the other hand, several studies propose (as we have detailed above) that trained immunity can promote a beneficial response in autoimmune conditions by inducing a macrophage response that can result in the apoptosis of active autoreactive T cells or by the promotion of an anti-inflammatory profile (96). Moreover, other reports indicate that by using helminths components (98, 99) or low LPS-dose (66), we could redirect trained immunity to regulate immune responses and reduce chronic inflammation (**Figure 1**). On the other hand, the observation that vaccination (more frequently documented with BCG) could be helpful to treat autoimmune and AIF diseases is encouraging since it has been widely used for many decades worldwide. Interestingly, although the duration of trained immunity has been reported for a few months, and even 1-5 years (39), transgenerational effects have recently been suggested (142, 143). Consequently, these strategies are interesting in the context of chronic diseases, as they promise a long-term beneficial effect.

CONCLUSIONS

This article aimed to review the cellular changes produced during trained immunity and weigh the effect of immune boost in the development/treatment of autoimmune and AIF conditions.

As we discuss, components that induce trained immunity aid in prevention and inducing antimicrobial therapy and mitigate some immune-mediated diseases. Thus, the use of stimuli that induce a trained immune response (enhanced or attenuated) may be beneficial in reducing the severity of various autoimmune diseases, as observed in SSc, MS, and T1D models. Along these lines, the consequences of this trained response will depend not only on the nature and concentration of the stimulus but also on the pathologic context (the type of autoimmunity/autoinflammation or infection).

In addition to the vaccination, β-glucans, helminth components, or pharmacological strategies for rebalancing the immune response, it is interesting to mention that other factors such as diet also affect the establishment of trained immunity (144). In this way, a set of environmental factors, such as diet and pollution, may be influencing the long-term immune response profile in the long term. Furthermore, already established "epigenetic scars" can be detected in autoimmunity, which opens the possibility of developing therapies that reverse this scenario (erasure or rewriting in the direction of an attenuated response). These events have been studied for decades in MS and T1D models; however, the warning arises to consider certain factors that can worsen conditions such as SLE and RA in response to some stimuli (**Table 1**).

This article intends to contrapose the controversial evidence concerning how trained immunity may impact autoimmune/autoinflammation conditions. Furthermore, understanding the mechanism of trained immunity raises new immunotherapy strategies aimed at long-term rebalancing immune responses. In this way, one could use the understanding to delete trained immunity marks if it favors the establishment of harmful chronic inflammation or increase it in the situation in which helps lowering the severity of autoimmune diseases.

AUTHOR CONTRIBUTIONS

SCF, MR, AF-F, and MDG wrote the manuscript. AK and MDG proofread the manuscript and corrected language use. SCF constructed the figure and table. SCF, MDG, and AK supervised the work and performed critical revision of the manuscript. All authors revised and approved the manuscript.

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REVIEW
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Induced Pluripotent Stem Cell-Derived Monocytes/Macrophages in Autoinflammatory Diseases

Takayuki Tanaka[1,2*], *Takeshi Shiba*[3], *Yoshitaka Honda*[4,5], *Kazushi Izawa*[1], *Takahiro Yasumi*[1], *Megumu K. Saito*[6] *and Ryuta Nishikomori*[7]

[1] Department of Pediatrics, Kyoto University Graduate School of Medicine, Kyoto, Japan, [2] Department of Pediatrics, Japanese Red Cross Otsu Hospital, Otsu, Japan, [3] Laboratory of Lymphocyte Activation and Susceptibility to EBV Infection, INSERM UMR 1163, Imagine Institute, Paris, France, [4] Institute for the Advanced Study of Human Biology (ASHBi), Kyoto University, Kyoto, Japan, [5] Department of Immunology, Graduate School of Medicine, Kyoto University, Kyoto, Japan, [6] Department of Clinical Application, Center for iPS Cell Research and Application, Kyoto University, Kyoto, Japan, [7] Department of Pediatrics and Child Health, Kurume University School of Medicine, Kurume, Japan

The concept of autoinflammation, first proposed in 1999, refers to a seemingly unprovoked episode of sterile inflammation manifesting as unexplained fever, skin rashes, and arthralgia. Autoinflammatory diseases are caused mainly by hereditary abnormalities of innate immunity, without the production of autoantibodies or autoreactive T cells. The revolutionary discovery of induced pluripotent stem cells (iPSCs), whereby a patient's somatic cells can be reprogrammed into an embryonic pluripotent state by forced expression of a defined set of transcription factors, has the transformative potential to enable *in vitro* disease modeling and drug candidate screening, as well as to provide a resource for cell replacement therapy. Recent reports demonstrate that recapitulating a disease phenotype *in vitro* is feasible for numerous monogenic diseases, including autoinflammatory diseases. In this review, we provide a comprehensive overview of current advances in research into autoinflammatory diseases involving iPSC-derived monocytes/macrophages. This review may aid in the planning of new studies of autoinflammatory diseases.

Keywords: autoinflammatory diseases, induced pluripotent stem cells, disease modeling, drug screening, monocytes, macrophages

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Hacettepe University, Turkey

***Correspondence:**
Takayuki Tanaka
tatanaka@kuhp.kyoto-u.ac.jp

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INTRODUCTION

The concept of autoinflammation, which was first proposed in 1999, refers to seemingly unprovoked and episodic sterile inflammation manifesting as unexplained fever, skin rashes, and arthralgia (1). Autoinflammatory diseases are caused mainly by hereditary abnormalities of innate immunity, without the production of autoantibodies or autoreactive T cells. Analysis of blood cells from patients with autoinflammatory diseases has expanded our understanding of these conditions; however, there are several limitations to this approach: i) collecting enough patient blood samples for analysis is difficult because autoinflammatory diseases are rare and, to make matters worse, many patients are infants, and ii) the *in vitro* phenotype of hematopoietic cells in these patients is affected by existing inflammation or by the prescribed drugs.

The revolutionary discovery of induced pluripotent stem cells (iPSCs), whereby a patient's somatic cells can be reprogrammed into an embryonic pluripotent state by forced expression of a defined set of transcription factors (2, 3), has the transformative potential to enable *in vitro* disease modeling and drug candidate screening, as well as to provide a resource for cell replacement therapy. iPSC-derived monocytes/macrophages provide an opportunity to analyze the effect of genetic variants in the absence of the limitations described above. Recent reports demonstrate that recapitulating a disease phenotype *in vitro* is feasible for numerous monogenic diseases, including autoinflammatory diseases (4–16).

One of the main obstacles to disease studies based on iPSCs is that directed differentiation of iPSCs is time- and labor-intensive, and the results of functional analysis usually show high variation (even among iPSC clones). To overcome these issues and to obtain a stable and scalable number of mature monocytic cells from iPSC clones, immortalized proliferating myeloid cell lines have been utilized (6, 10). Recent advances in genome editing technology, such as the CRISPR system (17), facilitate functional comparisons between isogenic pairs of mutant and control iPSC clones.

In this review, we provide a comprehensive overview of current advances in research into the role of iPSC-derived monocytes/macrophages in autoinflammatory diseases. We will outline how iPSC-derived blood cells contribute to i) elucidation of disease pathogenesis, ii) functional analyses to facilitate correct diagnosis, iii) evaluation of the disease relevancies of newly identified mutations, and iv) discovery of new drug candidates.

ADVANTAGES AND CHARACTERISTICS OF PLURIPOTENT STEM CELL-DERIVED MACROPHAGES

Monocyte-Derived Macrophages and Immortalized Cell Lines

The availability of tissue-resident macrophages isolated directly from human tissues is limited due to ethical issues; therefore, monocyte-derived macrophages (MDMs) are used widely for research into human macrophages. This approach involves isolating CD14+ monocytes from peripheral blood mononuclear cells (PBMCs) and exposing them to macrophage colony-stimulating factor (M-CSF) to induce differentiation into macrophages (**Figure 1**) (18). An advantage of the MDM model is that human peripheral blood samples can be obtained without an invasive procedure; indeed, several million monocytes can be obtained from a single venipuncture. Because the *in vitro* culture period is only 1 week, MDMs are most likely free from artifacts that appear after long-term culture; therefore, they should be more representative of the patients' macrophages. However, MDMs have drawbacks, including limited proliferative capacity and a short culture period, which limit the options when it comes to genetic modification. In an era when pluripotent stem cell (PSC)-derived macrophages (PSC-MPs) are available, MDMs remain an important research tool for autoinflammatory diseases because they are likely more "physiological" than PSC-MPs. For example, MDMs have been used to examine the clinical relevance of findings obtained using PSC-MPs (6) and to examine functional differences between primary monocytes and macrophages with respect to cytokine secretion (12) (see below).

The immortalized cell lines THP-1 and U937 are used as alternative sources of macrophages because they expand spontaneously and are amenable to genetic manipulation. These cell lines originate from the peripheral blood of patients with acute monocytic leukemia and contain highly proliferative floating CD14 + "monocyte-like" cells that can differentiate into "macrophage-like" cells upon culture in the presence of phorbol myristate acetate or M-CSF (19). In the field of autoinflammatory disease research, THP-1 cells are used to analyze cell death caused by the expression of mutant *NLRP3* (20, 21). However, as these cells are derived from malignant tumor cells, their biological relevance to non-malignant monocytes/macrophages is limited (22). For example, THP-1 cells secrete only small amounts of cytokines in response to lipopolysaccharide (LPS) stimulation (22), and there are no reports describing increased inflammasome activation in these immortalized cell lines.

Pluripotent Stem Cell-Derived Macrophages

To overcome the limitations described above, several methods have been developed to generate macrophages from PSCs. In this approach, the culture conditions drive embryonic stem cells or iPSCs to differentiate through a pathway that recapitulates embryonic hematopoiesis (23–26). The advantages of PSC-MPs include easy availability, scalability, standardizability, and easy genetic manipulation (27, 28).

While a few reports have described protocols for differentiating PSCs into monocytes (29), more research has been performed with PSC-MPs than with PSC-derived monocytes, particularly within the field of autoinflammatory diseases (**Table 1**). Many studies report that PSC-MPs and MDMs have similar phenotypes, functions, and transcriptomes (26, 30–33). However, stable differences between PSC-MPs and MDMs were also identified; indeed, it is suggested that PSC-MPs recapitulate embryonic-origin macrophages rather than MDMs, which are derived from definitive hematopoiesis (23–26). Therefore, even though PSC-MPs have been applied successfully to the functional analysis of macrophages in the context of many diseases (**Table 1**), we need to keep in mind that the phenotype of PSC-MPs sometimes differs from that of MDMs or tissue-resident macrophages; where necessary, the validity of findings based on PSC-MPs should be confirmed using MDMs.

In early studies, whenever we needed PSC-MPs, we repeated the whole process of differentiating PSCs into macrophages; this process took almost 1 month to generate enough cells for our experiments (4). The differentiation efficiency was not consistent, and the protocol was laborious. To improve the efficiency of differentiation and to standardize macrophage products, we developed a method of cryopreserving PSC-MP (34). For this purpose, we established PSC-derived immortalized myeloid cell

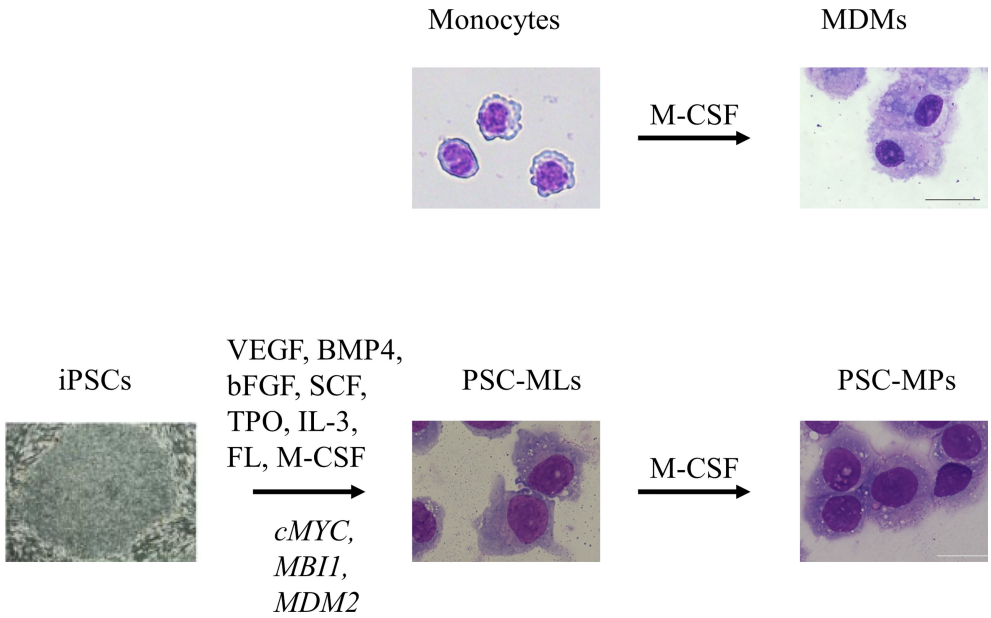

FIGURE 1 | *In vitro* culture of primary monocytes for 7 days in the presence of macrophage colony-stimulating factor (M-CSF) gives rise to adherent monocyte-derived macrophages (MDMs). Sequential stimulation with VEGF, BMP4, bFGF, SCF, TPO, IL-3, FL, and M-CSF differentiates induced pluripotent stem cells (iPSCs) into floating monocyte-like cells. After introduction of three transgenes, namely, *cMYC*, *MBI1*, and *MDM2*, into the floating cells, PSC-derived immortalized myeloid cell lines (PSC-MLs) begin to proliferate. *In vitro* culture of PSC-MLs for 7 days in the presence of M-CSF gives rise to adherent PSC-derived macrophages (PSC-MPs). Scale bars, 20 μm. VEGF, vascular endothelial growth factor; BMP4, bone morphogenic protein type 4; bFGF, basic fibroblast growth factor; SCF, stem cell factor; TPO, thrombopoietin; IL-3, interleukin-3; FL, FLT3 ligand.

lines (PSC-MLs) by introducing *MYC*, *BMI1*, and *MDM2* into iPSC-derived floating monocytic cells (**Figure 1**) (6, 10). The resulting PSC-MLs proliferated vigorously and continuously and were amenable to freeze-and-thaw cycles. After a 1-week culture in the presence of M-CSF, PSC-MLs differentiated into adherent macrophages. Both PSC-MLs and PSC-MPs expressed monocyte/macrophage markers CD45, CD11b, and CD14 and secreted cytokines in response to various stimuli. While immature PSC-MLs were more proliferative than PSC-MPs, terminally differentiated PSC-MPs secreted higher levels of cytokines than PSC-MLs. Therefore, we used both PSC-MLs and PSC-MPs for our research, depending on the goal of each experiment (4, 6–8, 10–12, 15, 16) (**Table 1**). Next, we will outline how PSC-derived blood cells contribute to i) elucidation of disease pathogenesis, ii) functional analysis to facilitate correct diagnosis, iii) evaluation of the disease relevancies of newly identified mutations, and iv) discovery of new drug candidates.

ELUCIDATION OF THE DISEASE PATHOGENESIS

Chronic Infantile Neurologic Cutaneous and Articular Syndrome

Chronic infantile neurologic cutaneous and articular (CINCA; MIM 607115) syndrome, also known as a neonatal-onset multisystem inflammatory disease (NOMID), is an autoinflammatory syndrome characterized by systemic inflammation accompanied by an urticarial rash, neurologic manifestations, and arthropathy that begins during the neonatal period (35, 36); it is the most severe form of the autoinflammatory spectrum called cryopyrin-associated periodic fever syndrome. Patients carry a heterozygous gain-of-function mutation in *NLRP3* gene and present with systemic inflammation due to excessive IL-1β production caused by hyperactivation of the NLRP3 inflammasome (37, 38). While approximately half of CINCA patients carry heterozygous gain-of-function mutations in *NLRP3* gene (39), 30%–40% harbor *NLRP3* mutations in only a small number of somatic cells (4.2%–35.8% of blood cells) (40, 41). Despite the small percentage of mutant cells, the clinical phenotype of mosaic patients is comparable with that of patients with germline mutations. Therefore, it remains controversial whether these low-frequency *NLRP3* mutant-positive cells alone are responsible for the disease phenotype or whether cells other than those harboring *NLRP3* mutations also contribute to pathogenesis.

Since each iPSC clone originates from a single cell (42), iPSC lines can be used as a discovery tool to evaluate the impact of low-frequency somatic mosaicism mutations. Taking advantage of this feature, *NLRP3*-mutant and wild-type (WT) iPSCs were established from patients with CINCA syndrome harboring a somatic *NLRP3* mutation (4). When these iPSCs were differentiated into macrophages and their phenotypes were compared, only *NLRP3*-mutant macrophages produced a large amount of IL-1β. Interestingly, when mutant macrophages were

TABLE 1 | Disease modeling and application of iPSCs to autoinflammatory diseases.

Primary objective	Gene	Disease	Target cell type	Reference
Disease modeling	*NLRP3*	CINCA syndrome	Macrophages, chondrocytes	(4, 5)
	NOD2	Blau syndrome	Macrophages	(6, 7)
	PSMB8	Nakajo–Nishimura syndrome	Myeloid cell lines	(8)
	IL-10RB	Inflammatory bowel diseases	Macrophages	(9)
Diagnosis	*NLRC4*	CINCA syndrome	Myeloid cell lines	(10)
	NEMO	Immunodeficiency without obvious ectodermal dysplasia	Myeloid cell lines	(11)
	MEFV	Familial Mediterranean fever	Macrophages	(12)
Disease relevancies	*OAS1*	OPAID	Macrophages	(13)
	NFKB1A	Autoinflammation with immunodeficiencies	Macrophages	(14)
Drug screening	*NLRP3*	CINCA syndrome	Macrophages	(15)
	PSMB8	Nakajo–Nishimura syndrome	Myeloid cell lines	(16)

CINCA, chronic infantile neurologic cutaneous and articular; OPAID, OAS1-associated polymorphic autoinflammatory immunodeficiency.

co-cultured with WT macrophages to create a pseudo-mosaic state, the production of IL-1β was significantly higher than that of mutant macrophages alone. In other words, in cases of somatic mosaicism, *NLRP3*-mutant cells are the main producers of IL-1β, although WT cells also contribute to inflammation in some way. Later, Baroja-Mazo et al. used patient-derived MDMs to show that upon activation of caspase-1, oligomeric NLRP3 inflammasome particles are released from activated macrophages and phagocytosed by surrounding macrophages, leading to further activation of caspase-1 (43). Thus, iPSCs can be used for a detailed analysis of the unique pathology associated with somatic mosaicism.

Regarding the pathogenesis of cartilage overgrowth in CINCA syndrome patients, different methods have been used to assess the contribution of chondrocytes and hematopoietic cells. After differentiating WT or mutant iPSCs into chondrocytes, we compared the size of the chondrocyte tissues produced; we found that mutant iPSCs produced larger chondrocyte masses than WT iPSCs owing to the overproduction of glycosaminoglycans, which correlated with increased expression of the chondrocyte master regulator SOX9; this was independent of caspase 1 and IL-1 and, thus, the NLRP3 inflammasome (5). By contrast, Wang et al. used a model mouse exhibiting global NLRP3 activation and several characteristics of the human disease (i.e., systemic inflammation and cartilage dysplasia) to show that activation of NLRP3 in myeloid cells, but not in mesenchymal cells, triggers chronic inflammation, which ultimately causes growth plate and epiphyseal dysplasia (44). Mechanistically, inflammation causes severe anemia and hypoxia in the bone environment but downregulates the HIF-1α pathway in chondrocytes, thereby promoting the demise of these cells. It is theoretically possible to obtain both PSC-derived chondrocytes and macrophages and evaluate their interaction *in vitro* co-cultures; however, mouse models may provide the opportunity to observe physiological interactions over a longer term than PSC-derived somatic cell models.

Blau Syndrome

Blau syndrome (MIM 186580) is a disease caused by a heterozygous gain-of-function mutation in *NOD2* gene, which leads to granulomatous lesions in the skin, joints, and eyes during childhood and can cause severe complications such as blindness and joint contractures later in life (45, 46). The NOD2 protein is an intracellular pathogen recognition receptor, which upon recognition of the ligand MDP activates the nuclear factor-κB (NF-κB) pathway, thereby upregulating the production of proinflammatory cytokines and chemokines. Although pathological studies reveal that granulomas in Blau syndrome patients are accompanied by a prominent expression of IFN-γ (47), the details regarding the molecular mechanism (s) by which *NOD2* mutations drive the pathogenesis of Blau syndrome are unclear. The treatment for Blau syndrome has long been non-specific immunosuppressive therapies such as corticosteroids and/or methotrexate; however, recent studies report the effectiveness of biologics targeting TNF, IL-6, and IL-1 (48–53). Among these, anti-TNFα agents are used most widely, although the pharmacologic mechanism is unknown. Therefore, investigation of the cellular phenotypes of patients is necessary to evaluate the mechanism(s) underlying anti-TNFα therapy.

Studies based on mouse models have not reproduced the disease-related phenotype sufficiently (54). Therefore, we investigated the phenotypes of human macrophages carrying mutant *NOD2* by establishing iPSCs from patients with a *NOD2* mutation and obtaining isogenic iPSC clones in which the mutation was repaired by CRISPR/Cas9; we then differentiated them into macrophages (6). We found that IFN-γ-primed PSC-MPs harboring mutant *NOD2* demonstrated ligand-independent activation of NF-kB translocation to the nucleus, followed by the production of proinflammatory cytokines such as IL-6 and IL-8. We also confirmed this phenotype in more physiological cells; indeed, MDMs derived from Blau syndrome patients showed IFN-γ-induced ligand-independent activation of NF-kB translocation to the nucleus and subsequent cytokine production.

Next, we tried to clarify how anti-TNF treatment helps to control inflammation by comparing characteristics such as transcriptome profiling and cytokine secretion by MDMs from untreated and anti-TNF-treated Blau syndrome patients (7). We found that TNF-dependent NF-kB signaling reduces the threshold for IFN-γ-mediated inflammatory responses in Blau syndrome and that resetting of this primed state by anti-TNF treatment contributes to the prevention of the autoinflammatory loop, even in the presence of a *NOD2* mutation and IFN-γ

stimulation. Thus, the iPSC-based macrophage study enabled us to elucidate disease pathogenesis and to identify the mechanism underlying the efficacy of anti-TNF treatment at the cellular level. To ascertain whether blocking IFN-γ signaling is a potential treatment for chronic inflammation in Blau syndrome patients, we still need to determine whether the IFN-γ pathway is actually activated in these patients and whether IFN-γ signaling is the principal priming pathway among the stimulants known to upregulate NOD2 expression (i.e., TNF-α, LPS, and other Toll-like receptor ligands) (55–57).

Nakajo–Nishimura Syndrome

Nakajo–Nishimura syndrome (NNS)/chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature (CANDLE) syndrome/joint contractures, muscular atrophy, microcytic anemia, and panniculitis-induced lipodystrophy (JMP) syndrome is a form of proteasome-associated autoinflammatory syndrome (PRAAS1/MIM 256040) characterized by chronic inflammation and lipomuscular atrophy caused by homozygous loss-of-function mutations in *PSMB8* gene encoding β5i, a component of the immunoproteasome (58, 59). Based on the finding of a strong type I interferon (IFN) response gene signature in patient peripheral blood cells (60, 61), Janus kinase (JAK) inhibitors are an effective treatment for PRAAS (62) because they inhibit the JAK/STAT pathway, the principal signaling pathway downstream of cytokines and growth factor receptors (including the IFN-α/β receptor) (63, 64). However, the precise mechanism underlying autoinflammation remains unclear. To elucidate the impact of *PSMB8* mutations on monocyte/macrophage function, we generated iPSCs from an NNS patient and repaired the PSMB8 mutation using the CRISPR/Cas9 system (8). We then generated iPSC lines that share the same genetic background but without the *PSMB8* mutation. When immunoproteasome assembly in PSC-MLs was induced by IFN-γ and TNF-α, immunoproteasome activity in *PSMB8*-mutant PSC-MLs was impaired significantly compared with that in the WT counterparts. As a consequence, secretion of the proinflammatory cytokine IL-6, and chemokines MCP-1 and IP-10, by mutant PSC-MLs increased. Furthermore, we revealed that the production of intracellular reactive oxygen species also increased, that mutant cells had higher levels of p38 MAPK and phosphorylated STAT1, and that addition of antioxidants, a p38 MAPK inhibitor, or JAK inhibitors suppressed the production of proinflammatory cytokines and chemokines. This demonstrates that PSC-MLs is a useful tool for modeling proteasome-associated autoinflammatory diseases.

Several different mechanisms have been postulated to explain the lipodystrophy in PRAAS. On the one hand, lipophagia can result from the proinflammatory state of adipose tissue macrophages (65, 66). Alternatively, high IFN levels may be toxic to adipocytes (67). PSC-derived blood cells alone cannot reproduce the complex interactions within the human body. Verhoeven et al. reported that hematopoietic stem cell transplantation halted the progression of lipodystrophy in a PRAAS patient during a 7-year follow-up, demonstrating that hematopoietic cells play a role in the lipodystrophy (68). It is theoretically possible to obtain both macrophages and adipocytes from PSCs and to evaluate their interaction in *in vitro* co-culture; however, to examine long-term effects, clinical observation of the patients may be more appropriate.

Inflammatory Bowel Disease Caused by Loss of IL-10 Signaling

IL-10, one of the most important cytokines for the maintenance of intestinal homeostasis, regulates inflammation by inhibiting macrophage activation (69, 70). While the protective role of IL-10 is relatively well established in the context of inflammatory bowel disease (IBD) and other inflammatory diseases, its role in susceptibility to infections is less well understood. To study the impact of IL-10 on the inflammatory and microbicidal activities of macrophages, Mukhopadhyay et al. established iPSCs from a patient with homozygous loss-of-function mutations in the IL-10 receptor β (IL-10RB) and differentiated them into macrophages (9). They showed that IL-10RB−/− patient PSC-MPs were deficient in the IL-10 signaling pathway and that suppression of proinflammatory cytokine secretion was not observed upon simultaneous stimulation with IL-10 and LPS. IL-10RB−/− macrophages also exhibited a defect in bactericidal activity. Genes involved in synthesis and receptor pathways for PGE2 were more highly induced in IL-10RB−/− PSC-MPs, and these macrophages produced more PGE2 than controls after LPS stimulation. Furthermore, combined inhibition of PGE2 synthesis and receptor binding increased bactericidal activity. These results indicate the presence of crosstalk between the IL-10 and PGE2 pathways, dysregulation of which may drive aberrant macrophage activation and impaired host defense, thereby contributing to IBD pathogenesis.

FUNCTIONAL ANALYSIS TO FACILITATE A CORRECT DIAGNOSIS

Diagnosis of Somatic *NLRC4* Mosaicism in a Patient With Chronic Infantile Neurologic Cutaneous and Articular Syndrome

As mentioned above, while about 90% of CINCA syndrome patients have constitutive or somatic mosaic mutations in *NLRP3*, the remaining 10% do not (41). Since most CINCA patients lacking *NLRP3* mutations respond to anti-IL-1 therapy, activation of some kind of inflammasome is suspected. Therefore, we established iPSCs from a CINCA syndrome patient in whom an *NLRP3* mutation was not identified by conventional Sanger sequencing, differentiated them into PSC-MLs, and measured the production of IL-1β in response to NLRP3 inflammasome activation (10). PSC-ML clones were categorized as "normal" clones that secreted IL-1β after LPS and ATP stimulation and "pathological" clones that secreted IL-1β after LPS stimulation alone. To elucidate the phenotypic heterogeneity of IL-1β secretion among the clones, we performed whole-exome sequencing of representative iPSC clones and identified a novel mutation in *NLRC4* gene in the diseased

clones. The mutant allele was observed in the patient's fibroblasts (34.1%) and PBMCs (30.3%). When we knocked out the mutant *NLRC4* gene in PSC-MLs, the production of IL-1β normalized. These results show that somatic mosaicism of the *NLRC4* gene mutation caused the clinical phenotype of CINCA syndrome in this patient. To date, no other case of somatic mosaicism of *NLRC4* has been reported. Collectively, these data show that iPSC technology can be used to diagnose a novel somatic mosaic mutation.

Diagnosis of Cell Type-Dependent Quantitative NF-κB Essential Modulator Deficiency Caused by a Deep Intronic Mutation

NF-κB essential modulator (NEMO), also known as an inhibitor of NF-κB kinase subunit gamma (IKK-γ), encoded by *IKBKG* gene (71, 72), is the third regulatory subunit of the IκB kinase (IKK) complex (73, 74). Amorphic mutations of *IKBKG*, which abolish canonical NF-κB activation, are lethal in men, whereas in women, they cause X-linked dominant incontinentia pigmenti (IP) (MIM 308300), a multisystem disorder affecting the skin and its appendages (75, 76). Hypomorphic *IKBKG* mutations that impair IκBα phosphorylation and sequential NF-κB activation cause X-linked recessive (XR) anhidrotic ectodermal dysplasia with immunodeficiency (EDA-ID) (MIM 300291) (77, 78). Affected men display typical signs of EDA, including sparse hair, eyebrows, and eyelashes; hypohidrosis; hypodontia; and conical incisors, together with immunodeficiency or inflammatory colitis (79). The main immune phenotype of EDA-ID is immunodeficiency rather than inflammation, but both EDA-ID and autoinflammatory diseases are categorized within the broad spectrum of primary immunodeficiency (80). Since iPSC-derived macrophages and ectodermal cells contributed substantially to establishing a correct diagnosis, we would like to describe the following study. While most variants underlying XR-EDA-ID are missense mutations or in-frame indels, approximately 10% of sporadic and familial cases of EDA-ID remain genetically unexplained. Therefore, we investigated three male patients from two families whose ID phenotype was much more severe than the manifestations of EDA (11), leading to an early death before the age of 1 year. Whole-genome sequencing identified the same deep intronic mutation in *IKBKG*. Next, we found that this deep intronic *IKBKG* mutation created a novel splicing donor site for a pseudoexon inclusion, which led to a severe decrease in NEMO protein expression and inflammatory cytokine secretion by patient PBMCs. Using patient-derived iPSCs, we revealed a cell type-dependent effect of the mutation on aberrant *IKBKG* splicing, which explains the reason for the discrepancy between the severe ID phenotype and the more subtle EDA symptoms. When we measured the levels of WT and alternative *IKBKG* transcripts in undifferentiated iPSCs, PSC-MLs, and iPSC-derived neuronal precursor cells (iPSC-NPs), we found that iPSCs produced 17% WT transcripts, PSC-MLs produced only 3% WT transcripts, and iPSC-NPs produced 35%. Patient-derived PSC-MLs showed much lower WT NEMO protein levels, along with impaired NF-κB activation upon LPS stimulation. Complementation of patient-iPSCs with WT NEMO restored NF-κB pathway activation in PSC-MLs. Thus, iPSCs contribute to the correct diagnosis of the deep intronic *IKBKG* mutation and to the identification of the cell type-dependent quantitative NEMO deficiency, thereby expanding our understanding of this disease.

Functional Evaluation of the Pathological Significance of *MEFV* Variants

Monocytes and macrophages play similar roles in the pathogenesis of most inflammatory diseases. For example, both *NLRP3*-mutant monocytes (37) and macrophages (4) exhibit spontaneous NLRP3 inflammasome activation without secondary signals and secrete IL-1β after priming with LPS alone. However, cytokine secretion by monocytes and macrophages from familial Mediterranean fever (FMF) patients is clearly different (12).

FMF, the most common hereditary autoinflammatory disorder, is characterized by recurrent episodes of fever, polyserositis, and abdominal pain (MIM 249100). FMF is associated with mutations in *MEFV* gene, which encodes the inflammasome adaptor pyrin (81). Pyrin is an inflammasome sensor that detects imbalances in Rho GTPase activity, which can be caused by bacterial effectors or bacterial toxins (82). Almost 400 *MEFV* variants have been recorded in *Infevers*, an online database of autoinflammatory disease mutations. Among *MEFV* variants, a systematic review revealed that M694V and M694I in exon 10 are related to a severe phenotype of FMF (83). Other *MEFV* variants are associated with variable clinical phenotypes, including pyrin-associated autoinflammation with neutrophilic dermatosis (84, 85) and autosomal-dominant FMF-like diseases (86–88). Consequently, the novel umbrella term, pyrin-associated autoinflammatory diseases, has been proposed to define all autoinflammatory diseases caused by *MEFV* mutations (89). Although a consensus-driven pathogenicity classification was proposed recently to support the uniform diagnosis of FMF worldwide (90), the complexity of the clinical phenotype and its association with *MEFV* variants led to difficulty in assessing the pathogenicity of variants identified in clinical settings. Despite the successful use of colchicine and IL-1β-blocking therapies as treatments for FMF, *in vitro* pyrin inflammasome activation (and its inhibition by colchicine) in patients' hematopoietic cells remains controversial (91–94).

To clarify this issue, we evaluated cytokine secretion by primary monocytes and MDMs obtained from FMF patients carrying the heterozygous M694I mutation. In response to TcdA stimulation, levels of IL-1β secreted by FMF monocytes were similar to those of control monocytes, and colchicine failed to inhibit IL-1β secretion by FMF monocytes. By contrast, IL-1β secretion by FMF MDMs was significantly higher than that by control MDMs in response to LPS and TcdA, and IL-1β secretion by FMF MDMs was inhibited by colchicine. These results suggest that MDMs, rather than monocytes, reflect the clinical features of FMF patients (e.g., hyperactivation of the pyrin inflammasome and subsequent inhibition by colchicine).

After confirming that macrophages derived from patients' iPSCs (PSC-MPs) recapitulate the phenotype of FMF MDMs, we evaluated two rare *MEFV* variants, T577N and N679H, identified in two families in which autoinflammatory disease with dominant inheritance was suspected (95, 96). No T577N patients met the Tel-Hashomer criteria, whereas two N679H patients fulfilled the criteria (97). Whereas the amount of IL-1β secreted by T577N iPS-MPs was comparable with that secreted by WT cells, N679H iPS-MPs secreted significantly more IL-1β (like the M694I variant). Thus, *MEFV* variants causing FMF, namely, N679H and M694I, induced IL-1β secretion after pyrin inflammasome activation. Thus, we established a method for evaluating *MEFV* variants by obtaining mutant PSC-MPs and measuring cytokine secretion in response to pyrin inflammasome stimulation (12).

In addition, we characterized cytokine secretion by primary monocytes and macrophages isolated from typical FMF patients. Gene expression differs considerably between monocytes and macrophages, including expression of the tubulin-related genes (98). Given the vital role of microtubule polymerization in pyrin inflammasome activation (99), we speculate that greater expression of tubulin-related genes in macrophages might be related to the differential response to colchicine inhibition between monocytes and macrophages. Moreover, monocytes and macrophages are somewhat different in terms of inflammasome activation pathways. For example, although both cell types use the canonical NLRP3 inflammasome activation pathway, the alternative (100) or non-canonical (101) NLRP3 inflammasome pathway is functional only in monocytes. It is possible that an undiscovered pyrin inflammasome activation pathway is functioning in either monocytes or macrophages, but not in both. The precise reason underlying the distinct mechanisms of pyrin inflammasome activation in these cells remains to be elucidated.

Evaluation of pyrin inflammasome activation using PSC-MPs led to the discovery of a role for enhanced pyrin inflammasome activation in the pathogenesis of CDC42-associated autoinflammation (102). Nishitani-Isa et al. used PSC-MPs to show that aberrant palmitoylation of CDC42 protein carrying the mutation caused its retention in the Golgi apparatus and triggered overactivation of the pyrin inflammasome. By contrast, subsequent *ex vivo* or *in vitro* studies established methods for functional evaluation of FMF-related *MEFV* variants. THP-1 cells transfected with FMF-related *MEFV* variants showed higher levels of UCN-01/TcdA-induced cell death than THP-1 cells expressing other *MEFV* variants (103). Similar studies focusing on the evaluation of primary cells have been reported; indeed, Magnotti et al. showed that UCN-01-induced cell death was much faster in FMF monocytes than in monocytes from healthy donors or patients suffering from other inflammatory disorders. They also established an assay that can be used for rapid diagnosis of FMF with high sensitivity and specificity (104). By focusing on unresponsiveness to colchicine inhibition, van Gorp et al. reported an assay that robustly segregated FMF patients from healthy donors and patients with other inflammatory disorders (105). One advantage of this assay is that the test may be performed on PBMCs and even whole blood. While cell line-based approaches are free from the influence of existing inflammation or prescribed drugs, as well as being more suitable for the evaluation of a specific mutation, primary cell assays have an advantage in that they take less time and the results reflect the influence of the genetic background, or the combined effects of multiple *MEFV* variants, in a single patient. Therefore, we need to select the most appropriate approach for each situation.

EVALUATION OF THE DISEASE RELEVANCIES OF NOVEL MUTATIONS

Autoinflammatory Immunodeficiency Caused by Heterozygous *OAS1* Gain-of-Function Variants

Type I IFN-inducible oligoadenylate synthetase 1 (OAS1) initiates an antiviral immune response upon recognition of cytoplasmic viral double-stranded RNA (dsRNA) (106, 107). OAS1 is a template-independent nucleotidyltransferase that produces the second messengers 2′-5′-oligoadenylate (2-5A) (108, 109). In turn, 2-5A activates ribonuclease L (RNase L), which degrades viral and cellular RNA, thereby interfering with viral propagation (110).

Okano et al. described a polymorphic autoinflammatory immunodeficiency with recurrent fever, dermatitis, IBD, PAP, and hypogammaglobulinemia caused by *de novo* heterozygous OAS1 gain-of-function mutations; they named the disease OAS1-associated polymorphic autoinflammatory immunodeficiency (OPAID) (13). The expression of mutant OAS1 in response to common infectious agents resulted in an inappropriate synthesis of 2-5A independent of dsRNA binding; this induced RNase L-mediated cleavage of cellular RNA, leading to transcriptomic alteration, translational arrest, and dysfunction and apoptosis of primary monocytes, PSC-MPs, and B cells. To overcome the scarcity of primary monocytes in that study, the authors differentiated patient-derived iPSCs into macrophages (i.e., PSC-MPs). IFNα-stimulated PSC-MPs carrying mutant OAS1 displayed impaired cell adhesion and clustering, scavenger receptor expression, and phagocytosis in an RNase L-dependent manner. While mutant OAS1-knock-in mice failed to reproduce the disease phenotype (111), the characteristics of PSC-MPs were consistent with those of primary monocytes. Given the reported differences in immune responses between species (112), human iPSC-derived hematopoietic cells may be a more relevant source of primary patient-derived cells than animal models for research into certain diseases.

Paradoxical Autoinflammation Caused by a Dominant-Negative *NFKB1A* Mutation

The NF-κB protein complex is integral to the initiation of inflammation, and NF-κB activation is controlled by inhibitors of κB (IκBα, IκBβ, and IκBϵ) and by the IκB kinase (IKK) complex, which comprises NEMO, IKK1, and IKK2 (described above). Patients with genetic defects (e.g., in *NEMO* and *NFKBIA*) in the NF-κB signaling pathway usually display

severe immunodeficiency, with impaired cellular responses to immune stimuli such as LPS or TNF-α (113, 114).

Tan et al. reported an infant with a clinical pathology comprising neutrophil-mediated autoinflammation and recurrent bacterial infections caused by a *de novo* heterozygous missense mutation in *NFKBIA* (14). The resulting L34P IκBα variant caused a severe reduction in NF-κB nuclear translocation and, consequently, downstream production of IL-6 or IL-8 by the patient's fibroblasts. Paradoxically, IL-1β concentrations in the patient's blood were elevated. To determine whether myeloid cells were the major source of elevated IL-1β levels, they generated iPSC-derived macrophages from the patient's fibroblasts. Despite the patient's PSC-MPs showing defective nuclear translocation of NF-κB in response to LPS stimulation, they produced significantly more IL-1β than control PSC-MPs. The patient's hypersecretion of IL-1β correlated with activated neutrophilia and liver fibrosis with neutrophil accumulation. Hematopoietic stem cell transplantation reversed the neutrophilia, restored neutrophils to a resting state, and normalized IL-1β release from stimulated leukocytes. These data suggest that NF-κB in humans plays an unexpected role as an anti-inflammatory agent by regulating IL-1β secretion, thereby preventing myeloid inflammation.

DISCOVERY OF DRUG CANDIDATES

Why are iPSC-derived monocytes/macrophages a useful platform for high-throughput screening of drug candidates? Because of their pluripotency and proliferative potential, iPSCs can serve as an unlimited source of patient-derived somatic cells. Two studies provide proof of the concept that iPSC-derived monocytes/macrophages are a useful tool for drug screening (15, 16).

In one study, we searched for compounds that inhibit NLRP3 inflammasome activation in PSC-MPs (15). The NLPR3 inflammasome is an attractive drug target because NLRP3 inflammasome activation is associated not only with rare autoinflammatory disorders such as CINCA syndrome but also with the pathogenesis of various chronic inflammatory conditions (115). *NLRP3*-mutant macrophages were used for this assay because LPS-mediated stimulation in the absence of a second signal was sufficient to activate the NLRP3 inflammasome (4). High-throughput screening of 4,825 compounds, including Food and Drug Administration (FDA)-approved drugs and compounds with known bioactivity, identified seven that selectively inhibited IL-1β secretion without affecting IL-6 production. All seven compounds inhibit the NLRP3 inflammasome (116–119). Before selecting the cell types for high-throughput screening, we compared three types of PSC-derived blood cells in terms of the coefficient of variation (CV) for IL-1β secretion; this is because blood cells with a low CV value enabled us to accurately assess the potency of candidate compounds. The first type of PSC-derived floating cells was obtained after a 2-week culture of iPSCs in a differentiation medium. The second cell type of PSC-MLs was established by the lentiviral-based introduction of three genes into PSC-derived floating cells (6, 10). After a 1-week culture of PSC-MLs in a fetal calf serum (FCS)-containing medium in the presence of M-CSF, we obtained a third type of terminally differentiated PSC-MP (**Figure 1**). The levels of released cytokines were more consistent, and the CV value became lower as differentiation progressed; the CV value was lowest for PSC-MPs. Therefore, we used PSC-MPs to screen NLRP3 inhibitors (15).

In another study, we screened potential therapeutic candidates using PSC-MLs derived from NNS patients (16); screening was based on consistent overproduction of MCP-1 and IL-10 from PSC-MLs derived from NNS patients in the preceding study (8). We screened 5,821 compounds, including FDA-approved drugs, kinase inhibitors, and bioactive chemicals, and we identified CUDC-907 as an effective inhibitor of MCP-1 and IP-10 release (16). While hit compound CUDC-907 seemed a promising drug candidate in terms of efficacy, there were concerns regarding adverse effects because CUDC-907 inhibited NNS fibroblast proliferation during a 2-week culture. Therefore, CUDC-907 was not directly applicable to the clinical study.

In both studies, we started screening compound libraries with known bioactivity and provided proof of concept that PSC-derived monocytes/macrophages can serve as an effective tool for screening drug candidates, although the hit compounds could not be applied directly to clinical studies. Combining high-throughput screening using PSC-monocytes/macrophages with pharmaceutical techniques (to generate a more potent compound from known substances by modifying the chemical structure) may pave the way to novel drug discovery.

LIMITATIONS OF THESE APPROACHES

The PSC–macrophage system would not necessarily be suitable for modeling all autoinflammatory diseases. Therefore, we would like to mention two points that should be considered before starting a study using PSC-MPs.

Elucidation of the Pathologic Interaction Between Hematopoietic and Non-Hematopoietic Cells

PSC-derived macrophages are a homogeneous population, and modeling the whole human body using only macrophages is impossible. For example, RELA (120) or RIPK 1 (121) mutations were identified among patients with early-onset inflammatory diseases. Both proteins are involved in the NF-κB activation pathway in response to TNF stimulation (122). Since they noted enhanced cytotoxicity caused by TNF stimulation in fibroblasts derived from the RELA-haploinsufficiency patient (but not in hematopoietic cells) (120), PSC-MPs alone would be insufficient for modeling such diseases; rather, studies of the interaction among epithelial cells, stromal cells, and hematopoietic cells would be necessary. As described above, clinical observation provided novel findings of lipodystrophy in a PRAAS patient

(68), and a knock-in mouse model revealed the contribution of *NLRP3*-mutant hematopoietic cells to cartilage overgrowth (44). Thus, elucidation of the pathologic interactions between hematopoietic and non-hematopoietic cells is usually difficult when using PSC-derived somatic cells alone.

Similarities and Differences Between Monocyte-Derived Macrophages and Pluripotent Stem Cell-Derived Macrophages

Many researchers have identified similarities between MDMs and PSC-MPs with regard to global gene expression (23, 24, 26, 30, 32, 33), cytokine secretion (26), and phagocytosis of infectious organisms (32). Polarization of macrophages from M0 to M1 or M2 is accompanied by changes in gene expression similar to those observed in blood-derived counterparts (26, 33). However, certain differences in the expression of genes involved in chemokine production, antigen presentation, and tissue remodeling were identified (30). We need to be cautious when applying PSC-MPs for disease modeling because these differences may affect their responses.

FUTURE DIRECTIONS

The list of autoinflammatory diseases continues to expand and now includes over 40 genetically defined disorders categorized according to defects affecting the inflammasome, type 1 interferonopathies, and non-inflammasome-related conditions (123). Although the genetic basis of many autoinflammatory diseases is now known, the molecular etiology frequently remains unclear. Given the rapid progress in the application of PSC-MPs to research into autoinflammatory diseases, further discoveries are expected. Takata et al. reported differentiation of PSCs into tissue-resident macrophage-like cells upon receipt of organ-specific cues (24). Co-culturing human PSC-MPs with iPSC-derived neurons *in vitro* promoted differentiation into microglia-like cells. Furthermore, murine PSC-MPs differentiated *in vivo* into functional alveolar macrophages after engraftment in the lung. Novel methods of driving differentiation into tissue-resident macrophages will enable modeling and elucidation of organ-specific inflammation. In addition to monocytes/macrophages, neutrophils are important innate immune cells that are involved in the pathogenesis of autoinflammatory disorders. While it is now possible to cryopreserve PSC-MPs at the progenitor level (6, 10), or as the final product (34), cryopreservation of PSC-derived neutrophils has yet to be reported. Improvements in the differentiation protocol may enable the utilization of PSC-derived neutrophils for autoinflammatory disease research. We would like to describe two other promising examples of PSC-MP applications.

Drug Screening to Identify Alternative Pyrin Inflammasome Inhibitors

The scalability and standardizability of PSC-MPs make them particularly suitable for screening drug candidates. In addition to the diseases described above (15, 16), PSC-MPs may be useful for identifying pyrin inflammasome inhibitors other than the traditional colchicine. Colchicine has been the standard treatment for FMF for more than 100 years. However, in 5%–10% of patients, it is either ineffective or associated with unacceptable side effects (124). The efficacy and safety of canakinumab, an anti-IL-1β monoclonal antibody, have been shown in patients with colchicine-resistant FMF (125). However, given the drawbacks of canakinumab, such as high cost and limited safety information about administration to pregnant women, novel alternative treatments are needed. Recent reports about the association between microtubule polymerization and pyrin inflammasome activation (99, 126) have focused attention on microtubule inhibitors. Microtubules are the target of anticancer drugs such as paclitaxel or vinblastine. Potent and safe colchicine binding-site inhibitors such as CA4P and ABT-751 have entered clinical trials as anticancer agents (127). Although many other candidates have not entered clinical trials due to toxicity at the high concentrations needed for anticancer treatment, they may be effective and safe pyrin inflammasome inhibitors when used at lower concentrations. Among the tubulin inhibitors that were previously developed as anticancer medicines, we may be able to identify a more potent and less toxic pyrin inflammasome inhibitor than conventional colchicine.

Investigation of the Interaction Between Innate and Adaptive Immunity in Patients With Interferonopathies

Originally, "autoinflammatory disorders" covered inborn errors of innate immunity (1). Classification of autoinflammatory diseases has been updated periodically and now covers not only abnormalities in innate immunity but also those in adaptive immunity, including STING-associated vasculopathy with onset in infancy (SAVI) or COPA syndrome. A severe inflammatory phenotype of SAVI is induced by gain-of-function mutations in *STING1*, which encodes STING (stimulator of IFN genes); this phenotype is characterized clinically by skin vasculopathy, systemic inflammation, and lung involvement (e.g., interstitial lung disease or diffuse alveolar hemorrhage), which is associated with high morbidity and mortality (128, 129). In addition to constitutive activation of the type I IFN pathway, an autoimmune component (e.g., high titers of autoantibodies) is frequently detected in SAVI (130). Self-DNA sensing through cGAS-STING is involved in many processes, including autoimmunity (131); however, the precise mechanism linking STING gain of function to the production of autoantibodies has not yet been defined.

While various groups have established methods for obtaining innate immune macrophages from PSCs (as described above), differentiating PSCs into T cells is difficult. However, a recent report describes a protocol for differentiating PSCs to T-cell receptor (TCR)-expressing innate lymphoid-like helper cells (132). These innate lymphoid helper-like cells induce bcr-abl-specific TCR signaling, which mediates effective anti-leukemic cytotoxic T lymphocyte responses *via* dendritic cell (DC) maturation. Further deciphering of STING-mediated autoimmunity is awaited, and

further investigations into PSC-derived macrophages and T cells may provide an opportunity to study aberrant interactions between innate and adaptive immune cells.

CONCLUSIONS

In this review, we provide a comprehensive overview of current advances in the use of iPSC-derived monocytes/macrophages for research into autoinflammatory diseases. We describe the advantages and characteristics of PSC-MPs, current applications to research into autoinflammatory diseases, and future directions. We hope that this review will provide clues that facilitate further research into autoinflammatory diseases and contribute to the development of new treatments for patients.

AUTHOR CONTRIBUTIONS

This article is mainly written by TT. YH wrote part of the manuscript. TS, KI, TY, and MS proofread the manuscript. RN revised the manuscript. The corresponding author had final responsibility for the decision to submit for publication. All authors contributed to the article and approved the submitted version.

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Lessons From Transcriptome Analysis of Autoimmune Diseases

Yasuo Nagafuchi[1,2*], *Haruyuki Yanaoka*[3] *and Keishi Fujio*[1]

[1] Department of Allergy and Rheumatology, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan, [2] Department of Functional Genomics and Immunological Diseases, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan, [3] Immuno-Rheumatology Center, St. Luke's International Hospital, St. Luke's International University, Tokyo, Japan

Various immune cell types, including monocytes, macrophages, and adaptive immune T and B cells, play major roles in inflammation in systemic autoimmune diseases. However, the precise contribution of these cells to autoimmunity remains elusive. Transcriptome analysis has added a new dimension to biology and medicine. It enables us to observe the dynamics of gene expression in different cell types in patients with diverse diseases as well as in healthy individuals, which cannot be achieved with genomic information alone. In this review, we summarize how transcriptome analysis has improved our understanding of the pathological roles of immune cells in autoimmune diseases with a focus on the ImmuNexUT database we reported. We will also discuss the common experimental and analytical design of transcriptome analyses. Recently, single-cell RNA-seq analysis has provided atlases of infiltrating immune cells, such as pro-inflammatory monocytes and macrophages, peripheral helper T cells, and age or autoimmune-associated B cells in various autoimmune disease lesions. With the integration of genomic data, expression quantitative trait locus (eQTL) analysis can help identify candidate causal genes and immune cells. Finally, we also mention how the information obtained from these analyses can be used practically to predict patient prognosis.

Keywords: transcriptome, eQTL, autoimmune disease, immune cell, monocytes, macrophages, systemic lupus erythematosus, rheumatoid arthritis

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***Correspondence:**
Yasuo Nagafuchi
nagafuchi@g.ecc.u-tokyo.ac.jp

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INTRODUCTION

Autoimmune reactions and chronic inflammation are hallmarks of systemic autoimmune diseases or rheumatic diseases. The presence of autoantibodies and autoreactive T and B cells in these diseases indicates that the adaptive immune system is critical for their pathogenesis. The innate immune response also plays an indispensable role. The infiltration of monocytes and macrophages is always observed in the affected tissues of patients with autoimmune diseases (1). These cells stimulate and recruit other immune cells to diseased tissues by secreting pro-inflammatory cytokines and chemokines. Macrophages are important phagocytes acting against pathogens and serve as antigen-presenting cells that activate adaptive immune responses (2). Monocytes, macrophages, and adaptive T and B cells cooperatively contribute to chronic inflammation in autoimmune diseases.

The majority of systemic autoimmune diseases are multifactorial or polygenic; i.e., no single variant or gene can fully explain disease development. Genetic studies have revealed cumulative

polygenic effects of numerous risk (or protective) variants with weak effect sizes on the susceptibility to developing autoimmune diseases. In the example of systemic lupus erythematosus (SLE), a prototypic autoimmune disease characterized by a broad spectrum of clinical symptoms and autoantibodies (3), patients in the highest polygenic risk score decile had a higher disease risk (odds ratio 30.3) compared with those in the lowest decile (4). In addition to polygenic risk factors, environmental factors are also critical in the development of autoimmune diseases. Concordance rates of 20–30% in monozygotic twins emphasize the importance of environmental factors in the development of autoimmune diseases (5). Transcriptome analysis of autoimmune diseases can capture dynamic genome-wide gene expression changes in immune cells reflecting both genetic and environmental stimulations.

In this review, we summarize how transcriptome analyses have improved our understanding of pathological immune cells, pathways, and genes in autoimmune diseases. We will introduce our peripheral blood transcriptome analyses on autoimmune diseases by the ImmuNexUT (Immune Cell Gene Expression Atlas from the University of Tokyo) consortium (6). We will also discuss the common experimental and analytical designs of transcriptome analyses and how transcriptome analysis can contribute to clinical decision-making for patient care.

BLOOD TRANSCRIPTOME AND IDENTIFICATION OF INTERFERON SIGNATURES

Microarray studies, the pioneer transcriptome analyses conducted in autoimmune diseases, involve the hybridization of fluorescently labeled cDNA samples to probes on microarrays. Identification of the prominent interferon (IFN) genes involved in SLE by microarray analysis of blood mononuclear cells in patients and healthy controls in 2003 was an early hallmark discovery (7, 8) (**Table 1**). Using this differential gene expression approach, we compared the transcriptomes of diseased patients and a control group and statistically created a list of disease signature genes. Physiologically, plasmacytoid dendritic cells sense viral nucleic acids *via* TLR7 and TLR9 and produce type I IFN. Type I IFN increases the expression of major histocompatibility complex genes, induces chemokines and cytokines to recruit immune cells, and activates both innate and adaptive immune cells into an antiviral state (16). A recent genome-wide association study found that genetic variants in type I IFN gene clusters are associated with SLE risk (4). Elevation of the serum IFN-α concentration in blood cells is diagnostic of SLE (17). Several clinical trials and identification of the IFN signature led to the approval of anifrolumab, a human monoclonal antibody against IFNAR1 that significantly decreases the expression of type I IFN-induced genes, for the treatment of SLE in 2021 (18, 19). Enhanced expression of type I IFN signature genes in SLE is thus pivotal in the pathophysiology of SLE.

Peripheral blood IFN signature genes are expressed not only in SLE but also in other autoimmune diseases, including rheumatoid arthritis (RA), dermatomyositis, systemic sclerosis (SSc), and Sjögren's syndrome (20–23). In RA, the most prevalent autoimmune disease, characterized by chronic autoimmune inflammation in the joints (24), the preclinical IFN signature predicts the development of arthritis (20). Moreover, a third to a half of established RA patients express IFN signature genes (21, 22). In a comparative study of five

TABLE 1 | Key immune cell transcriptome reports in SLE.

Authors	Reported year	Main experimental method	Main analytic method	Key findings	Reference
Bennett et al.	2003	Microarray of PBMC	DEG	IFN and granulopoiesis signature were elevated in SLE.	(7)
Baechler et al.	2003	Microarray of PBMC	DEG	IFN signature was elevated in SLE and it was related to more severe SLE.	(8)
Chaussabel et al.	2008	Microarray of PBMC	modular	IFN signatures and neutrophil signatures were correlated with SLE disease activity.	(9)
Lyons et al.	2010	Microarray of PBMC, CD4 and CD8 T cells, B cells, monocytes, and neutrophils	DEG, hierarchical clustering	Transcriptome differences observed in the PBMC largely reflected changes in their cellular composition. High IFN signatures in monocytes distinguished SLE from AAV and healthy controls.	(10)
McKinney et al.	2015	Microarray of CD4 and CD8 T cells	modular	Enhanced CD8 T-cell exhaustion and reduced CD4 T-cell co-stimulation signatures indicated a better prognosis in SLE and AAV patients.	(11)
Banchereau et al.	2016	Microarray of PBMC	modular	Plasmablast gene signature was the robust biomarker of disease activity. The neutrophil signature was correlated to active nephritis.	(12)
Arazi et al.	2019	single-cell RNA-seq of kidneys	graph-based clustering, trajectory analysis	IFN signatures were correlated between matched blood and kidney samples. Inflammatory blood monocytes gradually progressed to a phagocytic and then an M2-like macrophage. Naïve B cells differentiated to activated B cells with gradual elevation of ABC signature.	(13)
Nehar-Belaid et al.	2020	single-cell RNA-seq of PBMC	graph-based clustering	Subpopulations of major immune cells expressed high levels of IFN signatures. DN2 B cells were expanded in SLE.	(14)
Perez et al.	2022	single-cell RNA-seq of PBMC	Louvain clustering, modular, eQTL	Naïve $CD4^+$ T cells are decreased and $GZMH^+CD8^+$ T cells are increased in SLE. Classical monocytes expressed the highest levels of IFN signature.	(15)

PBMC, peripheral blood mononuclear cells; IFN, interferon; DEG, differentially expressed genes; AAV, antineutrophil cytoplasmic antibody-associated vasculitis; ABC, age-associated B cell; eQTL, expression quantitative trait locus.

autoimmune diseases, the proportion of patients positive for type I IFN signature genes was 73% for SLE, 66% for dermatomyositis, 61% for polymyositis, 68% for SSc, and 33% for RA (22). These data suggest variable contributions of IFN signature genes to the pathogenesis of autoimmune diseases.

MODULAR ANALYSIS OF THE BLOOD TRANSCRIPTOME AND CELL-TYPE GENE SIGNATURES

Analysis of differentially expressed genes at the transcriptome level is generally permissive to noise because of the large number of genes (usually > 10,000) analyzed and relatively limited sample numbers (usually ~100 and at most ~1,000). To overcome this "curse of dimensionality", Chaussabel et al. applied modular analysis to peripheral blood mononuclear cell (PBMC) microarray data from 239 individuals and found two SLE disease activity-related transcriptional modules: IFN-signature and neutrophil genes (9). In modular analysis, sets of coordinately expressed genes are identified, and modules are constructed in a data-driven way. The modules typically represent functionally associated genes, such as plasma cell-type genes and IFN signature genes.

Banchereau et al. applied this modular analysis to data from the whole blood of 156 pediatric SLE patients and identified the plasmablast cell-type gene signature as the most robust biomarker of disease activity (12). The neutrophil module was activated in a subset of patients with active nephritis. The authors proposed a model of gradual disease progression, with early increases in IFN responses and B-cell differentiation into plasmablasts, followed by development of kidney disease and full-blown systemic inflammation fueled by myeloid cells, including neutrophils.

The most valuable lesson from these early transcriptome analyses is that blood transcriptomic differences are largely driven by compositional changes in immune cell populations. For example, blood transcriptome studies revealed that peripheral neutrophils are increased in lupus patients with active nephritis, but they could not determine the qualitative changes in neutrophils in detail.

BULK IMMUNE CELL TRANSCRIPTOME AND PURIFIED CELL-TYPE SPECIFIC SIGNATURES

Lyons et al. isolated CD4 and CD8 T cells, B cells, monocytes, and neutrophils from patients with SLE or ANCA-associated vasculitis (AAV) and performed microarray analysis in comparison with microarray analysis of whole PBMCs (10). In their study, a substantial number of differentially expressed genes were identified only in the purified immune cells samples. Transcriptomic data from the monocytes differentiated AAV and SLE patients from each other and from controls more robustly compared with the PBMC transcriptomic data. Especially, IFN signature gene levels in monocytes distinguished the two diseases.

McKinney et al. analyzed the transcriptomes of purified CD8 and CD4 T cells using a module analysis approach (11). They found that enhanced CD8 T-cell exhaustion and reduced CD4 T-cell co-stimulation gene signatures indicated a better prognosis in SLE and AAV patients. In contrast, the CD8 T-cell exhaustion signature was associated with poor outcomes in patients with viral infections.

IMMUNEXUT; BULK RNA-SEQ ACROSS IMMUNE CELLS AND IMMUNE-MEDIATED DISEASES

Recently, we reported ImmuNexUT, a large database containing immune cell gene expression data from various immune-mediated diseases and many types of immune cells, in addition to healthy controls. In the first flagship study (6), we performed RNA sequencing (RNA-seq) in 28 types of purified bulk immune cells from healthy samples and 10 immune-mediated diseases (https://www.immunexut.org/). RNA-seq directly determines cDNA sequences using next-generation sequencers, independent of prior knowledge of genomic sequences, and the dynamic range to quantify gene expression levels is superior to that of microarrays (25). Modular analysis revealed that characteristically expressed gene modules in autoimmune diseases overlap IFN signature gene sets, and those in autoinflammatory diseases overlap IL-18- or IL-1β-activated gene sets (**Figure 1**). When comparing gene expression among individuals, we observed remarkable heterogeneity within diseases. Especially, patients with idiopathic inflammatory myopathy (IIM) with anti-MDA5 antibodies expressed high levels of IFN signature genes comparable with those in SLE patients, while the immune cell transcriptomes of the other IIM patients were heterogeneous. Anti-MDA5 antibodies in IIM are associated with life-threatening, rapidly progressing interstitial lung diseases (27). Because MDA5 is a cytosolic sensor of double-stranded RNA regulating IFN signaling, IFN signaling could have pathophysiological relevance, especially in this subtype of IIM.

IMMUNEXUT SUB-ANALYSIS OF SSC

SSc is an intractable autoimmune disease characterized by skin and internal organ fibrosis and vasculopathy (28). The rate of disease-related mortality is higher in SSc patients than in other autoimmune disease patients (26). Treatment approaches such as immunosuppressive drugs, vasodilation, and antifibrotic therapy only partially ameliorate the disease. Bulk transcriptome analysis of the affected skin showed adaptive immune cell signatures were associated with early-phase disease, while fibroblast and macrophage cell type signatures were associated with advanced fibrosis (29). Valenzi et al. have performed a single-cell RNA-seq

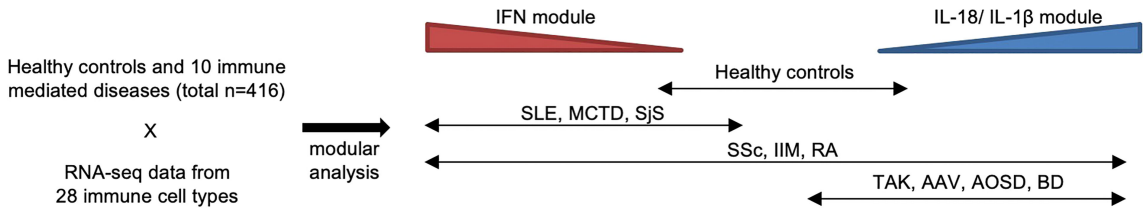

FIGURE 1 | Heterogeneity of immune-mediated diseases in ImmuNexUT. In the ImmuNexUT flagship article, we applied weighted gene correlation network analysis (26) to immune cell gene expression data and systemically characterized the gene modules related to immune-mediated diseases. When we compared the expression of these modules between autoimmune disease patients and healthy controls, gene modules enriched with IFN-induced gene sets were overexpressed in autoimmune disease patients. SLE, mixed connective tissue disease (MCTD), Sjögren's syndrome (SjS), systemic sclerosis (SSc), idiopathic inflammatory myopathy (IIM), and rheumatoid arthritis (RA). Gene modules enriched with IL-18 or IL-1β-induced gene sets were overexpressed in patients with autoinflammatory diseases: Behçet's disease (BD) and adult-onset Still's disease (AOSD). Takayasu arteritis (TAK) or ANCA-associated vasculitis (AAV) patients showed similar expression patterns to those in autoinflammatory disease patients. SSc, IIM, and RA patients were more heterogeneous compared with the other diseases.

analysis of the affected lung tissue from SSc patients and have identified a large proliferating myofibroblast population (30). However, little was known about the transcriptomic changes of peripheral blood immune cells in SSc.

In a sub-analysis of SSc patients from ImmuNexUT, we performed a modular analysis of gene expression data from each immune cell type to compare SSc and healthy controls (31). Using a machine learning approach, random forest, we prioritized the most important gene co-expression module for discriminating SSc from healthy controls. An inflammatory gene module in $CD16^+$ monocytes, including *KLF10*, *PLAUR*, *JUNB*, and *JUND* genes, showed the greatest capacity for discrimination. Integration with single-cell RNA-seq data from peripheral blood and interstitial lung disease lesional monocytes revealed a significant overlap of the gene module with a subgroup of monocytes. Because monocytes and their subpopulations are involved in tissue fibrosis (32–34), the pro-inflammatory monocyte subpopulation identified in that study might have a profibrotic capacity as well.

In addition, in a subsequent analysis comparing the transcriptomes of early-stage SSc (disease duration < 5 years) and late phase SSc (35), we revealed distinct differentially expressed genes in regulatory T cells (Tregs). In an integrative analysis with single-cell RNA-seq data, we performed deconvolution (36), a method to statistically estimate the proportions of cell subpopulations based on mixed cell data. Again, we found expansion of an activated subpopulation of Tregs in early-stage SSc (and also in IIM) with marked differences in gene expression. The role of Tregs in fibrosis is controversial because whether Tregs are profibrotic or antifibrotic depends on the experimental animal model (37). Our data suggest a profibrotic role of Tregs during the early phase of fibrotic autoimmune diseases.

In sharp contrast, a similar analysis of ImmuNexUT data in AAV patients versus healthy controls identified upregulation of a gene module related to neutrophil extracellular trap formation (NETosis) in AAV patients (38), revealing the importance of NETosis in the pathogenesis of AAV. In fact, the neutrophil enzymes MPO and PR3 are released by NETosis and are targets of autoantibodies in AAV (39, 40).

Because these analyses are performed using a data-driven hypothesis-free approach, the identified candidate immune cell populations, pro-inflammatory monocytes, and activated Tregs, could be characteristic of SSc and worth further investigation. These results also taught us that even the gene expression data from highly purified bulk immune cells in ImmuNexUT are influenced by changes in the sizes of target immune cell subpopulations.

SINGLE-CELL RNA-SEQ OF BLOOD IMMUNE CELLS IN SLE

The complex immune system involves interactions among multiple types of immune cells. Advances in single-cell RNA-seq technology have allowed comprehensive identification and characterization of distinct immune cell subpopulations at a single-cell resolution (41–44). However, currently available single-cell RNA-seq technologies capture only a few thousand of the most highly expressed genes per cell, resulting in a sparse gene expression picture with higher technical noise compared with bulk-cell RNA-seq technologies.

Nehar-Belaid et al. reported large-scale single-cell RNA-seq profiles (> 350,000 single cells) in the PBMCs of child and adult SLE patients (14), comprising 33 child and 11 adult patients. That study revealed that a small subpopulation of monocytes and other major immune cells expressing high levels of IFN signature genes was expanded and capable of distinguishing SLE from healthy controls. The results imply that enhanced expression of IFN signature genes in peripheral blood in many autoimmune diseases is, in fact, derived from a small subpopulation of high IFN-expressing cells. They also report an expansion of the B cell subpopulation expressing both IFN signatures and extrafollicular, potentially autoreactive, double negative switched memory B cell phenotype ($CD27^-IgD^-CXCR5^-CD11c^+$ DN2 cells) (45). This B cell subpopulation was enriched with monogenic lupus-associated genes, which suggest a causal role of this subpopulation in the SLE. DN2s are closely related to age-associated B cells (ABC), sharing their surface marker CD11c (45). ABCs are implicated in both

aging and autoimmunity. In addition, a fraction of SLE $CD8^+$ T cells showed upregulation of cytotoxic genes.

Recently, Perez et al. also reported larger-scale single-cell RNA-seq profiles (>1.2 million single cells) in the PBMC of 162 SLE patients and healthy controls both from European and Asian ancestries (15). They revealed a reduction in naïve $CD4^+$ T cells and an increase of $GZMH^+CD8^+$ T cells in SLE. $GZMH^+CD8^+$ T cells expressed cytotoxic, exhaustion, and IFN signatures. $GZMH^+CD8^+$ T cells were clonally expanded and restricted, and they may have a pathogenic role in the disease process. The presence of an atypical B cell population, sharing some ABC markers ($CD11c^+TBX21^+$) was confirmed. Classical monocytes expressed the highest levels of IFN signature. These results support the utility of single-cell RNA-seq technology to identify and characterize disease-relevant subpopulations of immune cells.

SINGLE-CELL RNA-SEQ ANALYSIS OF TISSUE AND PROINFLAMMATORY MACROPHAGES

Peripheral blood immune cells are popular cell models for transcriptome analyses of autoimmune diseases, probably because of the better access to such cell samples in comparison with immune cells infiltrating affected organs, such as the synovium in RA. However, whether peripheral blood immune cells accurately capture the immunological abnormalities of the affected organs is debated.

In a single-cell profiling experiment of immune cells, Wu et al. simultaneously analyzed peripheral blood and synovial $CD45^+$ mononuclear cells from RA patients with and without anti-citrullinated peptide antibodies (ACPAs), hallmark autoantibodies of RA (46). Peripheral blood analysis revealed important characteristics of RA, such as an expansion of cytotoxic CD4 T cell population (47, 48), while synovial gene profiling revealed correlates of ACPA status, including up-regulation of *CCL13*, *CCL18*, and *MMP3* in myeloid cell subsets of ACPA-negative RA compared with ACPA-positive RA. Although reports directly comparing the utility of single-cell RNA-seq analysis in blood versus synovium are scarce, synovial immune cell profiling seems more sensitive than peripheral blood profiling in the detection of immunological correlates of RA subpopulations.

As part of the Accelerating Medicines Partnership consortium, Zhang et al. profiled 51 synovial tissue samples from both RA and osteoarthritis patients by combining single-cell RNA-seq, bulk RNA-seq, and mass cytometry data (49). They found expansion of $IL1B^+$ pro-inflammatory monocytes, $ITGAX^+TBX21^+$ autoimmune-associated B cells, $PDCD1^+$ peripheral helper T cells (Tph), and follicular helper T cells (Tfh) in the RA synovium. Tph cells are a new subset of helper T cells previously identified in the RA synovium, which can induce plasma cell differentiation *in vitro* (50). In contrast to classical $CXCR5^+$ Tfh cells, Tph cells lack surface CXCR5 and produce CXCL13 and IL-21 to recruit both Tfh and B cells. Therefore, Tph cells appear to have an important role in the local autoantibody production in the inflamed tissues.

Kuo et al. focused on synovial macrophages and, using single-cell RNA-seq, identified $HBEGF^+$ pro-inflammatory macrophages enriched in the RA synovium (51). This macrophage subpopulation has the capacity to promote fibroblast invasiveness in an EGF receptor-dependent manner. Also, synovial fibroblasts can promote the HBEGF+ inflammatory phenotype of macrophages. The crosstalk between pro-inflammatory macrophages and synovial fibroblasts might be important in the pathophysiology of chronic inflammation in RA joints. Interestingly, synovial pro-inflammatory macrophages express some common gene markers, such as *PLAUR*, *NR4A2*, and *CXCL2*, to those expressed in inflammatory monocytes that we identified in the peripheral blood of patients with SSc (31). This may imply similarities between monocytes and macrophages in the pathophysiology of RA or SSc. Zhang et al. performed integrative single-cell RNA-seq analysis of > 300,000 cells in bronchoalveolar lavage samples from COVID-19, RA, and other inflammatory diseases, all of which exhibited expansion of $CXCL10^+CCL2^+$ inflammatory macrophages, experimentally driven by a combination of the pro-inflammatory cytokines IFN-γ and tumor necrosis factor (TNF)-α (52). That study is a good example demonstrating the utility of cross-disease data analysis to reveal shared disease processes, in this case, the expansion of inflammatory macrophage subpopulations in various disease conditions.

SINGLE CELL RNA-SEQ ANALYSIS OF LUPUS NEPHRITIS

In the Accelerating Medicines Partnership consortium, Arazi et al. reported immune cell clusters in lupus nephritis samples (13). Trajectory analysis, based on the similarity of single-cell gene expression, suggested a gradual progression of inflammatory blood monocytes to a phagocytic and then an alternatively activated (M2-like) macrophage phenotype in the kidney. Alternatively, activated (M2) macrophages have an anti-inflammatory function and regulate wound healing (53). Trajectory analysis also revealed a gradual differentiation from naïve B cells to activated B cells with an incremental elevation of the ABC gene signature. They compared the $CD8^+$ T cell exhaustion signatures, previously reported to be associated with lower lupus flares (11), between blood and kidney. The $CD8^+$ T cell exhaustion signature of patients with lupus nephritis was high in blood but not in the kidney. In that report, IFN signature gene expression was correlated between matched blood and kidney samples (n = 10), implying that the IFN response may be an extrarenal process. When comparing urine and kidney leukocytes, the urine samples had a higher frequency of phagocytic macrophages, and high transcriptomic correlations were observed between the samples. Urine cells may serve, at least partially, as an alternative to their kidney counterparts. Der et al. reported tubular and keratinocyte single-cell RNA-seq data

(54). They showed that the IFN signatures in tubular cells and keratinocytes distinguished patients with lupus nephritis from healthy control subjects. Moreover, high IFN and fibrotic gene signatures in tubular cells were associated with failure to respond to treatment.

DYNAMIC EQTL ANALYSIS OF AUTOIMMUNE DISEASES

Genome-wide association studies (GWASs) have identified tens of thousands of gene variants significantly associated with diseases (55). In RA and SLE, large-scale GWASs have identified more than a hundred robust genetic variants associated with each disease (4, 56, 57). However, most of the identified causal genetic variants of these autoimmune diseases are located in non-coding enhancer regions of the genome (58, 59), and their biological mechanisms in autoimmunity are not self-explanatory.

Expression quantitative trait locus (eQTL) analysis evaluates associations between genetic variants and gene expression levels (**Figure 2**) *via* linear regression of normalized gene expression levels and the tested allele dosages. Genetic variants associated with diseases have greater eQTL effects compared with other variants (60). The variants can alter the binding capacity of transcription factors to the enhancers or promotors of genes and thus regulate the expression of nearby genes (61). Importantly, eQTL analysis provides directional information on the effects of the tested single nucleotide polymorphisms (SNPs) on target gene expression. In the example in **Figure 2**, if the G allele of gene X is a GWAS risk SNP, enhanced expression of gene X, especially in stimulated monocytes, is a candidate biological mechanism. The following are limitations of eQTL analysis: large-scale transcriptomic data with typically more than 50–100 samples are required (62); eQTL effects are variable among immune cell types (63, 64) and stimulations (65, 66); overlap of GWAS and eQTL signals do not guarantee causality of the identified genes (67); and only common SNPs (allele frequency > 0.01–0.05) can be tested, while rare variants cannot.

ImmuNexUT includes samples from more than 400 individuals comprising both healthy controls and immune-mediated disease patients (unstimulated and stimulated immune cells *in vivo*) and 28 immune cell types (**Figure 1**). Using eQTL analysis of ImmuNexUT data (6), we identified immune cell-type specific and disease-specific eQTLs. A median of 7,092 genes was significantly regulated by eQTL in each immune cell type. Partitioning of GWAS heritability with cell-type-specific eQTL data revealed candidate causal immune cells in autoimmune diseases: effector Tregs in RA and naïve and unswitched memory B cells in SLE. Colocalization analysis between SLE GWAS and ImmuNexUT eQTL data identified candidate causal genes. For example, decreased expression of *ARHGAP31* in plasmablasts was associated with SLE. Those results highlight the power of eQTL analysis in elucidating autoimmune disease mechanisms.

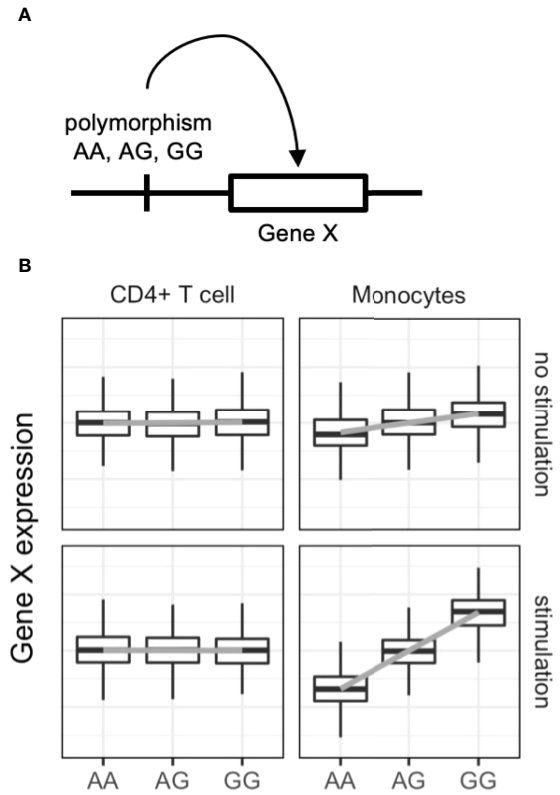

FIGURE 2 | Dynamic eQTL of the immune cells. **(A)** Schematic representation of expression quantitative trait locus (eQTL) analysis. eQTL is an association test between common single nucleotide polymorphisms and nearby (in most cases) gene expression. **(B)** An example of dynamic eQTL in immune cells. eQTL effect sizes, expressed as standardized linear regression coefficients, are cell-type and cell-state dependent. In this example stimulated monocytes have four times more eQTL effects compared with unstimulated monocytes.

In the OneK1K cohort, Yazar et al. collected PBMC single-cell RNA seq data of 1.27 million cells from 982 healthy donors (68). They identified 14 major immune cell populations and eQTL analysis identified variable number of independent eQTLs (399 in plasma cells and 6473 in naïve and central memory $CD4^+$ T cells). They showed the overlap of various autoimmune GWAS loci and immune cell eQTLs. Also, using single-cell RNA-seq data of SLE patients, Perez et al. performed eQTL analysis in the eight most abundant cell types (15). Their analysis identified 3331 genes with at least one eQTL in a cell type. The advantage of these single-cell RNA-seq based approaches over a bulk RNA-seq based approach is the ability to compute dynamic transcriptional transitions of cellular state using pseudotime analysis. However, cell-type-specific effects of the population with a small fraction size could not be estimated because of the limited number of cells. For example, the cell-type-specific eQTL effects of Treg were not examined in these single-cell RNA-seq eQTL studies, despite the importance of Treg in its ability to regulate autoimmune reactions. Further, Yazar et al. detected fewer eQTL signals in each immune cell type despite the higher

number of analyzed individuals (n=982) in comparison to the ImmuNexUT project (n=416). Therefore, the bulk RNA-seq based approach has some advantages in the detection power of immune cell eQTL analysis over the single-cell RNA-seq based approach.

CLINICAL APPLICATION OF TRANSCRIPTOME ANALYSES

In clinical trials of anifrolumab for SLE, higher baseline levels of IFN signature genes were associated with a better clinical response (18, 19). In those reports, IFN gene signatures, classified as either high or low, were estimated by whole-blood quantitative PCR-based analysis of four genes (*IFI27*, *IFI44*, *IFI44L*, and *RSAD2*). These results are consistent with the specific binding of anifrolumab to INFAR1. Similarly, baseline protein levels of TNF and soluble interleukin-6 (IL-6) receptor are associated with the response to their respective targeted treatment in RA patients (69, 70). In addition, the B cell subpopulation levels are associated with the treatment responses of B-cell-targeted anti-CD20 rituximab therapy in AAV or SLE patients (71, 72). As seen in these examples, gene or protein expression levels or cell populations appear to be promising candidate biomarkers for predicting treatment responses. However, these biomarkers do not have enough sensitivity or specificity for routine clinical use. One reason might be the use of blood for estimating the biological activity of cytokines because they typically act locally *via* both autocrine and paracrine manners on other cells (73).

Interestingly, peripheral blood levels of IFN signature genes are associated with the response to RA treatment. For example, high IFN signature gene levels are associated with better responses to treatments with a TNF-α inhibitor (74), the IL-6 receptor inhibitor tocilizumab (75), and the T-cell-blocking CTLA4-Ig abatacept (76). Meanwhile, IFN signature gene levels were associated with non-responsiveness to methotrexate (77) and B-cell-targeting rituximab (78, 79). Those reports imply that the IFN signature status could be used for immunological stratification of RA patients, thereby affecting treatment choice.

Transcriptome analysis of blood samples can capture both cytokine signaling gene expression (e.g., IFN signature) and immune cell-type signature gene expression (e.g., plasmablast signature). Therefore, peripheral blood transcriptome profiling is a candidate approach in precision medicine based on biologically targeted therapies. In addition, the peripheral blood transcriptome can be used to predict the natural history of various autoimmune diseases. In the report by McKinney et al., the "exhaustion signature" of purified $CD8^+$ T cells was associated with a lower number of disease flares in SLE and AAV patients (11). Serial peripheral blood transcriptome analyses have also identified gene signatures preceding RA flares, associated with B cell activation and subsequent expansion of $CD45^-CD31^-PDPN^+$ pre-inflammatory mesenchymal cells, which show gene expression features similar to those of inflammatory synovial fibroblasts (80). With the establishment of accurate and robust prognostic prediction by transcriptome analysis, treatments can be tailored based on the obtained transcriptomic data.

In RA, several studies have shown that gene expression analysis of joint synovial tissues can identify disease subtypes and predict treatment responses (81–84). Humby et al. combined gene expression with immunohistological analyses of the synovium in treatment-naïve early-stage RA patients (82). Three groups of patients were identified according to the synovial tissue phenotype: pauci-immune fibroid, diffuse-myeloid, and lympho-myeloid patients. Elevation of myeloid- and lymphoid-associated gene expression was strongly correlated with disease activity and the treatment (90% methotrexate) response at six months. Lewis et al. also profiled early RA-associated genes using RNA-seq and showed that the gene signature of synovial plasma cells predicts future joint damage (83). In addition, they compared the blood and synovial RNA-seq data and showed that synovial genes are more differentially expressed among the different synovial tissue phenotypes. In addition, the blood IFN signature was associated with synovial B and plasma cell infiltration, which may at least partially explain the correlation between the blood IFN signature and treatment effects (78). Finally, Humby et al. reported a clinical trial comparing the effects of the anti-CD20 monoclonal antibody rituximab and anti-IL6 receptor tocilizumab in non-responders to anti-TNF therapy (84). Baseline synovial biopsies were subjected to classical histological and RNA-seq analyses to classify the samples as B-cell rich or poor. In B-cell-poor RA, tocilizumab treatment had a better response rate than that of B-cell targeted rituximab therapy. An important finding of that study was that RNA-seq-based classification was better correlated to the treatment response than was histological classification. Currently, synovial biopsies are not standard clinical practice for RA, but they might become such, considering the limited invasiveness of the biopsy procedure and potential benefits of precision medicine based on the molecular disease subtype.

CONCLUSION

Here, we reviewed how the transcriptome analysis of immune cells has improved our understanding of the pathophysiology of autoimmune diseases with a focus on the ImmuNexUT analysis that we recently reported. Single-cell RNA-seq analyses have been expanding our knowledge of the immune cells infiltrating disease lesions. Inflammatory monocytes and macrophages are expanded across various autoimmune and inflammatory diseases. IFN pathway, neutrophils, and B cell subpopulations have been robust signatures in SLE in different experimental and analytic methods (**Table 1**). These cell populations could have pathophysiological relevance to autoimmune diseases. Expression QTL analysis, integrated with GWAS data, has identified candidate causal immune cells and genes. Genes identified by transcriptome analyses of the peripheral blood or the RA synovium can be used as clinical predictive or prognostic biomarkers. Therefore, transcriptome analysis of autoimmune diseases is not only a useful tool for the investigation of disease mechanisms but also has the potential for direct clinical application in future precision medicine.

AUTHOR CONTRIBUTIONS

YN, HY, and KF conceived and designed the study concept. YN wrote the original manuscript draft. HY and KF reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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The Role of M1/M2 Macrophage Polarization in Rheumatoid Arthritis Synovitis

Maurizio Cutolo, Rosanna Campitiello, Emanuele Gotelli and Stefano Soldano*

Laboratory of Experimental Rheumatology and Academic Division of Clinical Rheumatology, Department of Internal Medicine and Specialties (DIMI), University of Genova, Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) San Martino Polyclinic Hospital, Genoa, Italy

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Edited by:
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***Correspondence:**
Maurizio Cutolo
mcutolo@unige.it

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Innate and adaptive immunity represent a harmonic counterbalanced system involved in the induction, progression, and possibly resolution of the inflammatory reaction that characterize autoimmune rheumatic diseases (ARDs), including rheumatoid arthritis (RA). Although the immunopathophysiological mechanisms of the ARDs are not fully clarified, they are often associated with an inappropriate macrophage/T-cell interaction, where classical (M1) or alternative (M2) macrophage activation may influence the occurrence of T-helper (Th)1 or Th2 responses. In RA patients, M1/Th1 activation occurs in an inflammatory environment dominated by Toll-like receptor (TLR) and interferon (IFN) signaling, and it promotes a massive production of pro-inflammatory cytokines [i.e., tumor necrosis factor-α (TNFα), interleukin (IL)-1, IL-12, IL-18, and IFNγ], chemotactic factors, and matrix metalloproteinases resulting in osteoclastogenesis, erosion, and progressive joint destruction. On the other hand, the activation of M2/Th2 response determines the release of growth factors and cytokines [i.e., IL-4, IL-10, IL-13, and transforming growth factor (TGF)-β] involved in the anti-inflammatory process leading to the clinical remission of RA. Several subtypes of macrophages have been described. Five polarization states from M1 to M2 have been confirmed in *in vitro* studies analyzing morphological characteristics, gene expression of phenotype markers (CD80, CD86, TLR2, TLR4, or CD206, CD204, CD163, MerTK), and functional aspect, including the production of reactive oxygen species (ROS). An M1 and M2 macrophage imbalance may induce pathological consequences and contribute to several diseases, such as asthma or osteoclastogenesis in RA patients. In addition, the macrophage dynamic polarization from M1 to M2 includes the presence of intermediate polarity stages distinguished by the expression of specific surface markers and the production/release of distinct molecules (i.e., nitric oxide, cytokines), which characterize their morphological and functional state. This suggests a "continuum" of macrophage activation states playing an important role

during inflammation and its resolution. This review discusses the importance of the delicate M1/M2 imbalance in the different phases of the inflammatory process together with the identification of specific pathways, cytokines, and chemokines involved, and its clinical outcomes in RA. The analysis of these aspects could shed a light on the abnormal inflammatory activation, leading to novel therapeutical approaches which may contribute to restore the M1/M2 balance.

Keywords: Macrophage polarization, Rheumatoid anhritis, Inflammation, Synovitis, bDMARD therapy

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic systemic autoimmune inflammatory condition affecting approximately 1% of the population worldwide with considerable regional variation and an incidence rate higher in female than in male (1). Recognized as one of the most common autoimmune rheumatic diseases (ARDs) predominantly observed in the elderly population, RA is characterized by polyarticular synovitis at the level of small- and medium-sized joints, symmetrical joint swelling, tenderness, and redness as a result of the synovial lining layer inflammation, leading to joint damage and progressive disability (2–4). In this frame, multiorgan manifestations may arise during disease progression showing classical circadian rhythms (5).

Uncontrolled RA lowers life expectancy, and RA patients may have a roughly double average risk for developing malignancy and cardiovascular diseases (6). Although RA pathophysiology remains elusive, the presence of a complex interplay among genotype, epigenetic changes, and environmental factors underlying chronic inflammation is abundantly described (7, 8).

It is well established that among different risk factors, cigarette smoking, ozone exposure, and traffic-related air pollution are environmental elements significantly correlated to RA susceptibility, especially in those patients seropositive to rheumatoid factor (FR), anti-citrullinated peptide antibodies (ACPA), and anti-carbamylated protein antibodies.

Toxic components in smoke may enhance the activation of peptidylarginine deiminase (PAD) enzymes leading to a massive lung-protein citrullination. Additionally, smoke recalls antigen-presenting cells (APC), followed by T-helper-1 (Th1) activation, and finally anti-citrullinated peptide antibodies (ACPA)-specific B-cell memory formation (9, 10) .

A growing scientific interest is currently directed to highlight the role of intestinal microbiota and nutritional habits in RA patients (11, 12). In fact, diet may critically shape and alter the human gut microbiota composition, creating a "dysbiotic state," which modulates the immune regulatory function and promotes a pro-inflammatory status (13). Of note, the extra virgin olive oil, a crucial component of Mediterranean diet, seems to reduce both presence and function of pro-inflammatory M1 macrophages and increase that of anti-inflammatory M2 macrophages (11, 12).

In recent years, the pathophysiological roles of innate immune system in RA have been investigated. In RA, the delicate balance among Th1/M1 and Th2/M2 system is lost giving way to an aberrant and uncontrolled Th1/M1 activation leading to organ damage (14). Astonishing steps have been made towards a better understanding of the central role of macrophages in RA chronic inflammation on-set and progression.

This review focus on the monocyte/macrophage contribution in RA pathogenesis primarily highlighting the immune-pathophysiological impact and imbalance of M1 and M2 macrophages and their precursors monocytes, and the identification of specific pathways, cytokines, and chemokines involved in mediating the abnormal inflammatory activation. Finally, the impact of current therapies that might contribute to reprogram macrophages, promoting their polarization from a pro-inflammatory M1 phenotype into an anti-inflammatory M2 phenotype as possible new strategy in the resolution of RA inflammatory process, is also analyzed.

CIRCULATING MONOCYTES IN RA

Monocytes are circulating cells belonging to the mononuclear phagocytic system and known as the second line of defense in the innate immune system (15). Monocytes can play an important role in the initiation and maintenance of inflammation in the synovial tissue of RA patients: in fact, these cells are recruited from the circulation into the synovial tissue by chemotaxis through the interaction with fibroblast-like synoviocytes (FLSs) and other autoimmune cells (15).

Monocytes are classified into three subsets: classical monocytes ($CD14^{++}CD16^{-}$), intermediate monocytes ($CD14^{++}CD16^{+}$), and non-classical monocytes ($CD14^{dim}CD16^{++}$) (**Figure 1**) (16, 17). In RA synovial joints, classical monocytes seem to be the circulating precursors of osteoclasts involved in bone erosion (**Figure 1**) (18).

Moreover, the expression of CD14 and CD16 is upregulated on the monocyte cell membrane, and the percentage of intermediate monocyte subset is higher in both peripheral blood and synovial tissue of RA patients (19, 20). These intermediate monocytes secrete pro-inflammatory cytokines, such as tumor necrosis factor-α (TNFα), interleukine-1β (IL-1β), and IL-6, and they can differentiate into pro-inflammatory M1 macrophages, contributing to the local synovial inflammation (**Figure 1**) (21).

RA intermediate monocytes are characterized by an increased expression of HLA-DR compared to the other two monocyte subsets, and this increased expression seems to determine a high production of TNFα (15, 19, 22). In addition, $HLA\text{-}DR^{+}$ intermediate monocytes express high level of the costimulatory

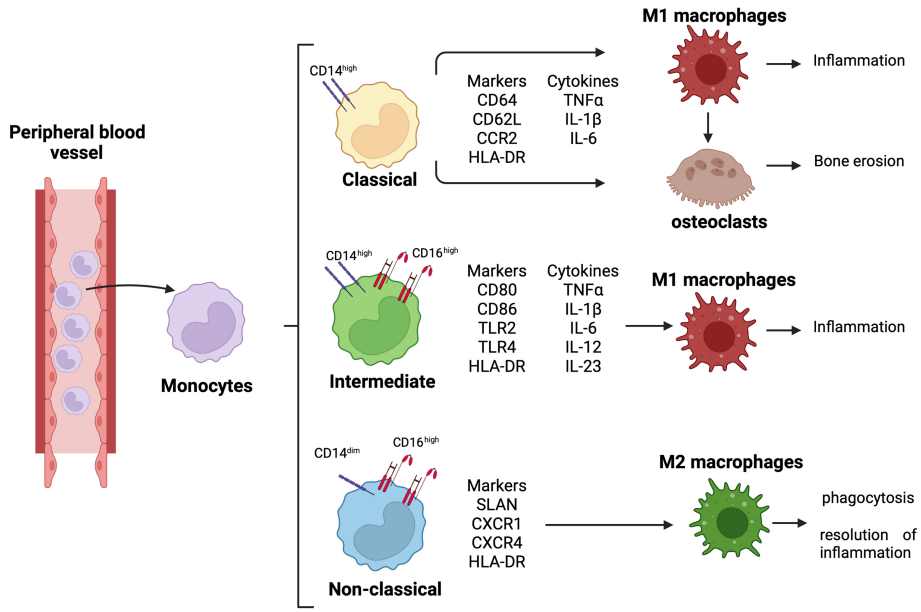

FIGURE 1 | Monocyte differentiation and related role in RA pathogenesis. Differentiation of circulating monocytes in their three subsets, classical ($CD14^{high}$), intermediate ($CD14^{high}CD16^{high}$), and non-classical ($CD14^{dim}CD16^{high}$) monocytes. Classical monocytes can differentiate into pro-inflammatory macrophages and osteoclasts, contributing to synovial tissue inflammation and bone erosion; intermediate monocytes differentiate into pro-inflammatory macrophages contributing to tissue inflammation; non-classical monocytes differentiate into anti-inflammatory macrophages promoting phagocytosis and resolution of inflammation.

molecules CD80 and CD86 promoting the induction of $IL17^{+}CD4^{+}$ T cells (**Figure 1**) (15).

Therefore, in the peripheral blood and synovial fluid of RA patients, the predominance of intermediate monocytes seems to suggest their functional role in modulating Th17 cell responses through the production of IL-12, which stimulates $CD4^{+}$ Th1 cell polarization, and the release of IL-6, IL-1β, and IL-23 (15). These cytokines drive Th17 cell polarization and the release of IL-17 by $CD4^{+}$ T cells (15, 23).

As described in a previous study, the intermediate monocyte subset is the major subset to undergo differentiation into pro-inflammatory M1 macrophages (**Figure 1**) (24). Together with classical monocytes, the intermediate monocytes express Toll-like receptor-2 (TLR2) on their surface membrane in both peripheral blood and synovial tissue of RA patients. However, compared to classical monocytes, intermediate monocytes highly express TLR2, which activates the signaling pathway responsible for the production of the pro-inflammatory cytokines IL-1β, IL-6, and TNFα (25, 26).

Conversely, even if non-classical monocytes seem to participate in the early inflammatory response, they differentiate into resident M2 macrophages taking part in the resolution of inflammation (**Figure 1**) (27).

As is well-demonstrated, monocytes are essential players in the pathology of several inflammatory diseases, including RA, in which these cells are one of the two major contributors to the damage at synovial tissue level, together with macrophages (28). This fundamental role of monocytes is also related to their plasticity, which is also achieved by a highly responsive epigenome: this epigenomic plasticity of monocytes is determined by the occurrence of relevant DNA methylation changes (28).

Several studies revealed how the high expression levels of *de novo* DNA methyltransferase 3A (DNMT3A) and the methylcytosine dioxygenase ten–eleven traslocation-2 (TET2) in monocytes are essential for the differentiation and activation of these cells during inflammatory responses, suggesting how DNA methylation represents the major epigenetic mechanism that potentially reflects the influence of disease-associated inflammation in monocytes (29, 30).

The important role of methylation in monocyte pathophysiology is highlighted by a recent study, which demonstrated a difference in DNA methylation profiling between monocytes isolated from RA patients and healthy subjects: the study revealed how RA monocytes are characterized by hypermethylated CpG sites related to several genes, including IFN and TNF, suggesting a potential implication of these cytokines and their signaling pathways in the acquisition of a further aberrant DNA methylation signature in RA patients (28).

Therefore, in RA patients, the high percentage of monocytes, primarily belonging to the intermediate subset, and their increased DNA methylation are linked to the inflammatory environment in the blood, correlating with the high disease activity (evaluated by 28-joint Disease Activity Scale—DAS28), serum level of C-reactive protein (CRP), and erythrocyte sedimentation rate (ESR) (28). All these observations suggest a role of monocytes as additional biomarker for high disease activity in RA patients (31).

Moreover, circulating RA monocytes also express high levels of several chemokines, including CCR7, which interact with CCL19; the upregulation of CCR7/CCL19 correlates with disease activity (DAS28) and the radiographic progression of joint damage (32).

MACROPHAGES: POLARIZATION AND SIGNALING PATHWAYS INVOLVED IN RA

Macrophages were described for the first time in 1882 by Metchnikov as the "big eater" of the immune system and represent the frontier soldiers of immune system, thanks to their capability to recognize, engulf, and destroy pathogens through the activation of TLRs and the production of pro- and anti-inflammatory mediators (33, 34).

As APCs, macrophages contribute to induce a Th1- or Th2-mediated immune response through the presentation of non-self-antigens to naive T cells and the release of cytokines and growth factors, confirming that their interplay with T lymphocytes represents a vital check point for T-cell maturation; this is a fundamental function in the regulation of inflammation and in the maintenance of homeostasis (35, 36).

Indeed, plasticity is a key feature also of macrophages, which are capable of presenting heterogeneous phenotypes creating various subpopulations; therefore, these cells are not only involved in the propagation of inflammation but also in its resolution, depending on their activation state (M1 or M2) (37). Therefore, it is becoming increasingly apparent that M1 and M2 phenotypes represent the extremes of a macrophage activated spectrum, which is characterized by the presence of "intermediate" phenotypes involved in the immuno-regulation or in tissue repair and defined by different metabolic pathways, surface markers, and cytokine production (37–40).

Due to the advanced research, science has made unbelievable progress during the past years, shedding light on the role of these cells in the immune response that characterizes RA.

In RA, the inflammatory process is mediated and sustained by M1 macrophages both in peripheral blood and in synovial tissue (**Figure 2**). Indeed, M1 macrophages are pro-inflammatory cells characterized by the high expression of major histocompatibility complex (MHC) class II, CD80, CD86, CD38, and TLR4, and the

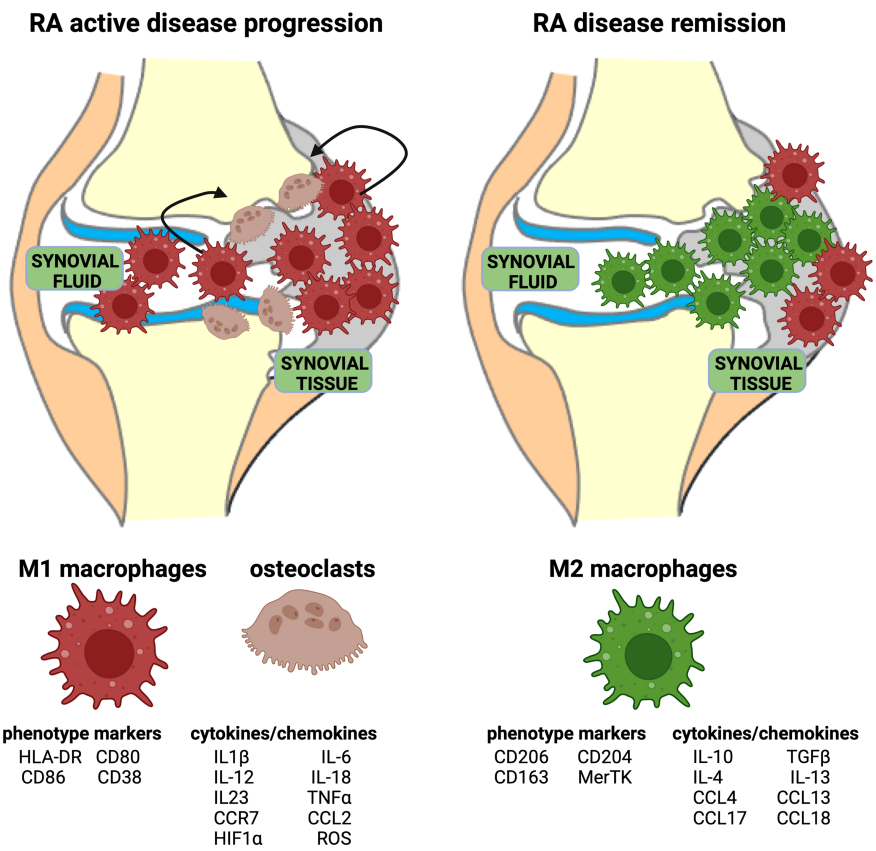

FIGURE 2 | Representation of acute RA inflammation and remission. Acute RA inflammatory phase is characterized by an imbalance in M1–M2 ratio in synovial fluid and tissue. This phase is dominated by a higher percentage of pro-inflammatory M1 macrophages, which display specific phenotype markers and release cytokines/chemokines. Moreover, the activation of osteoclasts contributes to bone erosion. RA disease remission is characterized by a high percentage of anti-inflammatory M2 macrophages, which display specific phenotype markers and release anti-inflammatory cytokines/chemokines.

secretion of pro-inflammatory cytokines, primarily IL-1β, IL-6, and TNFα, and chemokines, such as CCR7 (**Figure 2**) (35, 41).

Their prompt production of inflammatory cytokines stimulates the immune system enabling an efficient pathogen eradication. When self-tolerance is lost, inflammation persists evolving to a chronic maladaptive immune response. CD80/CD86 are costimulatory molecules present on these macrophages (among other cells) in response to activating signals finalized to pathogen suppression; these surface proteins bind to CD28 on naive T cells increasing sensitivity to T-cell receptor (TCR) stimulation and T-cell survival (42).

TLRs belong to a heterogenous receptor family distributed on the cell membrane or cytosol of APCs, including macrophages, natural killers, lymphocytes, endothelial and epithelial cells, and fibroblasts (43).

TLRs are one of the most ancient immunity tolls for host defense against infection recognizing pathogen-associated molecular patterns (PAMPs), and TLR2 and TLR4 are primarily involved in pathogen recognition (44).

Moreover, the expression of TLR4 on macrophages permits to recognize endogenous ligands relevant in RA, such as native articular proteins and citrullinated peptides, and subsequently induces intracellular signal transduction finalized to a prompt expression of pro-inflammatory genes through the activation of nuclear factor kappa B (NF-kB) signaling pathway (33, 45): in fact, the activation of TLR4-induced NF-kB signaling pathway mediates the pro-inflammatory activity of M1 macrophages through the production and release of IL-6, TNFα, and IL-1β in monocyte-derived and synovial macrophages obtained from RA patients (**Figure 3**) (46, 47).

The polarization of macrophages toward an M1 phenotype can be induced by several pro-inflammatory stimuli, including the activation of IRF5 expression (48).

The upregulation of IRF5 activates the intracellular signaling pathway, which induces the transcription of several subunits of IL-12 and the repression of IL-10, with the subsequent induction of Th17 differentiation of T cells (48). M1 macrophages also express high levels of IL-15, which promotes MHC class II overexpression and SOCS3 suppression, contributing to the activation of the proliferation of $CD4^{+}$T cells (49).

In RA, another important pathway linked with M1 macrophage-induced inflammation involves the activation of stress-activated protein kinases/mitogen-activated protein kinases (SAPK/MAPK) and Janus kinase/signal transducer and activators of transcription (JAK/STAT), which are activated by pro-inflammatory cytokines and promote both proliferation and survival of macrophages (**Figure 3**) (50). TNFα, IL-1β, and IL-6 also promote the activation of MAPK signaling pathways

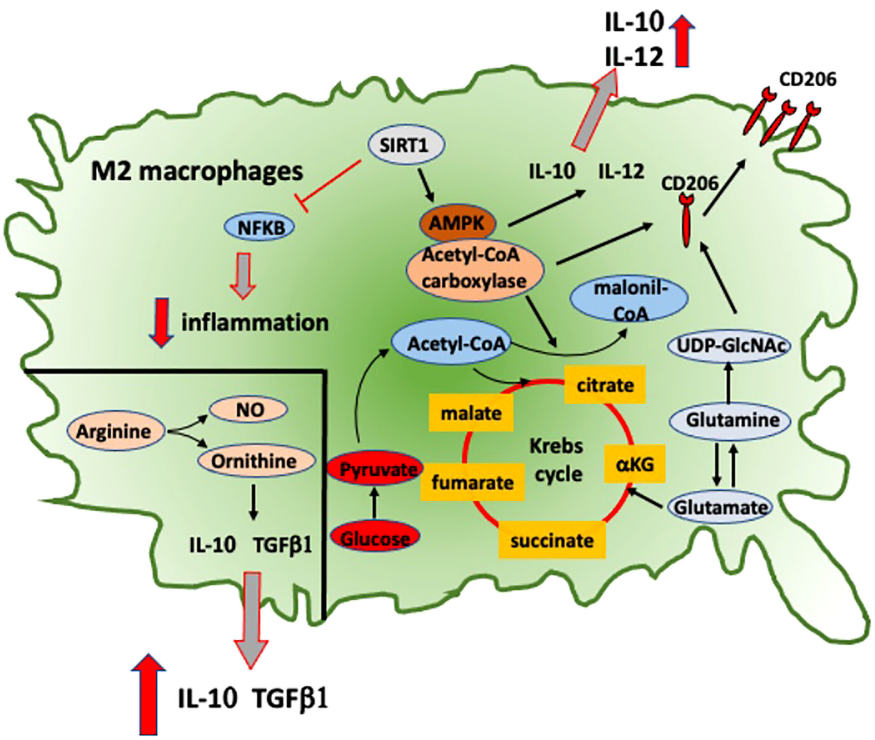

FIGURE 3 | Intracellular signaling and metabolic pathways activated into RA anti-inflammatory M2 macrophages. Metabolic pathways activated in M2 macrophages that contribute to their anti-inflammatory role in RA. NFkB, nuclear factor-kB; SIRT1, sirtuin-1; AMPK, adenosine monophosphate-activated protein kinase; IL-10, interleukine-10; IL-12, interleukine-12; TGFβ1, transforming growth factor-β1; NO, nitric oxide; CD206, mannose receptor-1; UDP-GlcNAc, uridine diphosphate N-acetylglucosamine.

through the phosphorylation of ERK1/2, JNK, and p38 kinase in synovial cells derived from patients with chronic RA (**Figure 3**) (51, 52).

In the contest of different biological activities of macrophages that contribute to tissue homeostasis and disease pathogenesis, an interesting macrophage subset, called arthritis-associated osteoclastogenic macrophages (AtoMs), was recently identified in the synovial fluid and tissue of RA patients (53).

These macrophages are characterized as $CX3CR1^{+}HLA\text{-}DR^{high}CD11c^{+}CD86^{+}$ cells, and they have a high osteoclastogenic potential (53). CX3 chemokine receptor-1 (CX3CR1) is a fractalkine receptor and marker of monocyte-lineage cells including a population of osteoclast precursor in the bone marrow under homeostatic condition, and it is also an osteoclast precursor marker in inflamed synovium (53).

Nevertheless, the expression of CD11c and MHC class II implies that AtoMs may share functional characteristics of both macrophages and dendritic cells. Of note, the expression of CD80 and CD86 indicates that these cells may be involved in antigen presentation in local foci of arthritic joints.

Moreover, together with their capability to differentiate into osteoclasts, AtoMs are efficient in inducing the activation of TNF-producing $CD4^{+}$T cells, contributing to the amplification of inflammation and bone destruction (53, 54).

These cells were also identified in a collagen-induced arthritis (CIA) mouse model, where their differentiation into osteoclasts seems to be mediated by the activation of receptor activator of NF-kB ligand (RANKL) signaling pathways and boosted by TNFα stimulation (53). This pathway involves the activation of the transcription factor Forkhead box M1 (FoxM1), whose inhibition blocks the differentiation of AtoMs into osteoclasts attenuating their inflammatory cytokine production in the synovium and reduces the articular bone erosion (53).

The "anti-inflammatory" M2 macrophages are phenotypically characterized by the expression of surface markers including macrophage scavenger receptors (CD163, CD204), mannose receptor-1 (CD206), and the MER proto-oncogene, tyrosine kinase (MerTK) (**Figure 2**). To fulfill their main role in tissue homeostasis preservation, these so-called "alternative activated" macrophages support proliferation, wound healing, and angiogenesis, and they mitigate inflammatory process. M2 macrophages are responsible for apoptotic cell clearance, production of extracellular matrix (ECM) components, and angiogenic and chemotactic factors (55, 56).

Additionally, IL-10 and TGFβ are molecules endogenously produced by M2 macrophages shifting the immune activation toward a tissue repair pattern (**Figure 2**) (55, 56). CD163 is a hemoglobin scavenger soluble or membrane-bound receptor highly expressed in resident tissue macrophages, which contributes to the anti-inflammatory local response lowering hemoglobin levels and promoting inflammation-resolving heme metabolites (57, 58).

CD206 is a mannose scavenger receptor mainly present in M2 macrophages and dendritic cells, known to be involved in collagen internalization and degradation (59). MerTK is a tumor-associated macrophage (TAM) receptor predominantly expressed in M2 macrophages during immunomodulation processes (60, 61). Through the interaction with the bridging ligands Gas6 and protein S, MerTK recognizes apoptotic cells facilitating their phagocytosis; this physiological process of clearance is fundamental for the maintenance of immune tolerance (60–63).

Moreover, MerTK-induced Gas6 expression amplifies IL-10 production reinforcing an M2 positive feedback (64). Recent data have shown a significant correlation in RA patients between the low relative proportion of $MerTK^{+}$ to $MerTK^{-}$ synovial tissue macrophages with disease flare upon drug withdrawal, suggesting a potential role of this molecule as biomarker (65). In RA macrophages, a signaling pathway described to promote the induction of M2 polarization is the adenosine-monophosphate-activated protein kinase (AMPK)/a-acetyl-CoA carboxylase, which promotes the upregulation of macrophage-derived chemokine (MDC), CD206, and IL-10 (**Figure 3**) (66).

This pathway is induced by sirtuin-1, which downregulates the pro-inflammatory IL-12, CCL2, and iNOS through the inhibition of NF-kB signaling pathway and promotes the polarization toward an anti-inflammatory M2 phenotype in cultured macrophages obtained from RA patients and CIA mouse model (**Figure 3**) (66).

From a metabolic point of view, M1 and M2 macrophages show opposed metabolic profiles: M1 macrophages use preferentially aerobic glycolysis, while M2 macrophages relay on oxidative phosphorylation (**Figures 3**, **4**) (67). Therefore, during articular inflammation, synovial "pannus" formation and the presence of a hypoxic inflammatory environment drastically increase glycolytic activity in macrophages, which are polarized towards a M1 phenotype (**Figure 4**). Indeed, M1 cells more than other cell populations commonly present in synovial inflammatory tissues are responsible for cartilage damage (**Figure 2**) (68).

In inflamed joints, oxygen levels rapidly drop, while a raise in hypoxia factor 1α (HIF-1α) and reactive oxygen species (ROS) production occurs followed by the activation of inflammatory genes (IL-1β and IL-6), which promotes a massive oxidative tissue damage (**Figure 4**) (69). Additional elements may actively contribute to macrophages metabolic switch: for example, TLR4 activates aerobic glycolysis finalized to provide sufficient bioenergetic resources to support cell mature state (**Figure 4**) (70).

Moreover, succinate is a transformation product of glycolysis highly present in lipopolysaccharide (LPS)-activated M1 macrophages and able to stabilize HIF-1α and influences IL-1β expression (**Figure 4**) (71).

Ornithine and nitric oxide (NO) are the most characteristic molecules of macrophage polarization toward M1 or M2 active state, respectively. Both of these molecules are metabolites obtained through L-arginine cleavage. Ornithine promotes cell proliferation, tissue healing, and fibrosis through the deposition of polyamines and collagen. NO instead inhibits cell proliferation and a raise in IL-12/23 and IL-18 levels (**Figure 3**) (71).

Based on these observations, a metabolic reprogramming through the inhibition of glycolysis seems to modulate the polarization of macrophages from an M1 to an M2 phenotype: the glycolysis inhibitor 2-deoxyglucose ameliorates adjuvant-

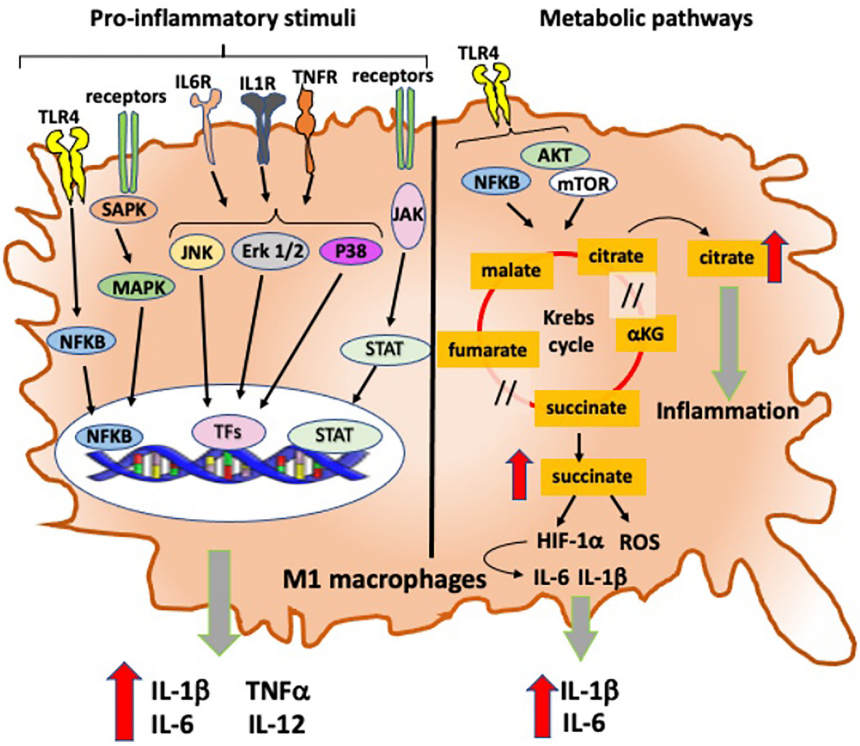

FIGURE 4 | Intracellular signaling and metabolic pathways activated into RA pro-inflammatory M1 macrophages. Intracellular signaling and metabolic pathways activated in M1 macrophages that contribute to their pro-inflammatory role in the inflammatory process in RA. TLR4, Toll-like receptor 4; IL-6R, interleukine-6 receptor; IL-1βR, interleukine-1β receptor; TNFR, tumor necrosis factor receptor; NFkB, nuclear factor-kB; SAPK: stress-activated protein kinases; MAPK, mitogen-activated protein kinases; JAK, Janus kinase; STAT, signal transducer and activators of transcription; Erk1/2, extracellular signal-regulated protein kinases 1 and 2; JNK, Jun N-terminal kinase; TFs, transcription factors; AKT, protein kinase B; mTOR, mechanistic target of rapamycin; HIF-1α, hypoxia-inducible factor-1α; ROS, reactive oxygen species; /, break in Krebs cycle.

induced arthritis by regulating macrophage polarization in an AMPK-dependent manner (**Figure 3**) (72).

EFFECTS OF M1 AND M2 MACROPHAGES IN RA SYNOVITIS

Synovial tissue is the major district of joint inflammation in RA patients, and the persistent chronic synovitis leads to an irreversible damage of cartilage and bone (73). The specialized structure of synovium is composed of two layers: the lining layer, which is populated by macrophages and FLSs, and a sublining layer constituted by vascularized connective tissue (74).

The synovial lining layer is a protective barrier, and synovial fluid is vital for physiological motion, maintaining cartilage and joints well hydrated. The absence of an epithelial basement membrane in the synovial lining contributes to its permeability and the diffusion of different compounds (73). Macrophages of the lining layer are resident cells involved in the maintenance of tissue homeostasis; these cells express CX3CR1, forming a protective tight-junction cell layer that avoids the infiltration of inflammatory cells responsible for arthritis development (75).

In the setting of synovitis, synovial tissue cellularity rises, and synovial thickening is commonly reported as radiographic feature. Moreover, most resident macrophages are still characterized by the expression of CD206, MerTK, and T-cell immunoglobulin and mucin domain containing 4 (TIMD-4) (75, 76).

Synovial macrophages along with infiltrating monocytes-derived macrophages are fundamental cells in the initiation and chronicity of RA synovitis through their capability to orchestrate the immune response releasing cytokines and enzymes involved in the inflammatory cascade, which in turn activate osteoclasts and fibroblasts, leading to joint destruction and disease perpetuation (**Figure 2**) (35, 77, 78).

These macrophages express TLRs, primarily TLR2, and activate local danger signals and modulate their activity (79).

In RA synovial tissue, the interaction between activated M1 macrophages and Th1 cells fosters the production of several pro-inflammatory mediators, including IL-1β, IL-6, TNFα, IL-23, CXCLs, and CCLs; this crosstalk is mediated first by MHC class II and secondarily by costimulatory molecules CD80/CD86, which are overexpressed in RA M1 macrophages (**Figure 2**) (77, 80, 81). In the early-stage of RA, these mediators contribute to the recall and activation of monocyte-derived macrophages

from peripheral blood into synovial tissue promoting and sustaining inflammation (82).

During disease progression, the synovial tissue responds to inflammatory insults with a maladaptive wound healing undergoing profound changes: inflammatory and mesenchymal cells infiltration, inner-layer hyperplasia, neovascularization, and pannus formation resulting in cartilage destruction.

As previously discussed, CCR7 signaling pathway was demonstrated to induce monocyte migration and M1 polarization: in fact, CCL21/CCR7 signaling mediates the migration of $CD14^+CD86^+$ monocytes, which polarize into M1 macrophages with a consequential pro-inflammatory cytokine production, primarily IL-6 and IL-23 (83).

RA macrophages and primarily pro-inflammatory M1 macrophages are characterized by a high expression of CCR7, and these CCR7-expressing macrophages induce and amplify the differentiation of Th17 cells (83). Moreover, the activation of CCL21/CCR7 signaling pathway in these macrophages determines their differentiation into osteoclasts in a process that involves the induction of Th17 polarization (83). CCR7 expression on monocytes is enhanced by IFNγ and TNFα, whereas its drastic reduction has been observed in the presence of IL-4, a typical Th2/M2 mediator (83).

Together with CCL21, IL-23 is another important cytokine mainly secreted by activated macrophages in the synovial tissue, which induces the differentiation of αβ $CD4^+$ naive T cells into Th17 cells (23). These $CD4^+$ T cells are the major producers of IL-17, which characterize the synovial compartment of RA patients and contribute to the pathogenesis of the disease (23). This cytokine interacts with its receptor on the surface membrane of several cell types, including monocytes/macrophages, activating several intracellular signaling pathways involved in the inflammation, such as those mediated by Erk1/2, JNK, p38, STATs, and JAK activation (23). As is well-demonstrated, the synergic effect of IL-17 and TNFα induces the production of pro-inflammatory mediators by macrophages, including IL-6, IL-1β, and matrix metalloproteinases (MMPs) that contribute to the progression of an early inflammation toward a chronic arthritis (84).

Moreover, these inflammatory macrophages are involved in the turnover of connective tissue and erosion of articular surface through their production and release of MMPs (85). The massive release of pro-inflammatory cytokines and chemokines determines a drastic change in the synovial microenvironment and allows an efficient activation of cytotoxic cells (85).

As a hallmark of inflammation, the abundant presence of macrophages (M1) in RA synovitis reflects disease activity, and therefore, their depletion at the level of target organ may be a good biomarker of therapeutic response (**Figure 2**) (86).

Many studies have confirmed the different origin of resident macrophages and monocyte-derived macrophages (86).

In the synovial tissue of RA patients and healthy subjects, resident macrophages are identified as CD68- and CD163-positive cells, able to proliferate and maintain themselves locally: these cells remain relatively quiescent, while they are activated during disease flares (75, 87). CD68 was shown to bind oxidized low-density lipoprotein and to be involved in the cell–cell interaction. In the synovial sublining, changes in the number of $CD68^+$ macrophage correlates with clinical outcomes evaluated using DAS28, representing a possible further reliable biomarker of therapeutic efficacy (87, 88).

In a recent study involving long-standing RA patients, the analysis of transcriptome profile of highly inflamed synovial tissue demonstrated the upregulation of transcripts related to the signaling pathways mediated by TLR, TNF, IFN, and IL-6 receptors and related to chemotactic and inflammatory processes, overlapping with those monocyte/macrophage patterns activated by bacterial and fungal pathogens, such as LPS (79).

As is well-demonstrated, *in vitro* stimulation of circulating human monocytes with LPS induces their differentiation and polarization into a pro-inflamatory M1 phenotype, characterized by the expression of specific surface markers CD80, CD86, HL-DR, TLR2, and 4, and the release of IL-1β, TNFα, and IL-6 (**Figure 2**) (42, 89). In RA synovitis, the best represented and upregulated genes and the secreted proteins are those correlated to M1 macrophages (79).

Moreover, among these secreted proteins, sCD14, S100A8/A9, S100P, LBP, CXCL13, MMP-3, and CCL18 showed a good correlation between their concentration and the DAS28/ESR (79).

In the synovial tissue and fluid of RA patients, $CD86^{high}$AtoMs characterized by an increased FoxM1 gene expression show a high osteoclastogenic potential compared to $CD86^{low}$AtoMs, contributing to the inflammatory process and bone erosion in RA (53).

Conversely, $MerTK^+CD206^+$ synovial tissue macrophages (STMs) are highly expressed in RA patients during the remission state (**Figure 2**) (64). $MerTK^-CD206^-$ STMs are the main source of pro-inflammatory cytokines in synovitis and the cell–cell interactions between macrophages and fibroblasts (64).

MerTK is a member of transmembrane receptor tyrosine kinase family, expressed on the surface membrane of macrophages and dendritic cells. After activation by its ligand Gas6 and protein S, MerTK plays an anti-inflammatory action inducing the phagocytosis of apoptotic cells, a key process for tissue repair and the maintenance of tissue homeostasis (90).

In human synovial tissue, $MerTK^+$ synovial macrophages are characterized by a specific regulatory signature depending on the disease state (healthy, active RA, or remission): in particular, RA patients who underwent remission show the upregulation of the genes encoding for the transcription factors Krüppel-like factor 2 (KLF2), KLF4, nuclear receptor subfamily 4 group A member 1 (NR4A1), NR4A2, or the dual-specificity phosphatase1 (DUSP1), representing negative regulators of inflammation that actively participate to restore tissue homeostasis, through lipid mediators such as resolvins (64).

OLD AND NEW THERAPEUTIC STRATEGIES INDUCING THE M1–M2 POLARIZATION AND FUTURE PERSPECTIVES

In RA patients, the high expression of pro-inflammatory molecules induces monocytes, primarily those belonging to the

intermediate subset, to migrate to synovial tissue and differentiate into M1 macrophages.

It is evident that the increased presence of activated pro-inflammatory macrophages in synovial tissue is considered an early hallmark of RA, and it is correlated with the higher proportion of M1 macrophages compared to M2 macrophages (**Figure 2**) (91, 92).

Therefore, an early inhibition of macrophages activation may be considered as an effective and well-tolerated therapeutic strategy in the management of RA (93, 94).

As is well-demonstrated, a prompt diagnosis followed by an early treatment is mandatory to prevent debilitating bone erosions, functional decline, and premature mortality in RA patients (95). Achieving early remission within the "therapeutic window of opportunity" determines better clinical outcomes and consequently future treatment avoidance (96).

Conversely, a delay in starting treatment results in prolonged symptom duration and poorer outcomes (97).

The identification of specific biochemical markers reflecting macrophage populations could be a useful tool to identify disease activation state and represent possible targets for RA treatment, such as the aforementioned MerTK (58).

Interestingly, in RA patients in disease remission, a high presence of $MerTK^+CD206^+CD163^+$ M2 macrophages has been detected in the synovial tissue, where they formed a tight lining layer; the increased presence of these cells was negatively correlated with DAS28/CRP, synovial hypertrophy, and vasculitis (64).

Of note, the presence of these cells was also observed in healthy synovial tissues. Conversely, active RA patients were characterized by the presence of $MerTK^-CD206^-$ macrophages in the lining layer of the synovial tissue (64).

Interestingly, this study confirmed that in RA patients where it was possible to taper and then discontinue biological treatment before the investigation of synovial tissue macrophages, the disease remission was maintained in those patients characterized by a high percentage and proportion of $MerTK^+CD206^+$ macrophages (M2 macrophages) (**Figure 2**); conversely, in those RA patients who flared after biological treatment discontinuation, the percentage of these M2 macrophages was lower (64).

These results indicate that $MerTK^+$ macrophages showing an M2 phenotype seem to characterize the synovial tissue of RA patients under disease remission and healthy subjects (**Figure 2**): these cells produce lipid mediators implicated in the resolution of inflammation, and they overexpress transcription factors implicated in the control of local immune responses and homeostasis.

Therefore, based on this new evidence, the induction of the MerTK signaling pathway might be considered a promising approach in driving disease remission in RA patients (63).

Interestingly, compelling evidence have demonstrated a positive correlation between glucocorticoid therapy and the augmented MerTK expression on monocyte-derived macrophages surface membrane, revealing an additional role of this therapeutic approach in RA flare attenuation.

Furthermore, cellular metabolic reprogramming could be an innovative therapeutic strategy to reduce M1 macrophage growth and alter inflammatory milieu in favor of anti-inflammatory M2/Th2 pathways, restoring the correct balance in the M1–M2 ratio (98).

In the last decades, RA treatment has significantly been changed, highlighting the pivotal role of treat-to target strategies aiming to a patient tailored therapy for a better control of disease activity. Therefore, the acknowledge of RA pathophysiology has been a crucial guide for the development of effective and safe treatments. About that, in the past years an increased number of biological disease-modifying anti-rheumatic drugs (bDMARDs) have been developed with a proven efficacy (99).

Indeed, starting with bDMARDs treatment at a very early stage can modify or even reverse disease progression thanks to their ability to interfere with biologic processes (96). Although these drugs are structurally unrelated and have different pharmacodynamic and pharmacokinetic properties, their clinical development has been largely overlapping (99).

Currently, no drugs are specific for macrophages in the treatment of RA, but their effects are directed to inhibit some aspects of macrophage activation, in particular the production of inflammatory cytokines, including TNFα, IL-1β, and IL-6: monoclonal antibodies or soluble receptors have been used for many years, but novel agents targeting these molecules seem to be more efficient in the treatment of inflammatory phase in RA (92).

TNF inhibitors (including infliximab, etanercept, adalimumab, golimumab, and certolizumab) bind to soluble and membrane-associated TNFα, inhibiting the activation of those intracellular signaling pathways involved in mediating pro-inflammatory properties, including NF-kB and RANK ligand (**Table 1**) (2).

Tocilizumab inhibits the IL-6-mediated inflammation through the interaction with IL-6 receptors, whereas the immune and pro-inflammatory action of IL-1β is contrasted by the inhibition of the binding with its receptors mediated by anakinra, a non-glycosylated recombinant form of the physiological IL-1 receptor antagonist (**Table 1**) (2, 100).

As matter of fact, in a recent study, the contribution of some bDMARDs, in particular anti-TNF agents, on the impact of pro-inflammatory M1 macrophages obtained from RA patients revealed their indirect capability to modulate the polarization of these cells toward an M2 phenotype (**Table 1**) (101).

The mechanism that promotes this polarization involves the activation of Gas6 and SOCS3 and the subsequent increase in IL-10, a process mediated by the induction of STAT3 signaling pathway (**Table 1**) (101).

Conversely, this effect in promoting the polarization from a M1 to an M2 phenotype seems not to be induced by the treatment with anti-IL-6 receptor and anti-CD20 agents, which do not determine the upregulation of M2 markers in cultured macrophages (**Table 1**) (101).

TABLE 1 | Targets, effect, and signaling pathways of biological disease-modifying anti-rheumatic drugs (bDMARDs).

Treatment	Target	M1–M2 shift contribution	Signaling	Reference
CTLA4-Ig (abatacept)	CD80/ CD86	Downregulation of CD80, CD86, and TLR4 Upregulation of CD204, CD163 and CD206, MerTK	Inhibition of NFkB	(42) (46) (47)
TNF inhibitors (infliximab, etanercept, adalimumab, golimumab and certolizumab)	TNFα	Upregulation of IL-10, SOCS3, GAS6, CD16	Activation of STAT3 Inhibition of NFkB	(100)
Rituximab	Anti-CD20	Downregulation of CD40	–	(100)
Tocilizumab	Anti-IL-6R	Downregulation of CD40 Upregulation of CD206	–	(100)

Description of molecular targets, effect exerted on cells involved in the inflammatory process and signaling pathways modulated by biological disease-modifying anti-rheumatic drugs, such as CTLA4-Ig (abatacept), TNFα inhibitors (infliximab, etanercept, adalimumab, golimumab, certolizumab), anti-CD20 antibody (rituximab), and anti-IL-6 receptor antibody (anti-IL-6R, tocilizumab). TLR4, toll-like receptor 4; CD204 and CD163, macrophage scavenger receptors; CD206, mannose receptor 1; MerTK, MER proto-oncogene, tyrosine kinase; TNF, tumor necrosis factor; IL-10, interleukine-10; SOCS3, suppressor of cytokine signaling 3; GAS6: growth arrest-specific 6; STAT3, signal transducer and activator of transcription 3.

THE RECENT DISCOVERED ROLE OF CTLA4-IG (ABATACEPT) IN INDUCING THE M1–M2 POLARIZATION

The capability of a selected bDMARDs to promote the polarization of pro-inflammatory M1 macrophages to an anti-inflammatory M2 phenotype was recently tested *in vitro* for the CTLA4-Ig fusion protein in cultured monocyte-derived macrophages obtained from RA patients (**Table 1**) (42). These RA monocyte-derived macrophages, which were characterized by a pro-inflammatory M1 phenotype, as demonstrated by their upregulation of CD80, CD86, and TLR4 gene expression, acquired an anti-inflammatory M2 phenotype after treatment with CTLA4-Ig. This polarization is determined by the downregulation of the gene expression of M1 phenotype markers and the upregulation of the gene and protein expression of M2 cell surface markers CD204, CD163, and CD206 and MerTK, suggesting also an increased induction of their phagocytic activity (**Table 1**) (42).

However, this important result was anticipated by the demonstration that the inhibition of the CD80-CD86/CD28 co-stimulatory signaling pathway by CTLA4-Ig generally contributes to downregulate several pro-inflammatory mediators involved in the inflammatory cascade of RA (**Table 1**) (102–105).

In fact, in RA patients, the treatment with abatacept significantly reduced serum levels of IL-6, IL-12, IL-1β, and soluble E-selectin and ICAM-1, together with the reduction in IFNγ and MMP-1/3 gene expression (**Table 1**) (102, 103). This reduction of these important inflammatory mediators determines an improvement of disease outcomes. Of note, several *in vitro* studies demonstrated the capability of CTLA4-Ig to block the differentiation of monocytes into osteoclasts, reducing the expression of CD80 and CD86, without affecting mature osteoclasts, the functions of which are important in terms of physiological bone homoeostasis and bone turnover (106–108). On the contrary, this physiological effect is not induced by other bDMARDs (106–108).

RA patients with an inadequate response to bDMARDs have a significant reduction in the composite score of DAS28/CRP level and the patient's global assessment of disease activity after 12 weeks of treatment with abatacept (109).

More specifically, several *in vitro* studies highlighted the capability of CTLA4-Ig treatment to reduce the gene expression and release of pro-inflammatory cytokines IL-6, IL-1β, and TNFα directly interacting with CD86 on the surface membrane of APCs, primarily synovial macrophages and monocyte-derived macrophages isolated from RA patients (104, 105). This direct anti-inflammatory effect is mediated by the inhibition of NF-kB signaling pathway in a short time (**Table 1**) (46, 47).

CONCLUDING REMARKS

In the pathogenesis of RA, monocytes and macrophages are fundamental mediators of the inflammatory process, contributing to the T-cell activation and production and release of pro-inflammatory cytokines and chemokines responsible for the migration of circulating cells to the synovial tissue and promoting an aberrant immune response that leads to the perpetuation of inflammation and bone erosion.

The development of this inflammatory environment is primarily due to an imbalance in M1–M2 monocytes/macrophages both in the peripheral blood and synovial tissue with a predominant presence of M1 macrophages, which also contribute to osteoclastogenesis in RA patients with active disease (14).

Conversely, the synovial tissue of RA patients under remission is characterized by a higher presence of M2 macrophages with a phagocytic activity compared to patients with active disease. Considering that the regulation of M1/M2 imbalance in favor of anti-inflammatory M2 macrophages might represent a therapeutic goal to restore tissue homeostasis, the identification of molecules that may promote M1/M2 polarization of RA macrophages may represent valuable therapeutic targets and could lead to the development of novel drugs.

Based on the newest acknowledgments concerning the therapeutic strategies currently used in clinical practice, the treatment inducing not only the downregulation of pro-inflammatory cytokines/chemokines but also the polarization

from M1 into anti-inflammatory M2 macrophages might be an interesting approach to better control the aberrant inflammatory response in RA patients.

AUTHOR CONTRIBUTIONS

MC and SS conceptualized the argument of the review, collected the data, and wrote the manuscript. RC collected the data and wrote the manuscript. EG reviewed the manuscript for important intellectual content. All authors contributed to the article and approved the submitted version.

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REVIEW
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Dendritic Cells and Macrophages in the Pathogenesis of Psoriasis

Masahiro Kamata and Yayoi Tada *

Department of Dermatology, Teikyo University School of Medicine, Tokyo, Japan

Psoriasis is a chronic inflammatory skin disease characterized by scaly indurated erythema. This disease impairs patients' quality of life enormously. Pathological findings demonstrate proliferation and abnormal differentiation of keratinocytes and massive infiltration of inflammatory immune cells. The pathogenesis of psoriasis is complicated. Among immune cells, dendritic cells play a pivotal role in the development of psoriasis in both the initiation and the maintenance phases. In addition, it has been indicated that macrophages contribute to the pathogenesis of psoriasis especially in the initiation phase, although studies on macrophages are limited. In this article, we review the roles of dendritic cells and macrophages in the pathogenesis of psoriasis.

Keywords: dendritic cell (DC), macrophage - cell, monocyte - macrophage, langerhans cell (LC), psoriasis, psoriatic arthritis (PsA)

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***Correspondence:**
Yayoi Tada
ytada-tky@umin.ac.jp

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1 INTRODUCTION

Psoriasis is a chronic inflammatory skin disease characterized by scaly indurated erythema. This disease impairs patients' quality of life enormously. Pathological findings demonstrate proliferation and abnormal differentiation of keratinocytes and massive infiltration of inflammatory immune cells. The pathogenesis of psoriasis is complicated, but it has been revealed by intensive research. Among immune cells, dendritic cells (DC) play a pivotal role in the development of psoriasis in both the initiation and the maintenance phases. In addition, it has been indicated that macrophages contribute to the pathogenesis of psoriasis especially in the initiation phase, although studies on macrophages are limited. In this article, we review the roles of DC and macrophages in the pathogenesis of psoriasis. Since the contributions of DC to the pathogenesis of psoriasis have already been well-described in the previous literature (1, 2), we give a concise overview of the current understanding. Then we review findings on the involvement of macrophages in the pathogenesis of psoriasis.

2 OVERVIEW OF THE CURRENT UNDERSTANDING OF THE PATHOGENESIS OF PSORIASIS AND THE ROLES OF DENDRITIC CELLS AND MACROPHAGES

Previous review articles have provided detailed descriptions of the pathogenesis of psoriasis (3–5). We focus on DC and macrophages (**Figure 1**). Briefly, in early-phase psoriasis, nucleic acids and a variety of antimicrobial peptides released from damaged keratinocytes activate innate immune cells, including plasmacytoid DC (pDC) and macrophages, which produce interferon (IFN)-α and tumor necrosis factor

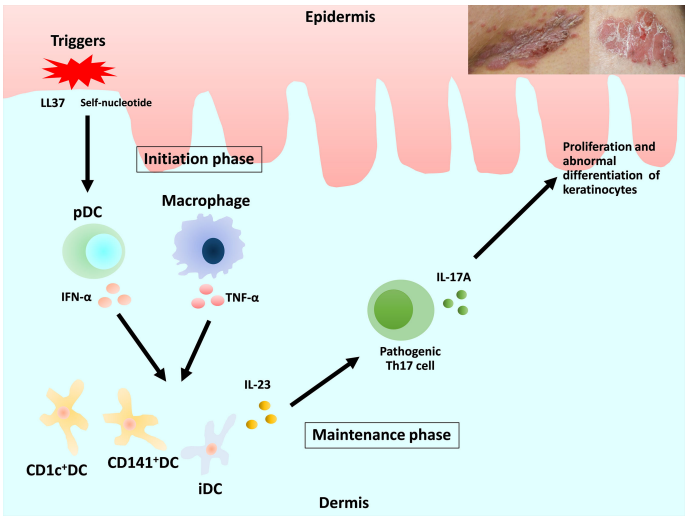

FIGURE 1 | Overview of the current understanding of the pathogenesis of psoriasis and the roles of dendritic cells and macrophages. DC, dendritic cells; pDC, plasmacytoid DC; iDC, inflammatory DC.

(TNF)-α. The release of IFN-α causes the maturation of resident dermal DC and the differentiation of monocytes into inflammatory DC (iDC). Mature resident DC and the rapidly increasing numbers of iDC produce interleukin (IL)-23, IL-12, TNF-α and other cytokines, which strongly activate the differentiation of naive T cells into Th1, Th17 and Th22. IL-23 maintains and promotes the proliferation of pathogenic Th17 cells. The release of IL-17 and IL-22 induces proliferation and abnormal differentiation of keratinocytes. Keratinocytes also act as immune cells by producing TNF-α, IL-8, vascular endothelial growth factor (VEGF), antimicrobial peptides, etc., some of which activate DC. This vicious inflammatory cycle causes the plaque to remain and deteriorate in the chronic phase of psoriasis (1, 2, 5, 6).

3 DENDRITIC CELLS

3.1 Dendritic Cells Under Steady-State Conditions

DC are heterogenous and are sub-classified based on location, origin, and function. Detected subtypes of DC are different in the steady state or in the inflammatory state (6). Furthermore, there is a little difference in surface marker expressions between human DC and mouse DC (1).

In human peripheral blood, three main subsets of DC can be identified: plasmacytoid DC (pDC), and two types of conventional DC (cDC), i.e., CD1c(BDCA-1)$^+$ cDC (cDC1) and CD141(BDCA-3)$^+$ cDC (cDC2) (7, 8), as shown in **Table 1** (6). Hierarchical clustering of mouse lymph nodes and human blood DC subsets by genome-wide expression profiling revealed clustering of human pDC with mouse pDC, human CD1c$^+$ cDC1 with mouse CD11b$^+$ DC, and human CD141$^+$ cDC2 with mouse CD8α$^+$ DC (9).

In the skin under steady-state conditions, two dermal DC subsets identical to CD1c$^+$ and CD141$^+$ blood cDC have been identified (10, 11) (**Table 2**). However, pDC are absent during steady-state conditions. Human tissues also harbor migratory CD14$^+$ DC, which do not have an identified murine equivalent (10, 11). Its phenotype and transcriptomic expression profiles show the characteristics of blood monocytes and tissue macrophages (10, 11), which raises the question of the origin of DC. Langerhans cells (LC) which are located in the epidermis, survey the epidermis for foreign antigens as antigen-presenting cells and activate T cells as needed (12).

TABLE 1 | Three main subsets of dendritic cells in human peripheral blood under steady-state conditions.

Human dendritic cells	Cell surface markers on the indicated DC	Equivalent cells in mice
Plasmacytoid DC (pDC)	CD11c$^+$ CD123$^+$ CD303(BDCA-2)$^+$ CD304(BDCA-4)$^+$	Mouse pDC
Conventional DC 1 (cDC1)	CD11c$^+$ CD1c(BDCA-1)$^+$	Mouse CD11b$^+$DC
Conventional DC 2 (cDC2)	CD11c$^+$ CD141(BDCA-3)$^+$	Mouse CD8α$^+$DC

DC, dendritic cells.

TABLE 2 | Human dendritic cells in the skin of normal individuals and in psoriatic skin.

Location	Human DC in the skin	Steady state	Cell surface markers in steady state*	Psoriatic skin	Cell surface markers in lesional skin**	Function in psoriasis	Equivalent cells in mice
Epidermis	LC	Present	$CD11c^+CD1a^+CD1c^+$(BDCA-1)$^+CD207$(Langerin)$^+$	Present	$CD1a^+CD1c^+$(BDCA-1)$^+CD11c^+$Langerin (CD207)$^+$	Controversial	Mouse LC
Dermis	$CD1c^+$DC (cDC1)	Present	$CD11c^+CD1a^+CD1c$(BDCA-1)$^+$	Decreased	$CD1a^+CD1c$(BDCA-1)$^+CD11c^+$	Induction and proliferation of Th1/17 cells and cytokine production	Mouse $CD11b^+$DC
	$CD141^+$DC (cCD2)	Present	$CD11^{low}$ $CD1a^+CD1c$(BDCA-1)$^{low/int}$ $CD141$(BDCA-3)$^+$	Increased	$CD11c^+CD141$(BDCA-3)$^+$	Induction and proliferation of Th1/17 cells and cytokine production	Mouse $CD103^+$DC
	pDC	Absent		Present	$CD11c^-CD123^+CD303$ (BDCA-2)$^+$	Production of IFN-α and activation and maturation of dermal DC	Mouse pDC
	iDC-Tip-DC-slan DC	Absent		Present	$CD11c^+CD206^+$	Production of TNF-α, iNOS, IL-23	Mouse iDC

DC, dendritic cells; LC, Langerhans cell; pDC, plasmacytoid cell; iDC, inflammatory cell.
**Cell surface markers on the indicated DC in the steady state.*
***Cell surface markers on the indicated DC in lesional skin.*

3.2 Dendritic Cells in the Skin of Psoriasis Patients

In psoriatic lesions, pDC and myeloid DC in addition to Th1/17 $CD4^+$ cells are observed in the dermis (13). Dermal DC in lesional skin can be divided into three subsets: $CD1c^+$ DC, $CD141^+$ DC, and $CD11c^+CD1c^-CD141^-$ inflammatory DC (iDC), including Tip-DC and 6-sulfo LacNAc DC (slanDC), as shown in **Table 2**. In inflammatory skin conditions including psoriasis, in addition to LC, $CD1c^+$ DC, and $CD141^+$ DC that are already present during the steady state, pDC and iDC migrate into the skin. pDC originate in the bone marrow and migrate to the skin under pathological conditions (14). The surface expression of CCR2, a chemokine receptor expressed by monocytes and required for their migration, on iDC indicates that iDC are derived from monocytes (15–17).

3.2.1 Plasmacytoid DC

Increased infiltration of pDC is observed not only in lesional skin but also in non-lesional skin of psoriasis patients, compared to normal skin from healthy controls (18–21). pDC recognize self-nucleic acids, thereby initiating inflammation of psoriasis through IFN-α production (18). Antimicrobial peptides in the epidermis of psoriasis patients, including LL-37, human β-defensin (hBD)-2, hBD-3 and lysozyme, bind self-DNA/RNA fragments released by stressed or injured keratinocytes, thereby inducing activation of pDC *via* TLR7/9 (22–27). Furthermore, DNA structures containing the neutrophil serine protease cathepsin G (CatG) and the secretory leukocyte protease inhibitor (SLPI), which are detected in lesional skin of psoriasis patients, induce the production of IFN-α in pDC. pDC play a role in early psoriasis (28).

IFN-α released by pDC activates dermal resident DC, and drives their maturation (29). Moreover, IFN-α induces rapid differentiation of human monocytes into iDC and polarizes $CD4^+$ T cells into Th1 and Th17 cells (30, 31). However, an anti-IFN-α monoclonal antibody failed to ameliorate plaque psoriasis in a phase I clinical trial (32), indicating that IFN-α is not important in the maintenance phase. It rather contributes to the development of psoriasis in the early phase.

3.2.2 Dermal DC

IFN-α and TNF-α released by pDC, macrophages, and other cells promote maturation and activation of myeloid DC, which play an important role in the chronic phase of psoriasis. In psoriasis patients, $CD11c^+$ DC are abundant in lesional skin, while there are relatively low numbers of these cells in non-lesional skin (33). Dermal DC derived from lesional skin induce proliferation of Th1 and polarization of Th17, and they are the source of IL-23 (33–40). As stated above, dermal DC in lesional skin can be divided into three subsets: $CD1c^+$ DC, $CD141^+$ DC, and iDC (**Table 2**). The number of $CD1c^+$ DC was lower in non-lesional and lesional skin of psoriasis patients than in normal skin, whereas the number of $CD141^+$ DC was higher. Lesional skin showed a considerable increase in infiltrating iDC compared with samples obtained from healthy controls (37), which mostly account for the total increase in $CD11c^+$ DC in lesional skin. Both the $CD1c^+$ DC and $CD1c^-$ DC populations from psoriatic skin strongly induced T-cell proliferation and production of IFN-γ and/or IL-17 to the same extent (37).

$CD11c^+CD1c^-CD141^-$ dermal iDC, including TNF-α and inducible nitric oxide synthase (iNOS)-producing DC (Tip-DC) and slanDC, have been identified in the dermis of psoriasis patients (41–43), and they seem to play a pivotal role in the pathogenesis of psoriasis (1). These iDC in psoriasis are identified as $CD11c^+CD1c^-$ DC, distinguishing them from resident cDC, and are assumed to be derived from monocytes (11, 37, 43, 44). Tip-DC express high levels of TNF-α and iNOS. TNF-α induces keratinocytes to express ICAM-1, CXCL8, and also pro-inflammatory cytokines including IL-1β and IL-6. iNOS in inflamed tissues catalyzes the production of nitric oxide (NO), which results in vasodilation of dermal blood vessels in the lesional skin of psoriasis patients (13). In addition, Tip-DC have been shown to produce high levels of IL-23 (6, 45, 46).

Through the expression of CX3CR1 and C5aR, slanDC are recruited by the increased expression CX3CL1 and C5a in psoriatic skin (42). The complete transcriptional overlap of blood slanDC with $CD16^+$ monocytes indicates that skin slanDC are derived from monocytes (11, 44). As with pDC, dermal slanDC are reactive to self-RNA-LL37 complexes (42)

and induce Th1/17 cells to produce IL-17, IL-22, TNF-α and IFN-γ (42, 43). In lesional skin of psoriasis patients, dermal slanDC express abundant IL-23-p19 and TNF-α (42, 47). Autocrine TNF-α stimulation of slanDC allows for higher production levels of IL-12, IL-23, IL-1β and IL-6 (48). Treatment with infliximab and dimethyl fumarate rapidly reduced the number of slanDC (1, 49, 50). Their phenotypic signatures suggest that dermal Tip-DC and slanDC represent the same inflammatory DC population although subpopulations may exist (6).

DC3 is a newly identified subset of inflammatory $CD5^{-}CD163^{+}CD14^{+}$ DC (51). Recently, single-cell analysis of human skin revealed that $CD14^{+}$ DC3 increased in psoriasis lesional skin, and they produced IL-1β and IL-23 (52), which could contribute to the pathogenesis of psoriasis.

Accumulating dermal iDC play a key role in the progression and sustenance of psoriasis by secreting large amounts of pro-inflammatory factors including iNOS, IL-23, and TNF-α (1, 6).

3.2.3 Langerhans Cells

LC are antigen-presenting cells residing in the epidermis. Once they recognize an antigen, they migrate into regional lymph nodes and present antigens. A Recent study reported identification of two steady-state (LC1 and LC2) and two activated LC subsets in the epidermis of human skin and in LC derived from $CD34^{+}$ hemopoietic stem cells (53). LC1 are characterized as classical LCs, mainly related to innate immunity and antigen processing. LC2 are involved in immune responses and leukocyte activation. LC1 remain stable under inflammatory microenvironment, whereas LC2 are prone to being activated and demonstrated elevated expression of immuno-suppressive molecules.

In the steady state, LC are continuously replaced from a resident precursor pool (54–56). However, in the inflammatory state, LC are repopulated by blood precursors (6, 57–60).

Their role in psoriasis has not yet been elucidated. The number of LCs in lesional skin of psoriasis patients was reported to be increased (61, 62), decreased (63, 64), or the same as the number of LC in control skin samples in various articles (65, 66).

The migration of LC is impaired in psoriatic patients (67, 68). Impaired LC migration in psoriasis is due to an altered keratinocyte phenotype induced by IL-17 (69).

LC play various roles in psoriasis according to previous studies. Some articles reported that LC play an anti-inflammatory role in psoriasis (53, 62, 70). In contrast, other studies indicated that LC are involved in the development of psoriasis (66, 71–74). Several studies demonstrated that LC produced IL-23 (66, 71, 72).

The discrepant data on LC are possibly due to different LC-deficient models, methods, or other factors (1). Further investigation is necessary to clarify the roles of LC in the pathogenesis of psoriasis.

The diversity of DC populations and different functions in psoriasis may be accounted for by plasticity of DC.

4 MACROPHAGES

4.1 Roles of Macrophages Under Steady-State Conditions and Inflammatory Conditions

Macrophages are categorized into two types: tissue-resident and infiltrating macrophages (6). Tissue-resident macrophages are long-lived non-migratory cells, and play an essential role in maintaining tissue homeostasis by clearing cell debris and promoting resolution of inflammation and wound healing (75). They are potent promoters of inflammation by producing chemokines, including CCL2, CXCL1, and macrophage inhibitory factor (MIF), and cytokines such as IL-6 and TNF-α, resulting in recruitment and activation of other immune cells (76, 77). Most tissue-resident macrophages are considered to be present from birth and are self-maintaining, independently from monocytes (78–81), except intestinal macrophages (82); however, this is still controversial.

Meanwhile, infiltrating macrophages are recruited to tissues in inflammatory conditions (6). Murine studies revealed that infiltrating macrophages originate from inflammatory monocytes. Infiltrating macrophages are divided into three populations based on function, displaying a pro-inflammatory profile (originally coined "classically activated" or "M1" macrophages), a regulatory profile, or a wound-healing profile (the latter two were originally grouped under the term "alternatively activated" or "M2" macrophages), depending on the tissue context and environmental stimuli (83, 84). According to their cell surface markers, secreted cytokines and biological functions, M2 macrophages are divided into M2a, M2b, M2c, and M2d subcategories (85). M1 polarization occurs in the presence of IFN-γ, LPS, and TNF-α. Cell surface markers of M1 macrophages are $CD14^{++}CD16^{-}$, CD40, and CD68. M1 macrophages produce IL-1β, IL-6, IL-12, IL-23, MCP-1, and TNF-α. In contrast, M2 polarization occurs in response to IL-4, IL-10, and IL-13. M2 macrophages express $CD14^{+}CD16^{++}$, CD163, and CD209 on their surface. M2 macrophages produce EGF, IL-10, PDGF, TGF-β, and VEGF (86). Among M2 macrophages, M2a macrophages, activated by IL-4 or IL-13, lead to the increased expression of IL-10, TGF-β, CCL17, CCL18, and CCL22 (85). These macrophages enhance the endocytic activity, promote cell growth and tissue repairing.

4.2 Roles of Macrophages in the Pathogenesis of Psoriasis

Murine studies demonstrated that depletion of macrophages improved psoriasis inflammation (87–89) and reduced the levels of Th1 cytokines, including IL-1α, IL-6, IL-23 and TNF-α to normal levels (90). These results underscore that macrophages contribute to the development and maintenance of psoriatic lesions (86).

Psoriasis patients have an increased level of circulating monocytes in peripheral blood (91, 92), and they favored the M1 phenotype (93). Furthermore, a considerable number of macrophages was observed in lesional skin (94). Immunofluorescence staining revealed that

$CD68^+iNOS^+$ M1 macrophages were increased and $CD68^+CD163^+$ M2 macrophages were decreased in human psoriasis lesional skin compared with skin samples from normal individuals (95). Another study demonstrated accumulation of dermal $CD68^+$ macrophages that expressed TNF-α in human psoriatic plaques (96). Other studies demonstrated that the number of $CD163^+$ macrophages increased in psoriatic lesional human skin, which decreased to non-lesional skin levels after an effective treatment with TNF-α inhibitors (33, 89, 97, 98). Moreover, it was indicated that $CD163^+$ macrophages produce IL-23p19 and IL-12/23p40 in addition to TNF-α and iNOS in human lesional skin (97, 99). Murine experiments revealed that in skin injected with IL-23, monocytes/macrophages characterized by the strong presence of $Ly6C^{hi}MHC\text{-}II^{hi}$ cells were the dominant immune population, particularly late in the model, and showed high expression of TNF-α but not IL-17A (100). In another murine study, when peritoneal macrophages freshly isolated from resting mice were treated with IL-23, they produced large amounts of IL-17A, IL-22 and IFN-γ, and expressed a distinctive gene expression profile compared with those of M1 and M2 macrophages (101). Under the condition of abundant IL-23 in psoriasis lesional skin, some macrophages possibly produce IL-17A, IL-22 and IFN-γ in addition to TNF-α. Since macrophage are highly plastic cells (102), the diversity of macrophage populations in psoriasis may reflect a heightened cellular plasticity.

Recently, the involvement of macrophage NLRP3 inflammasome activation in psoriasis has been reported (103–105).

4.3 Factors That Affect Macrophage Polarization in Psoriasis

The ratio of M1 to M2a macrophage polarization was higher in psoriatic patients comparing with that in controls (93). Treatment with TNF-α inhibitors decreased M1 phenotypes according to improvement of their clinical severity scores (88, 93).

Inappropriate and excessive activation of endosomal Toll-like receptors 7, 8, and 9 (TLRs 7-9) at the psoriasis lesion plays a pathogenic role in the onset of psoriasis. Murine experiments showed that treatment with a TLR7 agonist shifted macrophages in the psoriatic lesions to a higher M1/M2 ratio. Both exogenous and endogenous TLR7-9 ligands favored M1 macrophage polarization (106).

Blocking the signaling of 4-1BBL, a member of the TNF superfamily, reduced the expression of hallmark genes of M1 macrophages such as *Tnf*, *Nos2*, and *Il23* in imiquimod-treated mice. *In vitro* experiments revealed that deficiency of 4-1BBL resulted in reduced expression of *Tnf*, *Nos2*, *Il23*, *Il6*, and *Cxcl10* in LPS-and-IFN-γ–treated macrophages (M1), whereas the expression levels of *Il10*, *Arg1*, *Fizz1*, *Ym1*, *Egr2*, and *Mrc1* (*Cd206*) were increased in IL-4–treated 4-1BBL knock-out cells, suggesting that 4-1BBL favors the M1 polarization of macrophages (107).

Response gene to complement (RGC)-32 is important for M2 macrophage polarization and phagocytic activity, and inhibits the development of M1 macrophages (108). The level of *RGCC* (the gene encoding RGC-32) mRNA was significantly lower in lesional psoriasis than in samples from normal individuals (95). Furthermore, *Rgcc* expression was significantly reduced in the lesional skin of imiquimod-induced psoriasiform dermatitis. Considering that RGC-32 participates in M2 macrophage polarization, its reduced expression in psoriatic lesions possibly contributes to skewed macrophage polarization toward the M1 phenotype (95).

IL-35, known as an anti-inflammatory cytokine (109, 110), decreased the total number of macrophages and ratio of M1/M2 macrophages in three psoriasis models: a human keratinocyte cell line (HaCaT), a keratin 14-VEGF A-transgenic mouse model, and an imiquimod-induced psoriasis mouse model (111).

Hsa_circ_0004287, one of circular RNA (circRNA), inhibited M1 macrophage activation in an N 6-methyladenosine-dependent manner in atopic dermatitis and psoriasis (112).

Increased M1 polarization was associated with higher disease severity in psoriasis, returning to baseline after successful treatment by TNF-α inhibitors (93). TNF-α blockage inhibited M1 polarization through STAT1- and IRF-1-independent pathways.

4.4 Factors That Affect Macrophage Recruitment to the Skin in Psoriasis

Sphingosine-1-phosphate receptor 4 (S1PR4)-dependent CCL2 production may be involved in macrophage recruitment to the psoriatic lesion (113). In imiquimod-induced psoriasiform dermatitis, psoriasis severity was ameliorated in S1PR4-deficient mice without altered IL-17 production compared with those in psoriatic wild-type mice. Instead, deficiency of S1PR4 attenuated the production of CCL2, IL-6, and CXCL1 and subsequently reduced the number of infiltrating monocytes and granulocytes. Migration assays revealed reduced CCL2 production in murine skin and attenuation of monocyte migration under the conditions lacking S1PR4. S1PR4 signaling synergized with TLR signaling in resident macrophages to produce CCL2. They speculated that S1PR4 activation enhanced the TLR response of resident macrophages to increase CCL2 production, which attracted further macrophages.

Furthermore, the importance of the interaction between CX3CL1 and CX3CR1 has been postulated in psoriasis (114). CX3CR1, a receptor for CX3CL1, mediates migration of inflammatory cells. CX3CR1 deficiency attenuated imiquimod-induced psoriasis-like skin inflammation with decreased M1 macrophages.

4.5 Macrophage-Specific Soluble Factors in Psoriasis

Macrophage-specific soluble factors are involved in the pathogenesis of psoriasis. Macrophages produce monocyte chemoattractant protein-1 (MCP-1), which recruits Th1 inflammatory cells (86). MCP-1 and its receptor CCR2, expressed on dermal macrophages (115), are essential for monocyte recruitment from the circulation (116). Increased expression of MCP-1 is observed in psoriatic keratinocytes (115, 117). MCP-1 polymorphisms have been associated with an increased risk of psoriasis, and serum MCP-1 levels are higher in psoriatic patients (118) and in induced psoriatic lesions of murine models (88, 94). Local production of chemotactic MCP-1

correlated with macrophage accumulation in psoriasis, suggesting that MCP-1 dysregulation may contribute to the pathogenesis of psoriasis.

Macrophage migration inhibitory factor (MIF) is another cytokine implicated in the pathogenesis of psoriasis. MIF is produced by macrophages and recruits inflammatory cells (86). MIF polymorphisms have been associated with an increased risk of psoriasis (119–121). MIF drives murine psoriasiform dermatitis (122). Serum MIF levels were higher in psoriasis patients than in healthy controls, and the serum MIF level was positively correlated with the clinical severity score. Peripheral blood mononuclear cells from psoriatic patients spontaneously produced approximately ten-fold more MIF in *in vitro* culture, indicating an inherent overproduction of this cytokine in psoriatic patients (123). In MIF-null mice, severity of psoriasiform dermatitis was lower and macrophage recruitment was impaired (122). Thus, MIF may be involved in the recruitment of macrophages in psoriasis patients. However, in contrast to elevated serum MIF in psoriasis patients, MIF-positive cells were significantly decreased in the lesional psoriatic epidermis (124). Further studies are needed to clarify the involvement of MIF in the pathogenesis of psoriasis.

4.6 Involvement of Macrophages in Psoriatic Arthritis

Recently, studies on macrophages in the synovial fluid (SF) of arthritic joints in patients with psoriatic arthritis (PsA) have been reported.

PsA SF cells are dominated by monocytes/macrophages. $CD14^+CD16^-$ classical monocytes/macrophages were lower in PsA SF than in the SF of patients with rheumatoid arthritis (RA), while $CD14^+CD16^+$ intermediate monocytes/macrophages were more predominant in PsA SF compared to RA SF (125). Proteinase-activated receptor 2 (PAR2) and its activating proteinases, including tryptase-6, could be important mediators of inflammation in PsA (125).

In the synovial tissues of patients with PsA and RA, synovial tissue stromal cells and $CD163^+$ macrophages are the main source of granulocyte-macrophage colony-stimulating factor (GM-CSF) (126). Synovial tissue $CD163^+$ macrophages express pro-inflammatory polarization markers (activin A, TNF-α, and MMP12) and exhibit a predominantly GM-CSF-dependent pro-inflammatory polarization state.

Expression of the prolactin receptor (PRLR) is higher in synovial tissue from RA and PsA patients than in synovial tissue from osteoarthritis (OA) patients, and prolactin (PRL) cooperates with other pro-inflammatory stimuli such as CD40L and TNF to activate macrophages by increasing the expression of pro-inflammatory cytokines including IL-6, IL-8 and IL-12β (127). Although serum PRL levels were similar in female and male RA patients, PRLR expression was significantly higher in RA and PsA synovial tissue compared with OA synovial tissue. PRLR colocalized with synovial $CD68^+$ macrophages and von Willebrand $factor^+$ endothelial cells. An *in vitro* study showed that PRLR was prominently expressed in IFN-γ- and IL-10-polarized macrophages. The production of PRL by macrophages was increased by unknown components of RA and PsA SF (128), where PRL could contribute to disease progression.

Tie2 is a tyrosine kinase receptor essential for vascular development and blood vessel remodeling through interaction with its ligands, angiopoietin-1 (Ang-1) and Ang-2 (129). Tie2 and its ligands were expressed in RA and PsA synovial tissue at higher levels than in the synovial tissue of healthy controls and OA patients (130–132). In RA and PsA synovial tissue, Tie2 was expressed by fibroblast-like synoviocytes, endothelial cells and macrophages (131, 133). Kabala et al. revealed that Tie2 signaling enhanced TNF-dependent activation of macrophages in synovial inflammation in RA and PsA patients (134).

Some of the above-mentioned factors are not specific to PsA. The role of macrophages in arthritis might be common in many aspects between RA and PsA. Although the contribution of macrophages to the development of PsA is indicated, the data are limited at present.

5 CONCLUSION

pDC play an important role in the early phase of psoriasis by producing IFN-α, which causes the maturation of resident dermal DC and the differentiation of monocytes into iDC. Increased numbers of iDC produce key cytokines of psoriasis, including IL-23, which strongly activate the differentiation of naive T cells into Th17 and Th22. IL-23 contributes to the maintenance and proliferation of pathogenic Th17 cells. The contribution of LC to the pathogenesis of psoriasis is controversial.

M1 macrophages are considered to contribute to the development of psoriasis especially in early-phase psoriasis, by producing TNF-α. Recently, IL-23 production by $CD163^+$ macrophages has been reported. Further investigation is needed to clarify the involvement of macrophages in the pathogenesis of psoriasis.

AUTHOR CONTRIBUTIONS

MK wrote the first draft of the manuscript. YT contributed to conception, review and editing. All authors contributed to manuscript revision, read, and approved the submitted version.

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Keishi Fujio,
The University of Tokyo, Japan

REVIEWED BY
Hiroaki Niiro,
Kyushu University, Japan
Yannick van Sleen,
University Medical Center Groningen,
Netherlands

*CORRESPONDENCE
Ryu Watanabe
doctorwatanaberyu@yahoo.co.jp

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Pathogenic role of monocytes/macrophages in large vessel vasculitis

Ryu Watanabe* and Motomu Hashimoto

Department of Clinical Immunology, Osaka Metropolitan University Graduate School of Medicine, Osaka, Japan

Vasculitis is an autoimmune vascular inflammation with an unknown etiology and causes vessel wall destruction. Depending on the size of the blood vessels, it is classified as large, medium, and small vessel vasculitis. A wide variety of immune cells are involved in the pathogenesis of vasculitis. Among these immune cells, monocytes and macrophages are functionally characterized by their capacity for phagocytosis, antigen presentation, and cytokine/chemokine production. After a long debate, recent technological advances have revealed the cellular origin of tissue macrophages in the vessel wall. Tissue macrophages are mainly derived from embryonic progenitor cells under homeostatic conditions, whereas bone marrow-derived circulating monocytes are recruited under inflammatory conditions, and then differentiate into macrophages in the arterial wall. Such macrophages infiltrate into an otherwise immunoprotected vascular site, digest tissue matrix with abundant proteolytic enzymes, and further recruit inflammatory cells through cytokine/chemokine production. In this way, macrophages amplify the inflammatory cascade and eventually cause tissue destruction. Recent studies have also demonstrated that monocytes/macrophages can be divided into several subpopulations based on the cell surface markers and gene expression. In this review, the subpopulations of circulating monocytes and the ontogeny of tissue macrophages in the artery are discussed. We also update the immunopathology of large vessel vasculitis, with a special focus on giant cell arteritis, and outline how monocytes/macrophages participate in the disease process of vascular inflammation. Finally, we discuss limitations of the current research and provide future research perspectives, particularly in humans. Through these processes, we explore the possibility of therapeutic strategies targeting monocytes/macrophages in vasculitis.

KEYWORDS

giant cell arteritis, large vessel vasculitis, macrophages, monocytes, takayasu arteritis, vasculitis

1 Introduction

Monocytes are circulating blood leukocytes that play important roles in the inflammatory response, and represent 10% of leukocytes in human blood (1). Monocytes are functionally characterized by their capacity for phagocytosis, antigen presentation, and cytokine/chemokine production, and originate in the bone marrow from a hematopoietic precursor which is common for several subsets of macrophages and dendritic cells (DCs). These cells are not only part of the innate immune system, but also the monocytic lineage that support the activation of the adaptive immune system by antigen presentation (2). Monocytes/macrophages are deeply involved in vascular inflammation including atherosclerosis and vasculitis as well.

Vasculitis is an autoimmune and/or autoinflammatory vascular inflammation and causes breakdown of the blood vessel walls. Based on the distribution of vessel involvement, it is classified as large, medium, and small vessel vasculitis (3). Large vessel vasculitis affects the aorta and its major branches and include giant cell arteritis (GCA) and Takayasu arteritis (TAK). The hallmark of the two diseases is granulomatous inflammation, which is primarily composed of $CD4^+$ T cells and macrophages (4, 5). In GCA, name-giving multinucleated giant cells are often observed in the vascular tissue (Figure 1) and formed by Toll-like receptor (TLR) 2-induced fusion of macrophages (6). Thus, it is obvious that monocytes/macrophages are key players in the pathomechanisms of large vessel vasculitis. Since we have been working on the pathogenesis of GCA, this review will mainly focus on GCA.

The currently available treatments for GCA include glucocorticoids and tocilizumab (TCZ), an IL-6 receptor inhibitor. Even with the adequate use of glucocorticoids, inflammation of the temporal artery remains in about half of the patients after one year (7). Macrophages and giant cells also remain in one in four patients. On the other hand, TCZ reduces vascular inflammation detected by fluorodeoxyglucose-positron emission tomography (8) and flare-up of GCA (9). However, it is difficult to cure the disease, as shown by the flare-up after discontinuation of TCZ in most cases (8). Therefore, clinical unmet needs exist with the current therapies.

This review first summarizes the current knowledge of monocytes/macrophages subsets and the origin of tissue macrophages, particularly in the vascular tissue. Then, the pathogenic roles of monocytes/macrophages in the pathogenesis of large vessel vasculitis are presented. Finally, we discuss future perspectives for therapeutic options targeting monocytes/macrophages in large vessel vasculitis.

2 Monocytes/macrophages homeostasis under steady-state and inflammatory conditions

2.1 Circulating monocyte subsets

Monocytes differentiated from progenitor cells in the bone marrow reach the circulation. Currently, human circulating monocytes are divided into three subsets based on the expression of superficial CD14 (a cell co-receptor for lipopolysaccharide [LPS]) and CD16 (the low-affinity IgG receptor); "classical" $CD14^{++}CD16^-$ monocytes (≥90%), "intermediate" $CD14^{++}CD16^+$ monocytes, and "non-classical" $CD14^+$ $CD16^{++}$ monocytes (10). These subsets are characterized by different levels of cell surface markers and chemokine receptors, but there appears to be a developmental relationship between these cells (from classical by intermediate to non-classical) (10). The classical monocytes are involved in a variety

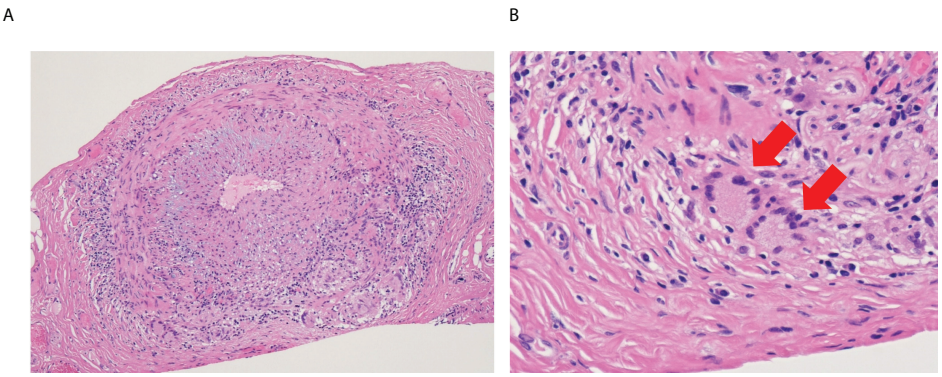

FIGURE 1
Microscopic image of giant cell arteritis. **(A)** Left temporal artery biopsy from 65-year-old woman with giant cell arteritis (Hematoxylin and eosin staining, x10). Lymphocytes and macrophages form granulomatous inflammation, and intimal hyperplasia causes narrowing of the blood lumen. **(B)** High power field image of the biopsy (Hematoxylin and eosin staining, x40). Red arrows show multinucleated giant cells.

of immune response such as inflammation and tissue repair. The intermediate monocytes are characterized by the highest TLR2, TLR4, and human leukocyte antigen-D related expression among monocyte subsets, and have the highest antigen presenting ability. They have superior reactive oxygen species production and have a role in angiogenesis. The non-classical monocytes are called "patrolling" monocytes and have high ability to stimulate $CD4^+$ T cells (11). The use of additional markers, such as C-C Chemokine Receptor 2 (CCR2) which is a key mediator of monocyte migration, for better delineation of monocyte subsets has been proposed (12), but its usefulness needs further study.

Conflicting data on cytokine production by the distinct monocyte subsets exist. We have previously reported that the intermediate monocytes treated with LPS produced the most IL-1β, IL-6, and TNFα (13). Wong et al. reported that non-classical monocytes produced the highest IL-1β and TNFα in response to LPS, but that equivalent amounts of IL-6 were secreted by the three subsets. (14). These inconsistencies are probably due to the different isolation methods used to purify the monocyte subsets (11).

An expansion of intermediate monocytes has been implicated in various inflammatory diseases and vascular diseases such as atherosclerosis (15), coronary artery disease (13), and antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (16). It has been suggested that the ability of intermediate monocytes to present antigens and produce proinflammatory cytokines may be involved in the pathogenesis of such diseases.

2.2 Tissue macrophage ontogeny

Tissue macrophages are derived from embryonic or adult hematopoietic stem cell (HSC) progenitor cells under homeostatic conditions (17). Representatives of tissue macrophages are alveolar macrophages (lung), Kupffer cells (liver), osteoclast (bone), microglia (central nervous system), and so on. They are remarkably heterogenous in terms of their surface markers, transcriptome, and epigenomes (18). Monocyte-derived cells also contribute to the macrophage population in the tissues but are mostly associated with a response to inflammatory conditions. It seems more likely that local environmental imprinting is the critical determinant for macrophage identity and function, irrespective of their origin (18).

Recently, the ontogeny of arterial macrophages has been revealed by an elegant method combining the fate-mapping analysis and single-cell RNA sequencing (19). Yolk sac erythro-myeloid progenitors (EMPs) migrate to the arterial adventitia and give rise to adventitial macrophages. Surprisingly, with aging, these adventitial macrophages decline in numbers, and are not replenished by bone marrow-derived monocytes. During vascular inflammation, bone marrow-derived monocytes are recruited to the vascular site and differentiate into adventitial macrophages, while EMP-derived macrophages show self-renewal and contribute to tissue regeneration (19). It has been reported that, during infection, monocytes are educated to be tissue-specific in the bone marrow by signals produced at the site of inflammation (20), but it remains unclear whether this is the case during vascular inflammation.

2.3 Macrophage activation and polarization

The most well-described paradigm of macrophage polarization is the M1/M2 polarization axis. M1 and M2 macrophages are also referred to as classically or alternatively activated macrophages, respectively (21). M1 macrophages are activated by the microbial products and proinflammatory cytokines (IFN-γ and/or LPS or TNFα) and characterized by an excess production of proinflammatory cytokines (IL-1β, IL-6, IL-12, IL-23), chemokines, nitric oxide, and reactive oxygen intermediates. In contrast, M2 macrophages are activated by IL-4, IL-10, IL-13, and express mannose receptor (CD206), scavenger receptor A (CD204), and chemokine receptors. High levels of IL-10 are produced by M2 macrophages (22). M2 macrophages are further classified into M2a (IL4/IL-13-induced), M2b (LPS/immune complexes-induced), M2c (IL-10/TGFβ/glucocorticoids-induced), and M2d (tumor-associated factors-induced) macrophages (23, 24).

However, macrophage activation is not that simple. It should be noted that M1 and M2 macrophages are not completely distinct subsets, but they are often overlapping; for example, in atherosclerotic plaque, macrophages expressing both M1 and M2 markers do exist (25). Thus, consensus on how to define macrophage activation *in vitro* and *in vivo* has not yet been fully established. In this context, a group of scientists proposed the updated nomenclature for macrophage activation and polarization (26). In this proposal, they described a set of standards encompassing three principles—the source of macrophages, definition of the activators, and markers to describe macrophage activation—with the goal of unifying experimental standards (26). Technological advances, such as single cell RNA sequencing, may reveal further new macrophage subsets in the future (27).

3 Pathogenic role of monocytes/macrophages in large vessel vasculitis

3.1 Giant cell arteritis (GCA)

3.1.1 Circulating monocyte population in GCA

An increased number of monocytes (monocytosis) is observed in the peripheral blood of active patients with GCA, and monocyte counts positively correlates with the C-reactive protein (CRP) levels (28). This observation is in line with the

report that monocyte-derived macrophages are dominant among tissue macrophages during vascular inflammation (19). Subpopulation analysis using flow cytometry demonstrated that monocytosis in the peripheral blood was attributable to classical monocytes and slightly intermediate monocytes (29). Interestingly, treatment with corticosteroids suppress the numbers of intermediate and non-classical monocytes, but the number of classical monocytes is unaffected (29). It has been reported that glucocorticoid-induced depletion of non-classical monocytes is mediated by caspase-dependent apoptosis (30).

Although monocytes can differentiate into DCs, it has been reported that the number of circulating DCs were comparable between GCA patients and healthy individuals (31). Most quiescent tissues contain resident DC population, but during inflammation, monocyte-derived DCs compensate resident population in the tissue (32). However, it remains elusive whether this is the case in GCA.

3.1.2 Proinflammatory cytokines

It is no doubt that research on monocytes/macrophages in GCA has dramatically progressed since the discovery of IL-6 (33). IL-6 acts on hepatocytes to produce acute phase proteins such as CRP and serum amyloid A (34). It was found that plasma IL-6 levels reflect the disease activity of GCA (35). Although 60–80% of circulating monocytes in patients with GCA can produce IL-6, the major source of IL-6 production was activated macrophages in the vascular lesion (36). Tissue macrophages are activated by IFN-γ released from $CD4^+$ T cells (4), and IL-6 shifts naïve $CD4^+$ T cell differentiation towards Th17 cells, while inhibiting regulatory T cell (Treg) differentiation (37). Other proinflammatory cytokines, including IL-1β and TNFα, were also localized to tissue macrophages and giant cells (38).

Treatment with corticosteroids diminish IL-1β and IL-6 production from tissue macrophages (39). In contrast, IL-6 receptor inhibitor tocilizumab (TCZ) increases plasma IL-6 levels in patients with GCA (40). TCZ may have little direct effect on suppressing macrophage activation in the vascular tissue and/or block clearance of released IL-6 through IL-6 receptor. However, TCZ reduces relapse and has a steroid-tapering effect on GCA (9) maybe because it restores not only the number of Tregs but also the function of these cells (41–43). Thus, TCZ is widely recommended in the treatment guidelines (44, 45).

IL-12, which is produced by M1 macrophages, is a heterodimeric proinflammatory cytokine that favours the differentiation of Th1 cells (46). Recently, it has been reported that IL-12 promotes conversion from Th17 cells into IFN-γ-producing Th1-like cells, called "non-classic Th1 cells" (47, 48). This transformation is governed by the transcription factor Eomes (49). Indeed, IL-12 is highly enriched in the biopsy-positive temporal arteries (50); therefore, IFN-γ found in the vascular tissues may be derived from Th1 or non-classic Th1 cells. However, ustekinumab, an IL-12 and IL-23 inhibitor, failed to show its efficacy in the treatment of GCA (51).

3.1.3 Proteolytic enzymes and proteinases

Monocytes/macrophages and giant cells not only produce proinflammatory cytokines, but also contribute to tissue destruction. They produce excess proteolytic enzymes and proteinases such as collagenases, cathepsins, and matrix metalloproteinase (MMP)-2 and MMP-9, disrupt external and internal elastic membranes and cause vessel wall destruction (52). Our recent work has revealed that MMP-9-producing macrophages/giant cells are mainly located at the intima-media border and monocyte-derived macrophages from patients with GCA outperformed producing MMP-2 and MMP-9 compared with those from healthy donors (53). Since MMP-2 cleaves the propeptide from the pro-MMP-9 to release enzymatically active MMP-9, this combination of MMPs allows vascular lesions as an active MMP-9-rich environment. We further demonstrated that, using an artificial basement membrane system composed of collagen I and collage IV, MMP-9 released from the circulating monocytes degrades basement membrane and enables $CD4^+$ T cell to invade into blood vessel. This study also showed that, using an experimental animal model of vasculitis, blocking MMP-9 was highly effective to protect vascular structure and homeostasis, suggesting that it may serve as a novel therapeutic option for large vessel vasculitis (53).

3.1.4 Colony-stimulating factors

A recent report showed that most of the MMP-9-producing macrophages were CD206 positive and induced by granulocyte macrophage-colony stimulating factor (GM-CSF) (54). Generally, GM-CSF is considered to induce M1 phenotype in macrophages (55, 56), but it may induce M1 plus M2 phenotypes in GCA macrophages. GM-CSF, which is produced by macrophages, T cells, myofibroblasts, and endothelial cells in GCA-affected arteries (57), is expected to be a promising therapeutic target in GCA in recent years. Indeed, treatment of *ex vivo* cultured GCA arteries with the anti-GM-CSF receptor antagonist mavrilimumab successfully ameliorated vascular inflammation through reducing T cell and macrophage infiltration and neoangiogenesis (57). Among T cells, mavrilimumab specifically reduced Th1 cells, but not Th17 cells. In addition, in a phase 2, randomised, double-blind, placebo-controlled trial, mavrilimumab showed superiority to placebo in the analyses of time to flare and sustained remission for patients with GCA (58). Therefore, GM-CSF is not only a macrophage differentiation factor, but is also fundamentally involved in vascular inflammation.

In contrast, macrophage-colony stimulating factor (M-CSF), which is generally considered to induce M2 phenotype in

macrophages (59, 60), is shown to skew macrophages into different phenotypes, namely folate receptor β (FRβ)-positive macrophages (54). M-CSF is mainly produced by CD206$^+$/MMP-9$^+$ macrophages at the intima-media borders. Collectively, it has been proposed that, at the initial stage of GCA, infiltrated monocytes from the vasa vasorum are primed by local GM-CSF and differentiate into CD206$^+$/MMP-9$^+$ macrophages. They migrate to media and media-intima junction and promote tissue destruction, while stimulating angiogenesis through IL-13Rα2 signaling (61). At the late stage of GCA, CD206$^+$/MMP-9$^+$ macrophages often fuse to form multinucleated giant cells and release M-CSF at the intima-media borders. Multiple cytokines (TNF, IL-6, IFN-γ, IL-4, etc) and TLR2 are thought to be involved in the formation of multinucleated giant cells, but the precise mechanism remains unclear (6). M-CSF-skewed FRβ$^+$ macrophages produce high concentrations of growth factors that activate myofibroblasts, leading to luminal occlusion (54, 62) (Figure 2). Anti-M-CSF antibodies have not been tested in patients with GCA so far.

3.1.5 Growth factors

Tissue macrophages produce growth factors such as transforming growth factor β1 (TGFβ1), platelet-derived growth factors (PDGF), and fibroblast growth factors (FGF) (63, 64). These growth factors are considered to induce an excessive fibroproliferative response leading to luminal occlusion. TGFβ1-expressing macrophages coproduce IL-1β and IL-6 and exhibit a strong preference for localization in the adventitia. Although not clearly proven, given the cytokine profile and localization of TGFβ1-producing macrophages, it is likely that they emerge at the initial disease stage and are activated by IFN-γ released from Th1 cells (62) (Figure 2). In contrast, FRβ$^+$ macrophages at the media-intima junction emerge at the late disease stage, and produce PDGF, which is closely associated with concentric intimal hyperplasia (54).

In addition, the number of newly formed blood vessel in the adventitia is associated with the production of vascular endothelial growth factor (VEGF), which is localized to tissue macrophages at the media-intima junction (65). VEGF production is augmented by IL-6 (66) and upregulates a NOTCH ligand, Jagged1, on the innermost microvascular endothelial cells. Jagged1 in turn stimulates NOTCH1 receptor on CD4$^+$ T cell, skewing CD4$^+$ T cell differentiation toward Th1 and Th17 (67). Therefore, anti-VEGF therapy may help to inhibit not only neoangiogenesis but also maldifferentiation of CD4$^+$ T cells (68).

3.1.6 Chemokines and chemokine receptors

Alteration in systemic and local chemokine production and chemokine receptor expression has been reported (Figure 3).

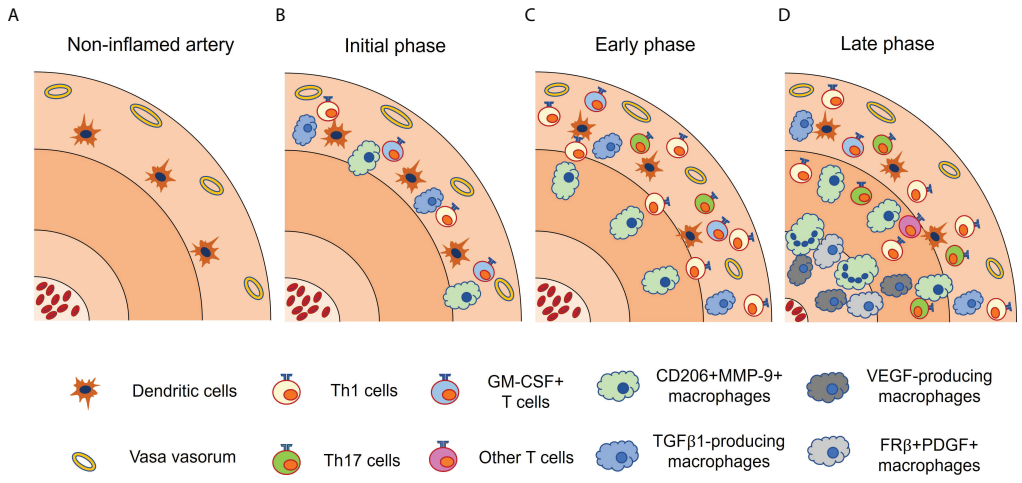

FIGURE 2
Functionally heterogenous macrophages in giant cell arteritis. Vascular lesion of giant cell arteritis contains a variety of macrophage subsets, each with a characteristic distribution. **(A)** In non-inflamed artery, vascular dendritic cells (vasDCs) reside in the media-adventitial border. **(B)** In the initial phase, vasDCs initiate inflammatory cascade, and recruits T cells and monocytes through chemokines. Infiltrated monocytes are differentiated into CD206+MMP-9+ macrophages by GM-CSF released from activated T cells. TGFβ1-producing macrophages are also present in the adventitia. **(C)** In the early phase, CD206+MMP-9+ macrophages migrate to the media and the media-intima border. Adventitial inflammation is increased. **(D)** In the late phase, CD206+MMP-9+ macrophages often fuse to form multinucleated giant cells and produce M-CSF, which gives rise to FRβ+ PDGF-producing macrophages at the media-intima border. Multiple cytokines and TLR2 are thought to be involved in the formation of multinucleated giant cells. VEGF-producing macrophages are preferentially located in the tunica media and intima. It should be noted that these macrophage subsets are not completely distinct. FRβ, folate receptor β; GM-CSF, granulocyte macrophage-colony stimulating factor; IFN, interferon; M-CSF, macrophage-colony stimulating factor; MMP, matrix metalloproteinase; PDGF, platelet-derived growth factor; TGFβ1, transforming growth factor β1; VEGF, vascular endothelial growth factor.

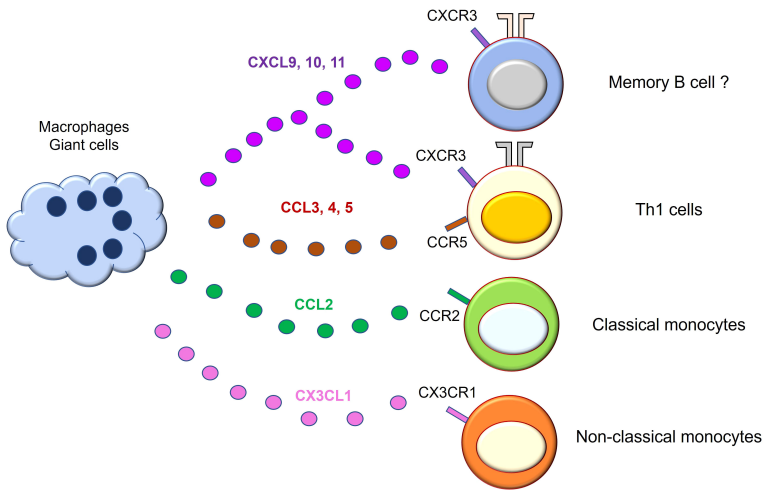

FIGURE 3
Macrophages/giant cells are professional chemokine producers in giant cell arteritis. Macrophages/giant cells in the vascular lesion of giant cell arteritis actively engage in chemokine production. The released chemokines amplify vascular inflammation by mobilizing cells that express the corresponding chemokine receptors. CXCL9, 10, and 11 recruit Th1 cells and memory B cells through CXCR3 receptor on the cell surface. CCL3, 4, and 5 recruit T cells expressing CCR5. CCL2 recruits CCR2-expressing classical monocytes. CX3CL1 mediates non-classical monocyte mobilization through CX3CR1 receptor. CCL, C-C motif Chemokine Ligand; CXCL, C-X-C motif Chemokine Ligand; CXCR, C-X-C Motif Chemokine Receptor.

Among them, C-X-C motif Chemokine Ligand 9 (CXCL9), CXCL10, and CXCL11 levels are elevated in the serum of GCA patients (69). These chemokines are produced by tissue macrophages in response to IFN-γ (70) and recruit Th1 cells through C-X-C Motif Chemokine Receptor 3 (CXCR3). C-C motif Chemokine Ligand 3 (CCL3), CCL4, and CCL5 are also overproduced from macrophages and recruit T cells through CCR5 (71). Not only T cells but also monocytes are recruited by chemokines. For example, classical monocytes are recruited into vascular tissue by the CCL2-CCR2 axis (72), while non-classical monocytes depend on the CX3CL1-CX3CR1 axis (29). These observations suggest that tissue macrophages attract T cells, particularly Th1 cells, and monocytes/macrophages through multiple chemokines, amplifying vascular inflammation. In addition, a recent report has demonstrated that CXCL9 attracts $CXCR3^+$ memory B cells, and CXCL13 recruits $CXCR5^+$ memory B cells into vascular tissue, respectively (73). Notably, not only tissue macrophages but also vascular DCs produce chemokines such as CCL19 and CCL21, rendering vascular tissue as a chemokine-rich microenvironment (74). Further study is needed to test whether blocking the chemokines and chemokine receptors could have a therapeutic potential.

3.1.7 Costimulatory and coinhibitory ligand expression

Not only costimulatory molecules like CD28, but also coinhibitory molecules, such as programmed death 1 (PD-1), are expressed on T cell surface, and the clinical significance of blocking the PD-1/programmed death ligand 1 (PD-L1) interaction has become clear in cancer immunotherapy. Surprisingly, vascular DCs residing at the media-adventitial boarder have defective PD-L1 expression, which is critically involved in the pathomechanisms of GCA (75). PD-L1-deficient DCs have an increased potential to activate T cells and polarize naïve $CD4^+$ T cell differentiation into Th1, Th17, and IL-21-producing T cells (76). Monocytes/macrophages derived from patients with GCA also had decreased expression of PD-L1 (70), although the significance of the deficient expression requires further elucidation. Taken together, PD-L1 immunoinhibitory mechanism to inhibit T cell hyperactivation is defective in myeloid lineage on vascular lesion in GCA. It is necessary to elucidate the mechanism of PD-L1 expression on vascular DCs and tissue macrophages. Also, testing the effect of PD-L1 signal-inducing agents, such as fusion proteins linking the extracellular domain of PD-L1 to the Fc portion of immunoglobulin (PD-L1 Fc), is warranted.

3.2 Takayasu arteritis (TAK)

Unlike GCA, it is difficult to perform biopsies of affected lesions in TAK, and only specimens that have undergone surgery are used for research. In addition, large amounts of steroids are often administered prior to surgery, making it rare to obtain an active untreated vascular sample. Thus, the pathogenesis of TAK

has not been fully elucidated. Although such bias is undeniable, M1 macrophages are dominant in aortic lesions of TAK (77, 78), which may be linked to excess IFN-γ produced by $CD4^+$ T cells, $CD8^+$ T cells, and natural killer cells (79). *In vitro* production of MMP-2 and MMP-9 in monocyte-derived macrophages is mildly increased compared with that from healthy donors (80). Steroid treatment transforms M1 macrophages into M2 macrophages and diminishes CCL2-expressing M1 macrophages (78). Thus, M2 macrophages dominate in treated aortic lesion and promote tissue remodeling with an excess fibrotic response.

Recently, single cell RNA sequencing was applied to examine the transcriptome of peripheral blood mononuclear cells from TAK patients (81). The study demonstrated that $CD14^+$ monocytes were increased, and gene expressions involved in oxidative stress were enriched. These monocytes may serve as a reservoir of tissue macrophages.

4 Discussion

We have reviewed the role of monocytes/macrophages in large vessel vasculitis, particularly in GCA. Of note, functionally distinct macrophage subsets have been increasingly identified in GCA (62), although there were no studies comparing monocytes/macrophages from cranial GCA and those from large vessel GCA. Also, it becomes clearer that circulating monocytes, rather than embryonic progenitor-derived macrophages, cause vascular inflammation by migrating and differentiating into the distinct subsets of macrophages, although it has not yet been fully investigated in human. In particular, since GCA only affects people over the age of 50, the number of tissue resident macrophages in the vasculature may be decreased. Furthermore, low grade inflammation caused by aging, which is called inflammaging, inevitably affects monocyte/macrophages, T cells, and vascular cells both in the circulation and in the vascular tissue (82, 83). Cellular senescence of immune cells is often linked to the senescence-associated secretory phenotype, which could be implicated in the pathomechanisms of GCA (62, 84).

Considering disease mechanisms mediated by monocytes/macrophages in GCA, inhibiting the migration of circulating monocytes or inhibiting their differentiation and function in the tissues may be therapeutic strategies targeting macrophages. The possible therapeutic options in GCA are summarized in Figure 4. Blockade of the chemokine and chemokine receptor interaction attracting circulating monocytes and T cells could be the preferential therapeutic option based on the pathomechanisms. However, it is speculated that by the time symptoms appear, a significant number of monocytes have already been recruited to the vascular tissues, and differentiated macrophages are refractory to the current therapies. Therefore, it is unclear to what extent chemokine blockade is effective. In fact, many attempts have been made to treat rheumatic diseases by blocking the chemokine-chemokine receptor interaction, but many of the results have been disappointing so far (85).

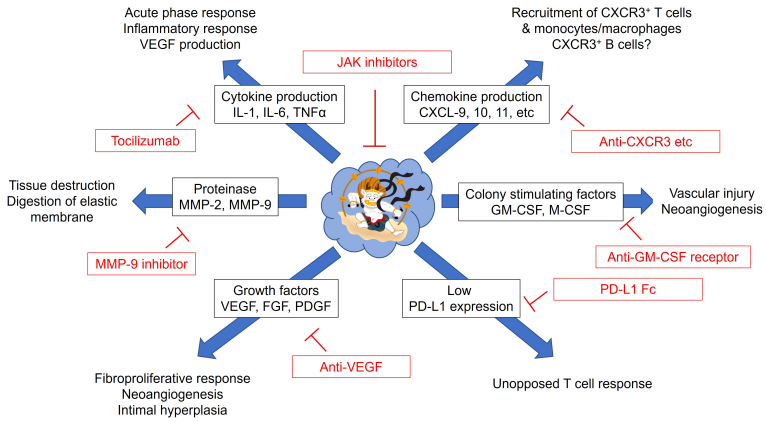

FIGURE 4
Possible therapeutic strategies for giant cell arteritis targeting monocytes and macrophages. Monocytes/macrophages from patients with giant cell arteritis have pleiotropic functions. Excess production of proinflammatory cytokines (IL-1β, IL-6, and TNFα), chemokines (CXCL9, 10, and 11), proteolytic enzymes (MMP-2 and MMP-9), colony stimulating factors (GM-CSF and M-CSF), growth factors (VEGF, FGF, PDGF) could be targeted by the corresponding inhibitors. Immune dysregulation by defective PD-L1 expression on monocytes/macrophages could be corrected by PD-L1 Fc. Janus kinase (JAK) inhibitors may directly suppress the function of monocytes/macrophages. CXCL, C-X-C motif Chemokine Ligand; CXCR, C-X-C Motif Chemokine Receptor; FGF, fibroblast growth factor; GM-CSF, granulocyte macrophage-colony stimulating factor; M-CSF, macrophage-colony stimulating factor; MMP, matrix metalloproteinase; PDGF, platelet-derived growth factor; PD-L1, programmed death ligand 1; VEGF, vascular endothelial growth factor.

Proinflammatory cytokines contribute profoundly to the exacerbation of vasculitis. The GiACTA trial have successfully demonstrated that blocking the IL-6 signal with TCZ suppresses flare of GCA and has steroid-sparing effect (9). However, as mentioned, recurrence after the discontinuation or even during TCZ therapy remains common. In addition, blocking TNFα with infliximab yielded disappointing results for GCA (86). Moreover, anakinra, an IL-1 receptor antagonist, has been shown the efficacy against GCA in case series (87), but its efficacy and safety have not fully been confirmed in the large-scale trials. Therefore, accumulating evidence shows that single cytokine inhibition may not be sufficient to completely diminish vascular inflammation.

As we have seen, treatment that suppresses a single therapeutic target, such as chemokines, proinflammatory cytokines, proteolytic enzymes, or growth factors, may not be sufficient for treating GCA. A combination of these or agents that inhibits the multiple cellular signaling, such as Janus kinase (JAK) inhibitors, may be effective (88). Multiple cytokines which are implicated in the pathomechanisms of GCA, such as IL-6, IFN-γ, IFN-α, GM-CSF, utilize the JAK-signal transducer and activator of transcription (STAT) pathway (89). Indeed, increased activities of the JAK-STAT pathway has been reported both in the vascular lesions and in circulating T cells (90, 91). In experimental animal model of large vessel vasculitis, JAK inhibitors not only reduced T cell infiltration and T cell-derived cytokine production, but also inhibited macrophage infiltration and growth factor production, resulting in reduced neoangiogenesis and intimal hyperplasia (90).

$CD4^+$ T cells from patients with TAK are also dependent on the JAK-STAT pathway (92). In addition, genome-wide association study has demonstrated that *IL-12B* is an susceptibility gene in TAK (93) and risk allele of *IL-12B* was associated with vascular damage in TAK (94). Since IL-12 utilizes the JAK-STAT pathway as a downstream signaling, JAK inhibitors could be promising agents for TAK as well (92, 95).

Finally, PD-L1 deficiency seems not specific to GCA monocytes. Monocytes derived from ANCA-associated vasculitis have the same defect (96). Lower PD-L1 expression leads to increased stimulatory capacity of monocytes, thus leading to overactivation of $CD4^+$ T cells. The defective PD-L1 expression was due to an enhanced lysosomal degradation of PD-L1 (96). As the efficacy of PD-L1 Fc has been shown in a mouse model of lupus (97), PD-L1 Fc may induce negative signals to overactivated T cells in vasculitis and ameliorate vascular inflammation. Alternatively, agents that inhibit PD-L1 degradation in lysosomes may have therapeutic potentials.

In conclusion, recent advances in the research have clarified the origin and various roles of monocytes/macrophages in vasculitis. Drugs that inhibit multiple therapeutic targets simultaneously, rather than a single target, or agents that block multiple cellular signaling may be effective; however, verification of the efficacy and the safety of such drugs is essential.

Author contributions

RW drafted the manuscript. MH revised and finalized the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that this review does not contain any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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EDITED BY
Naoki Iwamoto,
Nagasaki University Hospital, Japan

REVIEWED BY
Alexandre Wagner Silva De Souza,
Federal University of São Paulo, Brazil
Marc Hilhorst,
Academic Medical Center, Netherlands
Yafei Huang,
Huazhong University of Science and Technology, China

*CORRESPONDENCE
Xiaozhao Li
lixiaozhao@csu.edu.cn

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CX3CL1-induced CD16$^+$ monocytes extravasation in myeloperoxidase-ANCA-associated vasculitis correlates with renal damage

Jiale Tang[1,2], Zhonghua Liao[1], Liying Luo[1], Shuanglinzi Deng[2], Yuanyuan Jiang[2], Fangyuan Wang[1], Xinyue Hu[2], Hongling Yin[3], Guanghui Gong[3], Juntao Feng[2] and Xiaozhao Li[1]*

[1]Department of Nephrology, Xiangya Hospital, Central South University, Changsha, China, [2]Center of Respiratory Medicine, Xiangya Hospital, Central South University, Changsha, China, [3]Department of Pathology, Xiangya Hospital, Central South University, Changsha, China

Background: Monocytes are involved in the pathogenesis of ANCA-associated vasculitis (AAV). Monocyte/macrophages are the dominant infiltrating cells in the glomeruli of patients with myeloperoxidase-AAV (MPO-AAV). However, how human monocyte subsets extravasate to the kidney in MPO-AAV with renal damage is unclear.

Methods: 30 MPO-AAV patients with renal damage and 22 healthy controls were enrolled in this study. Monocyte subsets and monocyte-related chemokines in the blood and kidneys of MPO-AAV patients were detected. The chemoattractant activity of the CX3CL1-CX3CR1 axis on CD16$^+$ monocytes was observed. The effect of MPO-ANCA on the migration of CD16$^+$ monocytes to human glomerular endothelial cells (HGECs) was detected by flow cytometry and transwell migration assay.

Results: Compared with controls, CD16$^+$ monocytes were significantly decreased in the blood and increased in the glomeruli of MPO-AAV patients with renal damage. The level of CX3CL1, but not CCL2, was significantly increased in the plasma of MPO-AAV patients. CX3CL1 co-localized with glomerular endothelial cells in MPO-AAV patients with renal damage. Moreover, we initially found that MPO-ANCA promotes an increase of the chemokine CX3CL1 on HGECs, imposing recruitment on CD16$^+$ monocytes. Finally, the percentage of CD16$^+$ monocytes in the blood was found to be positively correlated with estimated glomerular filtration rate (eGFR) and negatively correlated with urinary protein creatinine ratio in MPO-AAV patients with renal damage. Furthermore, the urinary protein creatinine ratio was positively correlated with the infiltrating of CD14$^+$ and CD16$^+$ cells in the kidneys.

Conclusion: Enhanced extravasation of CD16$^+$ monocytes to the kidney *via* the CX3CL1-CX3CR1 axis may be involved in renal damage in MPO-AAV.

KEYWORDS

antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis, CD16+ monocyte, glomerular endothelial cells, CX3CL1, renal damage

Introduction

The anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is characterized by inflammation of blood vessels, endothelial injury and tissue damage (1). The AAV include microscopic polyangiitis (MPA), granulomatosis with polyangiitis (GPA) and eosinophilic granulomatosis with polyangiitis (EGPA). In addition, AAV is classified as myeloperoxidase (MPO)-ANCA vasculitis (MPO-AAV) and proteinase 3-ANCA vasculitis (PR3-AAV) based on autoantigen specificity (2).

Pathologically, AAV is characterized by inflammatory cells infiltrating the walls of small blood vessels, causing vascular damage and tissue necrosis. These inflammatory cells include neutrophils, T cells, macrophages, and monocytes (3). Although many studies have focused on neutrophils in the pathogenesis of AAV, monocytes also play an important role in AAV (4). Monocyte/macrophages are the dominant infiltrating cells in the glomeruli of patients with ANCA-associated glomerulonephritis (5). In a mouse model of anti-MPO antibody-induced necrotizing crescentic glomerulonephritis (NCGN), monocyte depletion significantly reduced glomerular necrosis and crescent formation (6). These studies suggest that monocytes are involved in the renal damage of AAV.

Human monocytes are divided into classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{+}CD16^{+}$), and non-classical ($CD14^{+}CD16^{++}$) subsets. Intermediate and non-classical monocytes are closely related in terms of gene expression profiles (7). Traditionally, human monocytes can be divided into $CD16^{-}$ monocytes ($CD14^{++}CD16^{-}$) and $CD16^{+}$ monocytes ($CD14^{+}CD16^{+}$ and $CD14^{+}CD16^{++}$) (8). In a mouse model of crescentic glomerulonephritis, non-classical monocytes were observed to migrate to the glomeruli and interact with neutrophils to promote acute glomerular injury (9). However, which monocyte subsets migrate to the kidney involved in renal damage in MPO-AAV is unclear.

Monocytes migration from blood to tissue requires monocyte-endothelial interactions involving rolling, adhesion and extravasation. The expression of CD11b was increased in monocytes in AAV patients, and serum soluble markers of adhesion molecules were increased (10). These results suggest enhanced rolling and adhesion of monocytes to endothelial cells in AAV. However, the process of monocyte extravasation has not yet been studied.

Extravasation of monocytes into target organs is mediated by soluble chemokines, mainly CCL2 and CX3CL1 (8). CX3CL1 was mainly expressed in glomerular endothelial cells, which was associated with $CD68^{+}$ macrophages but not with $CD3^{+}$ T cell infiltration in AAV patients (11). Some $CD68^{+}$ macrophages can be differentiated from peripheral blood monocytes (12). CX3CR1 is the only known corresponding CX3CL1 receptor (13). It suggests that the CX3CL1-CX3CR1 axis may be involved in monocytes extravasation to the kidney in MPO-AAV.

It is well known that ANCA plays an important role in the pathogenesis of AAV (14). Similar to neutrophils, monocytes also express MPO and PR3. Activated monocytes respond to ANCA by producing pro-inflammatory cytokines, chemokines, and reactive oxygen species (ROS) (15). *In vitro*, anti-MPO IgG induced CXCL-8 and CXCL-2 secretion by glomerular endothelial cells, leading to neutrophil chemotaxis (16). Whether MPO-ANCA can also affect the extravasation of monocyte subsets is unknown.

Here, we analyzed monocyte subset abundances and phenotypes in the blood and kidneys of MPO-AAV patients with renal damage, and explored the enhanced effect of the CX3CL1-CX3CR1 axis on glomerular endothelial cells recruiting $CD16^{+}$ monocytes from circulation to the glomeruli in MPO-AAV.

Materials and methods

Subjects

Complete data for the 143 AAV patients (69 females, 74 males, average age: 60 ± 14 years) and 176 healthy controls (HC) (90 females, 80 males, average age: 53 ± 11 years) were collected at Xiangya Hospital from December 2012 to June 2020 (the clinical characteristics of AAV and HC are summarized in Supplement Table S1). For follow-up *in vitro* experiments, peripheral blood samples were obtained from 30 patients positive for MPO-ANCA, diagnosed with MPA according to ACR/EULAR 2017 Provisional Classification Criteria, and 22 HC between March 2019 and June 2020. All patients had renal damage in the presence of hematuria, proteinuria, and/or an elevation of serum creatinine. Eight of them underwent renal

biopsy and renal tissues were collected. Clinical characteristics are shown in Table 1.

All MPO-AAV patients were not treated with glucocorticoids, immunosuppressants, or plasma exchange before samples collection. Patients were excluded if they had other immune-related diseases, malignancies, or infections. HC were obtained from the Physical Examination Center of Xiangya Hospital. They had no history of autoimmune disease, cancer, or any other inflammatory syndrome. Disease activity was scored according to the Birmingham vasculitis activity score (17, 18). The study was approved by the Ethics Committee of Xiangya Hospital, Central South University (2019030598).

Flow cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated according to density gradient (Lymphoprep™ STEMCELL). Peripheral monocyte subsets were detected by flow cytometry.

TABLE 1 Clinical and histopathological data of partial patients with blood drawing.

Characteristic	MPO-AAV Patients	HC	*p value*
N	30	22	
Age (years, mean ± SD)	63 ± 13	59 ± 15	*0.2413*
Gender (female/male)	14/16	12/10	
Monocytes (10^9/L)	0.57 ± 0.18	0.39 ± 0.10	*<0.0001*
$CD16^-$ monocytes (%)	81.81 ± 7.65	78.74 ± 7.66	*0.1671*
$CD14^{++}CD16^-$ monocytes (%)	81.81 ± 7.65	78.74 ± 7.66	*0.1671*
$CD16^+$ monocytes (%)	8.89 ± 4.81	13.61 ± 6.80	*0.0060*
$CD14^+CD16^+$ monocytes (%)	5.60 ± 2.76	5.10 ± 3.79	*0.5903*
$CD14^+CD16^{++}$ monocytes (%)	3.28 ± 2.91	8.58 ± 5.71	*<0.0001*
BVAS [M ± Q]	18 [4]		
MPO-ANCA titer (U/mL) [M ± Q]	52.19 [60.62]		
CRP (mg/L)	15.70 [33.14]		
ESR (mm/h)	94.00 [58.00]		
Scr (μmol/L)	279.00 [416.15]		
eGFR (ml/min/1.73m^2)	18.96 [19.85]		
Proteinuria (g/24h)	1.80 [3.95]		
Hematuria (n, %)	26 (87%)		
Urinary red blood cell (n/μL)	53.00 [181.25]		
Urinary protein/creatinine ratio (g/g)	2.83 [3.98]		
Extrarenal manifestations n (%)			
ENT	2 (7%)		
Pulmonary	26 (87%)		
Gastrointestinal	5 (17%)		
Nervous system	1 (3%)		

Data are expressed as the number, mean ± SD or M ± Q. M ± Q, median ± p75-p25; ANCA, myeloperoxidase- antineutrophil cytoplasmic antibody; MPO-AAV, MPO-ANCA vasculitis; HC, healthy control; BVAS, Birmingham Vasculitis Activity Score; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; Scr, serum creatinine; eGFR, estimated glomerular filtration rate; ENT, ear-nose-throat.

Surface staining was performed using a BD FACS Canto II flow cytometer. Data were analyzed with FlowJo software (version 10.0), and the gate strategy is described in Figure 1B. The following antibodies were used: Human TruStain FcX™, Zombie Aqua™ Fixable Viability Kit, anti-human CD45, CD16, CD14, CCR2, CX3CR1, CD40, CD80, and CD86 (all purchased from BD Biosciences or BioLegend). Negative thresholds for gating were set according to isotype-labeled controls.

Immunocytochemistry

To fully characterize the infiltration of monocytes in the kidney, we performed immunohistochemical staining of paraffin-embedded kidney tissue specimens from MPO-AAV patients, patients with minimal change disease (MCD), and normal control. Normal control tissue was obtained from renal space-occupying lesions, and there was no evidence of tumor infiltration in pathology. Slides were deparaffinized by incubation at 60°C for 2-4 hours, followed by rehydration, where slides were immersed twice in xylol (15 minutes, each), transferred to 100% ethanol (5 minutes, each), once in 90% ethanol (5 minutes), 80% ethanol (5 minutes), 70% ethanol (5 minutes), and finally flushed three times with phosphate buffered saline (PBS) to wash away the ethanol. Next, antigen retrieval was performed with citrate (pH 6.0); nonspecific binding was blocked by incubation in 3% bovine serum albumin for 60 min at room temperature, and then the slides were incubated with mouse anti-human CD14 antibodies (ab182032, Abcam) or rabbit anti-human CD16 antibodies (ab203883, Abcam) for 18 h at 4°C. Antibody labeling was detected using an SP-HRP goat IgG kit (PV-6000, ZSGB-Bio, China) according to the manufacturer's instructions. The chromogenic reaction solution contained 3,3'-diaminobenzidine (DAB) (ZLI-9018, ZSGB-Bio, China), and counterstaining was performed with Mayer's hematoxylin (Solarbio, Beijing, China). The slides were viewed under fluorescent microscopy (Olympus BX51; Olympus, Tokyo, Japan).

The semi-quantitative of $CD14^+$ and $CD16^+$ cell infiltrates in the kidneys of controls and MPO-AAV patients was measured through the Image J program, and Mean density was used for statistical analysis.

Immunofluorescence

Immunofluorescence staining was performed in kidney tissue embedded in paraffin according to standard pathology protocols. The primary antibodies used were mouse anti-human antibodies CD14 (ab182032, Abcam), CD31 (ab9498, Abcam), CCL2 (MABN712, Millipore), and rabbit anti-human antibodies

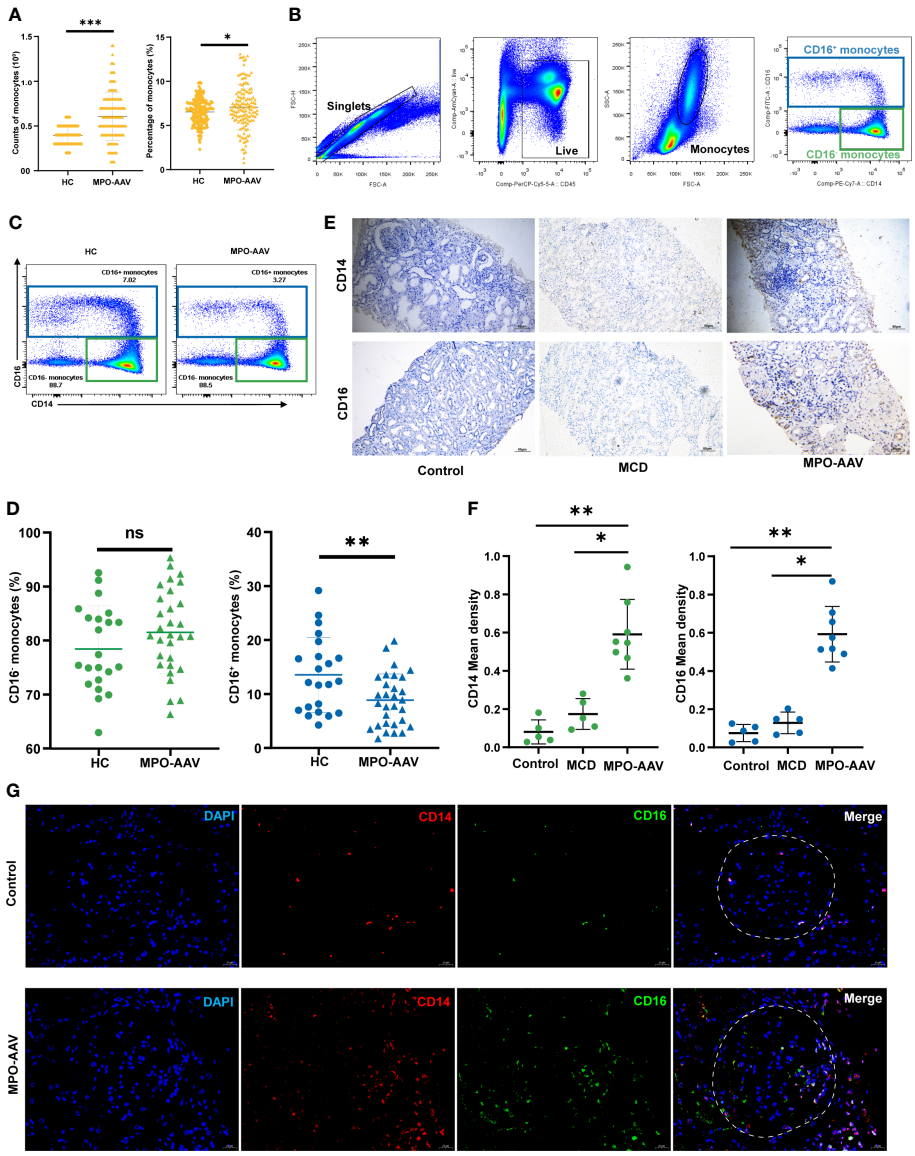

FIGURE 1
CD16$^+$ monocytes abundance in the peripheral blood and kidneys of MPO-AAV patients with renal damage. **(A)** Significant differences in the monocytes in the blood of AAV patients and healthy controls (HC) were observed. **(B)** The abundance of monocyte subsets in PBMC from MPO-AAV patients (n=30) and HC (n=22) was analyzed by flow cytometry. Monocytes were identified within the FSC-A^{hi} and SSC-A^{hi} cell populations and the gate strategy was based on CD14 and CD16 expression; **(C)** Representative flow cytometry dot plots show the subsets of monocytes in MPO-AAV patients and HC; **(D)** The percentage of CD16$^+$ and CD16$^-$ monocytes in the peripheral blood of MPO-AAV patients and HC was shown. **(E, F)** Representative kidney sections showing CD14$^+$ and CD16$^+$ cells in MPO-AAV patients, patients with MCD, and control; **(F)** Quantification of CD14$^+$ and CD16$^+$ cell infiltrates in the kidneys of MPO-AAV patients (n=8), patients with MCD (n=5), and control (n=5). **(G)** CD14$^+$CD16$^+$ cells (orange) abundance was significantly increased in the glomeruli (white dotted line) of MPO-AAV patients than in control. ns, not significant. *p<0.05; **p<0.01; ***p<0.001.

CD16 (ab203883, Abcam), CD31 (ab32457, Abcam), CX3CL1 (ab85034, Abcam). The slides were placed in a wet chamber followed by the addition of the appropriate primary antibodies at the concentration recommended by the manufacturer (double staining) and incubated overnight at 4°C. The slides were rinsed three times with PBS, and a 1:200 dilution of Alexa Fluor®594 goat anti-mouse IgG antibody (ab150116, Abcam) and Alexa Fluor®488 goat anti-rabbit IgG antibody were applied and incubated at 37°C (30 minutes) in the dark. DAPI mounting medium (ab104139, Abcam) was used for nuclear staining. Finally, the slides were viewed under fluorescent microscopy (Olympus BX51; Olympus, Tokyo, Japan).

Monocyte isolation

PBMCs from HC were extracted, and monocytes were isolated using magnetic CD14 microbeads (Miltenyi Biotech), according to the manufacturer's instructions. $CD16^+$ monocytes were isolated using CD16 microbeads (Miltenyi Biotech) after washing. The remaining cells were $CD16^-$ monocytes.

Purification of IgG

Blood or plasma exchange fluid was obtained from HC or MPO-AAV patients, and plasma was isolated and stored at –80°C. Control IgG and MPO-ANCA positive IgG were purified using protein G affinity chromatography. The preparation of IgG was performed according to previously described methods (19).

Cells stimulation

Isolated monocytes were resuspended at 1×10^6 cells/mL in RPMI 1640 medium (Gibco, Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal bovine serum (FBS, Gibco). Monocytes were cultured in 6-well plates with or without 100 ng/mL LPS (Peprotech), MPO-ANCA, or control IgG at a final concentration of 250 μg/mL. The cells were incubated at 37°C for 24 h, after which cells were collected and stained with an antibody mix containing Human TruStain FcX™, Zombie Aqua™ Fixable Viability Kit, anti-human CD45, CD16, CD14, CCR2, and CX3CR1 (BioLegend), followed by flow cytometry. Data acquisition was performed using a BD FACS Canto II flow cytometer. Data were analyzed using FlowJo software (version 10.0).

Human glomerular endothelial cells (HGECs, ScienCell, San Diego, CA, USA) were cultured in endothelial cell medium (ECM, ScienCell) supplemented with 10% FBS, 1% penicillin/streptomycin, and 1% endothelial cell growth factor. HGECs were cultured in 12-well plates with or without 10ng/mL TNF-α (Peprotech), MPO-ANCA, or control IgG at a final concentration of 500 μg/mL. The cells were incubated at 37°C for 24h, after which total RNA of HGECs was extracted for polymerase chain reaction (PCR) amplification, and supernatants were collected and stored at -80°C for subsequent ELISA detection.

Chemokine quantification

CCL2 and CX3CL1, measured by ELISA kits (eBioscience) according to the manufacturer's protocols, were used to quantify monocyte chemokine levels in plasma and HGECs culture supernatant.

Monocyte migration assay

Isolated monocytes (1×10^6) were seeded into a transwell (Corning Costar Transwell® 24 wells permeable 8 μM pore, Corning, NY, USA) to verify whether migration of monocyte subsets responded to different stimuli. Four different conditions were added to the lower chamber at 600 μl RPMI 1640 medium containing 10% FBS: chemokine free, 200 ng/mL CX3CL1 (C461, novoprotein), 200 ng/mL CCL2 (CM78, novoprotein) and 200ng/mL CX3CL1+2μg/mL anti-CX3CL1 monoclonal antibody (mAb) (AF365, R&D). Monocytes were incubated for 4 h at 37°C in a humidified atmosphere containing 5% CO2. Migratory cells were harvested and stained with an antibody mix containing Human TruStain FcX™, Zombie Aqua™ Fixable Viability Kit, anti-human CD45, CD16, and CD14, followed by flow cytometry. Data acquisition was performed using a BD FACS Canto II flow cytometer. Data were analyzed using FlowJo software (version 10.0). The migration index was calculated by dividing the number of monocytes that migrated in the chemokine-free group.

To further verify the migration effect of HGECs on $CD16^+$ monocytes, $CD16^+$ monocytes (6×10^5) were added to the top chamber, supernatants from HGECs cultures with or without 1μg/mL anti-CX3CL1 mAb were placed in the bottom chamber at a volume of 600 μl, and the chambers were incubated at 37°C in a 5% CO2 atmosphere for 12 h. After incubation, the non-migratory cells in the upper chamber were scraped off, and the membrane was washed gently with PBS. The migratory cells on the bottom surface of the transwell membrane were fixed in 4% paraformaldehyde for 10 min, stained with Wright, and then viewed and photographed under a digital microscope (Olympus BX51; Olympus, Tokyo, Japan). The chemotaxis index was calculated by dividing the number of monocytes that migrated in response to the ECM.

PCR

Total RNA was isolated using TRIzol reagent (Life Technologies, Ober-Olm, Germany) according to the manufacturer's instructions. After quantification of the RNA concentration with Nanodrop (Thermo Scientific, Darmstadt, Germany), RNA samples were reverse transcribed at equal concentrations using a Takara First Strand cDNA Synthesis kit (Ambion, Foster City, CA) and then subjected to real-time qPCR analysis using Power SYBR Green (Applied Biosystems® QuantStudio™ 7 Flex Real-time Fluorescent quantitative PCR system, Darmstadt, Germany). The comparative Ct method was used ($2^{-\Delta\Delta Ct}$) for quantification. The primer sequences are listed in Supplementary Table S2.

Statistical analysis

GraphPad Prism 8.0 software was used for statistical analysis. Data were expressed as the mean ± standard

deviation. We performed the independent-sample t test or Mann-Whitney-U test to compare data between the two groups, the one-way analysis of variance (ANOVA) followed by Tukey multiple comparison test for multi-groups, and Spearman's rank test for correlations. Experiments were repeated at least three times to ensure reproducibility, and differences were considered significant if p was less than 0.05.

Results

$CD16^+$ monocytes were significantly decreased in the blood of MPO-AAV patients with renal damage

We analyzed the number and percentage of monocytes in the peripheral blood of AAV patients and HC. As shown, significant increases in the number and percentage of monocytes were observed in AAV patients compared to those in HC (Figure 1A). To further explore the altered total blood monocyte populations, we analyzed the circulating monocyte subsets of 30 MPO-AAV patients with renal damage by flow cytometry (Figure 1B). We found that the percentage of $CD16^+$ monocytes in MPO-AAV patients was significantly decreased while the percentage of $CD16^-$ monocytes ($CD14^{++}CD16^-$) was not significantly different compared to HC (Figures 1C, D). MPO-AAV patients exhibited a significantly decreased percentage of non-classical monocytes ($CD14^+CD16^{++}$) and no significant difference in the proportions of intermediate monocytes ($CD14^+CD16^+$) compared to HC (Table 1).

Increased abundance of $CD16^+$ monocytes in the kidneys of MPO-AAV patients with renal damage

To fully characterize the infiltration of monocyte subsets in the kidney, we performed immunohistochemistry and immunofluorescence staining of renal biopsy specimens from 8 MPO-AAV patients, 5 patients with MCD, and 5 normal control, respectively. Compared with MCD and normal control, the number of $CD14^+$ and $CD16^+$ cells in the kidneys of MPO-AAV patients was significantly increased (Figures 1E, F). The number of $CD14^+$ $CD16^+$ myeloid cells was significantly increased in the glomeruli and periglomerular of MPO-AAV patients compared to normal control (Figure 1G).

The immunophenotype of monocyte subsets in MPO-AAV patients with renal damage

In order to investigate the reasons for changes in the distribution of monocyte subsets in MPO-AAV patients with renal damage, we explored the phenotypic differences of monocyte subsets in the peripheral blood.

Consistent with most studies, out results demonstrated that CCR2 was mainly expressed in $CD16^-$ monocytes, and CX3CR1 was mainly expressed in $CD16^+$ monocytes (Figure 2A). Moreover, we observed decreased expression of CX3CR1 in $CD16^+$ monocytes in MPO-AAV patients compared with HC. There was no significant difference in CCR2 expression in monocyte subsets from MPO-AAV patients compared with HC (Figure 2B and Supplementary Figure S1). Compared with $CD16^-$ monocytes, $CD16^+$ monocytes demonstrated higher expression of CD80 in MPO-AAV patients, and had an increased trend in the expression of CD86 and CD40. Overall, no differences were detected between the HC and MPO-AAV patients (Supplementary Figure S2).

CX3CL1 was increased in the plasma of MPO-AAV patients with renal damage and enhanced $CD16^+$ monocytes migration

We further detected the levels of CCL2 and CX3CL1 in the plasma of MPO-AAV patients with renal damage and HC. Compared with HC, CCL2 concentrations were not significantly different (Figure 2C), while CX3CL1 levels were significantly increased in the plasma of MPO-AAV patients (Figure 2D). Next, we evaluated the chemotaxis of CCL2 and CX3CL1 to monocyte subsets *in vitro* and receptor responsiveness to the ligand. We observed that CCL2 had no influence on $CD16^+$ monocyte migration (Figure 2E). CX3CL1 did not influence $CD16^-$ monocytes migration but increased $CD16^+$ monocyte migration, and this effect was reversed by blocking CX3CL1 with a mAb (Figure 2F).

MPO-ANCA can promote the recruitment of $CD16^+$ monocytes by the CX3CL1-CX3CR1 axis in glomerular endothelial cells

To examine the expression of monocyte-related chemokines in the kidneys of MPO-AAV patients, we performed immunofluorescence staining for CX3CL1 and CCL2. We observed that CX3CL1 was significantly expressed in glomerular endothelial cells, while CCL2 was expressed in small amounts (Figures 2G, H).

We further showed that the addition of MPO-ANCA led to a significant increase in CX3CL1 levels but no significant difference in CCL2 levels, in TNF-α-stimulated HGECs (Figures 3A, B). To explore the effect of MPO-ANCA on the migration of $CD16^+$ monocytes to HGECs, the culture

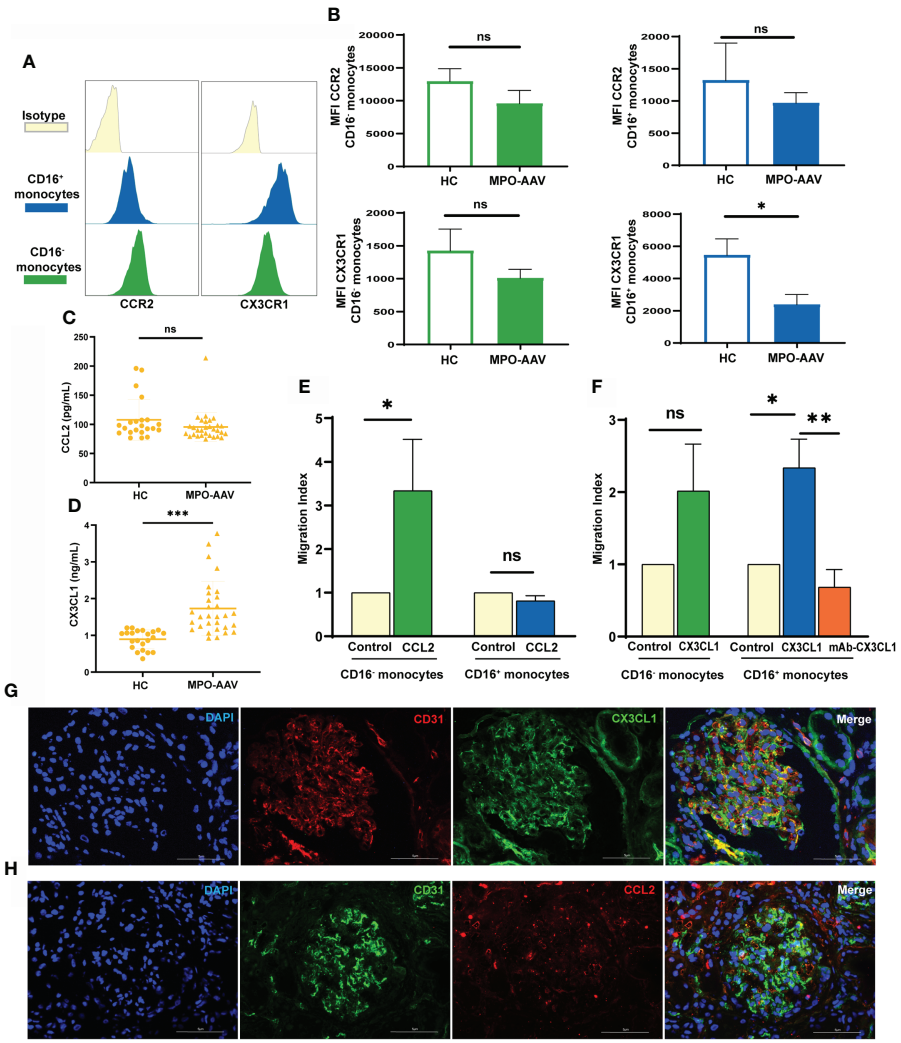

FIGURE 2
Altered CX3CL1-CX3CR1 axis and increased CD16$^+$ monocytes migration in MPO-AAV patients with renal damage. **(A, B)** Histogram with plot diagrams of flow cytometry analysis show the mean fluorescence intensity (MFI) of CCR2 and CX3CR1 in monocyte subsets from MPO-AAV patients and HC. **(C)** The concentrations of CCL2 and **(D)** CX3CL1 were measured in the plasma of MPO-AAV patients with renal damage and HC. **(E)** *In vitro* migration assay of isolated monocytes from HC (n=6) with CCL2 and **(F)** CX3CL1, control wells were used as an indicator of conversion efficiency to calculate migration index. ns, not significant. *p<0.05; **p<0.01; ***p< 0.001. **(G)** Double immunofluorescence staining was performed to show glomerular endothelial cells (CD31, red) expressing CX3CL1 (green) and **(H)** glomerular endothelial cells (CD31, green) expressing CCL2 (red) in MPO-AAV patients. Nuclei were stained with DAPI (blue).

supernatant of the above groups of HGECs was used in a chemotaxis assay. The culture supernatant of unstimulated HGECs had a strong chemoattraction of circulating CD16$^+$ monocytes, whereas anti-CX3CL1 mAb significantly inhibited monocyte chemotaxis. MPO-ANCA stimulation of HGECs increased CD16$^+$ monocyte migration, and CX3CL1 neutralization almost completely prevented the migration (Figures 3C, D). No such effect was observed in the Con-IgG stimulation group (Figure 3D).

CD16$^+$ monocytes show more pronounced CX3CR1 upregulation in response to MPO-ANCA *in vitro*

MPO in activated monocytes is transferred from the intracellular space to the surface and binds directly to ANCA. Since we observed differences in the expression of CCR2 and CX3CR1 in monocyte subsets between MPO-AAV patients and HC, we further explored the consequences of *in vitro* isolated

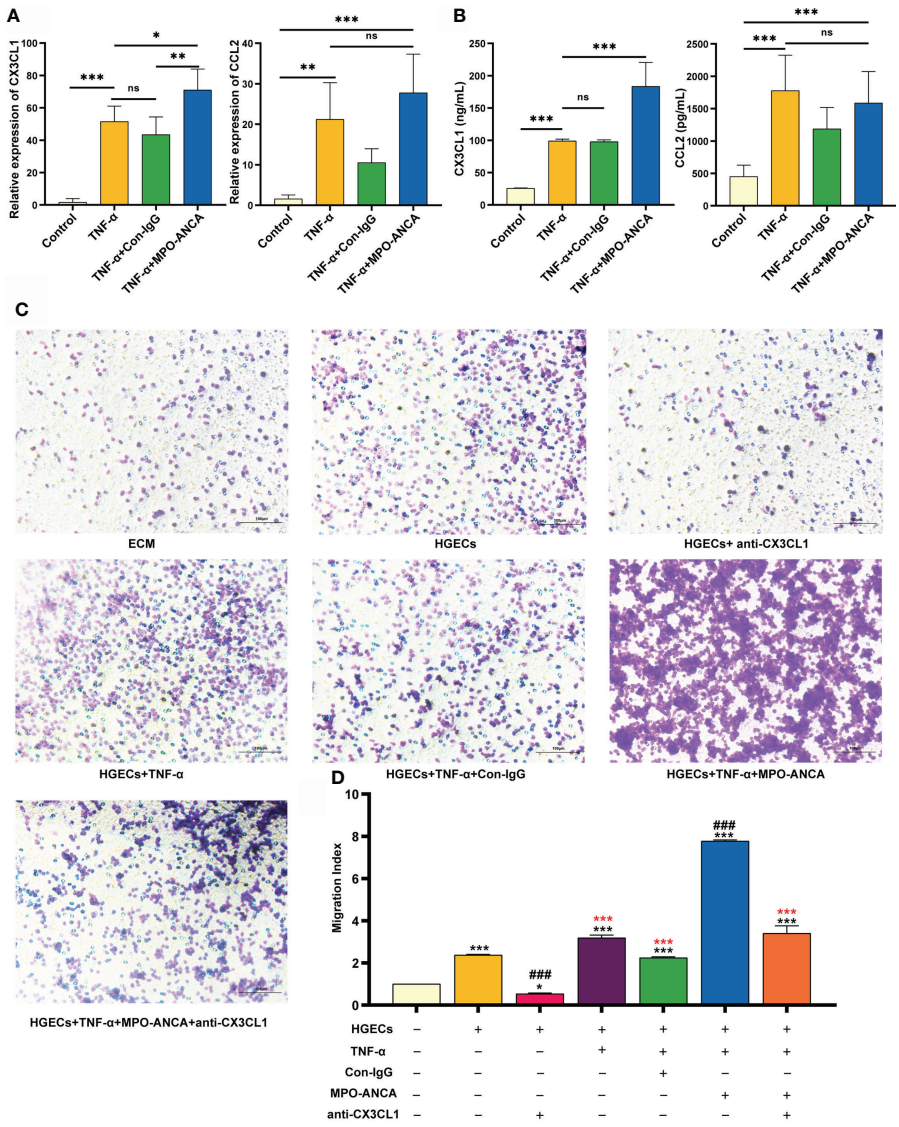

FIGURE 3
MPO-ANCA enhanced the recruitment of CD16$^+$ monocytes by the CX3CL1-CX3CR1 axis in glomerular endothelial cells. **(A)** The production of CX3CL1 and CCL2 by HGECs stimulated with TNF-α, MPO-ANCA, or Con-IgG was measured by RT-qPCR and **(B)** ELISA. ns, not significant. *p<0.05; **p<0.01; ***p<0.001. **(C)** *In vitro* migration assay of CD16$^+$ monocytes with the culture supernatants of HGECs, migrated CD16$^+$ monocytes were stained by Wright staining. **(D)** ECM wells were used as an indicator of conversion efficiency to obtain migration index; *vs. ECM group, *p<0.05, ***p<0.001; $^{\#}$vs. HGECs group, $^{\#\#\#}p$<0.001; *(red) vs. TNF-α+MPO-ANCA cultured HGECs group, ***(red)p<0.001. ECM, endothelial cell medium; HGECs, Human glomerular endothelial cells.

CD16$^+$ and CD16$^-$ monocytes exposed to LPS and MPO-ANCA. We observed that CCR2 and CX3CR1 in CD16$^-$ monocytes did not change significantly after stimulation (Figure 4A). However, the expression of chemokine receptors in CD16$^+$ monocytes is more prone to change under stimuli. Both CCR2 and CX3CR1 in CD16$^+$ monocytes were increased after LPS stimulation, and the increase in CCR2 and CX3CR1 was more significant after the combined addition of MPO-ANCA, while Con-IgG did not show this effect (Figure 4B and Supplementary Figure S3).

Extravasation of CD16$^+$ monocytes was associated with renal damage in MPO-AAV patients

Finally, we correlated the percentages of circulating monocytes with renal damage and disease activity. The low percentage of circulating CD16$^+$ monocytes was correlated with low estimated glomerular filtration rate (eGFR) and high urinary protein creatinine ratio in MPO-AAV patients,

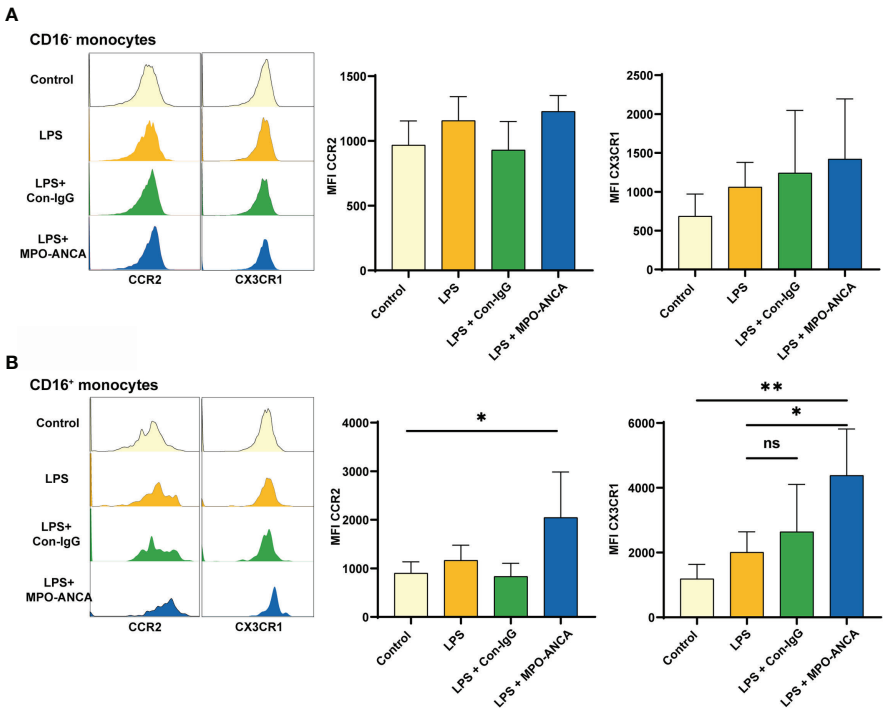

FIGURE 4
CD16$^+$ monocytes show more pronounced CX3CR1 upregulation in response to MPO-ANCA *in vitro*. Isolated CD16$^+$ and CD16$^-$ monocytes were treated with LPS or combined with MPO-ANCA and Con-IgG for 24h, respectively. Experiments were performed with monocytes from 5 healthy donors; **(A)** the MFI of CCR2 and CX3CR1 in CD16$^-$ monocytes after stimulation; **(B)** the MFI of CCR2 and CX3CR1 in CD16$^+$ monocytes after stimulation. *p<0.05; **p<0.01. ns, not significant.

but not with the percentage of CD16$^-$ monocytes (Figure 5A). The percentage of monocyte subsets did not correlate with BVAS, ANCA titers, C-reactive protein (CRP), or erythrocyte sedimentation rate (ESR) (Supplementary Figure S4). To further reveal the possible role of CD16$^+$ monocyte extravasation in renal damage, we analyzed the correlation between the extent of CD14$^+$ and CD16$^+$ cells infiltration in the kidneys and renal damage in MPO-AAV patients. The infiltration of CD14$^+$ and CD16$^+$ cells in the kidneys was positively correlated with urinary protein creatinine ratio in MPO-AAV patients with renal damage (Figure 5B). These data indicate that extravasation of CD16$^+$ monocytes may exacerbate renal damage in MPO-AAV patients.

Discussion

Even though it has been speculated that the CX3CL1-CX3CR1 axis may be involved in the evolution and progression of systemic vasculitis (20), the pathological role of the CX3CL1-CX3CR1 axis is poorly understood in AAV. Here, we demonstrate the functional implications of the CX3CL1-CX3CR1 axis on the abundance and recruitment of CD16$^+$ monocytes in MPO-AAV with renal damage. We find an increased level of CX3CL1 in the plasma of MPO-AAV patients compared to HC. Accordingly, we observe a decrease in CD16$^+$ monocytes in the peripheral blood and an increase in CD14$^+$CD16$^+$ cells infiltration in the kidneys of MPO-AAV patients compared with controls. Moreover, we document that MPO-ANCA promotes glomerular endothelial cell recruitment of CD16$^+$ monocytes by enhancing the effect of the CX3CL1-CX3CR1 axis.

We found that compared with HC, the number and percentage of monocytes in peripheral blood of MPO-AAV patients with renal damage were increased, while the percentage of CD16$^+$ monocytes was decreased. This discrepancy may be explained by the rapid extravasation of CD16$^+$ monocytes into tissues during inflammation, resulting in circulating CD16$^+$ monocytes consumption (13), and the release of classical monocytes from bone marrow to replenish circulating monocytes (21). In AAV patients, the percentage of monocyte subsets in the peripheral blood may vary in different clinical contexts (4). Previous studies on the percentage of circulating monocyte subsets were inconsistent with ours (22, 23),

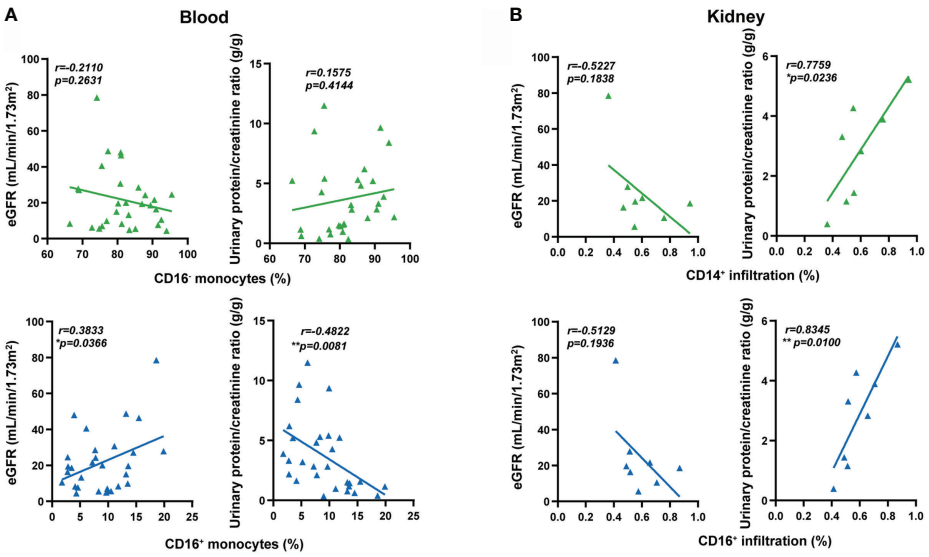

FIGURE 5
Extravasation of CD16$^+$ monocytes was associated with renal damage in MPO-AAV patients. **(A)** The percentage of monocyte subsets (CD16$^+$ and CD16$^-$ monocytes) correlated with estimated glomerular filtration rate (eGFR, mL/min/1.73m^2) and urinary protein/creatinine ratio (g/g) in MPO-AAV patients (n = 30); **(B)** The extent of CD14$^+$ and CD16$^+$ cell infiltrates in the kidneys of MPO-AAV patients (n = 8) correlated with eGFR and urinary protein/creatinine ratio, respectively. *p<0.05; **p<0.01.

which may be related to the cause that all MPO-AAV patients collected in this study had renal damage and were not receiving corticosteroids or immunosuppressants. It has been reported that the percentage of CD14^{++}CD16$^+$ intermediate monocytes is enriched by glucocorticoid treatment (24, 25).

Compared with HC, the expression of CX3CR1 was increased in PBMCs, CD4$^+$ T cells, and CD8$^+$T cells, but there was no significant difference in the expression of CX3CR1 on monocytes in GPA patients (26). Similar results were also found in MPA patients (27). However, the above study did not analyze CX3CR1 expression in monocyte subsets. We observed that the expression of CX3CR1 on CD16$^+$ monocytes was lower in MPO-AAV patients than in HC. In contrast, we found that MPO-ANCA can promote the surface expression of CX3CR1 on CD16$^+$ monocytes *in vitro*. The expression of some chemokine receptors was decreased on the cell surface when they participate in chemotaxis (28). The CX3CR1 MFI was reduction on T cells after the addition of CX3CL1 *in vitro* (29). This showed that in CX3CR1-expressing cells, ligand - receptor binding can result in decreased CX3CR1 surface expression. The significant decrease in CX3CR1 expression *in vivo* may be due to a loss of CX3CR1-expressing cells from the circulation or an interaction with CX3CL1. However, there are some flaws in our study. We did not explore CX3CR1 expression at the gene transcription level of CD16$^+$ monocytes in AAV patients or the specific mechanism of upregulation of CX3CR1 on monocytes under MPO-ANCA stimulation *in vitro*. Therefore, further studies are required to understand the mechanism of CX3CR1 expression on monocytes in MPO-AAV.

We observed that the expression of CD80, CD86 and CD40 was increased on CD16$^+$ monocytes compared with CD16$^-$ monocytes, although to varying degrees. Importantly, previous studies showed that CD14$^+$CD16$^+$ monocytes produce the highest quantity of IL-1β in response to anti-MPO antibody stimulation (30). In this study, correspondingly, we found MPO-ANCA significantly upregulated CX3CR1 expression on CD16$^+$ monocytes but had no effect on CD16$^-$ monocytes, suggesting activated CD16$^+$ monocytes react more strongly to MPO-ANCA than CD16$^-$ monocytes. CD16$^+$ monocytes are involved in many autoimmune diseases. The migration of circulating CD16$^+$ monocytes to synovial tissue was increased in rheumatoid arthritis patients and was associated with joint destruction (31). Non-classical monocytes represent major immune intravascular cells contributing to glomerular inflammation and kidney injury in various mouse models and patients with lupus nephritis (32). Similar to previous findings, we observed that the infiltration of CD16$^+$ monocytes in the kidneys was significantly increased compared with controls. Taken together, those results suggest that CD16$^+$ monocytes have more proinflammatory effects in MPO-AAV patients with renal damage.

We found no difference in the levels of CCL2 and significantly increased levels of CX3CL1 in the plasma of MPO-AAV patients with renal damage compared to HC. Monocyte recruitment responds to chemokine receptor-ligand

interactions (33). Our transwell migration assay demonstrated increased migration of $CD16^+$ monocytes in the presence of CX3CL1 only, and this effect was reversed by anti-CX3CL1 mAb. Our results suggest that CX3CL1 rather than CCL2 drives increased specific migration of $CD16^+$ monocytes.

Relative gene expression of CX3CL1 and CCL2 was upregulated in the kidney from the crescentic phase of anti-MPO IgG-treated mice compared with LPS-treated mice and was higher in glomeruli than in tubule-interstitial areas (34). High-CX3CL1 expression in the kidneys of AAV patients has been described (20). We further found that CX3CL1 was mainly expressed in glomerular endothelial cells in AAV patients. In addition, our *in vitro* results showed that MPO-ANCA from AAV patients contributed to the expression of mRNA and protein of CX3CL1 of glomerular endothelial cells induced by TNF-α. Subsequently, a migration assay further demonstrated that MPO-ANCA could enhance the recruitment of $CD16^+$ monocytes by glomerular endothelial cells through the CX3CL1-CX3CR1 axis. Collectively, our results logically supplement the reported phenomenon that CX3CL1 expression is associated with monocyte/macrophages infiltration in the glomeruli of AAV patients (11).

The migration of $CD16^+$ monocytes to tissues and the inflammatory responses they mediate, including the production of TNF-α and ROS, have the potential to aggravate autoimmune diseases such as lupus nephritis and rheumatoid arthritis (35). We found that decreased circulating $CD16^+$ monocytes in MPO-AAV patients were positively correlated with eGFR and negatively correlated with urinary protein creatinine ratio. In line with this, we observed that the infiltration of $CD14^+$ and $CD16^+$ cells in the kidneys was positively correlated with proteinuria. Inhibition of CX3CR1 can reduce glomerular leukocyte infiltration and crescent formation in a rat model of crescent nephritis, thereby improving renal function (36).These suggest that $CD16^+$ monocytes extravasate to the kidneys *via* the CX3CL1-CX3CR1 axis to participate in renal damage in MPO-AAV patients. However, to understand the role of $CD16^+$ monocytes in renal inflammation in MPO-AAV, further studies are required.

In conclusion, this study identified that MPO-ANCA enhanced the role of CX3CL1-CX3CR1 axis, inducing extravasation of $CD16^+$ monocytes to the kidneys to be involved in renal damage in MPO-AAV. Targeting the CX3CL1-CX3CR1 axis may aid in the management of MPO-AAV with renal damage.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of Xiangya Hospital, Central South University (2019030598). The patients/participants provided their written informed consent to participate in this study.

Author contributions

All authors contributed to the paper. JT, JF and XL designed research; JT, ZL, LL, SD, YJ, FW, XH and XL performed research; GG and HY contributed pathological analysis; XL supervised work; JT, ZL, XH, and SD analyzed data; and JT and XL wrote the paper. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.929244/full#supplementary-material

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EDITED BY
Atsushi Kawakami,
Nagasaki University, Japan

REVIEWED BY
Takao Fujii,
Wakayama Medical University, Japan
Yoshifumi Tada,
Saga University, Japan

*CORRESPONDENCE
Hiroto Tsuboi
Hiroto-Tsuboi@md.tsukuba.ac.jp

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Activation mechanisms of monocytes/macrophages in adult-onset Still disease

Hiroto Tsuboi*, Seiji Segawa, Mizuki Yagishita, Hirofumi Toko, Fumika Honda, Ayako Kitada, Haruka Miki, Ayako Ohyama, Shinya Hagiwara, Yuya Kondo, Isao Matsumoto and Takayuki Sumida

Department of Rheumatology, Faculty of Medicine, University of Tsukuba, Ibaraki, Japan

Adult onset Still disease (AOSD) is a systemic inflammatory disorder characterized by skin rash, spiking fever, arthritis, sore throat, lymphadenopathy, and hepatosplenomegaly. Although the etiology of this disease has not been fully clarified, both innate and acquired immune responses could contribute to its pathogenesis. Hyperactivation of macrophages and neutrophils along with low activation of natural killer (NK) cells in innate immunity, as well as hyperactivation of Th1 and Th17 cells, whereas low activation of regulatory T cells (Tregs) in acquired immunity are involved in the pathogenic process of AOSD. In innate immunity, activation of monocytes/macrophages might play central roles in the development of AOSD and macrophage activation syndrome (MAS), a severe life-threating complication of AOSD. Regarding the activation mechanisms of monocytes/macrophages in AOSD, in addition to type II interferon (IFN) stimulation, several pathways have recently been identified, such as the pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs)-pattern recognition receptors (PRRs) axis, and neutrophil extracellular traps (NETs)-DNA. These stimulations on monocytes/macrophages cause activation of the nucleotide-binding oligomerization domain, leucine-rich repeat, and pyrin domain (NLRP) 3 inflammasomes, which trigger capase-1 activation, resulting in conversion of pro-IL-1β and pro-IL-18 into mature forms. Thereafter, IL-1β and IL-18 produced by activated monocytes/macrophages contribute to various clinical features in AOSD. We identified placenta-specific 8 (PLAC8) as a specifically increased molecule in monocytes of active AOSD, which correlated with serum levels of CRP, ferritin, IL-1β, and IL-18. Interestingly, PLAC8 could suppress the synthesis of pro-IL-1β and pro-IL-18 *via* enhanced autophagy; thus, PLAC8 seems to be a regulatory molecule in AOSD. These findings for the activation mechanisms of monocytes/macrophages could shed light on the pathogenesis and development of a novel therapeutic strategy for AOSD.

KEYWORDS

adult-onset Still disease, monocytes/macrophages, inflammasome, placenta-specific 8, IL-1β, IL-18

Introduction

Adult onset Still disease (AOSD) is a rare systemic inflammatory disorder of unknown etiology that occurs at the age of 16 years or older and is characterized by skin rash, spiking fever, arthritis, sore throat, lymphadenopathy, and hepatosplenomegaly (1–3).

A previous nationwide epidemiologic survey of adult Still disease (ASD) in Japan revealed an estimated prevalence of ASD of 3.9 per 100,000 (4). Analysis of 169 ASD patients showed a mean age at onset of 46 years, and only 8 patients (4.8%) developed ASD when they were aged younger than 16 years, whilst the other 158 patients (95.2%) had AOSD. Of the 146 patients with available data on the clinical course, 58 (39.7%) and 50 (34.2%) patients showed monocyclic and polycyclic systemic patterns, whilst 15 (10.3%) and 23 (15.8%) patients showed monocyclic and polycyclic systemic patterns with chronic articular involvement, respectively. Regarding the complications, disseminated intravascular coagulation (DIC) and macrophage activation syndrome (MAS), which are severe life-threating complications of AOSD, were noted in 8 (6.3%) and 19 (15.0%) of 127 patients, respectively. At the last medical examination, 145 of 164 patients (88.4%) had achieved remission, whilst 66 of 169 patients (39.1%) experienced relapse during the observation period. Importantly, lymphadenopathy and MAS were significantly associated with increased risk of relapse. Regarding treatment, oral glucocorticoids were administered to 96% of the patients, whilst methotrexate and biologic agents were administered to 41% and 16% of the patients in that survey, respectively (4). In addition, a recent comprehensive systematic literature review (5) including the nationwide epidemiologic survey in Japan described above (4), showed that the estimated prevalence of AOSD was between 0.73 and 6.77 per 100,000 individuals, and for clinical course, monocyclic systemic/self-limited was 21.1-64.3%, polycyclic systemic/intermittent was 9.3-50.0%, and chronic articular was 11.9-55.6%, respectively. The review also reported that 1.7-23.5% of patients with AOSD developed MAS, and the mortality rate of patients with AOSD was 2.3-16% (5). From these findings, prevention of relapse and MAS seem to be the current unmet medical needs in the management of AOSD.

Intravenous tocilizumab (TCZ), a monoclonal antibody against the IL-6 receptor, has been officially approved for AOSD in Japan on the basis of promising results from a double-blind, randomized, placebo-controlled phase-III trial (6). In that trial, treatment responses such as American College of Rheumatology (ACR) 20 and ACR50 in the TCZ group were double those in the placebo group, and TCZ had a significantly stronger glucocorticoid-sparing effect than that of the placebo; moreover, the TCZ interval could be prolonged after disease control in several patients without flare (6). On the other hand, the possibility that TCZ could trigger the development of MAS in AOSD patients has been proposed (7). Although TCZ could be a hopeful treatment strategy against the prevention of relapse in AOSD, which is one of the unmet medical needs in the management of AOSD, TCZ does not seem to be a complete solution for the inhibition of MAS. For another promising biological agent, anakinra which is a recombinant human IL-1 receptor antagonist also has been examined for treatment of AOSD, while it has not been officially approved for AOSD in Japan. A systematic literature review, in which 15 manuscripts (one open randomized multicenter trial and 14 observational single-arm retrospective studies) were analyzed, showed that the majority of AOSD patients treated with anakinra could achieve a complete remission also in monotherapy, and the treatment with anakinra was associated with an important corticosteroids-sparing effect (8). Interestingly, a single center experience and systematic literature review clarified that the majority but not all of pediatric MAS patients associated with systemic juvenile idiopathic arthritis (sJIA) or autoinflammatory diseases (46 out of 50 reported cases) achieved remission by treatment with anakinra (9). Thus, revealing the accurate and comprehensive mechanisms for activation of monocytes/macrophages is necessary for the development of a new radical therapeutic strategy against AOSD complicated with MAS.

In this review, we survey the pathogenic process of AOSD, including the genetic background, triggers, innate and acquired immune systems, high production of ferritin, and proinflammatory cytokines. In particular, we focus on the activation mechanisms of monocytes/macrophages in the innate immune responses, which might play central roles in the development of AOSD and MAS.

Bird's eye view of the pathogenic process of AOSD

Table 1 summarizes the pathogenic process of AOSD including the genetic background, triggers, innate and acquired immune systems, high production of ferritin, and proinflammatory cytokines.

Genetic background

A genetic association between AOSD and human leukocyte antigen (HLA), including both HLA class I (HLA-B17, B18, and B35) and HLA class II (HLA-DR2, DR4, DRB1*12, and DRB1*15), has been reported (10, 11). Moreover, some associations of the HLA type with the clinical characteristics of AOSD, such as HLA-Bw35 and HLA-DRB1*14 with a mild, self-limiting disease; HLA-DRw6 with joint involvement; HLA-DRB1*1501 (DR2) and HLA-DRB1*1201 (DR5) with a

TABLE 1 Pathogenic process of AOSD.

Process	Factors	Pathogenic roles and clinical association	References
Genetic background	HLA class I (HLA-B17, B18, and B35) HLA class II (HLA-DR2, DR4, DRB1*12, and DRB1*15)	Disease susceptibility	(10, 11)
	HLA-Bw35 and HLA-DRB1*14	Mild, self-limiting disease	(12–14)
	HLA-DRw6	Joint involvement	
	HLA-DRB1*1501 (DR2) and HLA-DRB1*1201 (DR5)	Chronic disease course	
	HLA-DQB1*0602 (DQ1)	Chronic and systemic disease	
	IL-18	Higher production of IL-18	(15)
	MIF	Higher production of MIF, liver dysfunction	(16)
	MEFV, TNFRSF1A	Disease susceptibility, severe disease	(17)
	LILRA3	Disease susceptibility, inducing formation of NETs	(18)
Triggers	Viruses (rubella, measles, echovirus 7, coxsackievirus B4, cytomegalovirus, Epstein-Barr virus, parvovirus B19, hepatitis virus, influenza virus, adenovirus, and human immunodeficiency virus),	PAMPs	(19–22)
	SARS-CoV-2	Macrophage activation	(19)
	Bacteria (*Mycoplasma pneumonia*, *Chlamydia pneumonia*, *Yersinia enterocolitica*, *Brucella abortus*, and *Borrelia burgdorferi*)	PAMPs	(1, 23–25)
	Solid cancers and hematologic malignancies (necrotic tumor cells)	DAMPs	(1, 26, 27)
Innate immune system	Hyperactivated macrophages	Produce proinflammatory cytokines (IL-1β, IL-18, TNFα, and IL-6) and chemokines Enhanced phagocytosis Stimulate the release of ferritin	(19, 28)
	Hyperactivated neutrophils	Release cytokines/chemokines and communicate with macrophages NETs formation	(29, 30)
	Low activation of NK cells	MAS pathogenesis	(31–34)
Acquired immune system	Hyperactivated Th1 cells	Correlation with clinical activity score and serum IL-18 levels IFNγ activates macrophages	(28, 35)
	Hyperactivated Th17 cells	Correlation with severity score, serum ferritin levels, and proinflammatory cytokines	(36, 37)
	Low activation of Tregs	Disease activity affects the stability of Tregs	(38)
High production of ferritin	Increased ferritin from activated macrophages	Stimulate inflammatory pathways, correlation with disease activity	(1, 19, 39–43)
Proinflammatory cytokines	High levels of IL-18 and IL-1β High levels of IL-6 and TNFα High levels of IL-1β and IL-6 Increased IFNγ level compared with IL-18	Systemic disease Arthritis Fever and skin rash Development of MAS	(1, 3, 44, 45)
	IL-18	Triggers Th1 response inducing the secretion of IFNγ by cytotoxic $CD8^+$ and NK cells	
	PLAC8	Suppresses the synthesis of pro-IL-1β and pro-IL-18 *via* enhanced autophagy in monocytes	(46)
	IL-10	Inhibits the production of IL-1β, IL-6, and TNFα from monocytes induced by IFNγ	(47)
	IL-38	Inhibits the activation of NLRP3 inflammasome in macrophages	(48, 49)

MIF, macrophage migration inhibitory factor; MEF, Mediterranean fever; TNFRSF1A, tumor necrosis factor receptor superfamily member 1A; LILRA3, gene name for leukocyte immunoglobulin-like receptor A3; NETs, neutrophil extracellular traps; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; PAMPs, pathogen-associated molecular patterns; DAMPs, damage-associated molecular patterns; MAS, macrophage activation syndrome; Tregs, regulatory T cells; NK, natural killer; PLAC8, placenta-specific 8; NLRP3, nucleotide-binding oligomerization domain, leucine-rich repeat, and pyrin domain 3.

chronic disease course; and HLA-DQB1*0602 (DQ1) with chronic and systemic disease, have also been shown (12–14). Interestingly, polymorphisms in both the *IL-18* gene and the macrophage migration inhibitory factor (*MIF*) gene have been shown to contribute to the disease susceptibility *via* higher production of IL-18 and MIF (15, 16). For known hereditary periodic fever syndrome genes, a previous study showed that association with 3 rare variants in the Mediterranean fever (*MEFV*) gene, which encodes pyrin, and mutations in the tumor necrosis factor receptor superfamily member 1A (*TNFRSF1A*) gene were significant and that these genetic factors were associated with a severe disease course of AOSD (17). More recently, functional leukocyte immunoglobulin-like receptor A3 (LIR-A3; gene name *LILRA3*) has been identified as a novel genetic risk factor for the development of AOSD, and functional LIR-A3 might play a pathogenic role by inducing formation of neutrophil extracellular traps (NETs) (18).

Triggers

A large number of viruses, including the rubella, measles, echovirus 7, coxsackievirus B4, cytomegalovirus and Epstein-Barr, parvovirus B19, hepatitis, influenza, adenovirus, and human immunodeficiency viruses, have been suggested to trigger AOSD pathogenesis *via* activation of the aberrant response of the immune system as so-called pathogen-associated molecular patterns (PAMPs) (19–22). More recently, attention has been focused on an association with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and the common features of macrophage activation in coronavirus disease 2019 (COVID-19) and AOSD (19). In addition, some bacteria such as *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, *Yersinia enterocolitica*, *Brucella abortus*, and *Borrelia burgdorferi* have also been considered to be involved (1, 23–25). Other than these infectious pathogens, solid cancers and hematologic malignancies have been proposed as possible triggers of AOSD (1, 26). Damage-associated molecular patterns (DAMPs) typically released by necrotic tumor cells are recognized by innate immune receptors such as toll-like receptors (TLRs) and could stimulate innate immune responses within the tumor immune microenvironment (27), which might contribute to the development of AOSD. However, no disease-specific unique pathogenic trigger has been clearly identified, suggesting the possibility that multiple environmental triggers play a role in AOSD (1).

Innate immune systems

Activation of innate immune cells including macrophages and neutrophils plays a major pathogenic role in the development of AOSD (1). The activation mechanisms of monocytes/macrophages will be described in a later section. Hyperactivated macrophages could produce proinflammatory cytokines such as IL-1β, IL-18, TNFα and IL-6, as well as chemokines; enhanced phagocytosis; and stimulated the release of ferritin (19, 28). As for neutrophils, interaction between various PAMPs and DAMPs and a variety of specific receptors including pattern-recognition receptors (PRRs), such as TLRs and C-type lectin domain family 5-member A (CLEC5A), and Fc receptors on neutrophils promote neutrophil recruitment and activation (29). Activated neutrophils release more cytokines/chemokines and communicate with macrophages in the innate immune system. Neutrophil activation and mediator release form a positive-feedback loop that enhances neutrophil recruitment and amplifies inflammatory responses, acting as an axis of pathogenesis in AOSD (29). A previous study demonstrated that IL-18, a pivotal cytokine of AOSD, induces NETs by enhancing calcium influx into neutrophils (29). The protein components of NETs, like S100 proteins, act as ligands of TLR4 or receptors for AGEs (advanced glycation end products) (RAGE) to accelerate neutrophils and activate the release of proinflammatory cytokines, contributing to the systemic inflammation of AOSD (30). Thus, NETs formation can promote cytokine storms by linking neutrophils and macrophages, as described in a later section (29).

Contrastively, it was reported that the cytotoxic function of natural killer (NK) cells was defective in AOSD patients (31). The proinflammatory cytokines produced during AOSD, mainly IL-18, have been reported to decrease NK cell activity (32, 33). Importantly, impairment of NK cell cytotoxicity has been shown to play some roles in MAS pathogenesis (34).

Acquired immune system

A previous report showed that interferon (IFN) γ-producing helper T (Th) cells and the Th1/Th2 ratio in peripheral blood were significantly higher in patients with AOSD than in healthy controls (HCs) and that the percentages of IFNγ-producing Th cells and the Th1/Th2 ratio in peripheral blood correlated significantly with the clinical activity score and serum IL-18 levels in patients with AOSD (35). Importantly, IFNγ is known to increase the production of cytokines and chemokines, phagocytosis, and the intracellular killing of microbial pathogens by macrophages (28). Moreover, high frequencies of circulating Th17 cells were reported in active AOSD patients and correlated with the severity score, serum ferritin levels, and proinflammatory cytokines including IL-1β, IL-6, and IL-18 (36, 37). In contrast, a recent study clarified that the proportion of regulatory T cells (Tregs) was significantly lower in patients with acute AOSD patients than in the HCs and that the expression levels of IFNγ, IL-17, and IL-4 in Tregs were

significantly increased, whilst the suppressive function of Tregs was impaired in patients with acute AOSD (38). These results suggested that the disease activity might affect the stability of Tregs in AOSD (38).

High production of ferritin

Ferritin is an intracellular iron storage protein including 24 subunits: heavy (H) subunits and light (L) subunits, according to their molecular weight (1). It is well known that ferritin is a characteristic mediator of AOSD (19). In AOSD, H-ferritin and the number of macrophages expressing H-ferritin have been shown to be increased, suggesting a pathogenic role (39–42). Importantly, elevated H-ferritin expressions in the lymph nodes and skin were correlated with the severity of AOSD (41, 42). Ferritin synthesis is regulated, in addition to iron availability, by different cytokines such as IL-1β and IL-6, which are overexpressed in AOSD patients (1, 19). Moreover, ferritin can stimulate inflammatory pathways to amplify the inflammatory process, supporting a hypothesis that ferritin may not only act as a bystander of the acute-phase reaction (43). Thus, increased production of ferritin from macrophage activation might correlate with the disease activity of AOSD and might serve as an activity indicator of this disease (19).

Heme oxygenase-1 (HO-1), an inducible heme-degrading enzyme, is expressed by macrophages and endothelial cells in response to various stresses. Interestingly, it was reported that among patients with hemophagocytic syndrome (HPS) and AOSD, serum HO-1 correlated closely with serum ferritin but not CRP or lactate dehydrogenase (LDH) levels (50). These findings indicated an association between HO-1 and hyperferritinemia in patients with HPS and AOSD.

Moreover, several isoforms of ferritin have been described, one of which is glycosylated ferritin. A previous report revealed that glycosylated ferritin was significantly lower in AOSD patients than in the control patients including infection, liver disease, systemic inflammatory disease, fever of unknown origin, and neoplasia. The study concluded that increased ferritin and decreased glycosylated ferritin levels could be powerful diagnostic markers of AOSD (51).

Proinflammatory cytokines

The immune system activation described above leads to the production of several proinflammatory cytokines including IL-1β, IL-18, TNFα, IL-6, IFNγ, and IL-17 from activated macrophages, neutrophils, and Th1 and Th17 cells (1). Importantly, different cytokine profiles might be responsible for the distinct clinical manifestation of AOSD (3). For example, high levels of IL-18 and IL-1β are detected in systemic disease; high levels of IL-6 and TNFα, in arthritis; and high levels of IL-1β and IL-6, in fever and skin rash, respectively (1, 3). Regarding MAS, one of the most severe complications of AOSD, a previous study of sJIA showed that an increased plasma IFNγ level in comparison with IL-18 might raise suspicion about the development of MAS in sJIA (44). In addition, IL-6 has also been associated with MAS (3). Regarding the association between IL-18 and IFNγ, IL-18 might also trigger a Th1 response, thereby inducing the secretion of IFNγ by cytotoxic $CD8^+$ and NK cells (45). Although these correlations between the cytokine profiles and clinical features of AOSD might be clinically informative, serum cytokines are not routinely examined and might not widely affect our daily practice of individual AOSD patients (3).

Activation mechanisms of monocytes/macrophages in AOSD

In innate immunity, activation of monocytes/macrophages might play central roles in the development of AOSD and MAS, as described above. Regarding the activation mechanisms of monocytes/macrophages in AOSD, several pathways such as the PAMPs and DAMPs-PRRs axis and NETs-DNA have recently been identified (1, 19), in addition to type II IFN stimulation (28). Moreover, several inhibitory molecules that work against activation of monocytes/macrophages have been also reported. Figure 1 summarizes the activation mechanisms of monocytes/macrophages in AOSD.

mRNA expression pattern in monocytes from active AOSD patients and identification of PLAC8 as an active-AOSD-specific highly expressed gene which has suppressive ability on the synthesis of pro-IL-1β and pro-IL-18 via enhanced autophagy

We previously compared the gene expression pattern of peripheral monocytes of active-AOSD, inactive-AOSD patients (the same patients in the active and inactive phases), and HCs by DNA microarray to identify genes specifically associated with the active phase of the disease (46). The gene expression patterns in the 3 groups (active-AOSD, inactive-AOSD, and HC) showed distinct clusters specific to each group in principal components analysis (PCA). We identified 68 genes as active-AOSD-specific highly expressed genes, and focused on placenta-specific 8 (*PLAC8*) among them. Importantly, the *PLAC8* mRNA expression levels were significantly higher in the active-AOSD patients than in the HCs or the inactive-AOSD patients, as well as than in patients with rheumatoid arthritis (RA), Sjögren syndrome (SS), systemic lupus erythematosus (SLE), or

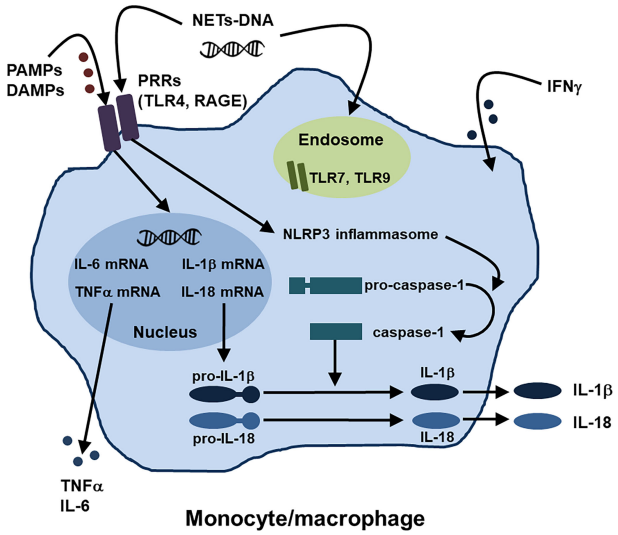

FIGURE 1
Activation mechanisms of monocytes/macrophages in AOSD. Regarding the activation mechanisms of monocytes/macrophages in AOSD, the PAMPs and DAMPs-PRRs axis and NETs-DNA have been identified, in addition to IFNγ stimulation. NETs, neutrophil extracellular traps; PAMPs, pathogen-associated molecular patterns; DAMPs, damage-associated molecular patterns; PRRs, pattern recognition receptors; TLR, toll-like receptor; receptor for AGEs (advanced glycation end products) RAGE; NLRP, nucleotide-binding oligomerization domain, leucine-rich repeat, and pyrin domain.

polymyositis (PM)/dermatomyositis (DM), indicating that the upregulation of *PLAC8* mRNA in monocytes was specific in the patients with active-AOSD. Interestingly, in patients with AOSD, the expression of *PLAC8* mRNA in peripheral monocytes was significantly correlated with serum CRP and ferritin levels. Moreover, serum IL-1β and IL-18 levels, but not IL-6 and TNFα levels, significantly correlated with *PLAC8* mRNA expression levels in the AOSD patients. Thus, these results suggested that the expression levels of *PLAC8* mRNA in peripheral monocytes are an activity or severity marker for AOSD (46).

Regarding the function of PLAC8, we indicated that upregulated PLAC8 acted on the synthesis of inactive precursors of IL-1β and IL-18 and suppressed the production of IL-1β and IL-18 through enhanced autophagy which was independent of caspase-1. Thus, PLAC8 seems to be a regulatory molecule in AOSD. Figure 2 summarizes the proposed suppressive function of PLAC8 in IL-1β and IL-18 production (46).

PAMPs and DAMPs-PRRs axis

Monocytes/macrophages could be activated through the recognition of various PAMPs and DAMPs by different types of PRRs such as TLRs, resulting in activation of inflammasomes responsible for pro-IL-1β and pro-IL-18 activation (1, 19). The nucleotide-binding oligomerization domain, leucine-rich repeat, and pyrin domain (NLRP) can form multimeric protein complexes in response to stimuli (19). NLRP3 inflammasomes trigger caspase-1 activation to convert pro-IL-1β and pro-IL-18 into mature IL-1β and IL-18, which contribute to the pathogenesis of AOSD (1, 19).

As for the roles of TLRs and their ligands in activation of monocytes/macrophages in AOSD, interaction between various DAMPs and TLRs, including S100 proteins with TLR4, high mobility group box-1 (HMGB1) with TLR4 and RAGE, and nucleic acids with TLR7, has been identified (19, 52).

NETs-DNA

As described in the innate immune system section, a previous study demonstrated that IL-18, a pivotal cytokine of AOSD, induces NETs by enhancing the calcium influx into neutrophils (29). Interestingly, Hu et al. showed that NETs-DNA complexes were significantly increased in the circulation of patients with AOSD when compared with that of HCs and that NETs-DNA from AOSD patients activated macrophages and increased the expression of IL-1β, IL-6, and TNFα *via* activation of the NLRP3 inflammasome (53). The authors hypothesized that AOSD neutrophils spontaneously release NETs-DNA, leading to an enhanced proinflammatory potential mainly *via* TLR9 (53). These findings indicated a novel link between neutrophils and macrophages by NETs formation in AOSD.

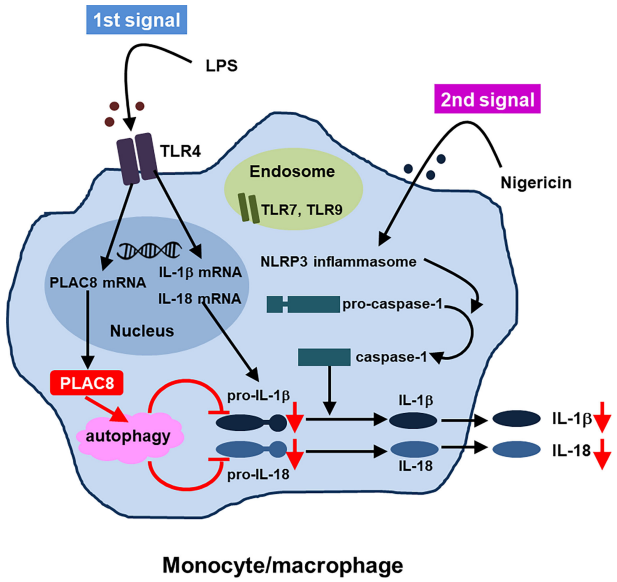

FIGURE 2
Proposed function of PLAC8 in IL-1β and IL-18 production (reference 46, modification)A schema illustrating how PLAC8 suppresses IL-1β and IL-18 production *via* enhancement of autophagy. Two steps might be needed for inhibition of IL-1β and IL-18 production by PLAC8 in primary monocytes. The first step is the upregulation of PLAC8, pro-IL-1β and pro-IL-18 in monocytes through LPS stimulation. The second step is the inhibition of pro-IL-1β and pro-IL-18 through the enhancement of autophagy by upregulated PLAC8, which is independent of caspase-1.

Type II IFN stimulation

IFNγ, type II IFN, would be the prototypic "macrophage-activating factor" which could increase cytokine and chemokine production, phagocytosis, and the intracellular killing of microbial pathogens by macrophages, thus playing important pathogenic roles in the development of AOSD and MAS (28, 54)

Inhibitory effect of IL-10 and IL-38 on proinflammatory cytokine production

A recent study showed that peripheral monocytes expressed IL-10 receptors as well as IL-6 receptors and gp130 (47). As expected, IFNγ enhanced the expression levels of proinflammatory cytokines including IL-1β, IL-6, and TNFα from monocytes (47). Interestingly, IL-10 clearly inhibited the production of these cytokines induced by IFNγ stimulation (47). Thus, IL-10 seems to have an inhibitory effect on proinflammatory cytokine production by monocytes.

IL-38 is a new member of the IL-1 family with multiple functions involved in infection and immunity. A recent study revealed that LPS upregulated IL-38 and its receptor IL-36 receptor, and IL-38 shifted macrophages from a M1 to M2 phenotype, as well as IL-38 dampened LPS induced activation of NLRP3 inflammasome in mouse peritoneal macrophages (48). Thus, IL-38 can significantly inhibit the activation of NLRP3 inflammasome, resulting in a potent anti-inflammatory activity (49).

Conclusion

Various factors including the genetic background, triggers, innate and acquired immune systems, high production of ferritin, and proinflammatory cytokines could contribute to the pathogenic process of AOSD. Among innate immunity, activation of monocytes/macrophages might play central roles in the development of AOSD and MAS. Regarding the activation mechanisms of monocytes/macrophages in AOSD, several pathways such as the PAMPs and DAMPs-PRRs axis and NETs-DNA have been identified, in addition to type II IFN stimulation. We identified PLAC8 as a specifically increased molecule in monocytes of active AOSD, which correlated with serum levels of CRP, ferritin, IL-1β, and IL-18. Interestingly, PLAC8 could suppress the synthesis of pro-IL-1β and pro-IL-18 *via* enhanced autophagy; thus, PLAC8 seems to be a regulatory

molecule in AOSD. These findings related to the activation mechanisms of monocytes/macrophages could shed light on the pathogenesis of AOSD and thus lead to the development of a novel therapeutic strategy for the disease.

Author contributions

Each author took part in the design of the study, collection of the data, and writing of the manuscript, and all agree to accept equal responsibility for the accuracy of its contents of this paper. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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EDITED BY
Atsushi Kawakami,
Nagasaki University, Japan

REVIEWED BY
Ryusuke Yoshimi,
Yokohama City University, Japan
Hua Ren,
East China Normal University, China
Beiyun Liu,
St. Jude Children's Research Hospital, United States

*CORRESPONDENCE
Zhenxing Chen
416158716@qq.com

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

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Immune Effects of Macrophages in Rheumatoid Arthritis: A Bibliometric Analysis From 2000 to 2021

YunLing Xu[1†], Zhongmin Zhang[2†], Jiaolong He[3] and Zhenxing Chen[2*]

[1]Department of Basic Medical, Zhejiang Academy of Traditional Chinese Medicine, Hangzhou, China, [2]College of Pharmacy, Guangxi University of Chinese Medicine, Nanning, China, [3]Department of Intensive Care, First Affiliated Hospital of Jishou University, Jishou, China

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease characterized by macrophage activation. The current characteristics, hotspots, and research frontiers of macrophage-related RA were analyzed using bibliometric analysis. Relatedpapers published from 2000 to 2021 in the Web of Science database were retrieved. The diagrams were generated and analyzed using the bibliometric software package. VOSviewer and CiteSpace were used to evaluate and visualize the research trends and hotspots in macrophage-related RA. A total of 7253 original articles were obtained. Global research on macrophage-related RA is in an advanced stage of development, with core authors, teams and research institutions emerging. United States has published the most papers, received the most citations, and had the highest H-index over the last 22 years. The University of Amsterdam and the journal of *Arthritis and Rheumatism* are the most productive research institutions and journals. Tak PP's (St Vincent's Hospital) paper has the highest publication and citation scores. The keywords "bone loss" and "polarization" have the highest frequency. Additionally, the study of macrophage polarization in RA has been research focus in recent years. This study demonstrates that research on macrophages in RA will continue. China is a significant producer, whereas the United States is an influential nation in this regard. In the last decade, most studies have concentrated on fundamental research. Recent studies have shown how macrophages play a role in controlling and weakening inflammation, and drug delivery and mechanism have come to the fore.

KEYWORDS

rheumatoid arthritis, macrophages, bibliometrics, hotspots, research status

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease associated with macrophages that manifests as a chronic inflammatory condition marked by severe joint damage and synovial joint destruction (1, 2). RA has a global prevalence of 0.3%–4.2% and is a significant global public health challenge (3, 4). Currently, the etiology of RA has not yet been fully clarified, and the disease cannot be cured entirely (5, 6). Macrophages are dynamic cells that contribute significantly to immune surveillance and respond to a variety of external stimuli (pathogenic microbes, damaged tissues, abnormal cells, etc.) (7, 8). They are abundant at the the synovium cartilage pannus junction during inflammation and are involved in the pathogenesis of RA. Macrophages are highly diverse and plastic, and they can be divided into two types: classically activated macrophage (M1) and alternatively activated macrophage (M2), each with distinct functional phenotypes (9). Thus, it is critical to quantitative analysis of the current state, its focus fields, and prospects for research on macrophage-related RA (10). Additionally, the field's hotspots and trends are updated continuously with the introduction of new technology and clinical diagnostic standards. While several scholars have made significant contributions to this field and published numerous papers, concise summaries are lacking. As a result, a comprehensive review and summary of this field are required to benefit the research participants.

Bibliometrics is a method for assessing and monitoring the progress of specific disciplines *via* statistical analysis of published data (11). Bibliometric analysis can be used to determine the outputs and citations of countries, institutions, and authors and the keyword frequency of research hotspots and frontiers in particular fields (12). Using bibliometric analysis techniques, a 22-year (2020 – 2021) longitudinal analysis was conducted to evolution the scientific literature on macrophage-related RA. The published literature was primarily analyzed using the following criteria: publication year, country, affiliation, journal, author, keyword, citation, and H-index. Finally, the bibliometric analysis results were combined with a traditional review conducted under the guidance of bibliometrics to demonstrate the evolution of the research on macrophage-related RA. This is the first attempt to conduct an in-depth to statistical analysis of literature on macrophage-related RA. In addition, it is supposed to give reliable data that can be used to inform experimental tactics and provide useful statistics for financing decisions.

Methods

Data collection

The Science Citation Index (SCI) Expanded Database of the Web of Science (WoS) was used to obtain bibliographic data. To avoid bias caused by daily database updates. All documents published between 2000 and 2021 were retrieved and downloaded from the WoS Core Collection (WoSCC) database on January 19, 2022. The search strategy applied was Title = (rheumatoid arthritis OR RA) AND Title = (macrophage OR macrophages). Only English-language research papers and review articles were retrieved. Two investigators (YLX and JLH) independently conducted the primary data search and discussed any discrepancies. The final agreement reached a value of 0.90, indicating a substantial agreement (13).

The data was saved and stored in download_txt format. For further analysis, only research articles and review articles chosen, as data acquisition flowchart presented in Figure 1. This study excluded abstracts from meeting, proceeding papers, editorial materials, early access, letters, book chapters, corrections, retracted publications, publication with expression of concern, reprints, and retractions.

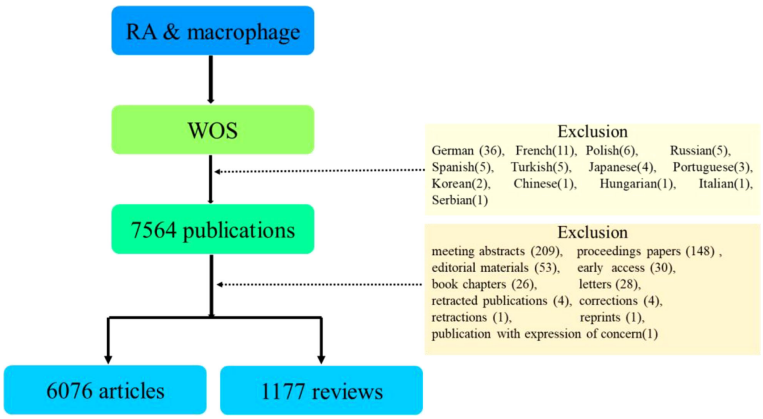

FIGURE 1
Flowchart of literature selection.

Bibliometric analysis

For visual analysis, all valid data collected from the WoSCC database was imported into CiteSpace (version 5.7) and OVSviewer (version 1.6.16) (14). CiteSpace was used to analyze the strongest citation bursts of references and keywords, investigate the research status, hotspots, and trend distribution maps over time, and determine the field's development trend (15). The collaborative networks between countries, institutions, journals and authors and co-citation of keyword clusters were visually analyzed using VOSviewer. The research status, hot spot and development trend in this field can fully understand by examining keyword frequency, centrality intensity, and prominence. A co-word network was constructed based on the co-occurrence of keyword co-occurrence, with each node representing a keyword. When two keywords appear in the same article, they form a co-occurrence relationship and are represented in the network as a single edge. A large mean value indicates that the node has significant representation in a particular subject field at a specific time. The degree of emergence suggests how much a node's collinear frequency and the number of co-citations increase over time. The greater the degree of emergence, the more evidence that the node was a research hotspot during a given period.

Results

Annual publication outputs

The total number of publications (NP) over a given period can objectively and quantitatively reflect a field's overall development trend. A total of 7253 publications were chosen based on the defined search terms. Among these publications, 6076 (83.77%) were original articles, and 6076 (16.23%) were reviews. The annual NP is depicted in Figure 2A. Despite some fluctuations, the NP increased from 211 in 2000 to 425 in 2021. Over the last 22 years, the growth rate has been relatively stable. Figure 2A also depicts a polynomial fitting curve for the publication's total annual growth trend. The annual NP trended upward and was highly correlated with the year of publication (R^2 = 0.9423). Overall, these findings indicate that the research on macrophage-related RA has gradually stabilized.

Distribution of countries/regions and institutions

The publications originated from 103 countries/regions, and 64 countries/regions had more than four publications. The top 10 most influential countries/regions are listed in Table 1, along with their NP, the total number of citations (NC), H-index, and average citation per item (AC). The top 10 countries/regions published 96% (7018/7253) of the publications. The United States was the leading country in terms of NP (28.7%,2079/7253), followed by China (13.2%,958/7253) and Japan (11.9%,866/7253). The top 3 countries with the highest NC were the United States (129061), England (38338), and Japan (36890). The top 3 countries with the highest AC were the United States (63.90), France (63.63), and the Netherlands (61.57). Figure 2B shows the top 10 most productive countries' annual NP from 2000 to 2021. The circle's size and colors correspond to the NP and citation values of the papers, respectively. Research on macrophage-related RA in the United States is at the core of global studies, and China's research has been more active in recent decades. Figure 2C depicts the global distribution of publications by country, and Supplementary Figure 1 contains the countries VOSviewer visualization map.

More than 5409 institutions have made contributions to this field, with 161 producing more than 18 papers. The visual cluster analysis (threshold > 18 papers) result is depicted in Figure 3. The 161 institutions that appeared more than 18 times were color-coded into eight clusters. A larger node indicated that more documents had been transmitted, and the largest node was highlighted in a green. The line illustrates the connection between the institutions. By and large, the institutions were concentrated around universities, and only a few were hospitals. According to the clustering results, University of Amsterdam, Karolinska Institutet, Leiden University, Imperial College of Science, University of Oxford, University of Glasgow, Northwestern University, Harvard University, University of Genoa, and the University of Tokyo collaborated and exchanged research on macrophages-in RA. Table 2 summarizes the top 10 most influential institutions. The institutions with the highest NP values were the University of Amsterdam (165), followed by the University of London (157), and the University of Oxford (154). The most significant AC scores were from Karolinska Institute (AC = 73.08), Harvard University (AC = 71.56), and Institut National de la Sante et de la Recherche Medicale (AC = 68.12).

Funding source

Funding support plays a vital role in scientific advancement. Table 3 summarizes the top 10 funding agencies and sponsors in this field. In the terms of funding, the United States' research funding agencies, including the Department of Health Human Services, the National Institutes of Health, the National Institute of Arthritis Musculoskeletal Skin Diseases, the National Institute of Allergy and Infectious Diseases, the National Heart Lung Blood Institute, and the NIH National Cancer Institute, occupied the primary positions that promoting to macrophage-related RA research. The remaining funds came

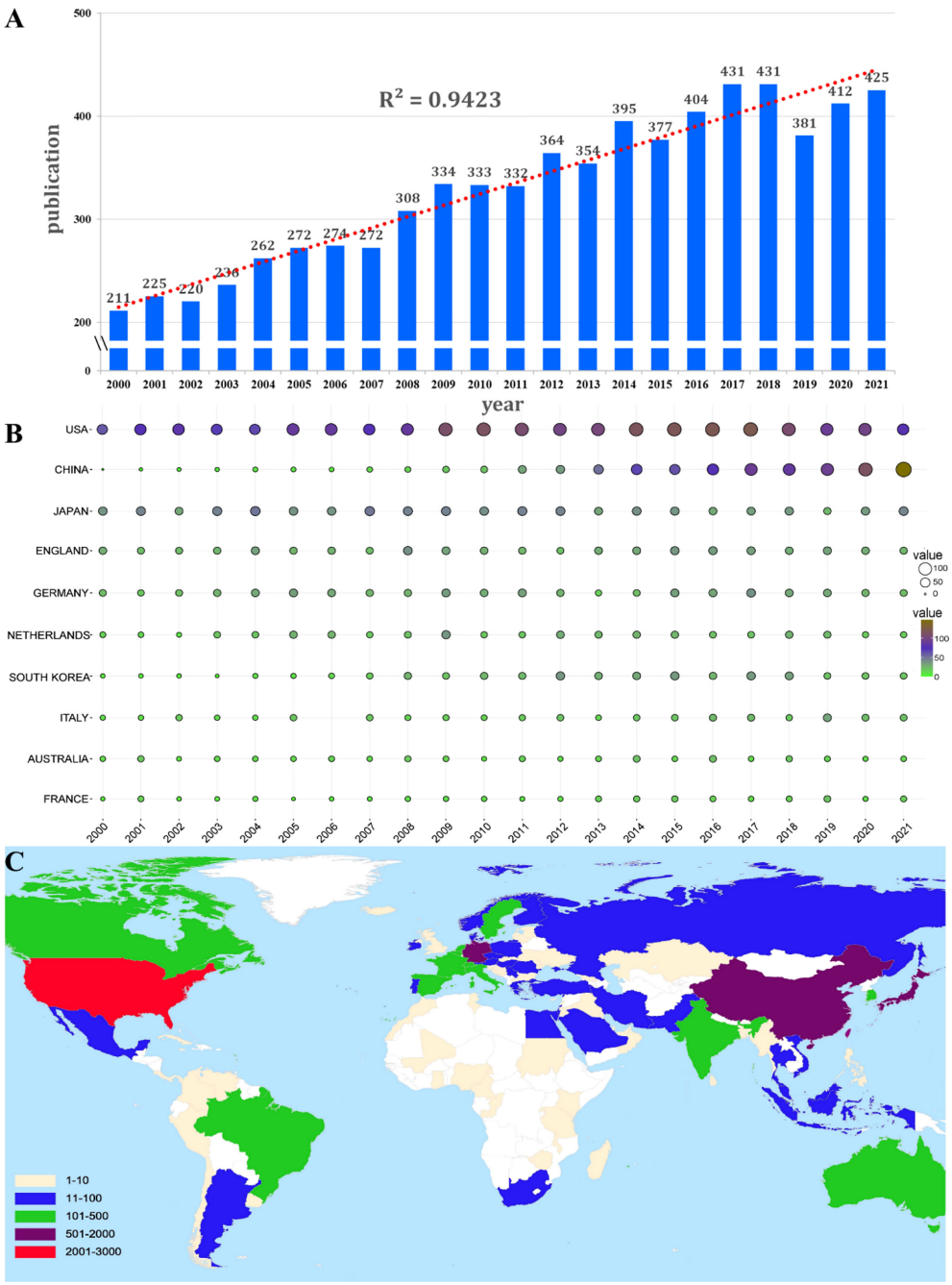

FIGURE 2
(A) The number of publications by year and curve fitting of the annual growth trend of publications (R^2 = 0.9423). **(B)** The annual number of publications in the top 10 most productive countries from 2000 to 2021. **(C)** Global distribution according to the country publications.

from the China's National Natural Science Foundation, the European Commission, Japan's Ministry of Education Culture Sports Science and Technology and the United Kingdom's Research Innovation. Along with established institution the United States has maintained at the forefront of research into macrophage-related RA research due to having sufficient funding.

Authors and co-citation authors

Over 36894 authors have authored in this field. Among them, 207 authors contributed at least eight papers to this collection. Table 4 summarizes the top 10 most productive authors. Tak PP of St. Vincent's Hospital was the most prolific author (NP = 110, NC = 7382) with the highest H-index (55),

TABLE 1 Top 10 most productive country in macrophages-related RA from 2000–2021.

Rank	Country	NP	NC	H-index	AC
1	USA	2079	129061	160	63.90
2	China	958	18191	62	19.76
3	Japan	866	36890	93	43.36
4	England	636	38338	99	61.47
5	Germany	613	32143	90	53.31
6	Netherlands	478	28419	93	61.57
7	South Korea	455	14087	60	31.50
8	Italy	384	21187	74	56.04
9	Australia	300	15034	71	52.16
10	France	249	15714	64	63.63

NP, total number of publications; NC, total number of citations; AC, Average Citations per Item.

and McInnes IB from the University of Glasgow had the most increased AC (94.47). A relationship of co-citation has been established between two documents that appear in the references of a third document (16). Co-cited authors are two or more authors who are simultaneously cited in one or more papers. These authors are related *via* co-citations. Among the 120146 cited authors, 140 (classified into seven clusters) had at least 106 citations. Based on the cluster summary, the authors devoted their minds to summarizing the evolving concepts, pathogenesis, related outcomes, and interventions of RA. Firestein GS ranked the highest in NC (798), followed by McInnes IB (770), Feldmann M (758), Tak PP (576), and Smolen JS (559). Supplementary Figures 2, 3 contain the co-authorship authors' and co-citation authors' VOSviewer visualization maps.

Source journals and co-citation journals

A total of 1382 journals published specific articles on macrophage-related RA. Among them, 160 journals contributed at least eight papers. Table 5 lists the top 10 most productive journals, their publishers, NP, impact factor (IF), NC, and H-index. Most of the journals specialize in arthritis and immunity. Four publishers are based in the the United States, while three are in England. *Arthritis and Rheumatism* was the most prolific journal (NP=347) with the highest NC (27563), and AC (80.76). *Annals of the Rheumatic Diseases* had the highest IF (27.973), followed by *Arthritis and Rheumatology* (15.483), respectively.

Co-cited journals are those in which two or more journals are cited concurrently by researchers. The threshold was met by 141 of met the 12023 cited references (minimum citations > 400). The *Journal of Immunology, Arthritis and Rheumatism*, the *Journal of Biological Chemistry*, *Journal of Experimental Medicine*, and *Annals of the Rheumatic Diseases* were the five most frequently and centrally cited journals. Supplementary Figure 4 illustrates the VOSviewer visualization map of the co-cited journals.

Citations documents and journals

Of the 7253 documents, 384 met the threshold of minimum citations >145. The top 10 articles with the highest NC are presented in Figure 4. The highest NC of the paper written by Joseph Keane in 2001 was 2681, ranking it first followed by Satish L. Deshmane's (NC =2097) and Toby Lawrence's (NC = 2010). In Joseph Keane's paper, the authors summarized the reports of Crohn's disease and RA after tumor necrosis factor α (TNF-α) neutralizing agent infliximab treatment(17). Furthermore, Satish L. Deshmane's work identified of monocyte chemoattractant protein-1 (MCP-1/CCL2) as one of the key chemokines that regulate monocytes/macrophages migration and infiltration. It summarized their biological processes and the structure and function of CCL2 (18). Toby Lawrence described how chronic inflammatory diseases like rheumatoid arthritis are linked to nuclear factor-kappa B (NF-κB) activation (19). He reveals complex roles for the NF-κB in inflammation that suggest both pro- and anti-inflammatory roles for this pathway. These documents made a big difference in studying macrophages in RA and could be called "seminal", which led to more research in this field.

Of the 1382 documents sources, 103 met the criterion (minimum number of documents of a source > 12). *Arthritis and Rheumatism* was ranked first in the terms of publications and citations (NP= 347, NC= 26634), follow by *Arthritis Research and Therapy* (NP=287, NC= 12435) and *Annals of the Rheumatic Diseases* (NP= 199, NC = 12633). Supplementary Figures 5, 6 show the VOSviewer visualization map of citations to document and journals in this field.

References citation bursts

Burst detection, an algorithm developed by Kleinberg (20), is an effective analytic tool for capturing rapid increases in the popularity of references or keywords over a specified time

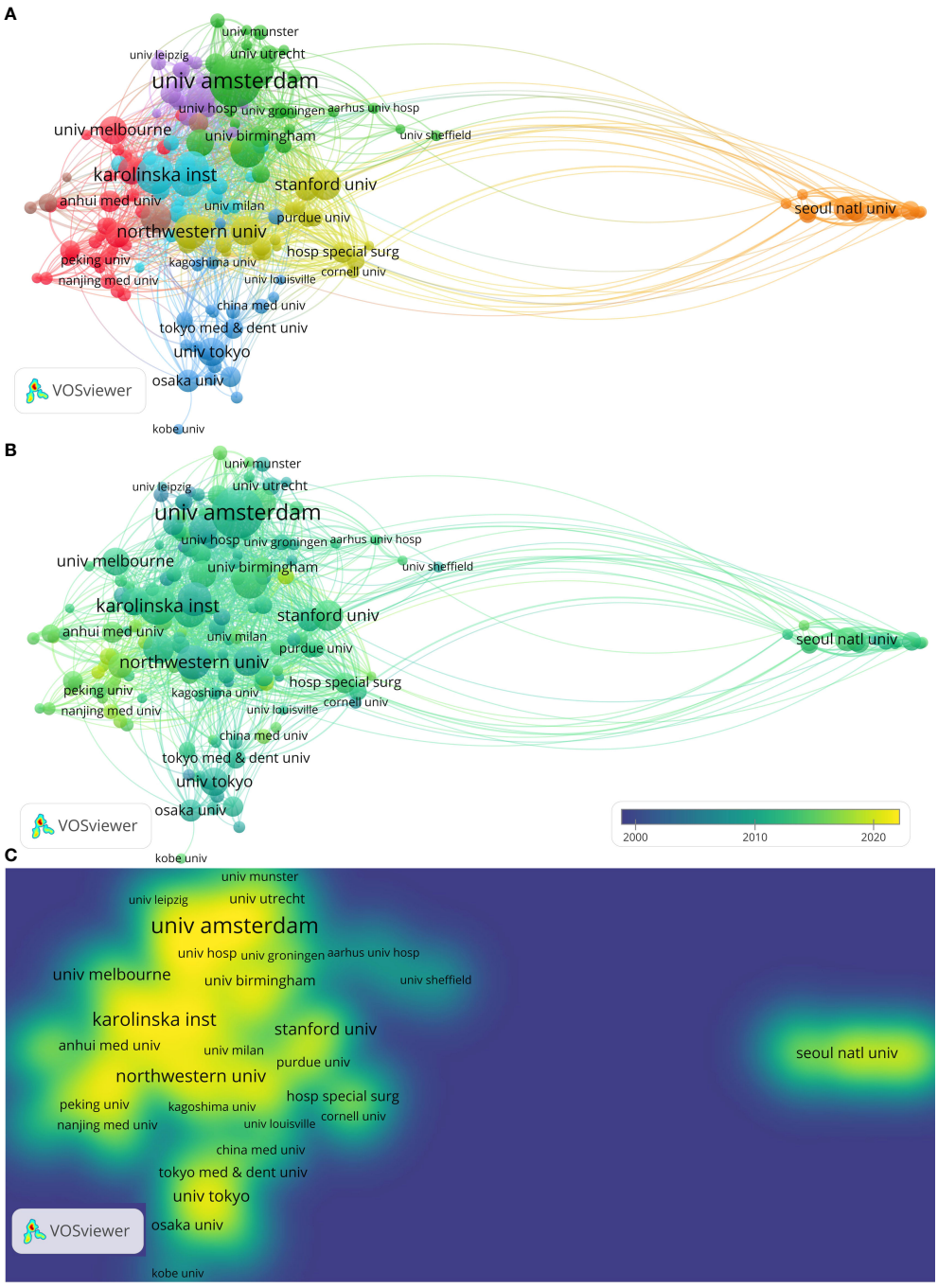

FIGURE 3
Cluster analysis of publications from different institutions from 2000-2021. **(A)** Cluster analysis of publications from different institutions. **(B)** Evolution of publications from different institutions. **(C)** Evolution of publications from different institutions frequency.

period. This function can quickly identify concepts or topic that are actively discussed during a specified period. The present study applied the burst detection algorithm to extract key references and keywords for macrophage-related RA research. Figure 5 illustrates the top 50 references with the strongest citation bursts. The blue lines represent the time period in this graph, and the red lines represent the period when the reference burst occurred. Among these references, four reference with the strong burst value was written by McInnes IB et al. In his articles summarized the crucial effector functions of immunological processes and key effector functions of cytokines in the pathogenesis of RA (21). Then give a conspectus of that

TABLE 2 Top 10 productive affiliations in macrophages-related RA from 2000–2021.

Rank	Affiliations	NP	Country	NC	H-index	AC
1	University of Amsterdam	165	Netherlands	9511	61	60.24
2	University of London	157	England	9510	55	61.04
3	University of Oxford	154	England	9511	57	62.76
4	University of California System	145	USA	9342	55	64.65
5	Harvard University	142	USA	10118	55	71.65
6	Institut National De La Sante Et De La Recherche Medicale	138	France	9338	50	68.12
7	Academic Medical Center Amsterdam	137	Netherlands	8907	60	67.77
8	Karolinska Institutet	128	Sweden	9258	46	73.08
9	Us Department of Veterans Affairs	125	USA	5376	45	44.20
10	Veterans Health Administration	122	USA	5259	44	44.24

NP, total number of publications; NC, total number of citations; AC, Average Citations per Item.

macrophage-derived cytokines (TNF-α, IL-1β, IL-6, IL-12 and IL-18) activate multiple pro-inflammatory pathways in synovial tissue and responsible for osteoclasts maturation and activation (22, 23). Furthermore, in RA patients' cytokines promote joint inflammation and destroy immunity and articular cartilage. NF-κB ligand receptor activator (RANKL), TNF, IL-17 and IL-1 play a graded role in this process.

Keyword co-occurrence and burst

Keyword analysis can also determine the time when keyword with a frequency change rate first appeared in a node, thereby defining the the research field's boundaries. Between 2000 and 2021,19174 keywords were used, with 140 of them appearing more than 69 times. These studies look at RA's clinicopathological diagnosis, RA related-inflammatory markers, the pathogenic mechanisms of RA, the therapeutic interventions, and how drugs work to treat RA. As illustrated in Figure 6, the top 10 keywords were "rheumatoid-arthritis", "expression", "inflammation", "macrophages", "activation", "collagen-induced arthritis", "NF-κB", "cytokines", "TNF-α"and "T cell". Among these keywords, the most concerned macrophage-derived cytokines are is TNF, other such as IFN-γ, colony-stimulating factor (CSF), IL-6, nitric oxide (NO), chemokines, growth-factor-beta (GF-β), migration inhibitory factor (MIF), lipopolysaccharide (LPS), endothelial growth-factor, granulocyte/macrophage colony stimulating factor (GM-CSF), monocyte chemoattractant protein-1(MCP-1), interleukin-1 (IL-1), proinflammatory cytokines, cyclooxygenase-2 (COX-2) have also attracted the attention of researchers (occurrences > 60, Table 6). Moreover, the high frequency keywords of RA-related macrophage function were: cytokines, activation, differentiation, apoptosis, chemokines, cytokine production, innate immunity, oxidative stress, immune-response, polarization, migration inhibitory factor, proinflammatory cytokines, metabolism, signal-transduction, migration (occurrences > 60, Table 6).

"Burst words" are words that are frequently cited in connection with a particular subject at a specific time. They enable the observation of emerging theories and the prediction of the research frontier base on the distribution of keywords with

TABLE 3 The top 10 funding source with most publications.

Rank	Funding source	NP	NC	H-index	AC
1	United States Department of Health Human Services	1,179	81689	134	70.69
2	National Institutes of Health (NIH)	1,176	81310	133	70.52
3	National Natural Science Foundation of China (NSFC)	510	8787	46	17.86
4	European Commission	497	27207	85	55.32
5	NIH National Institute of Arthritis Musculoskeletal Skin Diseases (NIAMSD)	463	32255	96	71.43
6	NIH National Institute of Allergy and Infectious Diseases (NIAID)	337	24590	86	73.85
7	NIH National Heart Lung Blood Institute (NHLBI)	260	21377	75	82.63
8	Ministry of Education Culture Sports Science and Technology Japan Mext	235	6416	42	27.55
9	UK Research Innovation (UKRI)	173	10955	57	63.96
10	NIH National Cancer Institute (NCI)	168	12786	58	76.38

NP, total number of publications; NC, total number of citations; AC, Average Citations per Item.

TABLE 4 The top 10 active authors in macrophages-related RA from 2000–2021.

Rank	Author	Affiliations	Country	NP	NC	H-index	AC
1	Tak PP	St Vincent's University Hospital	Ireland	110	7382	55	70.45
2	Van Den Berg WB	Radboud University Nijmegen Medical Centre	Netherlands	59	3766	32	65.46
3	Bucala R	Yale University	USA	55	3374	35	64.85
4	Pope RM	Northwestern University	USA	48	2794	30	61.65
5	Li J	University of Alabama at Birmingham	USA	47	1619	23	34.64
6	Wang Y	Anhui Medical University	China	47	1211	21	25.98
7	McInnes IB	University of Glasgow	Scotland	45	4209	29	94.47
8	Cutolo M	University of Genoa	Italy	44	2235	29	52.64
9	Zhang Y	Mudanjiang Medical University	China	41	1141	18	27.98
10	Feldmann M	Imperial College of Science, Technology and Medicine	England	40	2715	26	68.8

NP, total number of publications; NC, total number of citations; AC, Average Citations per Item.

the highest citation burst. Figure 7 lists the keywords with the strongest citation bursts. As shown, the keywords with strong bursts before 2013 were "TNF", "mRNA", "factor alpha", "necrosis factor-alpha", "human monocyte", "T lymphocyte", "adhesion molecule", "articular cartilage", "IL", "molecular cloning", "growth factor", "tissue", "smooth muscle cell", "proinflammatory cytokine", "cell infiltrate", "membrane" and "factor MIF". While the burst keywords after 2013 included "bone loss", "polarization," "nanoparticle", "collagen-induced arthritis", "macrophage polarization", "delivery," "drug delivery" and "mechanism", which represent the emerging trends.

Discussions

General information of macrophage-related RA research

A bibliometric analysis was performed to investigate the developmental trends and hotspots of research on macrophage-related RA from the SCI-Expanded database using VOSviewer and CiteSpace. A total of 7253 original articles and reviews published from 2000 to 2021 were obtained. According to the polynomial fitting curve, the annual NP was generally upward, but the growth stalled in the second half of the curve, particularly after 2015. The lack of high-impact, ground breaking publications was the main reason why the annual NP didn't move forward for recent three years (Figure 2A).

Among the top countries, the United States ranked first in terms of publication quality, indicating that the United States is a highly productive country in terms of macrophage-related RA (Figures 2B, 2C; Table 1). Four United States affiliations, six grants and three United States authors ranked among the top 10 affiliations, funding source and authors in the research of macrophage-related RA (Tables 2–4). This result indicates that the United States possess the world's most elite institutions, abundant funding and professional researchers, which partially explaining why the United States has developed rapidly in this field over the past 22 years. Similarly, Japan and high-income countries in Europe, such as England, Germany, the Netherlands, Italy, and France, consistently had high academic

TABLE 5 The top 10 most productive journals.

Rank	Journal	Country	ISSN	NP	IF (2021)	NC	H-index	AC
1	Arthritis & Rheumatology	United States	2326-5191	347	15.483	27563	95	80.76
2	Arthritis Research & Therapy	England	1478-6354	287	5.606	13351	62	47.13
3	Journal of Immunology	United States	0022-1767	257	5.426	19813	84	77.40
4	Annals of the Rheumatic Diseases	England	0003-4967	199	27.973	12891	70	65.48
5	Frontiers in Immunology	Switzerland	1664-3224	161	8.786	4490	35	28.17
6	PLoS One	United States	1932-6203	138	3.752	3352	33	24.36
7	Journal of Rheumatology	Canada	1499-2752	126	5.346	4796	42	38.32
8	Rheumatology	England	1462-0324	121	7.046	6840	49	56.90
9	International Immunopharmacology	Netherlands	1567-5769	82	5.714	1854	25	22.80
10	Scientific Reports	England	2045-2322	81	4.996	1776	25	20.38

NP, total number of publications; IF, Impact factor; NC, total number of citations; AC, Average Citations per Item.

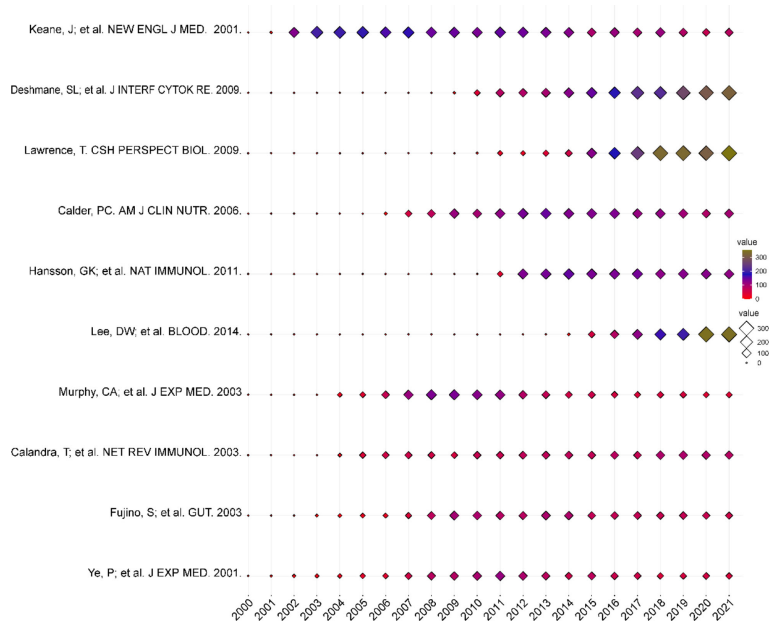

FIGURE 4
The yearly number of citations of papers with high citations.

productivity and an H-index of>90 each year, indicating the countries' continued commitment to and investment in the field. However, China's total number of articles published has increased dramatically over the last 22 years compared to the aforementioned countries. The country is now ranked second overall, though its H-index remains low. In this case, Chinese institutions and scholars need to do more to improve the research quality and make their research better. Parallel to China's economy development, financial support for medical research has continued to increase, which may be the one of the reasons for China's NP growth over the last 22 years. According to these data, it is foreseeable that China's academic productivity will get better and better, and its impact on global academic productivity will become greater in the near future.

Notably, of the top 10 most productive journals, 8 had high IF values (>5.0). This finding indicates that publishing research on macrophage-related RA in high-quality journals is not a challenging. The 50 articles with high NC were published in journals with a high-IF, indicating that these journals have published a greater number of potential breakthroughs in this field. As a result, researchers interested in this field should pay more attention to the news in these journals.

Research foci

There are many different immune cells affect in RA development. In addition to macrophages, immune cells such as T cell, B cell, dendritic cell, mast cell, natural killer cell are also involved in immune responses (24, 25). Of course, many articles have studied the relationship between these cells (Supplementary Table 1). In these publications, the highest frequency of other immune cells was described in the same paper with macrophage-related RA is T cell, accounting for 44.78% (3248/7253). Compared to other chronic diseases, RA shares numerous inflammatory pathological and immunological similarities. The expression of inflammation factors by excessive macrophage activation has long been a research hotspot in RA (Figure 6). In RA, hyperactivated macrophages increased the expression of toll-like receptors (TLRs), such as TLR2, TLR3, TLR4, and TLR7, which induce synovial inflammation and cartilage destruction by releasing chemokines, pro-inflammatory cytokines, and degradative enzymes have been recognized (26, 27). Activation of M1 macrophages, secrete a variety of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, chemokines) and activate inducible nitric oxide synthase to produce NO. Their key mechanisms of action in promoting inflammation and bone destruction have received the most extensively research in the context of RA-related macrophage functions (Table 6). M2 macrophages can secrete various anti-inflammatory factors, including transforming growth factor-β (TGF-β) and IL-10, and are critical players in the regression and repair of RA inflammation (28, 29). In patients with RA, the abnormal immune microenvironment promotes metabolic reprogramming, alters macrophage polarization states, disrupts the dynamic balance of M1 and M2 macrophages, and delays tissue inflammation. Aa a result, inhibiting M1 macrophage

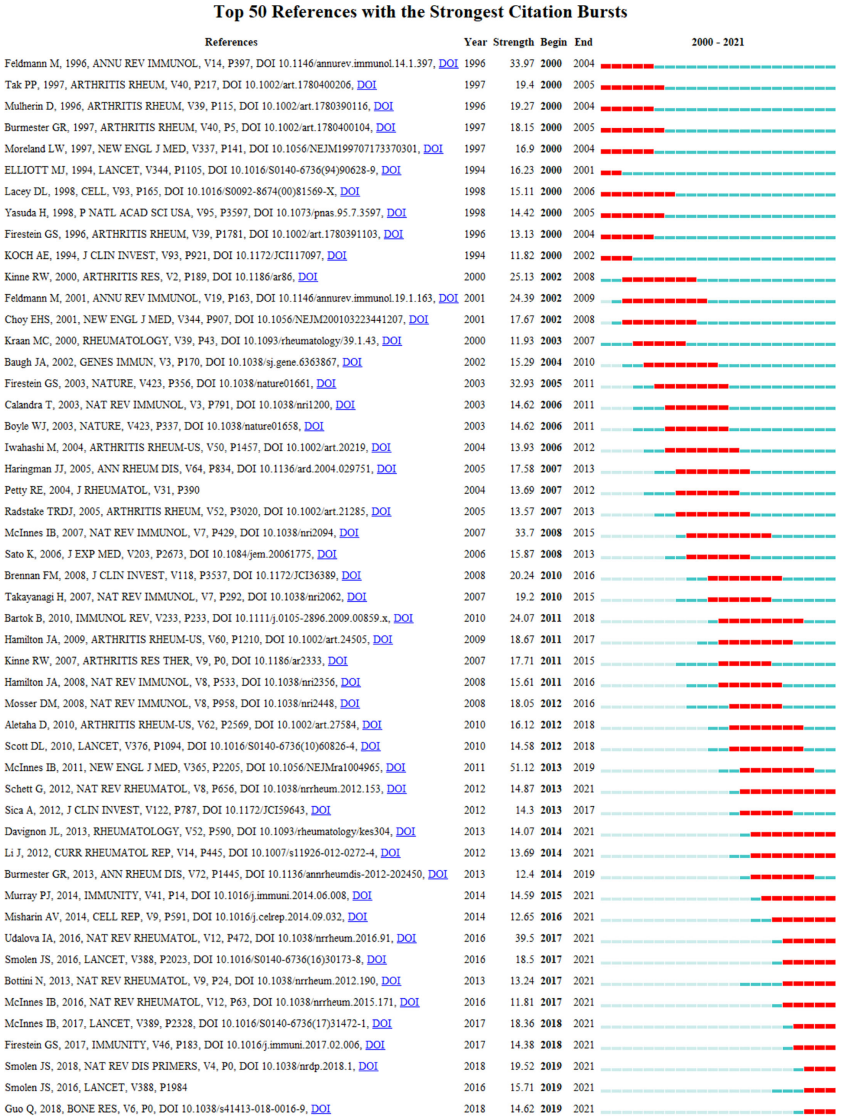

FIGURE 5
Visualization map of top 50 references with the strongest citation bursts involved in macrophage-related RA.

polarization and inducing M2 macrophage polarization are ideal drug research and development strategies in the treatment of RA (30, 31).

Synovial macrophage contributes to the synovial inflammatory response *via* TNF, IL-1 and other pro-inflammatory cytokines, as well as cell-to-cell contact, aggravating RA's disease (32). TNF is a well-established RA driver that regulates inflammation, autoimmunity, and joint destruction in RA patients' joints (33). As the statistics show, TNF is the most watched macrophage-derived cytokines, as 1275 of these publications were related to RA-treatment targeting to TNF (Supplementary Table 2). TNF inhibitor biologics have emerged as beneficial treatment options for amelioration of RA, and clinical remission has become a viable therapeutic target (34). Early intervention, such as TNF blockers, can increase the rate of clinical response and the likehood of clinical symptom improvement (35, 36). Additionally, biologics targeting on IL-1, IL-6, IL-10, IL-17 and GM-CSF are also favored by researchers, and relevant clinical trials are underway (37, 38).

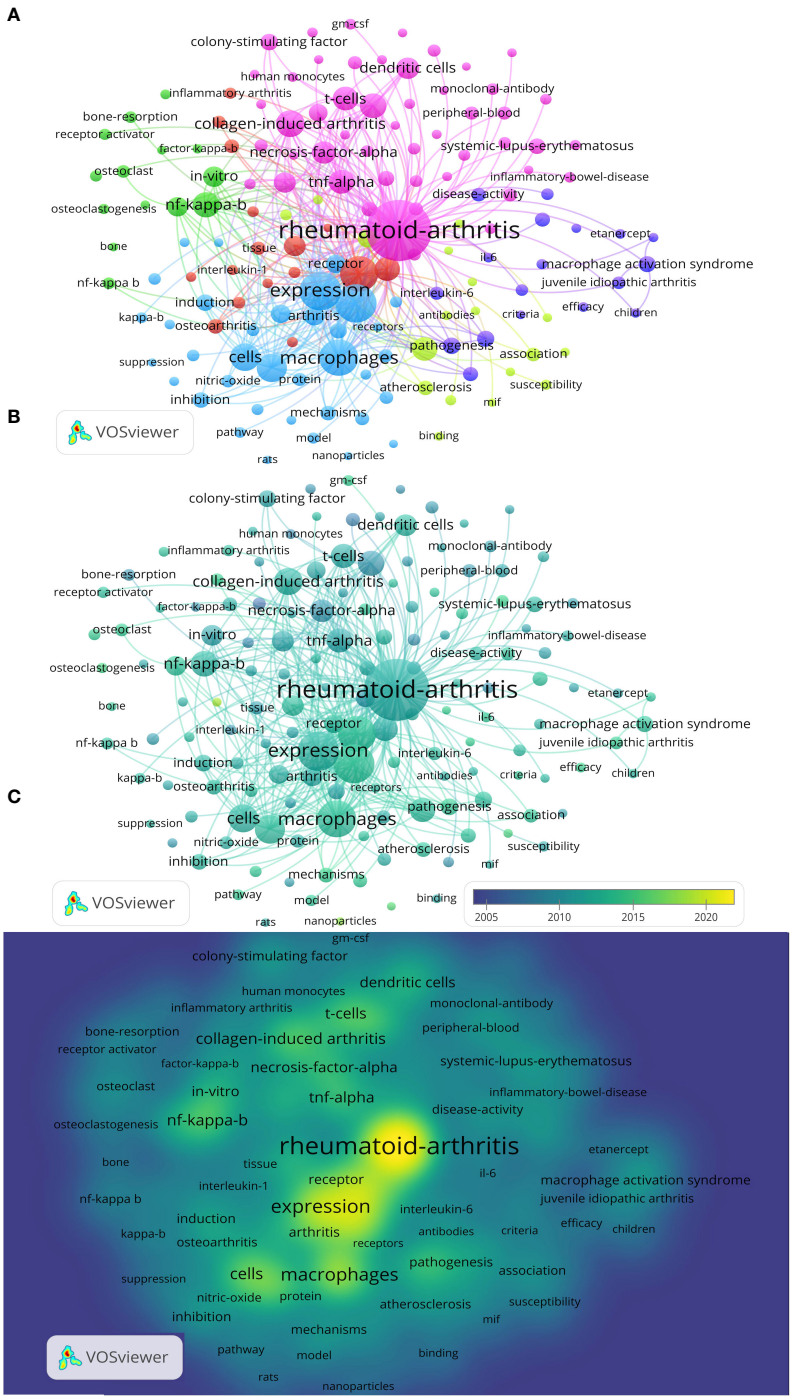

FIGURE 6
Cluster analysis of keywords from 2000-2021. **(A)** Cluster analysis of keywords. **(B)** Evolution of keywords. **(C)** Evolution of keywords frequency.

Currently, there is no complete cure for RA, existing treatments are limited by frequent drug administration, poor penetration across biological barriers, temporary remission, and severe long-term adverse effects (39, 40). In recent years, researchers have also increasingly invested in the field of drug deliver, such as bio-active nanoparticles and macrophage-derived macrovesicle-coated nanoparticle to overcome these obstacles (Figure 7), which can accumulate

TABLE 6 Top keywords of macrophage-derived cytokines and RA-related macrophage functions (occurrences> 60).

Rank	Macrophage-derived cytokines		RA-related macrophage functions	
	Keyword	Occurrences	Keyword	Occurrences
1	tumor-necrosis-factor (TNF-α)	1786	cytokines	825
2	interferon-gamma (IFN-γ)	288	activation	819
3	colony-stimulating factor (CSF)	259	differentiation	344
4	interleukin-6 (IL-6)	244	apoptosis	229
5	nitric oxide (NO)	231	chemokines	199
6	chemokines	199	cytokine production	128
7	growth-factor-beta (GF-β)	170	innate immunity	114
8	migration inhibitory factor (MIF)	165	oxidative stress	114
9	lipopolysaccharide (LPS)	123	immune-response	75
10	endothelial growth-factor	107	polarization	72
11	GM-CSF	107	migration inhibitory factor	70
12	monocyte chemoattractant protein-1(MCP-1)	100	proinflammatory cytokines	68
13	interleukin-1 (IL-1)	94	metabolism	65
14	proinflammatory cytokines	68	signal-transduction	64
15	cyclooxygenase-2 (COX-2)	63	migration	63

in chronic inflamed tissue by increasing drug permeation, increase drug concentration in inflamed joints, and actively binding to receptors overexpressed by cells in inflamed tissues, thereby maximizing efficacy and minimizing systemic adverse effects (41, 42). They are capable of effectively targeting drugs in inflamed joints to treat RA (43, 44).

Limitations

This study is based on bibliometric and visualization analyses, which may provide important evidence to the current research state and overall trend on the field's research academic frontiers. Additionally, this study employs NC as an indicator, which may help comprehend significant nodes in the trend in this field. Nonetheless, this study is bound to have some limitations. To begin with, we counted only English articles and reviews from SCI-Expanded-indexed journals. Second, some details may be omitted due to VOSviewer's inability to analyze the full text of publications. Finally, some newly published excellent papers with low NC may be excluded due to lag. We hope that future studies will look at more databases and get a complete picture of how macrophage-related RA is studied around the world.

Conclusions

This study used bibliometric analysis to summarized the articles on macrophages-related RA. It shed light on evolution of publications and their citations of macrophages-related RA over the last 22 years. The number of articles on macrophages-related RA were increasing. Clinical studies or clinical guidelines published in high-impact journals received a higher rate of citations in RA. The discovery of new targeted therapy drugs, exploration of therapeutic mechanism and drug delivery will remain the focus of future research. We hope this bibliometric analysis provides a beneficial reference to researchers to better comprehend the current state of macrophage-related RA research from a macro viewpoint.

FIGURE 7
CiteSpace visualization map of top 25 keywords with the strongest citation bursts involved in macrophage-related RA.

Data availability statement

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

Author contributions

YLX and ZXC conceived and designed the experiments. YLX and JLH performed the experiments. YLX and ZMZ wrote the paper. YLX and ZMZ contributed equally to this work. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary Material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.903771/full#supplementary-material

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