TARGETING LEUKOCYTE TRAFFICKING: INSIGHTS AND FUTURE DIRECTIONS

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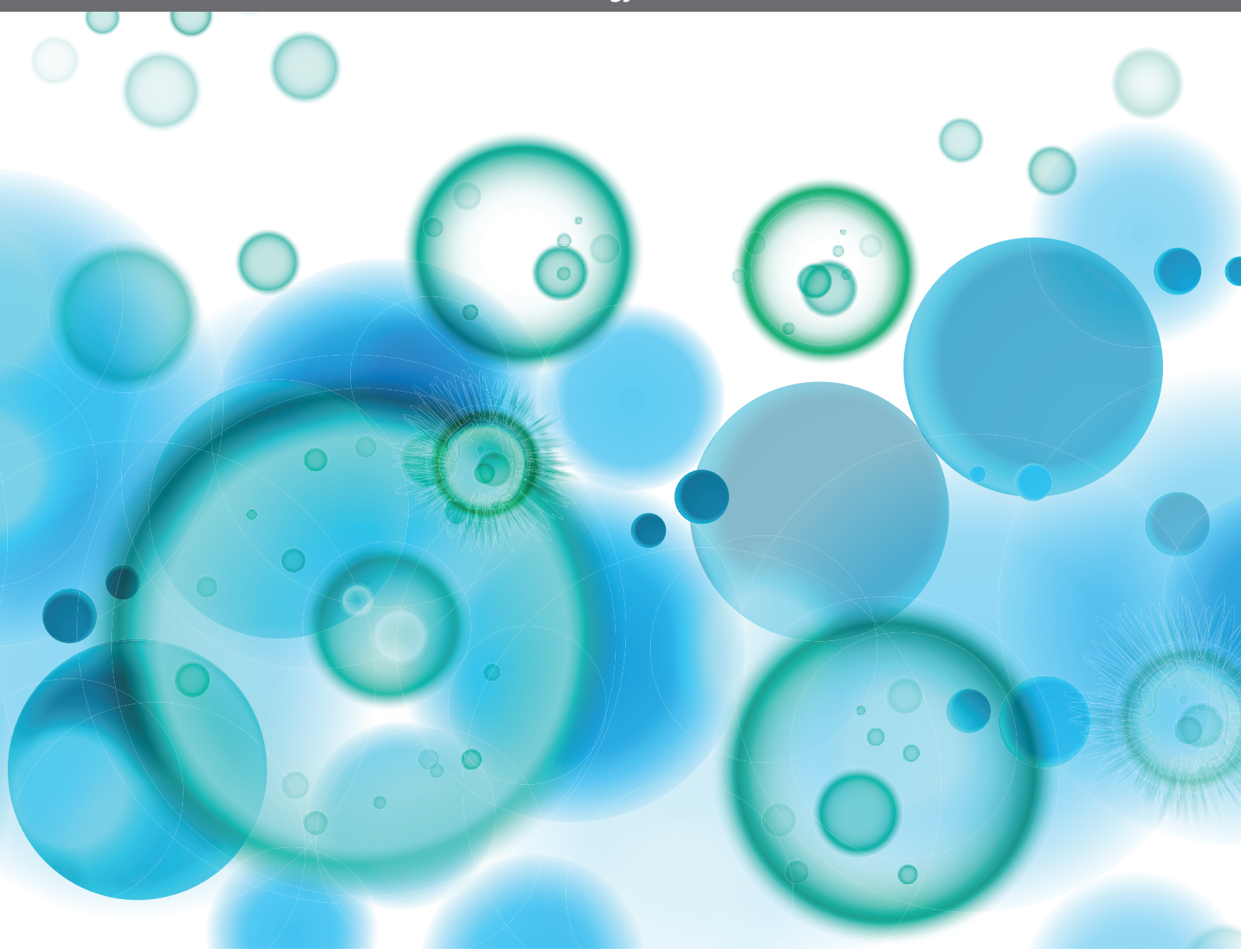

frontiers Research Topics

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TARGETING LEUKOCYTE TRAFFICKING: INSIGHTS AND FUTURE DIRECTIONS

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EDITORIAL
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Editorial: Targeting Leukocyte Trafficking: Insights and Future Directions

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Keywords: adhesion, ageing, chronic inflammatory diseases, Integrins, leukocyte, leukocyte recruitment, migration, T-cells

Editorial on the Research Topic

Targeting Leukocyte Trafficking: Insights and Future Directions

The ability of leukocytes to exit the vasculature (blood, lymphatic) and enter tissue (lymphoid, peripheral) is critical for the health and wellbeing of an organism. These processes form a tightly regulated cascade of sequential steps, each of which educates the migrating leukocyte. Whilst some believe that our knowledge of the leukocyte adhesion/migration cascade is complete, advances in imaging technologies and genetically modified animals and cell-lines, amongst others, are still revealing new insights to the original cascade described in 1995. For instance, unbiased omics approaches coupled with interactome mapping are expanding our appreciation of the cytoskeletal proteins, signalling events, and phosphorylation patterns coupled to integrin-mediated migration and effector function in leukocytes. Our current understanding of downstream inside-out and outside-in signalling through β_2-integrins in neutrophils and the consequence of phosphoinositide-3-kinase (PI3K) signalling in T-cell trafficking are elegantly reviewed by Bouti, et al. and Johansen, et al., respectively. Moreover, molecules traditionally considered to elicit effector functions are now being directly linked to the regulation of leukocyte migration. Here, myeloperoxidase (MPO) has been reported to negatively regulate neutrophil infiltration during inflammation and its absence significantly enhanced the migration capacity of neutrophils (Rehring, et al.).

Vascular endothelial cells are key gatekeepers of the tissue: with blood endothelial cells (BEC) facilitating leukocyte ingress and lymphatic endothelial cells (LEC) supporting their tissue egress. Renewed interest in glycan-binding proteins has revealed that specific expression patterns of galectin family members on BEC from different tissues or in response to different stimuli (e.g., reviewed by Lightfoot, et al.). Although there is still much to learn about galectins, such differences are likely to have a big impact on leukocyte-BEC interactions and thus leukocyte entry into inflamed tissues. Focusing on tissue egress Tadayon et al., have describe a dual role for Clever-1: (i) acting as a key adhesion molecule to support dendritic cell (DC) migration across LEC; whilst also (ii) negatively regulating the inflammatory phenotype of LEC (Tadayon, et al.). Consequently, LEC Clever-1 educates migrating DC, imparting an immunosuppressive signal that regulates the magnitude of immune response created in the draining lymph nodes.

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Leukocyte migration is unequivocally linked to their ability to mediate their effector functions – whether this is acting as first responders to invading pathogens and/or tissue injury in peripheral tissues; mounting highly specific antigen driven detection systems in lymphoid tissues; or regulating the degree and duration of such responses. Deficiencies in genes responsible for actin regulation, which is crucial for cellular movement, are associated with primary immunodeficiencies, including megakaryoblastic leukaemia (MKL1) (Sprenkeler, et al.). MKL1 deficiency is characterised by severe defects in neutrophil adhesion and migration, as well as the potential for myeloid cells to spread and form filipodia to sense chemotactic and haptotactic gradients (reviewed by Sprenkeler, et al.). Additionally, changes in adhesion molecule expression can provide novel insights into microbial responses, for example SARS-CoV-2 infection significantly reduced the expression of the gut-homing integrin, $\alpha_4\beta_7$, on circulating blood lymphocytes, indicative of priming in the GALT and potentially the preferential recruitment of these cells from blood into peripheral tissues (Müller, et al.).

Conversely, the inappropriate accumulation and activation of leukocytes underpins pathology and tissue damage in numerous immune-mediated inflammatory diseases, such as rheumatoid arthritis (Wright, et al.); chronic obstructive pulmonary disease (Belchamber, et al.); and inflammatory bowel disease (Wiendl, et al.). RNAseq analysis has revealed that rheumatoid synovial fluid fundamentally alters the migratory potential of healthy neutrophils, contributing to their accumulation, retention and the maintenance of an activated phenotype in the rheumatoid joint (Wright, et al.). Similarly, dyslipidaemia associated with atherosclerosis, in particular low levels of ApoA1, were correlated with elevated expression of integrin and chemokine receptors on circulating monocytes (Patel, et al.), thus supporting aberrant monocyte extravasation and potentially driving plaque progression.

Our ability to target leukocyte migration offers the opportunity to influence leukocyte trafficking profiles based on the underlying defect. For example, promoting recruitment in individuals where responses have diminished, e.g., in the primary immunodeficiencies (Sprenkeler, et al.), the elderly (Nathan, et al.) or in cancer (Bonilha, et al.). Additionally, one could envisage clinically taking advantage of the diurnal rhythmicity of leukocyte trafficking to maximise these responses (Nathan, et al.). Alternatively, treatment options might include limiting the trafficking of pathogenic effector leukocytes at sites of chronic inflammation, combined with agents that promote the migration of regulatory leukocytes to trigger resolution of inflammation and tissue repair. These concepts are elegantly highlighted by Bonilha et al., where antagonising junctional adhesion molecule-A (JAM-A) may control leukocyte trafficking in preclinical models of IMIDs, whilst the same treatment appears to enhance immune responses against cancerous cells in tumors (Bonilha, et al.).

Caution is required when designing any therapy that targets leukocyte adhesion and migration: Many of the molecules involved in these processes are ubiquitously expressed and utilised by all leukocyte's subtypes in their movement around the body. One of the major therapeutic success stories is vedolizumab, the anti-$\alpha_4\beta_7$-integrin antibody, used in IBD, which takes advantage of the unique adhesion molecule profile in the gut to specifically prevent the trafficking of gut-homing T-cells into the mucosal tissue (reviewed by Wiendl, et al.). Opportunistic infection and poor vaccine responses are common side-effects to many of the biologics in development or that have been developed, which is not surprising when considering the clinical attributes of primary immunodeficiencies. Moreover, attempts to target leukocyte accumulation by modulating their recruitment with chemokine receptor inhibitors has been unsuccessful in a number of contexts, including COPD (Belchamber, et al.), due to promiscuity and redundancy within this family. Further directions should focus on interactions that are unique to "disease" settings to minimise off-target effects, but also attempt to identify shared pathways hijacked across multiple conditions that might benefit from early, preclinical interventions.

The Frontiers Research Topic "*Targeting Leukocyte Trafficking: Insights and Future Directions*" includes a combination of reviews and original papers highlighting advances in our fundamental understanding of the molecular processes regulating leukocyte trafficking in health, during a day or over a lifespan, and in response to infection and disease. Each article further explores how this knowledge has and is being translated into novel pharmacological tools, providing current and future perspectives in this arena. The editorial team would like to thank all those who contributed to this collection.

AUTHOR CONTRIBUTIONS

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

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MINI REVIEW
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Targeting Opposing Immunological Roles of the Junctional Adhesion Molecule-A in Autoimmunity and Cancer

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The junctional adhesion molecule-A (JAM-A) is a cell surface adhesion molecule expressed on platelets, epithelial cells, endothelial cells and leukocytes (e. g. monocytes and dendritic cells). JAM-A plays a relevant role in leukocyte trafficking and its therapeutic potential has been studied in several pathological conditions due to its capacity to induce leukocyte migration out of inflamed sites or infiltration into tumor sites. However, disruption of JAM-A pathways may worsen clinical pathology in some cases. As such, the effects of JAM-A manipulation on modulating immune responses in the context of different diseases must be better understood. In this mini-review, we discuss the potential of JAM-A as a therapeutic target, summarizing findings from studies manipulating JAM-A in the context of inflammatory diseases (e.g. autoimmune diseases) and cancer and highlighting described mechanisms.

Keywords: autoimmune diseases, cell adhesion, epithelial barrier, F11 receptor, inflammation, junctional adhesion molecule-A

INTRODUCTION

The junctional adhesion molecule-A (JAM-A), also called junctional adhesion molecule-1 (JAM-1), is a member of the immunoglobulin superfamily and received its first denomination as F11 receptor (F11R), a molecule expressed on the surface of human platelets (1). Only a few years later, this molecule was detected in epithelial and endothelial intercellular tight junctions (2). JAM-A interactions with extracellular ligands assure firm cell-cell adhesion, playing important roles in endothelial cell migration (3–5) and proliferation (6) and epithelial cell barrier functions (7). Changes in barrier integrity caused by disruption of JAM-A pathways can indirectly modulate immune responses by modifying migratory patterns of antigen presenting cells (APC). However, JAM-A is also expressed by immune cells themselves, such as monocytes (2) and dendritic cells (DC) (8, 9). Thus, immune mediated processes are also likely to be directly influenced by JAM-A activity. As our understanding of the contributions of JAM-A to inflammatory process increases so does interest in the therapeutic value in targeting of JAM-A. In this regard, we summarize findings on the disruption of JAM-A pathways in murine models of inflammation and cancer, highlighting possible opposing immunological mechanisms mainly involving leukocyte migration.

JAM-A BIOLOGY

JAM-A is a transmembrane glycoprotein composed of a cytoplasmatic tail and an extra-cellular region consisting of a membrane-distal domain (D1) and a membrane-proximal domain (D2) (**Figure 1**). JAM-A homophilic binding (JAM-A-JAM-A) was first suggested to occur in epithelial-epithelial and endothelial-endothelial cell interactions, due to the presence of JAM-A in tight junctions (2). The detection of JAM-A dimers on the surface of JAM-A-transfected epithelial cells strengthened this hypothesis (11), being confirmed by protein- (11, 12) and cell-based (12, 13) assays. Homophilic ligation is mediated by JAM-A D1 domain and allows the formation of JAM-A dimers (14). Dimerization occurs by interaction of JAM-A monomers within the surface of the same cell (*cis* interactions). JAM-A dimers can then interact with other JAM-A dimers (*trans* interactions) to bridge epithelial cells in tight junctions (see **Figure 1C**).

Besides homophilic binding, JAM-A can also undergo heterophilic *cis* or *trans* interactions with other extracellular ligands (**Table 1**). JAM-A can bind to the β_2 chain of the lymphocyte function-associated antigen 1 (LFA-1), but not of the macrophage-1 antigen (MAC-1), *via* the JAM-A D2 domain (16, 18). The D2 domain was also found to be important in stabilizing homophilic interactions (13). However, the ligation of JAM-A D2 domain to LFA-1 was found to reduce the dynamic strength of JAM-A homophilic interactions in assays with immobilized JAM-A and Jurkat T cells (13), a human cell line expressing considerable levels of JAM-A (16). Immunoprecipitation assays revealed formation of JAM-A and β_3 integrin (CD61) aggregates in endothelial cell lysates (3), suggesting a direct interaction between these molecules. However, this interaction was later found to be dependent on CD9, as absence of this tetraspanin inhibited JAM-A coimmunoprecipitation with β_3 integrin (3). Other immunoprecipitation assays suggest that JAM-A can also bind to α_{IIb} integrin (CD41) (15), as well as the β_2 chain of LFA-1, as previously mentioned. However, CD9 is also known to interact with some of these integrins (19), which may point a dependence on this tetraspanin for the interaction of these integrins with JAM-A. In addition to these ligands, JAM-A can bind to another member of its family, the junctional adhesion molecule-B (JAM-B) (17), and can also work as a receptor for a few strains of murine and human viruses (14, 20–22). While JAM-A *cis* interactions are mainly responsible for cell signaling processes that may indirectly regulate cell migration (4, 15, 23), *trans* interactions directly mediate cell-cell adhesion and are essential for JAM-A role in leukocyte migration.

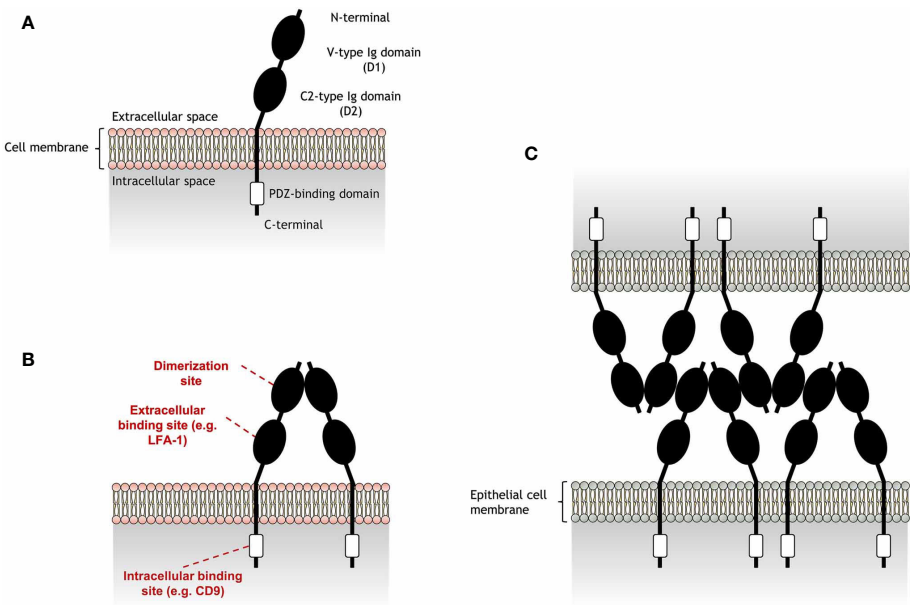

FIGURE 1 | Schematic representation of junctional adhesion molecule-A (JAM-A) structure and homophilic adhesion. **(A)** JAM-A is composed by an extracellular region and a cytoplasmatic tail, connected by a transmembrane portion. JAM-A extracellular portion is formed by a membrane-distal V-type Ig-like domain (D1), which also includes a N-terminal portion, and a membrane-proximal C2-type Ig domain (D2). These Ig-like domains are linked by a short connector region. JAM-A cytoplasmatic tail contains a PDZ-binding motif that is linked to a C-terminal portion. JAM-A can be expressed on the cell surface as monomers, but can also interact with JAM-A monomers in *cis* interactions to form **(B)** homodimers in a process called dimerization. While the D1 domain is the region in which JAM-A monomers interact with each other, the D2 region can bind to other extracellular ligands in *trans* interactions [e.g. lymphocyte function-associated antigen 1 (LFA-1)]. In addition, the PDZ-binding domain allows JAM-A to bind to scaffold proteins, such as CD9, responsible to link JAM-A to β_3 integrin. **(C)** Endothelial JAM-A homophilic adhesion consists of dimers on opposing cells forming contacts *via* the D1 domain and allows strong cell-cell adhesion in tight junctions for the formation of a molecular barrier that ensures the homeostasis of epithelial barrier integrity. JAM-A *trans* cell-cell arrangement was reported by Kostrewa et al. (10).

TABLE 1 | Junctional adhesion molecule-A (JAM-A) described extracellular ligands.

Protein	Family	Expression	Detection of interaction	Ligation conformation	References
$\alpha_{IIb}\beta_3$ (CD41/CD61)	Integrin	Platelets	Human platelet lysates by co- immunoprecipitation (co-IP)	*Cis*	(15)
$\alpha_L\beta_2$ (LFA-1 CD11a/CD18),	Integrin	Lymphocytes, dendritic cells, NK cells, neutrophils	JAM-A-transfected Chinese hamster ovary (CHO) cells with immobilized LFA-1 by adhesion assay	*Trans*	(16)
CD9	Tetraspanin	Platelets, endothelial cells, lymphocytes, monocytes, macrophages, dendritic cells, eosinophils, basophils, mast cells	HeLa cell lysates by co-IP Human umbilical vein endothelial cell (HUVEC); lysates by co-IP	*Cis*	(4)
JAM-A (JAM-1 F11R),	Junctional adhesion molecule	Platelets, epithelial cells, endothelial cells, monocytes, dendritic cells	Human platelet with immobilized JAM-A by adhesion assay; JAM-A-transfected CHO cells with immobilized JAM-A by adhesion assay	*Cis/trans*	(12, 13)
JAM-B (JAM-2 VE-JAM),	Junctional adhesion molecule	Endothelial cells	JAM-A- and JAM-B-transfected HEK293T cells by co-IP and proximity ligation assay	*Cis/trans*	(17)

JAM-A ROLE IN LEUKOCYTE TRAFFICKING

The first report of a role for JAM-A in leukocyte migration comes from Martìn-Padura et al. (2), in which JAM-A blockade was found to inhibit spontaneous and chemokine-induced monocyte transmigration through endothelial cell monolayers. Tumor necrosis factor alpha (TNF-α) and interferon-gamma (IFN-γ)-stimulated endothelial cells redistribute JAM-A on their surface from intercellular junctions to the apical region of the cells, increasing leukocyte adhesion to the inflamed endothelia (16, 24). As such, several other studies have shown endothelial and epithelial cell expression of JAM-A contributes to leukocyte trafficking.

JAM-A blockade was found to inhibit chemokine stimulated neutrophil transmigration across TNF-α and IFN-γ-inflamed endothelium, but not neutrophil arrest (16). Treatment with anti-JAM-A antibody or JAM-A-Fc fusion protein (human JAM-A extracellular domains fused to the Fc portion of human IgG1) inhibited transendothelial migration of human memory (CD45RO+) CD4+ T cells triggered by para-methoxyamphetamine (PMA) and CXC chemokine ligand 12a (CXCL12a) in a LFA-1-depedent manner (16, 25). Splenic CD11c+CD11b-B220+ plasmacytoid DCs (pDC) treated with a different anti-JAM-A mAb also had diminished transmigration through layers of high endothelial venule (HEV) cells, whereas cell adhesion remained unaffected (26). *In vivo*, treatment with anti-JAM-A mAb decreased leukocyte transendothelial migration through cremaster venules induced by IL-1β, but not by chemoattractants, leukotriene B4 (LTB4) or platelet-activating factor (PAF) (27). This same effect was found in JAM-A deficient mice in comparison with wild-type (WT) mice. This reduction in leukocyte transendothelial migration through venules was found to be mediated by JAM-A expressed on endothelial cells, but not on leukocytes.

Although studies in monocytes, neutrophils, memory T cells and pDCs suggest that JAM-A blockade could have an inhibitory effect on leukocyte migration, conventional DCs (cDC) show an increased propensity to cross lymphatic endothelial cells when JAM-A activity is lacking (8). While monocytes, neutrophils (2, 8, 9) and pDCs (26) express very low or undetectable levels of JAM-A, cDCs express high levels of this transmembrane protein (8, 9). Bone marrow-derived DCs (BMDC) treated with anti-JAM-A mAb, or originating from JAM-A knockout (KO) mice, showed increased random motility *in vitro* compared to their respective controls (8). These JAM-A-deficient BMDCs expressed similar levels of maturation markers (CD80 and CD86), surface molecules related to DC migration (CD11a, CD11b, CD11c, CD62L, JAM-B, and JAM-C) and antigen uptake capacity in comparison to WT BMDCs, suggesting that JAM-A may not participate in early stages of DC differentiation and maturation. However, JAM-A deficient BMDCs display increased transmigration across monolayers of lymphatic endothelial cells, but unaffected transmigration across microvascular endothelial cells. *In vivo*, BMDCs from JAM-A deficient mice had increased migration from FITC-painted skin to the LN. The selective transmigration of JAM-A-deleted DCs suggests that DC JAM-A plays a role in homing steps during DC trafficking to the LN from tissues but may not participate in DC tissue infiltration from the vasculature. When analyzing the role of endothelial JAM-A in DC migration, another study reported higher *in vitro* transmigration of JAM-A-expressing BMDCs through layers of JAM-A-deficient lung endothelial cells in comparison with endothelial cells from JAM-A$^{-/-}$ mice reconstituted with full-length JAM-A complementary DNA (cDNA) (28). These studies indicate that while DC JAM-A participates in DC trafficking through the lymphatics, endothelial JAM-A play a dominant role in DC arrest and migration functions through the vascular endothelium. The effects of JAM-A manipulation to the migration of DCs and

other immune cells have raised interest in potential JAM-A-targeted therapies for inflammatory diseases and cancer.

JAM-A MANIPULATION IN DISEASE MODELS OF INFLAMMATION

Due to its expression in different cell types (platelets, endothelial cells and leukocytes), its capacity to modulate cell adhesion and migration and its upregulation in inflamed tissues, JAM-A has been studied as a therapeutic target in a number of disease models.

Skin Inflammation

Inflammatory skin sites are characterized by proliferation of dermal cells, dilated blood vessels and accumulation of immune cells (29). In a model of skin inflammation, systemic treatment with an antagonistic anti-JAM-A mAb inhibited leukocyte infiltration upon chemokine administration in subcutaneous air pouches (2). However, in a model of ear skin inflammation driven in an antigen specific manner, JAM-A-deficient mice displayed enhanced contact hypersensitivity (8). The increase in ear swelling in this T-cell mediated model was linked to activity of JAM-A-deficient DCs, most likely arising through their augmented migration to LNs and enhancing activation of antigen specific T cells.

Vascular Disease

Adhesion molecules have been implicated in vascular wall integrity and play an important role in vascular diseases (30). JAM-A ability to control platelet aggregation (12) have triggered particular interest in this molecule in studies of vascular diseases such as atherosclerosis, a cardiovascular disease caused by the development of plaques on artery walls restricting or blocking blood flow to specific organs or regions of the body (31). In humans, elevated JAM-A gene expression was described in atherosclerotic plaques compared with artery segments of normal patients (32, 33) and in unstable carotid plaques in comparison to stable plaques (33). In addition, JAM-A has been found to be required for human platelet adhesion to inflamed endothelial cells (34).

In models of ischemia-reperfusion (I/R) injury, both JAM-A genetic depletion and blockade with anti-JAM-A mAb suppressed leukocyte infiltration in response to cremaster muscle (27) and liver I/R injury (35). However, no protective effects on microvascular and hepatocellular injury were reported, evidenced by unaffected levels of sinusoidal perfusion and liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST). The use of mice with JAM-A selective depletion in endothelial cells identified the requirement of endothelial JAM-A in the reduction of T cell, but not neutrophil, transmigration. This dependence on endothelial JAM-A was also reported in a model of heart I/R injury, in which JAM-A absence reduced leukocyte infiltration in the myocardium by affecting transendothelial migration (36).

In mouse models of atherosclerosis, increased *f11r* messenger ribonucleic acid (mRNA) expression was found in both early atherosclerotic endothelium of carotid arteries (25) and advanced atherosclerotic plaques (32). A JAM-A-Fc fusion protein was used in an *ex vivo* perfusion model to demonstrate JAM-A role in early atherosclerosis. Treatment with this fusion protein inhibited the arrest of human monocytes and memory CD4+ T cells in murine atherosclerotic endothelium under blockade of very late antigen 4 (VLA-4), an intercellular adhesion molecule 1 (ICAM-1) ligand (25). These results show the capacity of JAM-A targeting to modulate the recruitment of leukocytes to atherosclerotic endothelium by blocking pathways of competitor ligands.

In vitro, treatment with a JAM-A antagonistic peptide capable of blocking homophilic binding (peptide 4D), decreased platelet adhesion to inflamed endothelial cells, whereas agonistic reagents promoted platelet aggregation (12, 37). This antagonistic peptide also decreased plaque number and size and increased survival of mice in an atherosclerosis model in comparison with mice treated with a scrambled peptide (38), due to a reduction on platelet adhesion to the inflamed endothelium. However, in a murine model of thrombosis, lack of JAM-A resulted in increased thrombus formation due to enhanced aggregation of platelets (39). JAM-A$^{-/-}$ mice also had shorter tail-bleeding time and faster vessel occlusion. Although no studies have evaluated the capacity of the antagonistic peptide to block other JAM-A pathways, these studies suggest that, while JAM-A homophilic binding by platelets may be a useful target for avoiding formation of atheroma, other JAM-A pathways may have platelet stimulatory functions and promote blood coagulation that can obstruct and impair the homeostasis of the circulatory system.

Inflammatory Bowel Disease

The intestinal mucosa forms an important barrier against potential hostile microorganisms and other foreign antigens. The permeability of the intestinal epithelium is mediated by tight junctions, formed of molecular components, such as adhesion molecules, that link intestinal epithelial cells and exert a control on the passage of environmental molecules. Intestinal barrier defects have been associated with chronic mucosal inflammation and inflammatory bowel diseases (IBDs) (40). A few studies have addressed the role of JAM-A in the regulation of intestinal barrier functions during IBDs. Although JAM-A deficient mice displayed higher susceptibility for development of dextran sulfate sodium (DSS)-induced colitis in comparison with WT mice (41), JAM-A depletion increased epithelial proliferation, which resulted in faster repair of epithelial defects, evidenced by a reduction in damaged colonic mucosa. The increase in clinical disease was attributed to a higher permeability in the colonic mucosa of JAM-A$^{-/-}$ mice, supposedly enhancing its vulnerability to acute DSS-induced colitis (42). To address if this increased susceptibility was mediated by the higher number of B and T cells found in the lamina propria of JAM-A$^{-/-}$ mice, JAM-A-deficient mice crossed to recombination activating gene 1 (RAG1) knockout animals lacking B and T cell development were also investigated (43). Lack of adaptive immunity in JAM-A-deficient RAG1$^{-/-}$ mice promoted an even higher susceptibility for development of DSS-

induced colitis in comparison with JAM-A$^{-/-}$ animals. However, administration of antibiotics in JAM-A-deficient RAG1$^{-/-}$ mice reduced the susceptibility to disease development. These results suggest an important compensatory role of the adaptive immune system in JAM-A$^{-/-}$ mice that limits bacterial-driven colitis. CD4+ T cells played a relevant role in this compensatory mechanism, as depletion of these cells, but not CD8+ T cells, enhanced susceptibility to colitis. In addition, increased gene and protein expression of transforming growth factor beta 1 (TGF-β1) in colonic tissues pointed to a possible role of this growth factor in the compensatory mechanism. Treatment with anti-TGF-β1 mAb decreased body weight and increased disease activity index scores in JAM-A-deficient mice in comparison to anti-TGF-β1-treated WT mice and to isotype-treated JAM-A$^{-/-}$ mice, demonstrating a protective role of TGF-β1 in JAM-A-deficient mice from developing a more severe acute colonic inflammation.

Mice with selective loss of JAM-A in myelomonocytic cells, progenitors that can differentiate into monocytes, macrophages and subtypes of conventional DCs (44), were used to investigate the role of JAM-A expression on these cells during intestinal inflammation. These mice showed no difference in neutrophil recruitment into the peritoneum and macrophage chemokine production in response to lipopolysaccharides (LPS) or zymosan in comparison with control mice (45). However, these parameters were significantly reduced in global JAM-A$^{-/-}$ mice stimulated by these inflammatory mediators. Mice with selective loss of JAM-A on intestinal epithelial cells demonstrated increased intestinal permeability and reduced peritoneal neutrophil migration and macrophage chemokine production. These findings suggest that JAM-A expression in the epithelium is fundamental for JAM-A -mediated intestinal inflammation.

Exposure of human intestinal epithelial cells to cytokines (TNF-α, IFN-γ, IL-22, or IL-17A) was found to induce JAM-A cytoplasmatic tail tyrosine Y280 phosphorylation (46). Elevated levels of this phosphorylated form were also detected in the colonic mucosa of DSS-induced colitis mice and humans with ulcerative colitis in comparison to healthy mucosa. Further studies will investigate the role of JAM-A cytoplasmatic tail phosphorylation as a regulator of epithelial intestinal barrier functions during bowel inflammation.

Neurological Disorders

Disruption of the blood-brain barrier of the central nervous system (CNS) leads to leukocyte accumulation in the cerebrospinal fluid, a main component of brain disorders such as meningitis (47) and multiple sclerosis (MS) (48). Surface JAM-A is expressed in brain vessels of MS patients (49) and in vessels of the brain parenchyma and choroid plexus of mice subject to cytokine-induced meningitis (50). In this model of cytokine-induced meningitis, intravenous treatment with anti-JAM-A mAb attenuated meningeal inflammation (50). This therapeutic effect was attributed to inhibition of monocyte and neutrophil accumulation in the cerebrospinal fluid and neutrophil infiltration into the brain parenchyma arising from reduced blood–brain barrier permeability. Further pre-clinical models that aim to discover the effects of JAM-A blockade/promotion on other immunological components involved in neurological disorders may identify JAM-A as a potential target for disease treatment.

Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic systemic inflammatory autoimmune disease that mainly affects the joints, with an essential participation of the adaptive immune system in its induction phase (51). A breach of self-tolerance led by failure in immune regulatory mechanisms results in activation of immune responses (52) and production of several autoantibodies, against host proteins such as cartilage components, nuclear proteins, stress proteins and citrullinated proteins (53). At the center of these immune responses are leukocytes, that accumulate in the joint, contributing to chronic joint pain (54). Evidence for of JAM-A gene upregulation in human hypoxic cells (55, 56) suggest that the hypoxia present in the synovial tissue of RA patients (57–59) could potentially lead to increased JAM-A gene and protein expression on cells from inflamed joints of these patients. Recently, a study described increased expression of *f11r* mRNA on PMBCs of RA patients (60). Nevertheless, this upregulation could have been driven by the systemic inflammation itself, as healthy individuals were used as control. In addition, JAM-A is expressed in inflamed joints of K/BxN mice (61), animals expressing transgenic T cell receptor (TCR) and major histocompatibility complex class II (MHCII) that develop severe inflammatory arthritis (62).

In a model of RA in which autoantibodies from arthritogenic K/BxN drive inflammation and tissue destruction in serum-recipient mice, treatment with anti-JAM-A mAb delayed the disease onset and partially ameliorated overall disease (61). Similar effects were found on mice treated with anti-ICAM-1 mAb, and a more prominent amelioration was found on treatment with mAb targeting the alpha chain of LFA-1, but not the beta chain. ICAM-1 is another ligand for LFA-1 (63), which may suggest that the therapeutic effects in these arthritic mice treated with both anti-JAM-A and anti-ICAM-1 mAbs could have been caused by disruption of LFA-1 pathways. However, more studies are required to investigate the mechanisms involved in JAM-A blockade in models of autoimmune diseases, which may be related to a modulation on immune cells trafficking, as proposed in **Figure 2**.

JAM-A MANIPULATION IN CANCER MODELS

The six hallmarks of cancer are sustained proliferative signaling, resistance to cell death, replicative immortality, induction of angiogenesis, evasion of growth suppressors and activation of invasion and metastasis mechanisms (64). Interventions that aim to enhance immune responses to tumor cells, such as increasing tumor-derived antigen presentation to T cells are desirable for cancer treatment. Evidence from the literature suggest that circulating, soluble JAM-A could be used as a biomarker for the detection of some types of cancer, such as multiple myeloma (65) and head and neck squamous cell carcinoma (66). Although

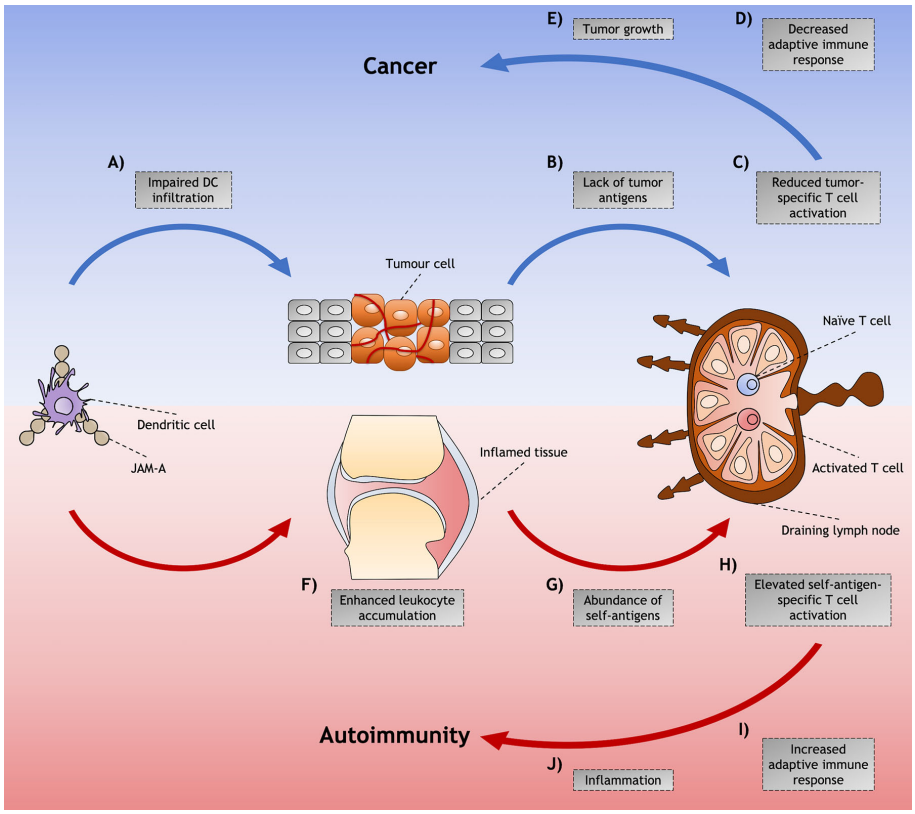

FIGURE 2 | Proposed model for junctional adhesion molecule-A (JAM-A)-mediated dendritic cell (DC) role in cancer and autoimmunity. Although JAM-A may contribute to disease through diverse cell types and signaling processes, mechanisms of DC trafficking mediated by JAM-A and their indirect effects in the immune response might play an important role in the induction and/or promotion of cancer and autoimmunity. Among these is the **(A)** impaired infiltration of DCs that is found in tumors that have achieved the hallmarks of cancer. **(B)** This diminished presence of DCs impairs the availability of tumor antigens that would be presented to tumor-specific T cells in the LNs, **(C)** leading to reduced T cell activation and **(D)** decreased adaptive immune responses against the abnormal cells. **(E)** The lack of immunity against these cells allows tumor to grow and cancer to be set. **(F)** On the other hand, in autoimmunity, JAM-A may assist the accumulation of immune cells in the inflamed tissues by enhancing leukocyte adhesion to the inflamed endothelia. **(G)** The accumulation of DCs increases the availability of self-antigen not only in peripheral tissues - such as arthritic joints of RA patients, where antigen presentation can occur - but also in the draining LN, by DCs carrying self-antigen captured in the affected tissue. **(H)** This abundance of self-antigens possibly leads to the activation of a higher proportion of self-antigen specific T cells. **(I)** The induction of adaptive immune responses against self-antigens leads to **(J)** inflammation and tissue destruction characteristic of autoimmune diseases.

few studies show that JAM-A can be downregulated in tumor sites of metastatic breast (67) and anaplastic thyroid carcinoma tissues (68), high levels of JAM-A protein expression in tumor tissues have been correlated with poor prognosis in breast cancer (69) and nasopharyngeal carcinoma (70) patients.

In vitro studies using human triple negative breast cancer or thyroid carcinoma cell lines showed increased tumor cell proliferation and migration under JAM-A gene silencing (67, 68). Transfection of a JAM-A plasmid to these cell lines impaired transendothelial migration and colony formation. However, treatment with a JAM-A antagonistic peptide inhibited transmigration of breast cancer cells through inflamed endothelium (71), an important step in the initiation of metastasis formation. Additionally, JAM-A inhibition increased apoptosis and decreased proliferation of multiple myeloma cells *in vitro* and inhibited the progression of this type of cancer *in vivo* (65). In RIP1Tag2 mice, which express the SV40 T-antigen under the rat insulin promoter resulting in carcinogenesis of β cells in pancreatic islets, JAM-A absence decreased growth and aggressiveness of tumors in comparison with control mice (28). An increased tumor specific immune response resulted in diminished angiogenesis and increased apoptosis that was attributed to a more efficient infiltration of JAM-A-deficient DCs, but not macrophages, into tumor sites. This mechanism was dependent on both CD4+ and CD8+ T cells. In a model of multifocal mammary adenocarcinoma, mice lacking JAM-A developed smaller mammary tumors than control mice (72). Disruption of JAM-A pathways increased cell apoptosis and decreased proliferation, however, no differences in angiogenesis or infiltration of DCs or macrophages were found. In addition, treatment with anti-JAM-A mAb suppressed progression of malignant tumors by impairing cell proliferation and angiogenesis in malignant myeloma xenograft murine models (73). Altogether, these studies suggest that although JAM-A expressed by tumor cells may have a protective role on progression of cancer, JAM-A antagonism could enhance

immune response against these abnormal cells by possibly facilitating leukocyte infiltration into tumors and DC egress to LNs (see **Figure 2**). Nevertheless, in a recent study, JAM-A-deficient female mice developed a more aggressive phenotype of brain tumor in comparison with WT females and JAM-A–deficient and WT males (74). This study highlights the impact of other factors, such as sex difference, in the therapeutic effects that JAM-A blockade might promote in the development of tumors and demonstrates the complexity and challenges involved in the potential development of a JAM-A-targeted drug for cancer treatment.

CONCLUSION AND FUTURE PERSPECTIVES

Upon recruitment of its extracellular ligands, JAM-A ensures firm adhesion of leukocytes and platelets to the endothelia and plays a definitive role in immune cell transmigration. Antagonistic JAM-A targeting in preclinical models of inflammatory diseases show promising results for controlling inflammation caused by leukocyte or platelet accumulation. In addition, in some cancer models, manipulation of JAM-A pathways achieved agonism of immune responses by affecting leukocyte infiltration into tumors, controlling the progression of the disease. However, the possible disruption in multiple pathways (endothelial cell-endothelial cell, leukocyte-endothelial cell, leukocyte-leukocyte, platelet-endothelial cell, platelet-leukocyte and platelet-platelet interactions) caused by JAM-A targeting suggests precaution in the interpretation of results from preclinical model studies. As such, studies with cell-selective JAM-A disruption that aim to distinguish pathway-specific effects in different pathological conditions will further our understanding of JAM-A role in autoimmunity and cancer and may highlight JAM-A as a potential therapeutic target for human disease.

AUTHOR CONTRIBUTIONS

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

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Conflict of Interest: Author RB was employed by the company Antibody Analytics Ltd.

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

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Rheumatoid Arthritis Synovial Fluid Neutrophils Drive Inflammation Through Production of Chemokines, Reactive Oxygen Species, and Neutrophil Extracellular Traps

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Rheumatoid arthritis (RA) is a chronic inflammatory disorder affecting synovial joints. Neutrophils are believed to play an important role in both the initiation and progression of RA, and large numbers of activated neutrophils are found within both synovial fluid (SF) and synovial tissue from RA joints. In this study we analyzed paired blood and SF neutrophils from patients with severe, active RA (DAS28>5.1, n=3) using RNA-seq. 772 genes were significantly different between blood and SF neutrophils. IPA analysis predicted that SF neutrophils had increased expression of chemokines and ROS production, delayed apoptosis, and activation of signaling cascades regulating the production of NETs. This activated phenotype was confirmed experimentally by incubating healthy control neutrophils in cell-free RA SF, which was able to delay apoptosis and induce ROS production in both unprimed and TNFα primed neutrophils ($p<0.05$). RA SF significantly increased neutrophil migration through 3μM transwell chambers ($p<0.05$) and also increased production of NETs by healthy control neutrophils ($p<0.001$), including exposure of myeloperoxidase (MPO) and citrullinated histone-H3-positive DNA NETs. IPA analysis predicted NET production was mediated by signaling networks including AKT, RAF1, SRC, and NF-κB. Our results expand the understanding of the molecular changes that take place in the neutrophil transcriptome during migration into inflamed joints in RA, and the altered phenotype in RA SF neutrophils. Specifically, RA SF neutrophils lose their migratory properties, residing within the joint to generate signals that promote joint damage, as well as inflammation *via* recruitment and activation of both innate and adaptive immune cells. We propose that this activated SF neutrophil phenotype contributes to the chronic inflammation and progressive damage to cartilage and bone observed in patients with RA.

Keywords: neutrophils, rheumatoid arthritis, synovial fluid, transcriptomics, neutrophil extracellular traps

INTRODUCTION

Rheumatoid arthritis (RA) is an inflammatory disorder characterized by systemic inflammation, including swelling and pain in synovial joints. Left untreated, uncontrolled inflammation will destroy joints, causing deformity and disability. Many studies have shown that blood neutrophils have an aberrant, activated phenotype in RA, characterized by increased production of ROS and cytokines, and delayed apoptosis (1–4). As well as having an activated phenotype in peripheral blood, activated neutrophils are found at high numbers in both synovial joints and tissues of patients with RA (5–7). Their presence within RA joints is accompanied by high levels of neutrophil granule proteins in synovial fluid, including myeloperoxidase (MPO), cathepsin G, proteinase 3, elastase, and lactoferrin (1, 8–12). These granule proteins contribute to the pathogenesis of RA through proteolytic cleavage and activation of proteins (including cytokines and chemokines), cleavage of soluble receptors to initiate trans-signaling (such as the IL-6 receptor) and degradation of cartilage (e.g. cleavage of collagen fibers) (1, 13–16). *Ex vivo* synovial fluid (SF) neutrophils have an altered phenotype compared to paired blood neutrophils. They produce higher levels of superoxide (O_2^-) and contain phosphorylated $p47^{phox}$, indicating assembly and activation of the NADPH oxidase (NOX2) *in vivo* (17). They also express the high-affinity FcγR1 receptor (CD64) and MHC Class II proteins (18–21). SF neutrophil lysates also have lower levels of granule proteins such as MPO, indicating they have undergone degranulation within the synovial joint (8). Animal studies and human case studies of early RA suggest an important role for neutrophils in the initiation of synovial inflammation in RA joints (5, 7, 22), possibly through the release of granule enzymes and production of VEGF, both of which enable fibroblast adhesion and growth of the inflammatory synovial pannus (23, 24). A key role for exposure of citrullinated antigens on neutrophil extracellular traps (NETs) has been proposed in the initiation of auto-immunity and development of anti-citrullinated peptide auto-antibodies (ACPA) in RA (1, 25). NET products are detectable in both RA serum and synovial fluid (12, 26) and NETs have been observed in RA synovial biopsy tissues by staining for CD15, elastase, MPO and citrullinated (cit) histone H3 (25, 27). It was recently shown that up to 70% of newly-diagnosed RA patients have auto-antibodies in their serum that recognize NET components (ANETA) (28).

We have extensively studied neutrophil phenotype in RA and shown that RA neutrophils have activated NF-κB signaling leading to delayed apoptosis (2). Additionally, we have shown that the neutrophil transcriptome is altered in RA compared to healthy controls (29) and that gene expression in RA patients pre-TNFi therapy can be used to stratify patients based on response or non-response to therapy (30). Whilst several studies have analyzed SF neutrophil functions, to our knowledge none have measured the transcriptome of RA SF neutrophils compared to paired blood neutrophils. In this study we first used RNA-seq to describe the changes that take place when blood neutrophils migrate into RA joints and then validated our bioinformatics predictions using healthy control neutrophils incubated in RA SF *in vitro*. We show that RA SF neutrophils have an altered phenotype, including decreased expression of genes associated with extravasation and migration, increased expression of chemokines, FcγR1 and MHC II, decreased apoptosis and increased ROS and NET production. We propose that these altered properties enable SF neutrophils to regulate inflammatory events by attracting and activating innate and adaptive immune cells, including other neutrophils, T cells, NK cells, monocytes, macrophages, and dendritic cells within diseased RA joints (31–33).

METHODS

Ethics Statement and Patient Selection

This study was approved by the University of Liverpool Committee on Research Ethics for healthy controls, and NRES Committee North West (Greater Manchester West, UK) for RA patients. All participants gave written, informed consent in accordance with the declaration of Helsinki. All patients fulfilled the ACR 2010 criteria for the diagnosis of RA (34).

Blood and Synovial Fluid Collection

Peripheral blood was drawn into heparinized vacutainers from healthy controls and RA patients. Synovial fluid was aspirated from the knee joint of RA patients (n=3) approximately 1 month prior to the start of the TNFi therapy Etanercept. All patients had a DAS28 score greater than 5.1 at the time of sample collection. Patients had a mean age of 59 years and an average disease duration of 20.6 years; two patients were female (**Supplementary Table 1**). Synovial fluid was decanted into universal tubes containing 50 µl heparin immediately upon collection and neutrophils were isolated within 1 h. Aliquots of un-diluted synovial fluid were centrifuged at 2000 g for 5 min to remove leukocytes. Cell-free synovial fluid was decanted and frozen at -20°C.

Neutrophil Isolation

Neutrophils were isolated from heparinized peripheral blood using Polymorphprep (Axis Shield) as previously described (2, 35). Erythrocytes were lysed using hypotonic lysis with ammonium chloride buffer. Synovial fluid was passed through gauze prior to dilution 1:1 with PBS. Neutrophils were isolated from diluted synovial fluid using Ficoll-Paque (GE Healthcare). Neutrophil purity from both peripheral blood and synovial fluid isolations was >97% as assessed by cytospin (**Supplementary Figure 1**). Neutrophils accounted for an average of 85.7% leukocytes in whole synovial fluid as assessed by cytospin (**Supplementary Figure 1**, **Supplementary Table 1**). Following isolation, neutrophils were resuspended in RPMI 1640 media (Life Technologies) containing L-glutamine (2 mM) and Hepes (25 mM) at a concentration of $5x10^6$/ml.

RNA Extraction

RNA was isolated from 10^7 neutrophils using Trizol-chloroform (Life Technologies), precipitated in isopropanol and cleaned

using the RNeasy kit (Qiagen) including a DNase digestion step. RNA was snap-frozen in liquid nitrogen and stored at -80°C. Total RNA concentration and integrity were assessed using the Agilent 2100 Bioanalyzer RNA Nano chip. RNA integrity (RIN) was ≥ 7.0.

RNA Sequencing

Total RNA was enriched for mRNA using poly-A selection. Standard Illumina protocols were used to generate 50 base pair single-end read libraries. Libraries were sequenced on the Illumina HiSeq 2000 platform. Reads were mapped to the human genome (hg38) using TopHat v2.0.4 (36) and gene expression (RPKM) values were calculated using Cufflinks v2.0.2 (37). A minimum RPKM threshold of expression of ≥ 0.3 was applied to the data in order to minimize the risk of including false positives against discarding true positives from the dataset (35, 38). Statistical analysis of differential gene expression was carried out using Cuffdiff (a program within Cufflinks) applying a 5% false discovery rate (FDR) for significance. The raw sequencing data reported in this manuscript have been deposited in the NCBI Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE154474.

Bioinformatics Analysis

Bioinformatics analysis was carried out using IPA (Ingenuity® Systems, www.ingenuity.com), as previously described, applying a 1.5-fold change in gene expression cut-off and a Benjamini-Hochberg correction to p-values for canonical pathway and upstream regulator analysis. Heatmaps were produced using MeV (39) with Euclidean clustering and average linkage. Multivariate partial least squares-discriminant analysis (PLS-DA) was performed on count data, generated using Rsubread (40) using mixOmics v6.10.9 (41) working in R v 3.6.3. Modular analysis of gene expression was carried out using the CRAN package *tmod* (42) and based on the modular framework for classifying blood genomics studies proposed by Chaussabel and colleagues (43).

Measurement of Apoptosis

Neutrophils ($5x10^5$/ml) were incubated at 37°C in 5% CO_2 in RPMI 1640 (+Hepes, +L-glutamine) for 18 h in the absence or presence of 10% AB serum (Sigma) or 10% cell-free synovial fluid. Following incubation, $2.5x10^4$ cells were diluted in 50 µl of HBSS (Life Technologies) containing 0.5 µl Annexin V-FITC (Life Technologies), and incubated in the dark at room temperature for 15 min. The total volume was then made up to 500 µl with HBSS containing propidium-iodide (PI, 1 µg/ml, Sigma Aldrich) before analysis by flow cytometry (>5,000 events analyzed) using a Guava EasyCyte flow cytometer.

Measurement of ROS Production

Neutrophils ($5x10^6$/ml) were incubated with or without TNFα (10 ng/ml, Merck) for 20 min. Following priming, neutrophils ($2.5x10^5$) were diluted in HBSS in the presence of 10µM luminol (Sigma Aldrich). ROS production was stimulated by either f-Met-Leu-Phe (fMLP, 10^{-6}M, Sigma Aldrich) or 25% cell-free synovial fluid. Luminol-enhanced chemiluminescence was measured continuously for 60 min on a Tecan plate reader.

Chemotaxis Assay

The chemotaxis assay was carried out in 24-well tissue culture plates (coated with 12 mg/ml poly-hema to prevent cell adhesion) using hanging chamber inserts with a 3 µM porous membrane (Merck), as previously described (44). Media containing fMLP (10^{-8}M), interleukin-8 (100 ng/ml, Sigma) or 25% (v/v) synovial fluid was added to the bottom chamber. Neutrophils (10^6/ml) were added to the top chamber and incubated for 90 min at 37°C and 5% CO_2. The number of migrated cells after 90 min incubation was measured using a Coulter Counter Multisizer-3 (Beckman Coulter).

Assay for Neutrophil Extracellular Trap Production

Neutrophils were seeded (at $2x10^5$ cells/500 µl) in RPMI media plus 2% AB serum in a 24-well plate containing poly-L-lysine coated coverslips, as previously described (45). Cells were allowed to adhere for 1 h prior to stimulation with phorbol 12-myristate 13-acetate (PMA, 50 nM, Sigma), A23187 (3.8 µM, Sigma) or 10% RA SF. Cells were incubated for a further 4 h to allow for NET production. Cells adhered to coverslips were fixed with 4% paraformaldehyde prior to immunofluorescent staining. Briefly, coverslips were removed from the plate and washed with PBS, permeabilized with 0.05% Tween 20 in TBS, fixed with TBS (2% BSA) and then stained for 30 min on drops of TBS (2% BSA) on parafilm stretched across a clean 24-well plate. Primary antibodies used were mouse anti-myeloperoxidase (1:1000, Abcam) and rabbit anti-citrullinated histone H3 (1:250, Abcam). Coverslips were washed 3 times with TBS prior to secondary antibody staining (anti-rabbit AlexaFluor488, 1:2000, anti-mouse AlexaFluor647, Life Technologies) in TBS (+2% BSA) for 30 min. Coverslips were washed prior to staining with DAPI (1 µg/ml, Sigma). Slides were imaged on an Epifluorescence microscope (Zeiss) using a 40X objective. Images were analyzed using ImageJ (46) and are presented with equal color balance.

Measurement of Cytokines in Synovial Fluid

The concentrations of granulocyte-colony stimulating factor (GCSF), interferon-γ (IFNG), interleukin-1β (IL1B), interleukin-6 (IL6), and tumor necrosis factor-α (TNF) in RA SF were measured as part of a previous study (47). Briefly, cell-free SF was pre-incubated with blocking buffer (40% mouse serum, 20% goat serum, 20% rabbit serum, all Sigma) for 30 min at room temperature and then centrifuged at 14,000g for 10 min to remove immune complexes. The multiplex cytokine assay (Biosource) was carried out in a 96-well filter plate as per manufacturer's instructions and as previously described (47). The plate was read in a Bio-Plex Suspension Array system, model Luminex 100 (Bio-Rad), and cytokine concentrations calculated by reference to a standard curve.

Statistical Analysis

Statistical analysis of experimental data was performed using a Student's t-test or ANOVA in GraphPad Prism (v5) as stated in the text.

RESULTS

Transcriptomic Alterations From Blood to Synovial Fluid

In order to determine the changes in RA neutrophil transcriptome induced by migration from peripheral blood to inflamed, synovial joints, we isolated paired blood and synovial fluid neutrophils from patients with RA (n=3) with active disease (DAS28>5.1) prior to commencement of biologic therapy with the TNF inhibitor etanercept. Transcriptome sequencing (RNAseq) identified 772 genes that were significantly different between peripheral blood neutrophils and synovial fluid (SF) neutrophils (FDR<0.05). Of these, 412 genes were significantly higher in blood neutrophils and 347 were higher in synovial fluid neutrophils. Multivariate partial least squares-discriminant analysis (PLS-DA) on the expressed neutrophil transcriptome (~14,000 genes) modelled the difference between PB and SF neutrophil transcriptomes with a ROC AUC = 1 (**Figure 1**, p<0.05, two components). Modular analysis of the transcriptional networks active in RA blood and SF neutrophils revealed activation of gene modules regulating localization, cytoskeletal remodeling, and cell signaling (ATP, small GTPases, phosphatidylinositol) in both blood and SF neutrophils. However, signaling in response to MHC, toll-like receptors, inflammasomes and type I interferons was higher in SF neutrophils (**Figure 1B**). Modules corresponding to integrin signaling and recruitment of neutrophils were higher in blood neutrophils compared to SF.

In order to determine the signaling pathways most altered in RA neutrophils following migration from peripheral blood to synovial joints, we carried out bioinformatics analysis using Ingenuity Pathway Analysis (IPA) applying a 1.5-fold change in gene expression cut-off. The pathways most significantly up-regulated in SF neutrophils were: Antigen Presentation Pathway ($p=10^{-8}$), Role of NFAT in the regulation of the immune response ($p=10^{-7}$), and Acute phase response signaling ($p=10^{-6}$). EIF2 Signaling ($p=10^{-8}$), STAT3 pathway ($p=10^{-8}$), Leukocyte Extravasation ($p=10^{-7}$) and Granulocyte Adhesion and Diapedesis ($p=10^{-6}$) were the most down-regulated pathways in SF neutrophils (**Figure 2A**). Chemokine signaling was up-regulated in RA SF neutrophils ($p=10^{-4}$). Chemokines up-regulated in RA SF (**Figure 2B**) included those associated with attraction of both innate and adaptive immune cells, including other neutrophils (CXCL8, CXCL1, CXCL2), T cells, monocytes and macrophages, natural killer cells, and dendritic cells (CXCL16, CXCL10, CCL2, CCL3, CCL4) (31–33). Genes which were up- or down-regulated in SF and associated with the Leukocyte Extravasation Signaling pathway are shown in **Figure 2C** ($p=10^{-7}$). HIF-1α signaling was up-regulated ($p=10^{-4}$) in SF neutrophils, in line with the known hypoxic state of the RA SF joint (48), and the HIF1α transcription factor complex was predicted to be activated in RA SF neutrophils ($p=5.8x10^{-12}$).

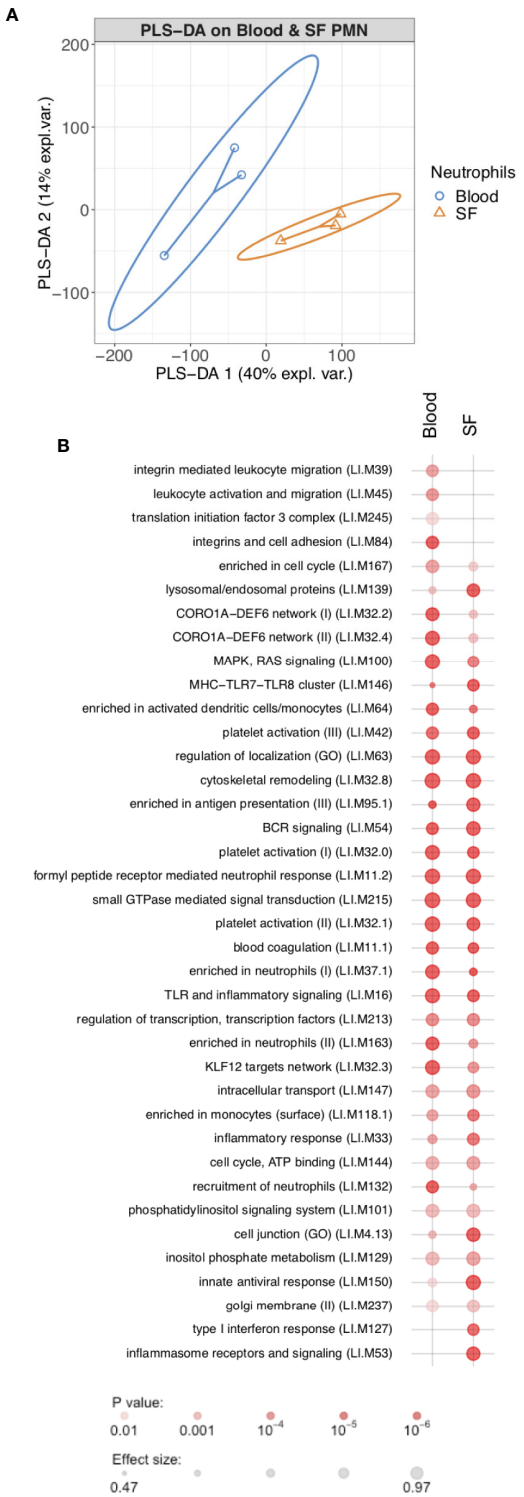

FIGURE 1 | Transcriptomic analysis of rheumatoid arthritis peripheral blood and synovial fluid (SF) neutrophils (PMN). **(A)** PLS-DA modeling of blood and SF neutrophil transcriptomes using mixOmics. Blood and SF neutrophils were discriminated based on two components with an AUC of 1.0 (p<0.05). **(B)** Modular analysis of gene expression networks in blood and SF neutrophils using *tmod* (AUC>0.8).

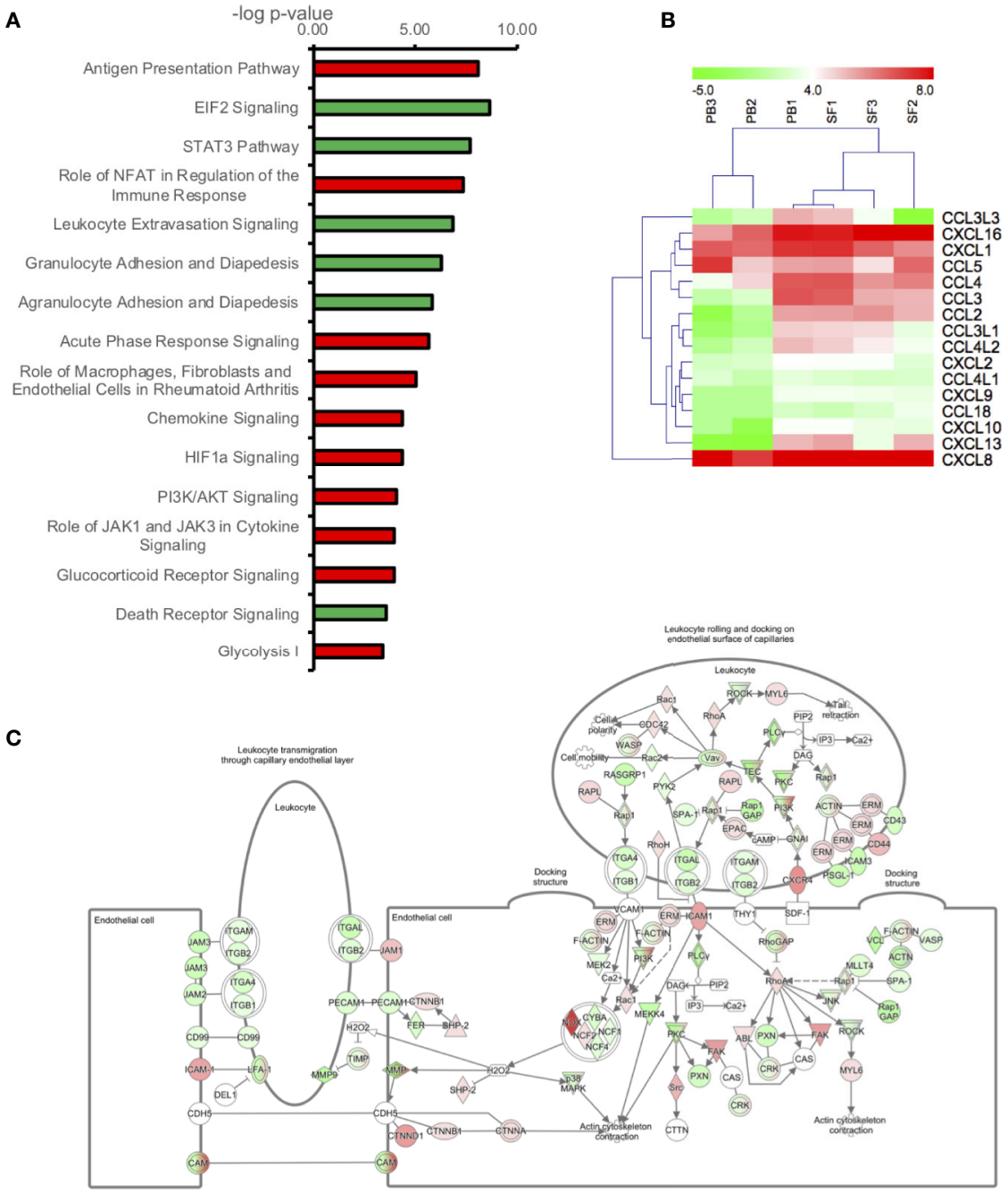

FIGURE 2 | Transcriptomic analysis of genes differently expressed (1.5-fold change) between peripheral blood (PB) and synovial fluid (SF) neutrophils. **(A)** IPA signaling pathways predicted to be up-regulated (red) or down-regulated (green) in SF neutrophils. **(B)** Heatmap showing chemokine gene expression in PB and SF neutrophils. **(C)** Leukocyte extravasation pathway ($p=1.41x10^{-7}$) showing genes up-regulated (red) or down-regulated (green) in SF neutrophils (white = not expressed).

The Antigen Presentation Pathway was predicted to be up-regulated ($p=10^{-8}$), and indeed genes for both MHC Class I (B2M, HLA-A, HLA-B, HLA-C, HLA-G) and MHC Class II (HLA-DMA, HLA-DMB, HLA-DQB1, HLA-DRA, HLA-DRB1, HLA-DRB5) were all significantly higher in SF neutrophils, in line with previous reports (18). Networks of genes regulating the migration and activation of antigen presenting cells were predicted to be up-regulated in SF neutrophils (**Figure 3**). In particular, these networks were predicted to be regulated by ERK (**Figure 3A**), MYD88 (**Figure 3B**), and TICAM1 signaling (**Figure 3C**). We also noted genes encoding the high affinity FcγR1 (FCGR1A,

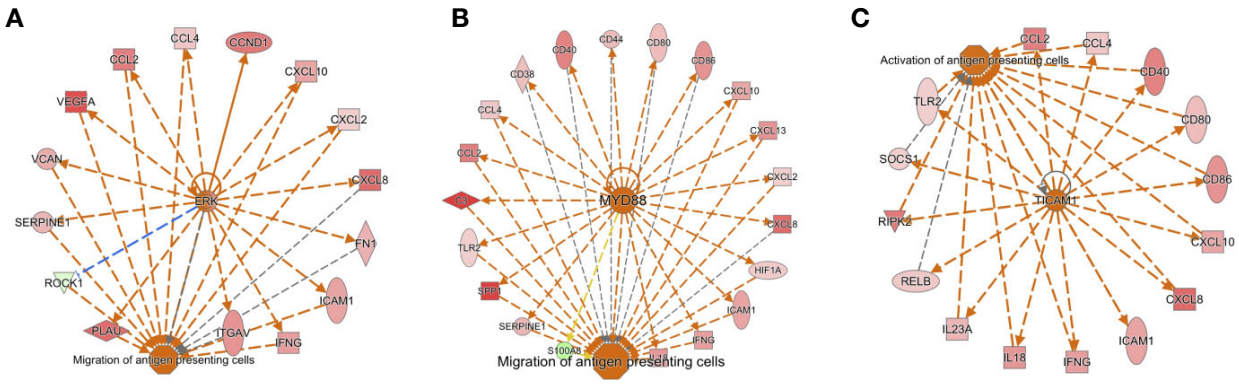

FIGURE 3 | Activation of gene networks in RA SF neutrophils that control migration and activation of antigen presenting cells. These networks are regulated by **(A)** ERK, **(B)** MYD88, and **(C)** TICAM1.

FCGR1B, FCGR1C) were significantly higher in RA SF neutrophils (FDR<0.01), as previously reported (19).

Glycolysis enzymes were highly expressed in RA SF neutrophils (glycolysis pathway activation, $p=10^{-3}$). This is in line with our recent metabolomics analysis of RA SF which identified lower levels of substrates for glycolysis in RA SF; this corresponds to higher levels of anaerobic cellular metabolism under hypoxic conditions (49). Lactate dehydrogenase enzymes were also higher in SF neutrophils (LDHA, LDHB; FDR<0.05). These enzymes catalyze the reduction of pyruvate to lactate producing NAD^+ and enabling continued energy production under the hypoxic cellular state (50).

Effect of Synovial Fluid on Neutrophil Migration, Apoptosis, and ROS Production

In order to validate the predictions of the IPA analysis, we tested the ability of RA SF to alter neutrophil function. We found that 25% v/v RA SF (three separate donors, all RF positive) could significantly induce healthy blood neutrophil migration through a 3 μM transwell membrane (**Figure 4A**, ANOVA p<0.0001) compared to both random migration (Tukey's post-hoc p<0.001), and known chemoattractants fMLP (10^{-8}M, Tukey's post-hoc p<0.001) and interleukin-8 (IL-8, 100 ng/ml, Tukey's post-hoc p<0.001). We also observed a delay in apoptosis (18 h) when we incubated healthy control neutrophils in 10% cell-free SF (**Figure 4B**). This delay in apoptosis was significant for all RA SF compared to untreated (media only) neutrophils (p<0.05). However, when the RA SF was compared to 10% AB serum, the delay in apoptosis was only significant for patient SF1 (p<0.05). Apoptosis was not significantly altered by migration into synovial fluid through a transwell membrane (3 μM, data not shown). IPA analysis indicated that this delay in apoptosis was *via* the up-regulation of the NFκB transcription factor complex ($p=2.9x10^{-8}$) in response to activation of TNF receptors 1 and 2 (TNFR1 signaling $p=10^{-2}$; TNFR2 signaling $p=10^{-3}$) in RA SF neutrophils (**Figure 4C**).

We next used IPA to predict which cytokines were regulating neutrophil gene expression in RA SF. The major cytokines predicted were interferon-gamma (IFNγ), TNFα, interleukin-1β (IL-1β), interleukin-6 (IL-6), and granulocyte-colony stimulating factor (G-CSF) (**Figure 5A**, $p<10^{-15}$). The levels of these cytokines had previously been measured in the donor RA SF as part of a parallel study (**Figure 5B**) (47). Evidence of RA SF neutrophil activation by cytokines can be demonstrated by measuring the respiratory burst in cytokine-primed and unprimed RA blood and SF neutrophils using luminol-enhanced chemiluminescence. When unprimed blood neutrophils were activated with fMLP, little or no ROS production was observed; this was greatly enhanced by priming for 20 min with TNFα (**Figure 5C**). However, unprimed RA SF neutrophils produced around 2-fold greater ROS compared to unprimed RA blood neutrophils, indicating the SF neutrophils have been primed *in vivo* by synovial cytokines (**Figure 5C**). Production of reactive oxygen species by RA SF neutrophils was predicted by IPA canonical pathway analysis ($p=10^{-3}$, **Supplementary Figure 2**).

IPA analysis also predicted that RA SF neutrophils had been activated by immunoglobulins (p<0.01, **Supplementary Figure 3**); this is likely to be immune complexes such as rheumatoid factor (RF) and/or anti-citrullinated protein antibodies (ACPA). The presence of immune complexes in RA SF can be demonstrated using luminol-enhanced chemiluminescence which detects ROS produced by cytokine-primed or unprimed neutrophils in response to the addition of RA SF (20). In our experiments, soluble immune complexes present in RA SF (25% v/v) activated a rapid respiratory burst in cytokine-primed neutrophils (but not unprimed neutrophils) (**Figure 5D**) whereas insoluble immune complexes present in RA SF (25% v/v) activated a slower and more sustained respiratory burst in both cytokine-primed and unprimed neutrophils (**Figure 5E**).

Effect of Synovial Fluid on NET Production

NETs are implicated in the pathology of RA by the exposure of antigenic proteins such as citrullinated histones (1, 25). Whilst the exact signaling mechanisms regulating NET production in have yet to be fully elucidated, several signaling pathways that contribute to NET production have been proposed, including: Raf-MEK-ERK, RIPK1-RIPK3-MLKL, AKT, p38-MAPK and

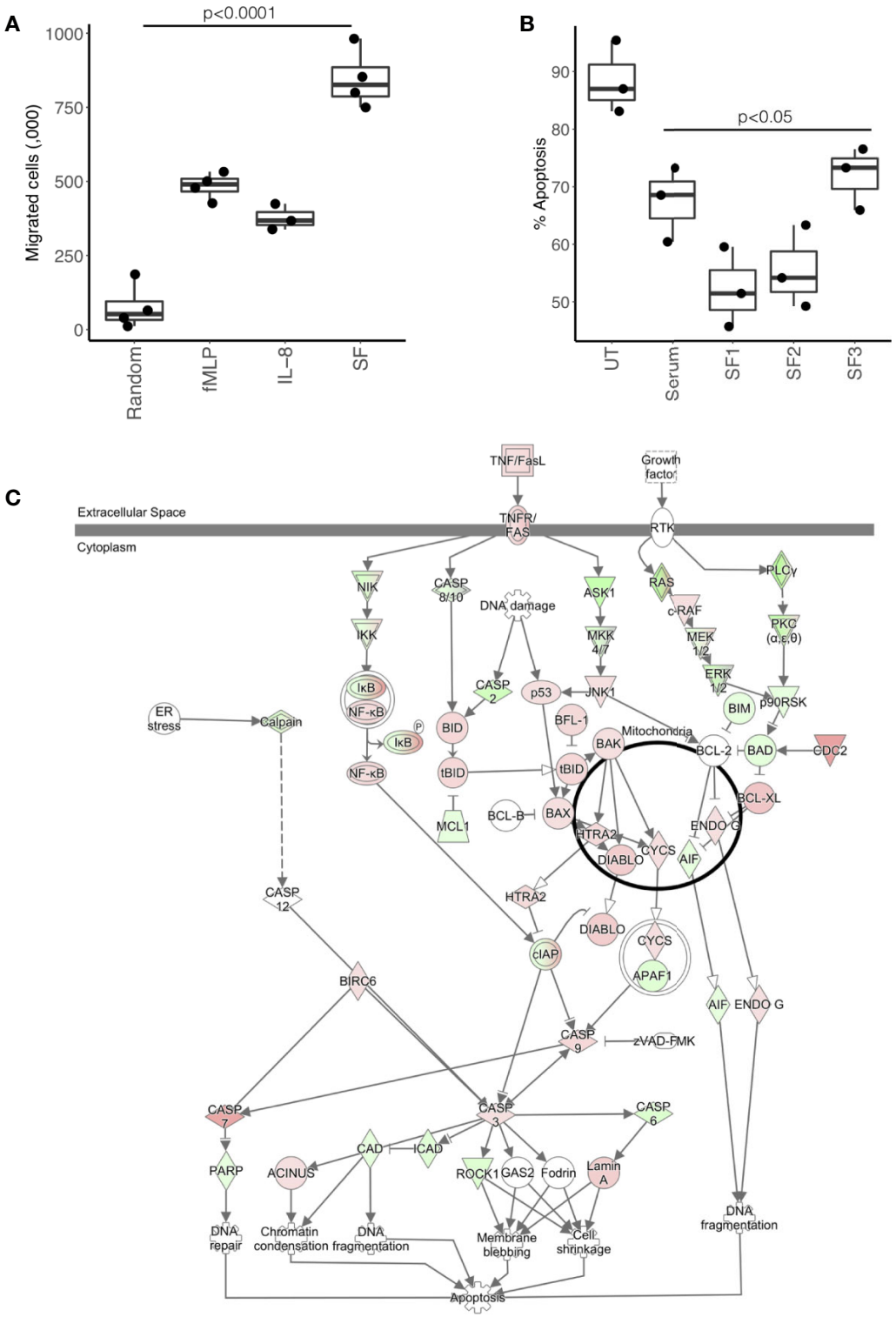

FIGURE 4 | Effect of RA SF on neutrophil migration and apoptosis. **(A)** Neutrophil chemotaxis was significantly increased towards fMLP, interleukin-8 (IL-8) and 10% RA SF ($p < 0.0001$). **(B)** Neutrophil apoptosis was significantly delayed by RA SF over 18 h ($p < 0.05$). **(C)** IPA apoptosis signaling pathway showing up-regulation (red) and down-regulation (green) of genes associated with regulation of apoptosis in SF neutrophils (white = not expressed).

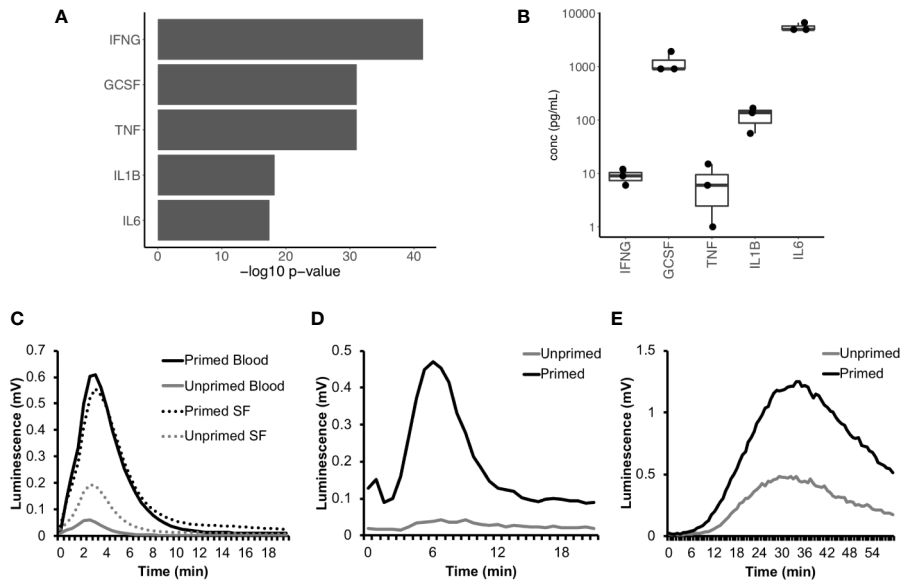

FIGURE 5 | Cytokines in RA SF prime the neutrophil respiratory burst. **(A)** IPA predicted up-stream cytokine regulators of gene expression in RA SF neutrophils (IFNG, interferon-γ; GCSF, granulocyte colony stimulating factor; TNF, tumor necrosis factor α; IL1B, interleukin 1β; IL6, interleukin 6). **(B)** Levels of cytokines measured in RA SF from which the neutrophils sequenced by RNA-seq were isolated. **(C)** ROS production by RA blood and SF neutrophils (with and without cytokine priming) to fMLP. RA SF (25% v/v) also activates ROS production in healthy neutrophils. Stimulation of neutrophils with RA SF containing soluble immune complexes **(D)** activates ROS production in cytokine primed neutrophils only, whereas RA SF containing insoluble immune complexes **(E)** activates ROS production in both primed and unprimed neutrophils.

cSrc (51–54). Interestingly, all these signaling pathways were predicted by IPA as up-stream regulators of gene expression in RA SF neutrophils ($p<0.01$). Signaling networks regulated by AKT, RAF1, and SRC are shown in **Figure 6A**.

We propose that activation of these kinases in RA SF neutrophils will lead to NET production, and therefore tested the ability of RA SF to activate NET production by healthy control neutrophils. We found that 10% RA SF increased NET production by neutrophils to levels similar to that of positive controls PMA and A23187 as shown by the concentration of DNA in culture supernatants ($p<0.001$), as well as the level of extracellular DNA (DAPI), myeloperoxidase (MPO) and citrullinated histone H3 visible on exposed on immunofluorescently labeled NET structures (**Figure 6B**).

DISCUSSION

In this study we have described for the first time the changes in gene expression that take place in RA neutrophils following migration from peripheral blood into inflamed joints. Using RNA-seq we have revealed an activated neutrophil phenotype in RA SF, characterized by increased expression of chemokines, delayed apoptosis and increased activation of kinases and transcription factors which may be implicated in the production of NETs.

Our experiments showed that RA SF decreased the rate of neutrophil apoptosis, which may be attributed to the high levels of inflammatory cytokines present in SF. We previously measured the levels of 13 cytokines in RA SF and found high levels of IL-1β, IL-1ra, IL-2, IL-4, IL-8, IL-10, IL-17, IFN-γ, G-CSF, GM-CSF, and TNF-α (47). Many of these cytokines have been demonstrated individually to delay neutrophil apoptosis *in vivo*, including G-CSF, GM-CSF, IL-1β, TNFα, and IFN-γ (55–62), although this is often at concentrations in excess of those found *in vivo*. GM-CSF secreted by synovial fibroblasts in response to IL-17 and TNFα has been shown to delay neutrophil apoptosis (63), however a separate study found that apoptosis delay induced by RA SF was not related to the TNF or GM-CSF content, but did correlate with adenosine (64). Lactoferrin present in RA SF also delays neutrophil apoptosis and may serve as a feed-back anti-apoptotic mechanism for activated neutrophils (10). In a separate study, RA SF was shown to be pro-apoptotic in overnight neutrophil cultures, but this effect was reversed when neutrophils were incubated with SF under conditions of hypoxia which more closely model the RA joint (48). Relatively few apoptotic neutrophils are present in freshly-isolated RA SF (65), and while SF is anti-apoptotic, the question remains as to the fate of SF neutrophils. One possibility is that they undergo efferocytosis by synovial macrophages, or another is that they may reverse migrate from the joint back into circulation, as demonstrated in zebrafish models of inflammation (66). The ability of *ex vivo* RA SF neutrophils to migrate in response to chemoattractants such as IL-8 *in vitro* should be investigated in future experiments.

RA neutrophils contain high intracellular levels of citrullinated proteins, including known auto-antibody targets: cit-actin, cit-histone H1.3, cit-histone H3, cit-vimentin (25, 67).

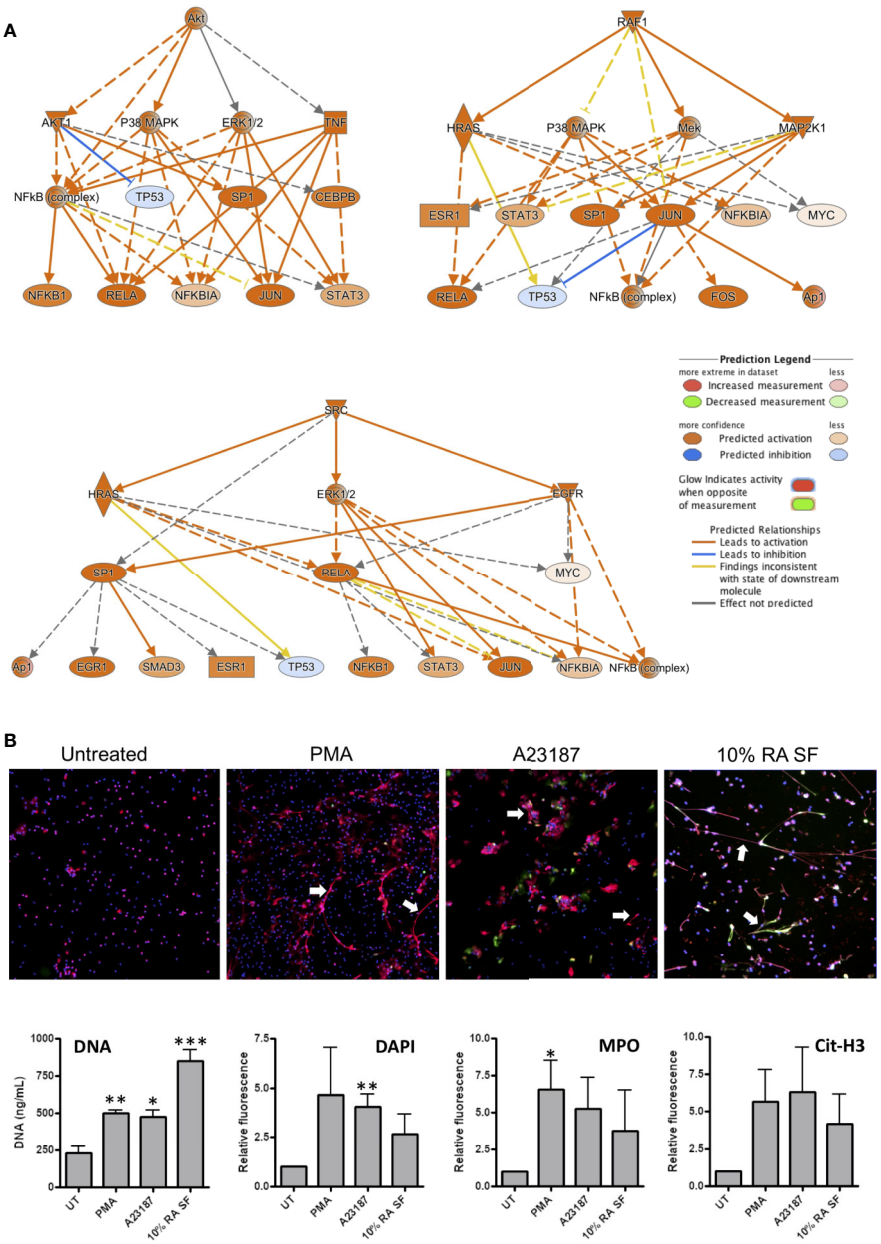

FIGURE 6 | Activation of NET production by RA SF. **(A)** IPA predicted activation of signaling cascades regulated by AKT, RAF1 and SRC which may regulate NET production. **(B)** Neutrophils were incubated for 4 h with PMA (50 nM), A23187 (3.8 uM), or 10% RA SF. NET production was increased by RA SF as measured by quantification of extracellular DNA in culture supernatants, DAPI staining of extracellular NET DNA (blue), and immunofluorescent staining for myeloperoxidase (MPO, red) and citrullinated histone H3 (cit-H3, green) on NET structures (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). White arrows indicate examples of NETs on each panel.

Several of these citrullinated auto-antigens are present on RA NETs (45). The RAGE-TLR9 pathway plays a key role in both the internalization and presentation of citrullinated NET peptides on MHC Class II in fibroblast-like synoviocytes (FLS). This leads to the development of ACPA specific to the citrullinated NET peptides and cartilage damage in mouse models of RA (68). A role for NET-derived elastase in cartilage destruction has also been proposed, whereby elastase contained in NET material disrupts the cartilage matrix and induces the release of PAD2 by fibroblast-like synoviocytes (FLS) leading to citrullination of cartilage fragments (69). These citrullinated cartilage fragments are subsequently internalized and presented by FLS and macrophage to antigen specific T-cells leading to the development of auto-immunity and ACPA in HLA-DRB1*04:01 transgenic mice (69).

Recent proteomic analysis of SF from RA patients and spondyloarthritis (SpA) patients identified elevated levels of many neutrophil proteins in RA SF, including MPO, cathepsin

G, annexin-A1 and NGAL. Interestingly, whilst the concentration of cell-free DNA did not differ between RA and SpA SF, the levels of 21 NET proteins were elevated in RA SF, including histones H2A, H2B and H4, MMP9, elastase, and α-enolase (12). It has previously been shown that levels of ACPA in RA SF correlate with neutrophil numbers and severe disease activity, and that SFs with high ACPA titers induce high levels of ROS and NET production (70). A separate study demonstrated that depletion of ACPA from RA SF inhibits NET production *in vitro* (71). A role for IgA immune complexes in RA SF has also been proposed, with SF rich in IgA inducing NET and ROS production, and release of lactoferrin by healthy control neutrophils in a mechanism that was inhibited by blockade of the FcαRI receptor (72). RA SF also contains high levels of extracellular DNA, concentrations of which correspond to neutrophil counts and PAD activity, and which have been attributed to NETs (27). Our recent proteomics study identified both PAD2 and PAD4 in RA NETs (45). PAD enzymes have also been identified by IHC in synovial biopsies, localized with MPO in necrotic areas of synovial tissue (6) that contain large areas of citrullinated and hypercitrullinated proteins, possibly indicating the presence of NET structures within synovial tissue.

Whilst the exact signaling mechanism(s) regulating NET production remain unclear, at least two types of NET production (NETosis) have been described: NOX2-dependent and NOX2-independent. NOX2-dependent NETosis occurs *via* activation of the NADPH-oxidase (NOX2) and production of ROS. This causes increased intracellular membrane permeability, elastase release into the nucleus and degradation of histones leading to chromatin decondensation and NET release (73). NOX2-independent NETosis does not require the production of ROS by the NADPH oxidase. In this case, mitochondrial ROS combine with increased intracellular calcium levels to activate PADs leading to hypercitrullination of histones, chromatin decondensation and NET release (74, 75). Signaling pathways including Raf-MEK-ERK, RIPK1-RIPK3-MLKL, AKT, p38-MAPK, and cSrc have been identified as some of the drivers of NETosis (51–54). In our study, all of these signaling pathways were predicted to be activated in RA SF neutrophils. Interestingly, all of the kinase activation networks predicted also included downstream activation of NFκB. This important signaling pathway, activated by cytokines such as TNFα and IL-1β, has not yet been implicated in NET production and would be an interesting candidate for future investigation, which should focus on the use of signaling inhibitors to understand the regulation of NET production in RA blood and SF neutrophils. Recent work has shown that the production of NETs by inflammatory neutrophils leads to a forward-feedback loop, where NET debris (DNA:protein complexes) further activates surrounding neutrophils *via* toll-like receptors (TLRs) 7, 8, and 9 (76). RNA:protein complexes have also been identified within human neutrophil NETs. Furthermore, RNA:LL37 complexes from NETs induced NET release and expression of cytokines (TNFα, IL-6, IL-1β, CCL4) *via* TLR8 (77). Interestingly, our informatics prediction of gene networks activating SF neutrophils identified MyD88 and TICAM1 (both downstream components of TLR signaling (78)) as key signaling proteins activated in RA SF neutrophils. This suggests that SF neutrophils may be activated by NET debris within RA SF, something that should be the focus of future investigation.

Our RNA-seq analysis identified increased expression of MHC Class II genes in RA SF neutrophils. We and others have previously shown that whilst healthy control neutrophils do not express MHC Class II, activated neutrophils can express both MHC Class II mRNA and protein and stimulate proliferation of T cells (18, 79–81) RA blood neutrophils express MHC Class II RNA, and RA SF neutrophils expression both RNA and MHC Class II protein, although the latter was contained within intracellular pools which were mobilized to the plasma membrane following overnight incubation (18). While expression of co-stimulator molecules CD80 and CD86 were detected only at very low levels, SF neutrophils were able to stimulate $CD4^+$ T-cell proliferation *via* a mechanism that was inhibited by an anti-MHC Class II antibody (18). Our transcriptomics analysis also detected increased expression of a number of chemokines in RA SF (shown in **Figure 2B**), including CCL3, CCL4, CCL10, CXCL16, CXCL2, and CXCL8. These chemokines play a key role in regulating the inflammatory response in the joint, not only through recruitment of other neutrophils (CXCL1, CXCL2, CXCL8), but also through the recruitment and activation of both innate and adaptive immune cells (31–33) as summarized in **Figure 7**. This increased production of chemokines within the joint, coupled with a down-regulation of adhesion receptors, suggests that RA SF neutrophils become resident within the joint to drive further inflammation through recruitment and activation of other immune cells.

Our findings provide novel insight into the multitude of ways that synovial neutrophils drive chronic inflammation in RA. This raises the possibility that aberrant neutrophil activation may be a target of future therapeutics in this chronic and life-limiting

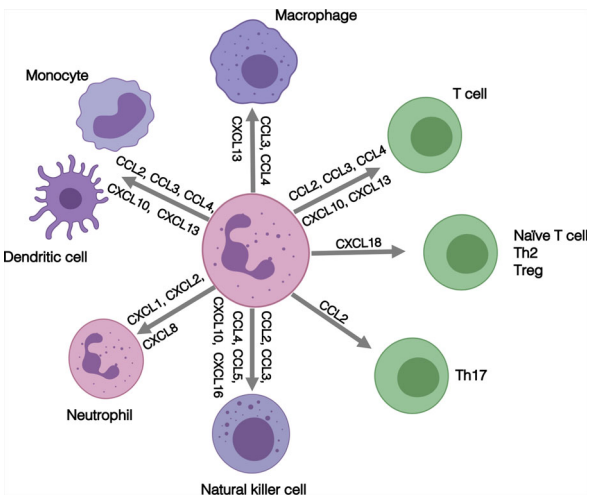

FIGURE 7 | Summary of the role of inflammatory role of chemokines expressed by RA SF neutrophils.

condition. A number of new therapies which directly or indirectly target neutrophil function have been proposed or are in clinical trial (82). Inhibitors of CXCR1/CXCR2, the receptor for CXCL8 (interleukin-8), have been demonstrated to reduce neutrophil adhesion and recruitment to synovial joints in murine models of inflammatory arthritis (83), an effect which was associated with lower TNFα levels and disease activity (84). JAK inhibitors including tofacitinib and baricitinib, which target signaling down-stream of cytokine receptors including IL-6, interferon-α and -γ, and GM-CSF receptors, are clinically effective in treating RA (85, 86) although many patients report transient neutropenia and increased infections. These drugs have been shown to inhibit cytokine signaling in neutrophils, including inhibition of migration and ROS production by RA neutrophils (87). The anti-GM-CSF therapy mavrilimumab was effective in decreasing RA disease activity in a Phase 2b clinical trial, with over 70% of RA patients achieving an ACR20 improvement in the group receiving the highest dosing regime (88). Anti-G-CSF therapy prevents neutrophil migration into joints, suppressing cytokine production and halting the progression of murine arthritis (89). Finally, a number of novel therapeutics which target neutrophil proteases, including MPO and elastase, have been effective in reducing neutrophil-driven inflammation in animal models of inflammatory disease (90) and human respiratory disease (91) respectively. These inhibitor drugs have potential to inhibit NET production and damage to cartilage associated with inflammation in RA synovial joints, and thus may be potential therapeutics for repurposing to target neutrophil-driven RA joint inflammation.

One limitation of our study is the use of an *in vitro* model of RA to validate our transcriptomics data. Whilst the incubation of healthy control neutrophils in RA SF is a widely used model of the disease (48, 70–72), future studies should focus on validating the RA SF neutrophil phenotype using *ex vivo* RA SF neutrophils. There have been a number of reports on the properties of RA SF neutrophils and in general these show an activated phenotype, in terms of surface receptor expression, ROS production and apoptosis (see *Introduction*). We show here for the first time that RA SF neutrophils also have an altered transcriptome compared to blood neutrophils, and that analysis of these transcriptional changes reveals that RA SF neutrophils not only have altered functions but are activated to express a range of chemokines that can exacerbate inflammation by the recruitment of more neutrophils and other leukocytes. While we have only analyzed the transcriptomes of paired blood and SF neutrophils from three patients with active disease, the data between these samples are consistent and entirely in line with other functional changes that have been measured. However, it would be interesting to expand this study to measure transcriptome changes in RA patients with different severity of disease and post-therapy to determine if disease improvement and resolution of inflammation is associated with reversal of these transcriptional signals.

In addition, future experiments should clarify whether the RA SF phenotype reported in our study is specific to inflammation within the RA joint, or whether this neutrophil phenotype is more reflective of a more generalized inflammatory activation. Neutrophils have been shown to be transcriptionally plastic during migration from bone marrow to peripheral blood and into inflammatory tissue. In a mouse model of peritoneal inflammation around a thousand neutrophil genes were differentially regulated during migration between these three compartments (92). Interestingly a number of chemokine and cytokine genes, including CCL3, CXCL1, CXCL2, and CXCL3, were increased in peritoneal neutrophils following migration. This is in line with our observation of an increase in chemokine production following migration to inflamed joints. These transcriptional changes during migration into the inflamed peritoneum were also mirrored by an increase in expression of anti-apoptotic genes (92). This suggests that at least some of the transcriptional changes we observed in our study are associated with the physical movement of neutrophils from peripheral blood into inflamed tissues, possible due to integrin signaling during adhesion and the physical act of squeezing and migrating through the endothelial layer of blood vessels and the basement membrane of connective tissues. This requires further investigation, although is challenging to test in a human model. Further work to examine the phenotype of synovial joint neutrophils from conditions such as gout or reactive arthritis would go some way to answering this question. Finally, further investigation of the signaling pathways regulating apoptosis and the production of NETs by RA SF, and the effect of small molecule inhibitors of intracellular signaling, would potentially identify novel therapeutic targets of NET production that would specifically target neutrophil activation within the RA joint.

In conclusion, we have used RNA-seq and experimental analysis of paired blood and SF RA neutrophils to describe a pro-inflammatory SF neutrophil phenotype which includes delayed apoptosis, production of ROS, and release of NETs. RA SF neutrophils down-regulate adhesion molecules to become resident in the joint and drive inflammation *via* increased production of chemokines that attract and activate both innate and adaptive immune cells. We propose this altered neutrophil phenotype contributes to the pro-inflammatory nature of active RA and explains the role of neutrophils in the pathogenesis of the disease.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of Liverpool Committee on Research Ethics for healthy controls, and NRES Committee North West

(Greater Manchester West, UK) for RA patients. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

HW designed the research, carried out the experiments, analyzed the data, and wrote the manuscript. ML carried out the experiments and analyzed the data. EC carried out the experiments, analyzed the data and revised the manuscript. RM revised the manuscript. SE analyzed the data and revised 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.2020.584116/full#supplementary-material

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REVIEW
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β2 Integrin Signaling Cascade in Neutrophils: More Than a Single Function

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Neutrophils are the most prevalent leukocytes in the human body. They have a pivotal role in the innate immune response against invading bacterial and fungal pathogens, while recent emerging evidence also demonstrates their role in cancer progression and anti-tumor responses. The efficient execution of many neutrophil effector responses requires the presence of β2 integrins, in particular CD11a/CD18 or CD11b/CD18 heterodimers. Although extensively studied at the molecular level, the exact signaling cascades downstream of β2 integrins still remain to be fully elucidated. In this review, we focus mainly on inside-out and outside-in signaling of these two β2 integrin members expressed on neutrophils and describe differences between various neutrophil stimuli with respect to integrin activation, integrin ligand binding, and the pertinent differences between mouse and human studies. Last, we discuss how integrin signaling studies could be used to explore the therapeutic potential of targeting β2 integrins and the intracellular signaling cascade in neutrophils in several, among other, inflammatory conditions in which neutrophil activity should be dampened to mitigate disease.

Keywords: CD11b/CD18 integrin, β2 integrin signaling, neutrophils, neutrophil function, therapeutic targets

Abbreviations: ADCC, Antibody-dependent cellular cytotoxicity; ADCP, Antibody-dependent cellular phagocytosis; EGF, Epidermal growth factor; ECM, Extracellular matrix; F-actin, Filamentous actin; FAK, Focal adhesion kinase; GPCRs, G-protein coupled receptors; HPK1, Hematopoietic progenitor kinase 1; dHL-60, HL-60 cell line differentiated towards neutrophil-like cells; ITAMs, Immunoreceptor tyrosine-based activation motifs; LAD, Leukocyte adhesion deficiency syndrome; fMLP, N-formylmethionine-leucyl-phenylalanine; NK cells, Natural killer cells; NETs, Neutrophil extracellular traps; PSI, Plexin–semaphorin–integrin; PI3K, Phosphoinositide 3-kinase; PMN, Polymorphonuclear; PKC, Protein kinase C; RACK-1, Receptor For Activated C Kinase 1; ROS, Reactive oxygen species; SYK, Spleen tyrosine kinase; TNFα, Tumor necrosis factor α; WASP, Wiskott–Aldrich syndrome protein.

INTRODUCTION

Neutrophils

Human neutrophils or polymorphonuclear cells (PMNs) form the frontline of the immune response against bacteria and fungi. Despite their fundamental role in inflammation, recent evidence gives prominence to the controversial role of neutrophils in cancer (1, 2). Whereas recent data using deuterium labeling *in vivo* suggest that human neutrophils can survive up to 5.4 days in the circulation (3), general belief based on traditional studies still is that the high number of neutrophils emerging from the bone marrow on a daily basis (10^{11} per day) is balanced with their short half-life in the circulation (up to about 12h) (4). In contrast, the abundancy of neutrophil population in humans, representing up to 70% of circulating leukocytes, is not reflected on mice in which the percentage of neutrophils varies from 10 to 25% of white blood cells (5, 6).

Apart from the daily extravasation to offer that first natural line of defense at the tissue level, neutrophils are able to directionally migrate at larger numbers out of the bloodstream at the site of infection and either clear the pathogen or induce the recruitment of adaptive immune cells by chemokine/cytokine production or antigen presentation (7–9). Neutrophils exit the circulation *via* trans-endothelial migration, which takes place in sequential steps (tethering, rolling, crawling, cell arrest/firm adhesion/transmigration); all have been thoroughly described (10, 11). At the site of infection, neutrophils exert their killing properties to eliminate the pathogen extracellularly or upon phagocytosis. Neutrophils have the capacity to kill the invaded microorganism by secreting their toxic granular content (degranulation), producing reactive oxygen species (ROS), or releasing their DNA and subsequently forming neutrophil extracellular traps (NETs) in a process of cell death known as NETosis (6, 12). These processes of neutrophil extravasation, migration, and neutrophil effector functions against infectious agents rely to a very large extent on integrins.

Integrins: Expression, Structure and Activation

Integrins are a family of ubiquitously expressed, transmembrane receptors. They anchor cells within their ambient extracellular matrix (ECM) and bind to counter-receptors expressed on other cells (13). Integrins are expressed on the cell surface as heterodimers of non-covalently associated α and β subunits (14, 15). So far, 24 different $\alpha\beta$ heterodimers have been described in mammals, which exhibit specific ligand binding properties. The extracellular part of the α subunits defines ligand specificity. It consists of a seven bladed β-propeller module, followed by the thigh and two calf-domains (16–18). The β subunits consist of an N-terminal βA or β-I like domain, a plexin–semaphorin–integrin (PSI) domain, and α hybrid domain, followed by four cysteine-rich epidermal growth factor (EGF) repeats (19). While for most integrins the β-propeller from the α subunit and the N-terminal βA domain from the β subunit form the ligand binding head, a subset of vertebrate integrin α subunits, among them are the β2 integrin family, the collagen, and some laminin-binding integrins, have an additional α-I domain inserted into a loop in the β-propeller domain, which employ ligand binding (20). A hallmark of integrins is their ability to reversibly switch between different ectodomain conformations with distinct affinities for the ligand. It is meanwhile well accepted that integrins can adopt at least three conformations: a low affinity bent conformation, an extended-closed conformation with intermediate affinity, and an extended-open conformation with high ligand affinity (21–23). Notably, the difference in affinity for the ligand between the low and high affinity states varies strongly between different integrins from several hundred to several thousand fold (24–26). The conformational changes are either induced and stabilized by the extracellular ligand (outside-in activation) or are triggered by tightly regulated intracellular signals (inside-out activation); the latter is particularly important for integrins expressed on blood cells and will be described in detail in the following sections.

Both integrin subunits have a single-pass transmembrane helical domain, which are non-covalently associated within the plasma membrane. The two transmembrane domains interact at two interfaces termed the "outer membrane clasp" and the "inner membrane clasp", which are mediated by helical packing of the N-terminal part and hydrophobic interactions and a salt bridge at the C-terminal part of the transmembrane domains, respectively (27–29). The transmembrane domains are essential for integrin function as they propagate conformational changes across the plasma membrane in both directions. These changes occur upon binding of the ligand to the ectodomain or intracellular adapter proteins to the cytoplasmic domain, which cause the disruption of the transmembrane domain interactions and a separation of the transmembrane and intracellular domains.

The intracellular domains represent the control modules of integrins. These rather short peptides serve as docking stations for numerous intracellular proteins, which regulate the integrin's activity state and execute their functions. The majority of studies were done on the β integrin tail, which has a length of only 30 to 70 amino acids with the exception of the much longer β4 integrin cytoplasmic domain. Most β-tails contain a membrane proximal NPxY/F and a membrane distal NxxY/F motif flanking a serine/threonine-rich motif (30). Many intracellular proteins interact with these motifs in a phosphorylation dependent or independent manner. The most prominent proteins that bind to these motifs are Talins and Kindlins, which are essential integrin activators and will be described in detail below (31, 32). The cytoplasmic tails of the different α chains show low homology but share a GFFXR sequence in the membrane-proximal region (33). Much less is known about protein interactors, yet the tails of these α chains are important in the regulation of proteins bound to their respective β subunits (34). One of the few proteins known to associate with integrin α chains, including CD11a, is SHARPIN, an integrin negative regulator (35). In addition, CD11a, CD11b, and CD11c cytoplasmic domains have all been reported to be phosphorylated on conserved serine residues, and these phosphorylation sites are important for integrin function (36, 37).

Integrin Activation by Talin and Kindlin

Intracellular signals that eventually trigger changes in integrin affinity for the ligand, also named as integrin inside-out activation, culminate in the binding of Talin and Kindlin to the β integrin tail. While Talin was long thought to be the sole integrin activator, studies in cells and animal models revealed that it requires assistance by Kindlin. Moreover, beside their essential function in integrin activation, both proteins initiate the formation of adhesion complexes that link integrins with the actin cytoskeleton and form signaling hubs that modulate many cellular processes (integrin outside-in signaling) (30, 31, 38, 39).

Talins are large cytoplasmic proteins consisting of an N-terminal head domain, which comprises an atypical FERM domain, and a C-terminal rod domain composed of 13 helical bundles. Talin1 is ubiquitously expressed and the major isoform expressed in hematopoietic cells, while the closely related Talin2 isoform shows a more restricted expression pattern (40). Binding of the Talin head to the membrane proximal NPxY/F motif within the integrin tail destabilizes the transmembrane interactions between the α and β subunits thereby inducing conformational changes of the integrin's ectodomain (41). Talin is a mechanically regulated protein and provides a direct link with the actin cytoskeleton (42). It contains two actin-binding sites and several binding sites for the actin binding protein Vinculin within its rod (43, 44). Tensile forces generated by the actin-myosin contractile apparatus are transmitted *via* Talin to the integrin and contribute to full integrin activation. Moreover, the mechanical forces result in stretching and partial unfolding of several bundles within the Talin rod thereby exposing cryptic Vinculin binding sites (45–48). Before Talin is capable to bind and activate integrins it also requires an activation process as intramolecular interactions between the Talin head and rod domains preclude integrin binding (49). Details in the intramolecular interactions were recently provided by the cryo-EM structure of Talin. These structural data also revealed that even in the autoinhibited state the N-terminal F0 and F1 domains are exposed allowing them to bind to activated, membrane-bound Rap1 (50). This recruitment pathway is further promoted by electrostatic interactions between multiple sites within the Talin head and the negatively charged membrane lipids, in particular PIP2 (51). Concurrently, charge repulsions between the plasma membrane and the Talin rod domain interfere with the Talin head–rod interaction and promote the release in autoinhibition (52). Interactions with other proteins such as RIAM, Vinculin, and KANK proteins may further contribute to Talin recruitment and Talin activation and are further discussed below (53–55).

Mammals have three *FERMT* genes encoding Kindlin protein, which are expressed in a tissue specific manner. While Kindlin-1 is primarily found in epithelial cells, Kindlin-2 is almost ubiquitously expressed, and Kindlin-3 expression is restricted to cells of hematopoietic origin (56). The Kindlin protein structure resembles that of the Talin head and formed by an atypical FERM domain consisting of an N-terminal F0 domain followed by the canonical FERM domains F1, F2, and F3. A striking difference to the Talin FERM domain is an inserted PH domain within the F2 domain of Kindlins (57). The PH domain and a long loop within the F1 domain make contacts with phospholipids of the plasma membrane (58, 59). However, the molecular signals that direct Kindlins to integrin adhesion sites are not known yet. The Kindlin F3 domain binds directly to the membrane distal NxxY/F and the more N-terminally located threonine rich motif of the β integrin cytoplasmic tail (60, 61). Mutations in these motifs abolished Kindlin–integrin interaction resulting in defective integrin activation and function. Whether Kindlins alone, like as it has been shown for Talin, can induce conformational changes of the integrin ectodomain is not known yet. It rather seems that Kindlins support Talin-mediated integrin activation by further destabilizing the interactions between the integrin α and β transmembrane domains, which lead to the fully activated, extended open conformation of integrins (62, 63). Kindlins may also promote integrin activation by clustering integrins and thereby increasing their ligand binding avidity rather than their affinity (64). This model is also supported by structural data, which showed that Kindlin-2 can dimerize (65). Whether dimerization is a general mechanism of all Kindlin members to cluster integrins remains to be shown. Interestingly, recent crystallization studies suggest the formation of trimeric or even higher Kindlin complexes, which are unable to bind integrins (66, 67). Future studies will show whether Kindlin dimer/multimerization does occur and have an impact on integrin activity regulation *in vivo* and how this is regulated by upstream signals. Irrespective of their crucial role in integrin activity regulation more recent studies began to unravel the important role of Kindlins in adhesion site assembly and stabilization. Meanwhile multiple Kindlin interactors such as Paxillin, Leupaxin, ILK, and actin have been identified, and differences in the interactome or affinities to these and other still unknown interactors may explain the specific features of the individual kindlin members (68–73).

Leukocyte Integrins and the β2 Integrin Family

In leukocytes, αMβ2 or MAC-1 (CD11b/CD18), CR3; αLβ2 or LFA-1 (CD11a/CD18); αDβ2 (CD11d/CD18); αXβ2 (CD11c/CD18); α4β1 or VLA-4 (CD49d/CD29); α5β1 or VLA-5 (CD49e/CD29); α6β1 or VLA-6 (CD49f/CD29); α4β7 (CD49d/HML-1), and αEβ7 (CD103/HML-1) have been described (30, 74). β2 integrins are only found on leukocytes. The four members of this family are expressed on different leukocyte subpopulations and fulfil different roles in immunity. CD11a/CD18 is expressed on all leukocytes, regulating cell–cell communication by binding to ICAM ligands; it has the most restricted ligand repertoire of all the β2 integrins and is structurally more distantly related to the other three. In contrast, only myeloid cells including neutrophils express CD11b/CD18 and CD11c/CD18 on most cells, while macrophages also have additional CD11d/CD18 proteins on their surface, most likely with similar ligand binding properties (75). The most prominent integrin on neutrophils is CD11b/CD18, which has a promiscuous binding capacity to more than 40 ligands, including ICAM, iC3b, fibrinogen, and other ECM

proteins (18), indicating its pleiotropic effect on multiple cellular processes.

The complexity of integrin biology is reflected in its intricate mechanism of activation. Recent reviews have proposed how β2 integrins get activated (31, 76). In brief, integrins may exist in three conformational stages, bent-inactive, extended-inactive, and extended-active conformation. Each of them is related to their binding affinity status and the subsequent efficacy of ligand binding. In fact, a recent report on β1 integrin suggests that all of the different integrin conformations can be present on the cell surface in a dynamic equilibrium in order to initiate or preserve cell motility (77). The "switchblade" and the "deadbolt" models have been predicted both describing the chronological order of integrin changes in affinity and ligand binding. However, there is no clear evidence supporting one of these models to date (31).

Focusing on the β2 integrins in leukocytes and, more specifically in neutrophils, neutrophils require the presence of Kindlin-3 protein for all CD11/CD18 integrin-mediated ligand binding (31). The importance of β2 integrin and their protein regulators has been demonstrated by the identification of human leukocyte adhesion deficiency syndromes (LADs) (78). Loss of β2 integrin in humans due to mutations in the gene *ITGB2* encoding the β2 subunit causes LAD-I. In contrast, LAD-III (or LAD-I/variant) arises from mutations in *FERMT3* encoding the Kindlin-3 protein (79–81) in the presence of normal levels of leukocyte integrins but a lack of high-affinity binding activity upon cell activation. Both syndromes result in similar patient phenotypic characteristics, showing susceptibility to bacterial infections due to the lack of neutrophil migration to the site of infection, poor wound healing, and late detachment of the umbilical cord in most affected children (82). In addition, many LAD-patients suffer from increased IL-17-driven periodontal tissue inflammation, leading to periodontitis, at least in part due to reduced recruitment of neutrophils (83). In LAD-III, the Kindlin-3 mutations also result in a clinical bleeding tendency due to the lack of β3 integrin activity on platelets (84). The role of Talin-1 in initiating intermediate integrin conformation and both Talin-1 and Kindlin-3 in inducing the high affinity state of the CD11a/CD18 integrin was subsequently reported in a murine model (63), very similar to CD11b/CD18 integrin activation by Kindlin-3 (85, 86). After initial activation, integrins engage with their ligands and form nascent clusters. Again, several models of clustering induction and stabilization have been suggested for β1, β2 or β3 integrins, yet the precise mechanism remains to be elucidated (87–90).

Although Talin-1 and Kindlin-3 are the primary proteins associated with β2 integrins in case of both inside-out and outside-in integrin activation, β2 integrins can only function effectively after recruitment of several additional proteins to the β2 integrin cytoplasmic tail, thus regulating or stabilizing its high-affinity state. Although recent technology can identify proteins associating with integrins (91), the heterogeneity among the different β integrin subunits as well as their diverse function in different cell types averts a general statement about integrin signaling and function. Although the proteins that can directly associate with β2 integrins or regulate their affinity and function have been reviewed (92), their contribution to downstream signaling of β2 integrins in the context of neutrophils is limited (38, 93–97). Examples of proteins interacting directly with the cytoplasmic tail of the β2 subunit and having a known function in neutrophils include Cytohesin-1 (98, 99), SYK (100, 101), and Filamin A (97) as we will also discuss below.

INSIDE-OUT SIGNALING DURING ENDOTHELIAL TRANSMIGRATION

Chemokine Mediated Signaling

For trans-endothelial migration to occur, leukocytes utilize CD11a/CD18 and CD11b/CD18 to adhere to the endothelial wall. As discussed, CD11a/CD18 and CD11b/CD18 are kept in an inactive state, and require activation through recruitment of Talin-1 and Kindlin-3. Activation of these β2 integrins increases their binding affinity, which allows adhesion of leukocytes to several substrates including endothelial membrane proteins ICAM-1, upregulated during inflammation, and ICAM-2. During selectin-mediated rolling and slow rolling, leukocytes engage with chemokines and cytokines presented on the vessel wall with several different receptors, of which most are G-protein coupled receptors (GPCRs), also known as seven transmembrane receptors. Engagement of chemokines, such as CXCL8 (better known as IL-8) and CXCL1 (also known as GRO-α, MGSA, or KC) with their respective receptors causes the activation of CD11a/CD18 and CD11b/CD18 through a common pathway which recruits Talin-1 and Kindlin-3 to the cytoplasmic tail of CD18. Note that Talin-1 can be recruited through both selectin and GPCR signaling.

This common pathway through GPCRs starts with $G\alpha_i$ and the Gβ/γ subunits, which split after chemokine engagement with the receptor and activate Phospholipase C β2 (PLCβ2) (**Figure 1A**) (102–104). The signaling then branches into two pathways, as has mostly been supported by gene knockout models in mice. The first is the activation of CalDAG-GEFI (RASGRP2) by PLCβ2 and to a minor extent PLCβ3 through the generation of DAG and a calcium influx by IP3 generation (105, 106), resulting in the activation of Rap1 (107). Activated Rap1 exposes a membrane targeting sequence, and upon interaction with the plasma membrane the N-terminus of Rap1 interacts with RIAM, which contains a Talin-binding sequence (108). A reduced fusion protein consisting of the Rap1 membrane targeting domain and the RIAM-Talin-binding sequence is sufficient to target Talin-1 to the plasma membrane, allowing engagement of Talin-1 with the NPXY motif in the cytoplasmic tail of CD18 (109) and resulting in β2 integrin activation (108). Interestingly, direct Rap1 interactions have also been described with the F0 and F1 domains of Talin-1, which in mice are pivotal for normal overall integrin activation, adhesion, and cell spreading, whereas the RIAM–Talin-1 interaction seems specific for β2 integrins in leukocytes (110–113).

The second pathway for β2 integrin activation downstream of PLC goes through Protein kinase C (PKC). PKC is activated by

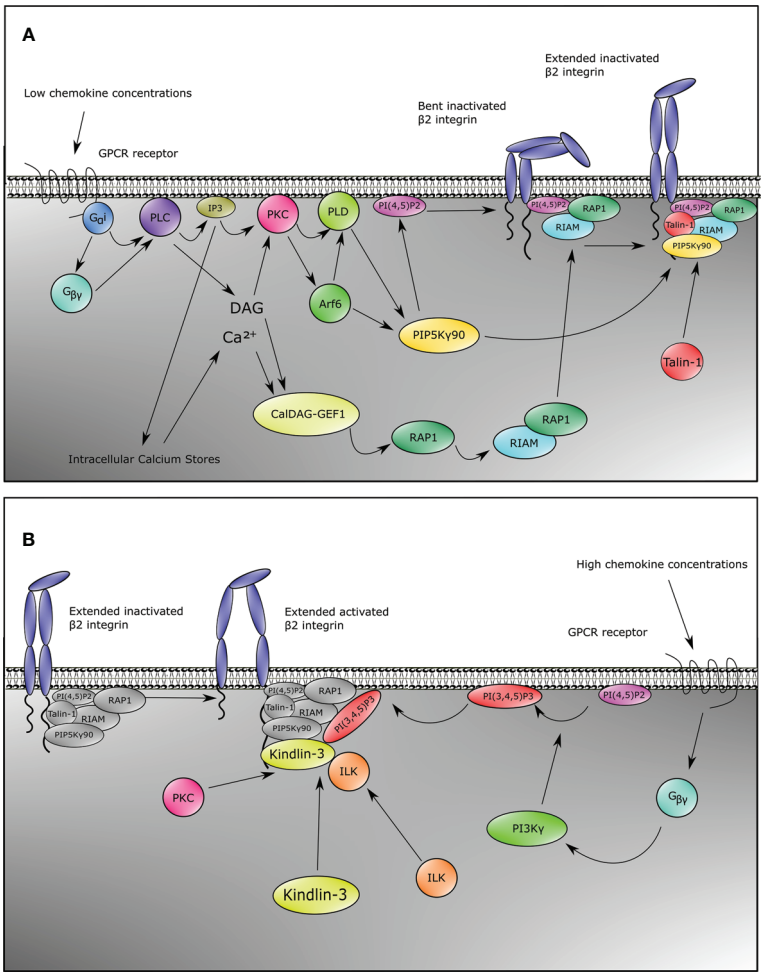

FIGURE 1 | Inside-out signaling recruits Talin-1 and Kindlin-3 to β2 integrin. Upon stimulation of neutrophils with chemokines, both Talin-1 and Kindlin-3 become rapidly recruited to activate β2 integrins. This can be divided into two pathways for Talin-1 and Kindlin-3. For Talin-1 recruitment **(A)**, GPCRs engage with their ligands which causes the heterotrimeric G-proteins to split into G_{α} and $G_{\beta\gamma}$. This initiates the activation of PLC by $G_{\alpha i}$, and $G_{\beta\gamma}$. Signaling through the second messengers' calcium which is released from the endoplasmic reticulum by IP_3 and diacylglycerol (DAG) is a critical element in many biological systems. Integration of calcium and DAG signals has been suggested to occur primarily through protein kinase C (PKC) family members, which bind both calcium and DAG. An alternative pathway involves CalDAG-GEF1, which has structural features (calcium-binding EF hands and DAG-binding C1 domains) and functions in calcium and DAG signal integration. This GEF activates RAP1, which then results into activation of RIAM and binding of RIAM to Talin-1. Together with catalyzation of PIP into $PI(4,5)P_2$ by PIP5K$_{\gamma}$90 through the PKC-PLD-Arf6 axis, these events recruit Talin-1 to β2 integrin, thus extending the integrin from the bent conformation. $G_{\beta\gamma}$ is also involved in Kindlin-3 recruitment **(B)**. In addition to activating PLC, $G_{\beta\gamma}$ also activates PI3K, which generates $PI(3,4,5)P_3$. This allows recruitment of Kindlin-3 to β2 integrin. Binding of Kindlin-3 after Talin-1 binding activates extended β2 integrins. Note that the Kindlin-3 recruitment cascade might not be complete.

both DAG and IP3 generated by PLC. PKC then elicits its function by activation of Phospholipase D (PLD) and Arf6 (**Figure 1A**) (114, 115). Both Arf6 and PLD subsequently activate PIPKIγ (also known as PIP5K1C) which increases phosphatidylinositol 4,5 (PIP_2) levels at the inner plasma membrane (104, 114–117), resulting in the recruitment and activation of Talin-1 *via* its PIP_2 binding F2 domain and subsequent binding to the NPXY motif of the CD18 cytoplasmic tail (**Figure 1A**) (104, 109, 112, 118). Also taking into account a direct interaction between the C-terminal domain of PIP5K(γ90) and Talin-1, this collectively suggests a feedforward loop of both recruitment of Talin-1 to the β2 integrin tail and activation of Talin-1 through local increase in PIP_2 levels close to the β2 integrin (118).

Although multiple studies have shown the importance of Kindlin-3 for β2 integrin activation (31, 63), the signaling pathway essential for activation of Kindlin-3 and recruitment to the CD18 cytoplasmic tail is as yet still less clear compared to what is known about Talin-1 activation (**Figure 1B**). Notwithstanding, Kindlin-3 recruitment to CD18 temporally precedes the high affinity conformation of β2 integrins (59). Additionally, structure function analyses have indicated that the pleckstrin homology domain of the F2 subdomain of the FERM domain is important for binding phosphatidylinositols at the

plasma membrane (119, 120), and that part of this protein population has been suggested to reside in an auto-inhibitory homotrimer (67). Moreover, high chemokine concentrations induce neutrophil arrest, which is mediated through high-affinity β2 integrin by Kindlin-3 associating with the cytoplasmic tail of the β2 subunit and PIP_3 increments upon strong activity of PI3K (**Figure 1B**) (104). Blocking PI3K has been suggested to decrease human neutrophil adhesion to fibrinogen, and adhesion to ICAM-1 is affected by specific blocking of PI3K with wortmannin (103, 121). ILK might also play a role in Kindlin-3 function in mice since genetic abblation resulted in reduced Kindlin-3 phosphorylation and a decrease in high-affinity CD11a/CD18 (122).

Despite these common pathways in β2 integrin activation, some chemokines trigger alternative pathways. For instance, in murine neutrophils, CXCL1 induces a strong calcium influx, which is an important signaling mediator (**Figure 1A**) for β2 integrin activation (123). This differential signaling effect is substantiated by the knockout of *TRPC6* encoding a plasma membrane calcium channel, resulting in lower calcium transient increases, thus leading to reduced Rap1 and β2 integrin activation by CXCL1 (124). Other chemokines remain to be tested for this pathway. Secondly, the actin-binding protein Hematopoietic cell-specific LYN Substrate 1 (HS1) knockout mice have reduced Rac1 and subsequent Rap1 activation upon CXCL1 stimulation (125), which indicates another integrin activation pathway parallel to the common GPCR pathways (**Figure 1A**) in at least CXCL1-mediated integrin activation. Additionally, IL-8 also branches towards another pathway *via* PI3K, which activates the serine/threonine kinases RAF-1 and B-RAF, from which RAF-1 is expressed in human neutrophils. Both RAF-1 and B-RAF can subsequently activate the MEK/ERK/p38-MAPK pathways (126), and blocking MAPK indeed reduces neutrophil adhesion to fibrinogen, indicating a role for this pathway in CD11b/CD18 activation (103).

There are some noticeable differences in the activation of CD11a/CD18 and CD11b/CD18, which can solely be attributed to the presence of either CD11a or CD11b. For example, Cytohesin-1 is known to activate CD11a/CD18 and increases firm adhesion, spreading, and transendothelial migration for mouse neutrophils, whereas the opposite effect was observed for CD11b/CD18 (98, 99, 127–129). Additionally, CD11a Ser^{1140} and CD11b Ser^{1126} phosphorylation are critical events in the activation of the respective CD18 heterodimers for binding of ICAM-1, to a lesser extent ICAM-2, but not for the non-cellular ligand inactivated complement 3 fragment b (iC3b) in case of CD11b/CD18 (36, 130). Furthermore, CD11a/CD18 in humans is more rapidly activated than CD11b/CD18, but the activation is more transient, being deactivated after 1 min. In contrast, CD11b/CD18 seems to become fully activated after 1 min and remains activated for up to more than at least 5 min after stimulation with fMLP (131). This may reflect different roles of CD11a/CD18 and CD11b/CD18 in trans-endothelial migration, where CD11a/CD18 facilitates slow rolling and early adhesion, whereas CD11b/CD18 has a more prominent role during firm adhesion and subsequent chemotaxis. These findings are speculative and should be interpreted against background expression levels of these integrins, being much higher for CD11b/CD18 on human but not murine neutrophils. Selective genetic defects for either of the two integrins in gene knockout models of the respective α-chain have not been fully explored and cannot be supported by human 'experiments of nature' with genetic mutations in patients as the cause of disease.

Selectin-Mediated Signaling

Aside from GPCRs, other mechanisms of inside-out signaling have been described. Selectin-mediated signaling has been thoroughly described in the past (132), and it is out of the scope of this review, yet we briefly discuss the concept in response to β2 integrin activation. During selectin-mediated rolling, the physical interactions between L-selectins on leukocytes with P- and E-selectins on inflamed endothelium induce recruitment of Talin-1 to the cytoplasmic tail of CD18 (133). Upon engagement of L-selectin with E-selectin, Src-family kinases (SFKs) are recruited and phosphorylate either FcRγ adapters or DAP12 (134) together with ERM proteins Moesin and Ezrin in an ITAM-motif based manner (135). This is followed by phosphorylation of Spleen tyrosine kinase (SYK) (136), which then recruits a complex of SLP76 together with ADAP (137). These two proteins subsequently activate Bruton tyrosine kinase (BTK) to phosphorylate p38-MAPK and also activate PLCγ2 (133) instead of PLCβ2 (as known for chemokine stimulation). Downstream signaling through selectins involves the activation of Rap1a and PIP5K(γ90) (**Figure 1A**) (104), thus leading to the convergence of selectin-mediated signaling with the common GPCR pathway resulting in the recruitment of Talin-1 to CD11a/CD18 and CD11b/CD18 (110, 111, 138). To date, selectin-mediated CD11a/CD18 activation has only been reported for murine and not human neutrophils, which coincides with a much lower expression of CD11a/CD18 on human neutrophils. The medium avidity state of CD11a/CD18 initiates slow rolling due to transient interactions with ICAM-1 (133, 138). This allows the neutrophil to sample chemokines presented by the endothelial cell wall, thus inducing chemokine mediated inside-out signaling, which leads to further activation of β2 integrins (138). Although it has been found that selectins are sufficient to cause β2 integrin-mediated arrest (139), both the presence of selectins and chemokine signals may be required for full activation of CD11a/CD18 and CD11b/CD18 in human blood vessels (138), as selectins fully activate β2 integrins cooperatively at low chemokine concentrations (104). In human neutrophils, chemokines but not selectins used PI3Kγ in cooperation with Rap1a to mediate integrin-dependent slow rolling (at low chemokine concentrations) and arrest (at high chemokine concentrations). High levels of chemokines activate β2 integrins without the need of selectin signals.

Role of β2 Integrin Downstream Signaling in Slow Rolling, Adhesion, Firm Adhesion, and Crawling

Upon interacting with the vascular wall, neutrophils first decrease their velocity, strengthen their interaction with the

endothelium, and finally sample the endothelium by crawling over it to find an appropriate spot to squeeze through and transmigrate into the subendothelial space. In humans, both CD11a/CD18 and CD11b/CD18 have partial redundancy (131), whereas CD11b/CD18 seems redundant in terms of adhesion compared to CD11a/CD18 in mice (140).

For adhesion strengthening, β2 integrins can upregulate their own affinity state. Upon engagement of β2 integrins with their ligands, SFKs are recruited in order to phosphorylate SYK, among other proteins. In mice, the absence of HCK and FGR proteins prevents continued adhesion to ICAM-1 (141), indicating that β2 integrins regulate themselves to maintain a high-affinity conformation. Another example is the binding of 14-3-3ζ isoform to β2 integrins upon phosphorylation of Thr578 of the CD18 cytoplasmic tail (142), although a membrane-proximal site of the cytoplasmic tail which does not require phosphorylation has also been described (143). The binding of 14-3-3ζ to phospho-Thr578 obscures the binding site for Filamin-A (142), a protein which keeps β2 integrins in an inactive state (144). Some of the signals may counter or act to fine-tune the process of adhesion strengthening. This has been indicated in mice by the promoting activity of PKCs (145), whereas hematopoietic progenitor kinase 1 (HPK1) in concert with mammalian actin binding protein 1 (mAbp1) specifically activates CD11a/CD18 (146), thereby negatively affecting intraluminal crawling and spreading of neutrophils.

Aside from regulating their own affinity state, CD11a/CD18 and CD11b/CD18 each also separately clusters with itself. This increases the overall binding avidity of the integrins, which is required for firm adhesion. Integrin clustering also facilitates outside-in signaling by grouping the CD18 cytoplasmic tails close together. Clustering likely occurs due to the linkage of CD11a/CD18 and CD11b/CD18 to the actin cytoskeleton, in which filamentous actin (F-actin) polymerization plays a pivotal role. The initial F-actin polymerization likely is not induced by direct stimulation of CD11a/CD18 or CD11b/CD18, but rather through surface receptors for tumor necrosis factor α (TNFα) and other chemokines (147). Blocking specifically actin polymerization with Cytochalasin D, a strong actin polymerization inhibitor, prevents adhesion strengthening and crawling but does not change CD11a/CD18 or CD11b/CD18 expression in human neutrophils (148). Prior to F-actin polymerization, the SFKs HCK and FGR as well as SYK relocate to the cytoplasmic tail of CD11a/CD18 or CD11b/CD18 (149), and mouse knockout models highlight the relevance of these proteins in spreading and F-actin polymerization (101, 141, 150–152). These studies collectively indicate that F-actin polymerization occurs prior to adhesion strengthening and clustering and that β2 integrins relay signals for actin polymerization. After initial F-actin polymerization, integrins recruit multiple actin binding proteins, including Vinculin and α-actinin (43, 153, 154). Neutrophils express the cytoskeletal protein Vinculin, although they do not form mature focal adhesions as macrophages or fibroblasts do. The role of Vinculin in β2 integrin-dependent neutrophil adhesion, migration, and recruitment was found to be not completely abrogated in knockout mice and more so under static than dynamic flow conditions, suggesting a dispensable 'mechanosensitive' function *in vivo* (155). Other proteins might also promote integrin clustering. The protein 14-3-3ζ, which binds the phosphorylated Thr^{578} in the CD18 cytoplasmic tail, forms dimers which indicates a role in clustering of β2 integrins (36, 92, 156). Moreover, Cytohesin-1 increases firm adhesion by CD11a/CD18 but decreases adhesion by CD11b/CD18, indicating a role for this protein in the regulation of integrin clustering (98, 99, 127–129). The recruitment of actin binding proteins and CD18 binding proteins ultimately packs the β2 integrins close together due to their collective binding to F-actin filaments close to the membrane, resulting in an increase in binding avidity. It remains to be shown which of these actin-binding proteins are non-redundant in neutrophil behavior. Interesting novel candidates involved in F-actin dynamics might be found in proteomics data from MKL1-deficient patients, which show downregulation of actin-associated proteins AIP1, ARHGAP9 and Profilin-1 (157), regulating actin polymerization and disassembly (AIP1, ARHGAP9), or inhibits polymerization of actin (ARHGAP9, PFN1).

Role of β2 Integrin Downstream Signaling in Cell Polarization, Spreading, Chemotaxis, and Migration

Once inside-out signaling facilitates β2 integrin intramolecular conformational changes, the outside-in β2 integrin signaling, which is subsequently initiated by adhesion, automatically takes place. Adhesion is followed by polarization of the cell for subsequent directed cell migration or chemotaxis by recognition of chemoattractants such as chemokines, lipid mediators, or chemotactic peptides (158).

The process of chemotaxis can be divided in three separate events: sensing of the chemoattractant, polarization, and cell motility or migration. Here we describe 2D chemotaxis. In fact, chemotaxis in a 3D environment is mechanistically a very different process and can occur even without integrin-mediated adhesion (159). Upon perceiving chemoattractants, leukocytes typically invoke changes in the actin cytoskeleton and activate their β2 integrins as discussed previously. These cytoskeletal rearrangements initiate the second step of cell polarization (160, 161) as initiated by translocation of intracellular and extracellular proteins towards the leading edge of the cell (160, 161). In contrast to tissue cells, leukocytes migrate by forming pseudopods or lamellipodia, actin-filled protrusions important for cell movement at the leading edge of the cell (162). At the trailing end, cells form a uropod which serves as the anchoring point of the cell. For motility to occur, the pseudopod needs to move towards the chemoattractant, adheres with β2 integrins closer to the chemoattractant, and creates a strong interaction between the actin cytoskeleton and the β2 integrins. Subsequently, the uropod pulls the trailing end of the cell towards the new site of adhesion, which requires these strong interactions between the actin cytoskeleton and the β2 integrins. Meanwhile, the uropod also degrades the F-actin at the trailing

end after this movement. Concluding, the turnover of β2 integrin interactions at the uropod is critical for proper regulation of motility [also reviewed in (160)].

Following adhesion, activated integrin induces tyrosine phosphorylation of SYK protein by SFKs, such as HCK, FGR, or LYN in neutrophils (101, 163) Activation of SYK results into provisional binding to CD18 molecule, which accordingly facilitates rearrangements of the actin cytoskeleton and subsequent neutrophil spreading (**Figure 2A**). In mice, neutrophil SYK deficiency resulted in defective adhesion-dependent responses, *i.e.* spreading, degranulation, and oxidative burst. Yet, neutrophil migration was not impaired in

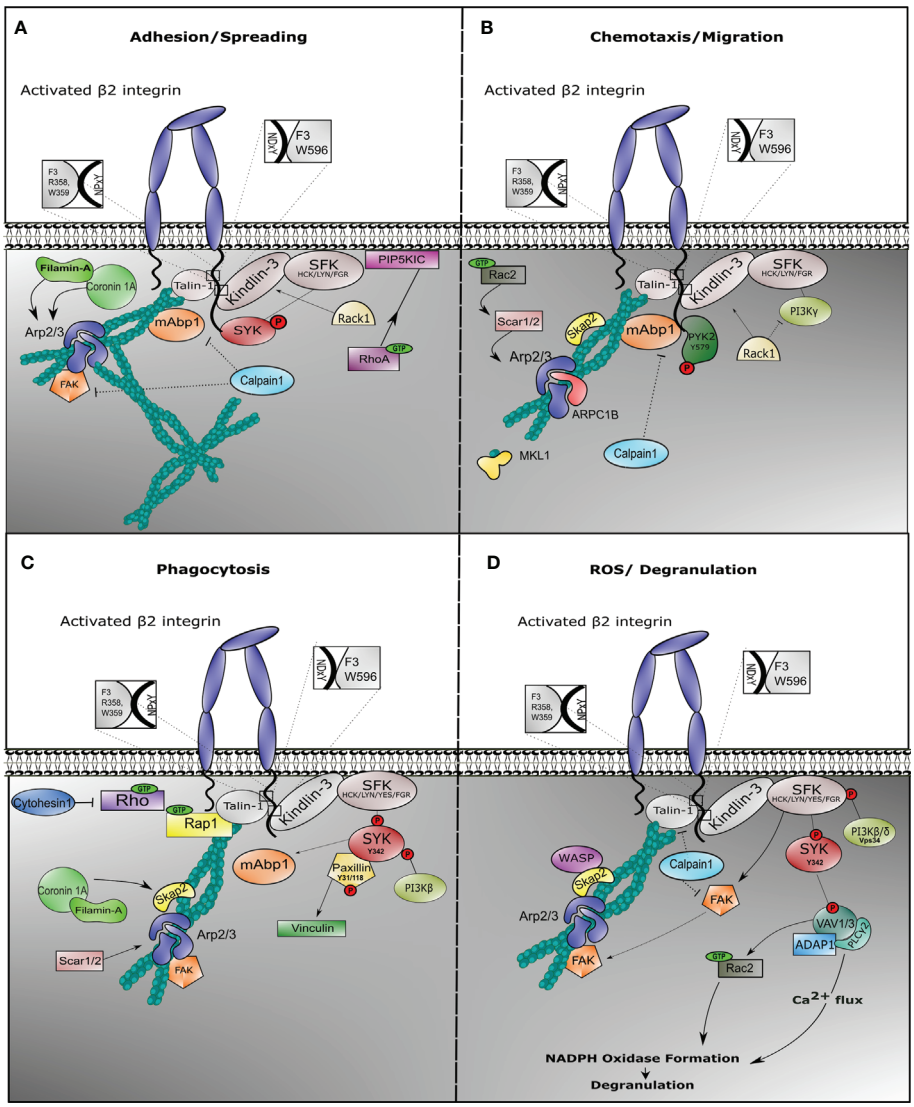

FIGURE 2 | Outside-in signaling involves different protein complexes in response to neutrophil effector function. Once β2 integrin is activated on neutrophils, outside-in signaling is facilitated in order to induce cell effector function. Neutrophils are endowed with multiple mechanism, all achieving target elimination. In all cases, integrin outside-in signaling involves stable interaction with Talin-1 and Kindlin-3, which recruits binding of the actin cytoskeleton and induction of signaling *via* Sarcoma family kinases (SFKs). During spreading **(A)**, SFKs phosphorylate and recruit SYK to the cytoplasmic tail of the β2 integrin, while Phosphatidylinositol 4-phosphate 5-kinase (PIP5KIC) induces activation of RhoA GTPase. Mitogen-activated binding protein 1 (mAbp1) is involved in stabilization of actin cytoskeleton, while Calpain-1 could play a role to negatively regulate its function. During migration **(B)**, phosphorylated PYK2 is recruited and the Arp2/3 complex is stabilized *via* SKAP2 and Rac2 GTPase. Filamin A and ARPC1B are important for migration. We do not show WASP protein because deficiency results in impaired neutrophil migration in murine models only. Particularly during phagocytosis **(C)**, phosphorylation of tyrosine protein kinase SYK induces binding of phosphorylated Paxillin with subsequent activation of Vinculin and activation of Phosphoinositide 3-kinase (PI3Kβ) and mAbp1. SCAR1/2 protein (also known as WAVE1/2) recruits Arp2/3 complex to stabilize actin cytoskeleton, while SKAP2 (or SKAP55R) functions as adaptor protein. During ROS production **(D)**, degranulation is facilitated through increase of calcium influx. Phosphorylation of SYK initiates VAV phosphorylation with ADAP1 and PLCγ2 complex formation, promoting oxidative burst. VAV induces Rac1 GTPase activity, which facilitates NAPDH oxidase formation. In this context, Calpain1 could additionally inhibit Talin-1 or Focal adhesion kinase (FAK) function.

the absence of SYK, indicative of the variation of integrin signaling in each neutrophil response (101).

During β2-mediated polarization of myeloid HL-60 cells differentiated towards neutrophil-like cells (dHL-60), mAbp1 was enriched towards the cell's leading edge, suggesting that mAbp1 is a positive regulator under β2 integrin-mediated chemotaxis by counteracting the more adhesion-strengthening signals, thus enabling cell motility (164, 165). HPK1, a protein colocalizing with mAbp1 and actin, was required for CXCL1-induced CD11a/CD18-mediated adhesion in dHL-60 cells, independent of CD11b/CD18 proteins (146). CD11a/CD18 is known to be endo- and exocytosed in a lipid-raft mediated coordination in the presence of chemokines in contrast to CD11b/CD18 (166), again supporting distinctive roles of CD11a/CD18 compared to CD11b/CD18 in the process of cell motility.

Filamin-A was found to be indispensable for dHL-60 spreading but was not essential for neutrophil migration (167). Although Filamin-A is shown to directly interact with Coronin-1A, deficiency of the actin-binding protein Coronin-1A resulted in reduced spreading and impaired chemotaxis, which indicates an independent role of these two proteins during neutrophil motility (96). This might be explained by the role of Coronin-1A in recruiting Arp2/3 complex to the F-actin polymerizing filament (168). Arp2/3 complex is essential to coordinate actin cytoskeleton rearrangement during chemotaxis, while Filamin A may act in more static conditions such as spreading and phagocytosis, and a negative regulator of motility (see below) (**Figure 2B**).

ARPC1B functions as a subunit of the Arp2/3 complex in hematopoietic cells, and its deficiency in neutrophils resulted in significant defective migration. Additional proteins have been shown to play a role in motility. For instance, human neutrophils lacking Megakaryoblastic leukemia 1 (MKL1) protein which normally sequesters monomeric G-actin, show normal adhesion under static conditions but impaired adhesion under flow conditions and defective chemotaxis (157).

SRC kinase-associated phosphoprotein 2 (SKAP2) and WASP, two adaptor proteins which are also linked to the rearrangements of the actin cytoskeleton, led to impaired neutrophil migration when absent in mice (169). However, neutrophils from WASP-deficient patients show no migratory defect, indicating that murine and human neutrophils may function differently in the context of migration (170). Defects in other cytoskeleton associated proteins also result in impaired neutrophil migratory functions. One such protein is Rac GTPase, a small guanine-nucleotide binding protein, involved in cytoskeleton rearrangement through signaling *via* SCAR1/2 (also known as WAVE1/2) and Arp2/3 complex (171). Rac2 deficiency in human neutrophils leads to significantly impaired integrin-dependent chemotaxis (172).

Proline-rich tyrosine kinase 2 (PYK2) has a more complex activity. It is known to interact with cytoskeleton-interacting proteins. It associates with β2 integrin cytoplasmic tail upon fMLP stimulation (173) and was shown to contribute to migration in all-trans retinoic acid (ATRA)-differentiated NB4 myeloid cells (174). Pyk2 deficiency in murine model showed impaired neutrophil migration, with normal phagocytosis and killing (175). PYK2 regulation may be related to differential phosphorylation of PYK2 itself. This was observed for murine CD11a/CD18 during chemotaxis and motility where decreased migration was the result of a defect in the detachment from ICAM-1 at the trailing edge when PYK2 function was inhibited (176).

Although the above mentioned proteins are mainly important to positively regulate β2 integrin during motility, negative regulators have also been identified with the exception of the activated C kinase 1 receptor (RACK1). RACK1, a binding partner of Gβγ heterotrimeric subunits, inhibits PI3Kγ activity (177), and RACK1 deficiency showed enhanced neutrophil chemotaxis (177). Calpain-1 acts as a negative regulator of neutrophil chemotaxis downstream of GPCRs. Even though inhibition of Calpain-1 promoted random migration of resting neutrophils through MAPK and PI3K activation, directed neutrophil chemotaxis was defective in the absence of Calpain-1 due to the lack of directionality (178, 179).

Filamin A has clearly been found to bind to β2 integrin tails at a site which overlaps with Talin and Kindlin-3 (142), and its binding is regulated by phosphorylation of the conserved threonine triplet, where Filamin A can only bind to the unphosphorylated integrin. Filamin A is a large cytoskeletal protein which interacts with a multitude of intracellular partners and has been reported to function as an integrin negative regulator by competing with Talin-1 (180). Indeed, murine neutrophils lacking Filamin A display increased integrin-mediated adhesion of T cells (97), confirming that Filamin A indeed functions as a negative regulator of β2 integrins in these cells. Moreover, neutrophil extravasation in a mouse peritonitis model is affected negatively by both conditional knockout of Filamin A or Talin-1; thus correct interplay between positive and negative regulators is crucial for normal neutrophil function and behavior (63, 181).

Interestingly, we have also found that SHARPIN, which binds to integrin α-tails, can negatively regulate β2 integrin-mediated neutrophil adhesion (Khan, Fagerholm, unpublished observation), indicating that integrin binding proteins indeed can directly regulate integrin function in both positive and negative ways in neutrophils.

It is undeniable that neutrophil function requires a well-balanced equilibrium of positive and negative regulation, yet further proteins that negatively regulate neutrophil chemotaxis in a integrin-dependent manner have not been established (182). Although protein kinases such as SYK and SFKs mediate PMN activation through ITAM signaling domains, SFKs also activate ITIM domains to negatively regulate PMN signal transduction pathways. ITIM phosphorylation by SRC family kinases (HCK, FGR and LYN) recruits SH2 domain-containing phosphatase (SHP) 1 and 2 as well as SHIP 1 and 2 that dephosphorylate substrates of ITAM mediated activating signaling pathways in order to downregulate PMN functional responses. These ITIM containing immunoreceptors may play key roles in negative regulation, including chemotaxis. Well known is the effect of a

point mutation in *Shp-1* that reduces expression of SHP-1 protein by 90% and displays a hyper-adhesive phenotype of macrophages, contributing to neutrophil inflammation, mostly of the skin, in humans with a spontaneous mutation in the *PTPN6* gene encoding SHP-1 (183, 184).

β2 INTEGRIN SIGNALING IN NEUTROPHIL-MEDIATED KILLING AND CYTOTOXICITY

Phagocytosis

Phagocytosis is the uptake of particles by a cell. Here, we describe the uptake of pathogens by professional phagocytes of the immune system (*i.e.* neutrophils, monocytes, macrophages, and dendritic cells). All phagocytes have the capacity to eliminate invading pathogens by engulfment and degradation (185). Pathogens are often coated with a number of soluble opsonins, which are successfully recognized by phagocytes, including neutrophils (186). Upon recognition of pathogens at the plasma membrane, protrusions encircle the pathogen and draw it into the phagocyte. After engulfing the pathogen, the so-called phagosome fuses with granules in order to degrade the engulfed pathogen. Neutrophil phagocytosis is efficiently initiated upon IgG or iC3b binding to Fcγ receptor or complement receptors, respectively, including CD11b/CD18 (or CR3), while no role for the CD11a/CD18 integrin is described in phagocytosis (187). Earlier studies have primarily focused on macrophage phagocytosis, where it was suggested that Fcγ receptor and CD11b/CD18 integrin-related induction of phagocytosis are two distinct mechanisms (188) (Freeman et al., 2016). Referring to neutrophil phagocytosis, cells from LAD-I patients failed to phagocytose C4b-opsonized sheep erythrocytes or iC3b-opsonized *S. aureus* due to the specific lack of CD11b/CD18 integrin (189). In fact, CD11b/CD18 integrins and downstream associated proteins are important in both Fc receptor and CR3-induced phagocytosis (190, 191). The extracellular binding site of CD11b/CD18 (CR3) for iC3b is different from the binding site of cellular ligands (REF). In macrophage cell lines the mechanism of phagocytosis by CD11b/CD18 involves Arp2/3 complex formation for an efficient Talin-1-dependent actin-induced internalization of the pathogen. Whether a similar role of Arp2/3 and SYK in phagocytosis applies to human neutrophils is doubted. Neutrophil phagocytosis in patients deficient in ARPC1B or MKL1 is unaffected (192). Also, Arp2/3-inhibited control neutrophils did not show a defect in phagocytosis. We believe that these Arp2/3 processes in macrophages and neutrophils differ. We know that the importance of Rap1 GTPase in phagocytosis is highlighted in murine macrophage cell lines (193, 194) as well as in murine neutrophils, in which disruption of the Rap1/Talin-1 interaction abolishes phagocytosis of serum-opsonized *E. coli* (111, 195). The complementary function of Talin-1 and Kindlin-3 is also reflected in neutrophil phagocytosis, where LAD-III-derived neutrophils were unable to phagocytize zymosan particles (196) (**Figure 2C**).

SYK protein initiates integrin inside-out activation through activation of the Rap1/Talin-1 complex, while it is also involved in β2 integrin-induced phagocytosis stabilizing the outside-in signaling (132, 197). Phosphorylated SYK (phospho-Tyr342) colocalizes in macrophage phagosomes with phosphorylated Paxillin (phospho-Tyr31 and phosphor-Tyr118), which leads to the recruitment of Vinculin (198) and subsequent anchorage of F-actin to the membrane to facilitate target internalization. Yet, the above mentioned phosphorylation sites, somehow anchorage points for actin rearrangements, have not yet been verified during neutrophil effector functions.

The process of adhesion strengthening, spreading, or phagocytosis indicates a role of the cytoskeleton in which β2 integrins must provide together with additional proteins anchorage points for membrane rearrangements. *In vitro* studies have shown localization of Filamin A at the phagocytic cup during phagocytosis (167), but at least murine neutrophil phagocytosis can occur without Filamin A (97). Nonetheless, *FLNA* mutations identified thus far in humans have not indicated a major impact, suspect of a clear impact and clinical manifestations suggesting a LAD-like phenotype (199).

In contrast, the Filamin A-binding protein Coronin-1A was present only during late phagosome formation (167) and most is likely to be redundant and dispensable (97). CD11b/CD18-dependent particle ingestion required the presence of the Arp2/3 protein complex in murine macrophages (200, 201), but human neutrophils from ARPC1B-, MKL-1- or WASP-deficient patients could efficiently phagocytize and kill pathogens *in vitro* (157, 170, 192), indicating that involvement of Arp2/3 or actin-binding proteins in neutrophil phagocytosis is redundant.

The role of focal adhesion kinase (FAK), also known as cytoplasmic protein tyrosine kinase-2 (PTK2), is interesting in this respect. FAK has been explored in knockout mice, showing impaired phagocytosis of opsonized *E. coli* (202). FAK deficiency did not result in abnormal cell migration, implicating a role for this protein in stabilization of the phagocytic cup. Human studies on FAK in neutrophils and macrophages are lacking to date. Recently the role of SKAP2 has been described during murine neutrophil phagocytosis, showing impaired phagocytosis of *E. coli* particles by SKAP2-deficient neutrophils (169). The role of additional proteins in this process still needs to be determined. In this context, signaling molecules such as SYK and PYK2 are important kinases that may contribute to the upstream integrin-mediated signaling required to initiate or stabilize in conjunction with FAK, the actin-based spreading or particle uptake in macrophages or neutrophils.

Last, in contrast to neutrophil chemotaxis, neutrophil phagocytosis is promoted in the presence of Calpain-1 (203). Human neutrophils incubated with Cytohesin-1 inhibitor SecinH3 resulted in significantly increased phagocytosis of unopsonized zymosan (204). Cytohesins are a class of small GEFs for ADP-ribosylation factors (ARFs), which regulate cytoskeletal organization, integrin activation, or integrin signaling, being ubiquitously expressed. In neutrophils, Cytohesin-1, in contrast to the above mentioned proteins,

seems to be a negative regulator of phagocytosis, maintaining the equilibrium in integrin regulation.

Antibody-Dependent Cellular Cytotoxicity

As mentioned, the presence of immunoglobulin can initiate Fc receptor activation and facilitate phagocytosis in an integrin-dependent way. When a target is too large to be phagocytized, neutrophils can exploit immunoglobulin binding to perform antibody-dependent cellular cytotoxicity (ADCC) (205). Besides antibody-opsonized bacteria and fungi, neutrophils can also kill antibody-opsonized covered targets such as cancer cells by extracellular toxicity (206, 207). Although Fcγ receptors have been traditionally invoked as the main receptors for antibody binding (208), also Fcα receptors are capable of initiating ADCC in human monocytes and neutrophils (209). Activation of these Fc receptor induces an intracellular signaling cascade with the immunoreceptor tyrosine-based activation motifs (ITAMs) of the Fc tail being phosphorylated, thus recruiting SYK protein binding, which eventually facilitates ADCC response. In ADCC of target cells, cell–cell interactions take place, gearing the response to a certain strength and outcome.

The β2 integrins are involved in ADCC either *via* inside-out or outside-in integrin regulation (210). CD11b/CD18 can become activated from Fcγ receptor IIa or IIIb through the recruitment and activation of PI3K. To date, the most solid evidence on β2 integrin and FcR involvement during ADCC has been collected from natural killer (NK) cells (211). Moreover, both CD11a/CD18 and CD11b/CD18 ligation to ICAM-1 positively regulates conjugate formation and cytolytic granule release (212, 213), whereas PD-1 signaling impairs outside-in integrin signaling and subsequent granule polarization (214). Yet, the killing effector function of NK cells substantially differs from neutrophils and macrophages, as it involves NK cell granule components.

We and others have clearly implicated the substantial role of CD11b/CD18 on neutrophils in ADCC against cancer cells (215, 216); however, current evidence on the integrin-associated ligands on tumor cells remains very limited. Although *in vitro* studies have shown that neutrophils adhere to melanoma cells *via* β2 integrin–ICAM-1 interactions (217), our recent findings have excluded ICAM-1, -2 or -3 as essential ligands for β2 integrin during neutrophil adhesion and cytotoxic activity in the case of breast cancer cells (218), indicating that the neutrophil interactions with different cell targets may vary in the use of counter/receptors or ligands.

ROS Production

Simultaneously with the process of phagosome formation, neutrophils consume an extreme amount of oxygen (respiratory burst), which enables them to produce reactive oxygen species (ROS) (219). The enzymatic machinery responsible for this function is the NADPH oxidase enzyme complex, which converts oxygen to superoxide and consequently hydrogen peroxide (220). As we have previously demonstrated, LAD-III syndrome causes impaired respiratory burst in response to zymosan, indicating a clear correlation between integrin activation and ROS production (196). These data are complementary with CD11b/CD18 deficient cells in mice, which resulted in defective respiratory burst (221). Integrin-mediated ROS induction (169) and Fcγ-receptor-mediated neutrophil activation required members of the VAV guanine nucleotide exchange factor and Rac small GTPase families (VAV1/3 and Rac2, respectively) (222, 223), and the PI3-kinase isoforms PI3Kβ and PI3Kδ instead of PI3Kγ, with a predominant role for PI3Kβ (224–226). SLP76/ADAP1 together with VAV isoforms (VAV1 and VAV3 in neutrophils) forms a complex with PLCγ2 which is also associated with immune complex-mediated signaling (222, 227, 228). VAV protein also functions as a Rac-GEF, enabling activation of Rac2 GTPase and thus inducing NADPH oxidase formation (229).

In an effort to further dissect the integrin-induced signaling cascade leading to NADPH oxidase in murine model systems, apart from the previously reported cascades, an indispensable role of the class III PI3K isoform (Vps34) was established in integrin-related ROS production in response to *Escherichia coli* or *Staphylococcus aureus* in neutrophils (**Figure 2D**). Vps34 is different from the above-mentioned class I PI3K isoforms (**Figure 2D**), and it is used for successful phagosome maturation and *Mycobacterium tuberculosis* destruction, while not essential for neutrophil chemotaxis or adhesion (230).

Outside-in signaling is important in adhesion-dependent responses so that neutrophils in suspension will not be able to perform, including degranulation and ROS production in case of TNF-induced effector function (231). Integrin outside-in signaling has been associated with neutrophil effector functions, including adhesion-dependent ROS production, which was dependent on SYK (101). Data on murine or human SFK-deficient neutrophils (lacking all three neutrophil-expressed SFK members, LYN, HCK and FGR) demonstrated the crucial role of SFKs during TNF-α-induced ROS production, accompanied by reduction of VAV protein phosphorylation in mice (232), which is a known regulator of oxidative burst (222).

THERAPEUTIC APPROACHES

With the emerging role of neutrophils in health and disease and because β2 integrins play such an integral role in the cellular functions of this cell type as discussed above, targeting β2 integrins on neutrophils specifically with small molecules could prove fruitful as a therapy. There are two considerations to use integrin-modulating drugs in the context of neutrophil function. Neutrophil activity could be dampened or enhanced through blockade or induction of integrin activity, respectively. For example, decreasing neutrophil extravasation could provide major benefits to patients suffering from inflammatory bowel disease such as Crohn's disease (233, 234), whereas LAD-III patients would benefit from an increase in neutrophil extravasation (80, 196, 235). One absolute requirement for such therapy in neutrophils would be the high specificity for

the β2 integrin molecule due to the multifunctional nature of β2 integrins, as well as of other proteins involved in its downstream signaling cascade. The way of intervening neutrophil function by altering β2 integrin activity could vary. A large selection of small molecules has been shown to interact with CD11b/CD18, giving rise to various candidates to be further explored as therapeutics (236), and low dosage of such compounds might also already impose a sufficient response with limited side effects on neutrophils and other leukocytes.

Another interesting possibility is the use of antibody-based therapies. A recent study has shown the use of a monoclonal antibody, named anti-M7 that specifically disrupted the interaction between CD11b/CD18 and CD40L and not any other tested ligand. In blocking this CD40L-binding activity of CD11b/CD18, downregulation of pathological inflammation in mice could be accomplished while keeping other functions intact (237). In contrast to general anti-CD11b antibodies (238), this anti-M7 against the ligand-binding I-domain of the CD11b subunit ameliorated instead of aggravated sepsis. Anti-M7 in fact enhanced iC3b binding and phagocytosis while blocking binding to endothelial or platelet CD40L (237). Such ligand-specific antibodies might prove useful during acute inflammatory responses or sepsis. One possible downside to the usage of antibodies is their relatively long half-life and possible off-target effects on other leukocytes, but this might be altered by tempering with the antibody structure, isotype, or using nanobodies which have a much shorter half-life. Humanized monoclonal antibody against CD11a (Efalizumab) has been thoroughly studied in a Phase III clinical trial in psoriasis patients, but the drug has been withdrawn due to a significant risk of developing multifocal leukoencephalopathy (239, 240). Collectively, intervening with β2 integrin-mediated interactions seem to be a promising avenue in both decreasing neutrophil extravasation as well as trying to increase neutrophil phagocytic activity, albeit requiring very specific targeting, dosage, or both, which seems a very unique characteristic to explore in case of the CD40L binding sequence in the I-domain of CD11b (237).

Another possibility in regulating neutrophil integrin function is by modulating other proteins involved in either inside-out or outside-in signaling. This would allow for intervention in integrin activation and thus function, but also modulation of specific processes, such as phagocytosis or ROS production, while excluding or limiting the effects on other cellular processes. For inside-out signaling, Kindlin-3 might be a promising candidate. However, this would require small cell-permeable Kindlin-3-antagonists which specifically interfere with β2 integrin activation while preserving activation of other integrins. This effect might be achieved by obscuring binding between the threonine triplet in the CD18 cytoplasmic tail, since threonine into alanine mutations affect the Kindlin-3 interaction with CD18 in T cells while retaining partial activity (241). Notably, this motive is absent next to the second NXXY motif of other β integrins and could be a specific target for β2 integrins (242), thus avoiding bleeding, a side effect also seen in LAD-III patients. Recent work has also elucidated for the first time the crystal structure of Kindlin-3 (67), which might partially reside as an auto-inhibitory homotrimer. Relaxation or enforcement of this homotrimeric state might be another way in which inside-out signaling can be influenced either positively or negatively, respectively.

Aside from Kindlin-3, Cytohesin-1 is an interesting molecule as a drug target since its inhibition results into neutrophil pre-activation, although its function is also implicated in phagocytosis (204). Since this effect might stem from the relief of negative regulation by Cytohesin-1 on the Arf6 GTPase (98), exploring specifically Arf6 inhibitors or activators could also prove to be a valid therapy as well as expand our current knowledge on its role in integrin activation. Although targeting inside-out signaling shows potential, targeting outside-in signaling proteins solely involved in one cellular process could also be of interest to retain other cellular functions associated with β2 integrins. For example, targeting PI3Kδ could be sufficient to downregulate ROS production and degranulation, although having a minor role compared to PI3Kβ (224–226). Moreover, Pyk2 could be a good target to reduce chemotaxis and migration while keeping other functions intact (175, 176). In conclusion, intracellular proteins regulating integrin activity could be explored as potent therapeutic targets; however, taking into account the multifunctional roles of the proteins in neutrophil response, more studies should be performed to assure maximal efficacy with limited off-target effects.

As mentioned above, Calpain-1 is a negative regulator of integrin-dependent chemotaxis and migration by cleaving a number of integrin-associated proteins such as Talin-1 (243). Calpains are intracellular cysteine proteases with very diverse physiological roles. *In vitro* studies with human neutrophils and small inhibitory compounds have shown that Calpain-1 is also involved in the β2 integrin signaling cascade of adhesion-dependent TNFα-induced oxidative burst (244). The potential use of therapeutic Calpain-1 inhibitors to counter predictable and unwanted inflammatory reactivity in which neutrophils play a major role, has been explored (244). Yet, aberrant activity of calpains—whether it be over- or under-activation—is often associated with pathological functions and thus with diseases. Effective calpain inhibition may be achieved by peptide and peptidomimetic inhibitors developed to increase the specificity and potency. Since calpains constitute a family of homologous proteins ubiquitously expressed, highly specific inhibition seems problematic. Calpain inhibitors have demonstrated efficacy in animal models of calpain-related diseases, but progression of the inhibitors into clinical trials has been hampered partly due to the lack of calpain isoform selectivity of these inhibitors.

Worth mentioning is the alternative approach for targeting β2 integrins with ICAM-1 blocking antibodies. Studies in patients with partial-thickness burn injury using the anti-ICAM-1 antibody (Enlimomab) have shown promising modulation of the inflammatory response and improved wound healing (245). However, application of the drug has also shown several side effects. Additional *ex vivo* studies have shown that the concentration of the antibody used in the initial clinical trials could promote complement-dependent neutrophil activation (246). For the treatment of Crohn's disease or ulcerative colitis, a 20-base antisense oligonucleotide inhibiting

ICAM-1 production (Alicarfosen) showed potential clinical benefit (247, 248). Clinical remission rates compared to placebo and whether the response can be maintained in the long-term in larger studies are as yet unknown.

DISCUSSION

We have reviewed and discussed most neutrophil functions requiring signaling through β2 integrins, either inside-out or outside-in integrin-dependent neutrophil activation. Although several proteins downstream of β2 integrins have been identified to play a role in phagocytosis, degranulation, or respiratory burst (**Table 1**), their exact role and sequential order or interdependence in integrin-mediated outside-in signaling cascade in killing and cytotoxicity remain to be determined. However, additional studies with innovative technologies are needed to develop a full picture of the CD11b/CD18 or CD11a/CD18 signaling cascades and their involvement in neutrophil effector function in humans.

The last decade, the technology of unbiased OMICS has improved our knowledge in cell biology. From transcriptomics to proteomics, we are now able to characterize RNA and protein expression levels in primary cells with an example of dissecting the composition of neutrophil cytoskeleton and the proteins associated with phagosome maturation (252, 253). In addition, large-scale studies include identification of signaling events and phosphorylation patterns after neutrophil stimulation, all of which have been nicely summarized elsewhere (254). Similar approaches have been followed in case of β1 adhesion receptors or intracellular signaling, where integrin-associated complexes have been identified by proteomic analysis (255–257). Recent methods focusing on interactome mapping based on proximity, such as Enhanced Ascorbate PeroXidase (APEX) or Biotin IDentification (BioID) have been used in order to anticipate function or downstream signaling cascade of β1, β4, and β5 integrins (258–262). Yet the complexity of leukocyte- associated β2 integrin has not allowed the investigation of its pathway with such method to date.

The increasing knowledge on the β2 interactome and integrin signaling in the context of neutrophil function allows it to be considered as a target for therapeutic intervention. Due to the short half-life of primary human neutrophils, research on β2 integrin-associated proteins upon activation is very limited. An alternative way to explore β2 integrin signaling is by exploiting neutrophil-like cell lines, such as HL-60 and NB4 cells. However, a considerable disadvantage of such cell lines is that they do not completely recapitulate neutrophil morphology or function, still unable to produce granules or migrate. Studies with these myeloid cell lines are useful for an initial orientation but will not be able to reveal the precise mechanism of signaling events in primary cells (263). Therefore, current efforts are focusing towards the generation of reproducible ways to generate neutrophils derived from induced pluripotent stem cells (iPSCs), being much closer to primary neutrophils (264).

In summary, we have discussed the role and signaling events of β2 integrins during neutrophil effector function. The relevance of this protein in this context is undisputed; however several questions remain unanswered. Although a detailed common pathway for extension of the β2 integrin is known, such a pathway for high-affinity activation of β2 integrins remains elusive; therefore we have summarized the current and most recent findings. We have also described differences regarding proteins involved with β2 integrins during different neutrophil functions such as adhesion, oxidative burst, phagocytosis or ADCC, highlighting the notion that β2 integrin outside-in signaling might include several signaling pathways, each distinctive per neutrophil effector response. Due to short neutrophil half-life, most studies have been focused on murine neutrophils, yet the differences compared to human neutrophils are substantial. To avoid possible confusion and emphasize the potential impact of each study, we have highlighted the data

TABLE 1 | Relevance of human and mouse CD11a/CD18 and CD11b/CD18 in neutrophil function.

	Human CD11a/CD18	*Human CD11b/CD18*	*Mouse CD11a/CD18*	*Mouse CD11b/CD18*
Adhesion/ Crawling	Initial adhesion of intermediate-avidity to endothelium following selectin-mediated rolling. After firm CD11b/CD18-mediated adhesion, a role in crawling over the endothelium (131)	Suggested to become fully active after CD11a/CD18. Pivotal in firm adhesion to the endothelial cell wall (131)	Most pivotal of the CD11/CD18 isoforms in mice, KO results in severe adhesion defects despite presence of CD11b/CD18 (249)	More redundant in mice, but adhesion and migration is affected in its absence (249).
Antibody derived cellular cytotoxicity (ADCC)	N/A	Activated *via* Fcγ-receptors, involved in downstream signaling (216).	N/A	N/A
Chemotaxis/ spreading (Two-dimensional)	Turnover of CD11a/CD18 during chemotaxis (166).	Increased avidity of CD11b/CD18 at cell-contact sites (250).	CD11a/CD18 seems more important compared to CD11b/ CD18 in mice (176).	N/A
Phagocytosis/ ROS production	No role described for CD11a/CD18 (251).	Binding of pathogens in a lectin-like way or once opsonized with iC3b and C4b (189). Recruits Fcγ-receptors for downstream signaling (196).	N/A	Binding of complement-opsonized zymosan. Recruits Fcγ-receptors for downstream signaling (221).

Summary list of human or murine studies regarding β2 integrin activation, inside-out or outside-in signaling during neutrophil effector function.

derived from mouse and human neutrophils. To conclude, usage of novel methods for both analysis and generation of neutrophils will be a key to further understanding the β2 integrin signaling. Such studies could lead to new therapeutic targets for modulating β2 integrin mediated neutrophil functions, or even leukocyte function in general.

AUTHOR CONTRIBUTIONS

HM and TK were the principle investigators that designed the work. SF, RA, MM, HM, and TK supervised the work on behalf of the LADOMICs consortium. PB, SW and MM wrote the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Molecular Mechanisms of Leukocyte Migration and Its Potential Targeting—Lessons Learned From MKL1/SRF-Related Primary Immunodeficiency Diseases

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Megakaryoblastic leukemia 1 (MKL1) deficiency is one of the most recently discovered primary immunodeficiencies (PIDs) caused by cytoskeletal abnormalities. These immunological "actinopathies" primarily affect hematopoietic cells, resulting in defects in both the innate immune system (phagocyte defects) and adaptive immune system (T-cell and B-cell defects). MKL1 is a transcriptional coactivator that operates together with serum response factor (SRF) to regulate gene transcription. The MKL/SRF pathway has been originally described to have important functions in actin regulation in cells. Recent results indicate that MKL1 also has very important roles in immune cells, and that MKL1 deficiency results in an immunodeficiency affecting the migration and function of primarily myeloid cells such as neutrophils. Interestingly, several actinopathies are caused by mutations in genes which are recognized MKL(1/2)-dependent SRF-target genes, namely *ACTB*, *WIPF1*, *WDR1*, and *MSN*. Here we summarize these and related (ARPC1B) actinopathies and their effects on immune cell function, especially focusing on their effects on leukocyte adhesion and migration. Furthermore, we summarize recent therapeutic efforts targeting the MKL/SRF pathway in disease.

Keywords: MKL1, SRF, neutrophil, migration, immunodeficiency—primary

INTRODUCTION

Leukocytes constantly traffic between different compartments in the body, and need to be able to employ different types of adhesion and migration modes in different types of environments. Briefly, leukocytes adhere to endothelial cells under shear flow conditions using adhesion receptors such as selectins, integrins, and intercellular adhesion molecules, utilize 2D migration modes on the

endothelial cell layer, and migrate in complex tissue environments utilizing 3D migration. Whereas 2D migration is strongly dependent on cell adhesion (i.e., ligand binding *via* integrins), the 3D amoeboid migration mode of leukocytes such as dendritic cells is associated with very low adhesion strength (i.e., integrin-independent) and relies on cytoskeletal deformation instead (1, 2).

Both during 2D and 3D migration, actin dynamics in migrating cells is complex and regulated by both positive and negative regulators. Actomyosin contraction at the cell rear end (uropod) aids in moving the cytoplasm and cell body, while the actin-related protein (ARP) 2/3 complex is an important mediator of actin polymerization at the leading edge (1). The small GTPases Rac and Cdc42 localize to the leading edge and regulate actin polymerization (2). In contrast, RhoA localizes to the uropod where it regulates actin cables (through the formin mDia) and actomyosin contractility (2). Proteins binding to G-actin, such as ADF/cofilin, and proteins severing and capping actin filaments, such as gelsolin, are also important factors regulating actin dynamics in migrating cells (3).

Primary immunodeficiencies (PIDs) are rare genetic disorders of the immune system, and lead to immune deficiencies of various severity. By studying primary immunodeficiencies, much has been learned about the molecular basis of immune system function, including leukocyte trafficking. In a classical example, Wiskott-Aldrich syndrome is caused by defects in the WAS protein (WASP), which plays essential roles in the regulation of the actin cytoskeleton upon cell activation. Consequently, WASP deficiency leads to fundamental defects of the immune system, including leukocyte migration, as well as a platelet defect with bleeding tendency because of reduced platelet counts (4).

Leukocyte adhesion deficiency type I-II and -III in turn are caused by defects in beta2-integrins, selectins, and kindlin-3, respectively, and result in severe defects in leukocyte (especially neutrophil) trafficking into sites of inflammation, as well as specific defects in adaptive immunity (5). Notably, all abovementioned defects (apart from LAD-II, which is a metabolic disorder which also affects leukocytes and neutrophil extravasation) represent typical hematopoietic disorders. This is explained by the lack of protein expression outside of the hematopoietic system and/or lack of redundancy in activity by homologous proteins that may substitute for the protein that is lacking or dysfunctional in activity. The selective expression of these proteins within the hematopoietic system also explains that curative treatment currently (still) consists of bone marrow transplantation.

Megakaryoblastic leukemia 1 (MKL1) deficiency is one of the most recently identified primary immunodeficiencies that causes a rare defect in actin-dependent processes, including leukocyte adhesion and migration. Here, we review what is known about MKL1 deficiency and other MKL/SRF (serum response factor)-related actin-based primary immunodeficiencies. In this review, we will focus on the proteins involved in this major pathway, their roles in immune cell migration and effector functions, and discuss potential lessons to be learned from these diseases and new opportunities with regards to therapeutic targeting of this pathway.

THE MKL1/SRF PATHWAY

MKL1, also called MRTF-A (myocardin-related transcription factor-A) or MAL (megakaryocytic acute leukemia), is a transcriptional co-regulator expressed in many cell types. It has long been known to have important roles in regulating actin and other cytoskeleton genes in many types of cells, together with the transcription factor SRF. There are two isoforms of MKL, MKL1, and MKL2, which have similar roles in cells. However, they also have non-redundant roles, as MKL2 knockout mice are embryonic lethal (6), while MKL1 knockout mice have a milder phenotype. MKL1 knockout mice show partial embryonic lethality, abnormal mammary gland function and reduced platelet count (7).

MKL1 is an interesting transcriptional coactivator, which is itself regulated by actin cytoskeletal dynamics (8). MKL1 is normally found in the cytoplasm, where it is bound to G-actin, and therefore excluded from the nucleus, which means it cannot regulate gene transcription. When cells receive an activating stimulus, such as serum stimulation, chemokine stimulation or other types of stimuli, RhoA is activated, leading to actin polymerization into F-actin. As a consequence, MKL1 is released from G-actin and transported into the nucleus (**Figure 1**). There, it encounters the transcription factor SRF and together the complex initiates gene transcription. In fact, SRF recruits two families of coactivators, the MKLs and the TCFs (ternary complex factors), to couple gene transcription to growth factor signaling. The MKL/SRF complex is involved in regulating cytoskeletal genes, such as actin, in many types of cells, including leukocytes, thereby influencing actin-dependent processes in cells (**Figure 1**). In macrophages, the SRF pathway indeed regulates the expression of cytoskeletal genes (9). In B cells, however, deletion of SRF also led to decreased expression of IgM, CXCR4 and CD19 (10). The specific role of MKL1 in immune cells is less explored. In human MKL1-deficient neutrophils, many adhesion and actin regulators were downregulated, including *CASS4, ALCAM, MYL9* (11); proteomic studies further identified *WDR* and actin itself. MKL1 also regulated expression of genes involved in interferon signaling, such as *STAT1* and several *IFIT* genes, but MKL1 deficiency also resulted in the upregulation of several genes (11). We have recently shown that in murine dendritic cells, MKL1 deletion did not only impact expression of cytoskeletal genes and genes of cytoskeletal regulators (*Fgr, Hck, Stmn1, Ckap2l, Anln, Tpm2, Tubb5*), but also genes encoding for proteins involved in many other cellular pathways, such as lipid metabolism (12). Therefore, the effect of MKL1 on gene expression in immune cells appears to be cell type-specific. However, it is clear that many MKL/SRF-target genes in leukocytes, as in other cell types, are actin and actin regulators, for example *ACTB*, *WIPF1*, *WDR1*, and *MSN* (13), which regulate actin cytoskeletal dynamics, and therefore affect actin-dependent processes in cells (**Figures 1** and **2**).

MKL1 DEFICIENCY

Interestingly, patients with MKL1 mutations have recently been reported. The first patient with a homozygous nonsense mutation in MKL1 was reported in 2015 (14), while two more cases (siblings)

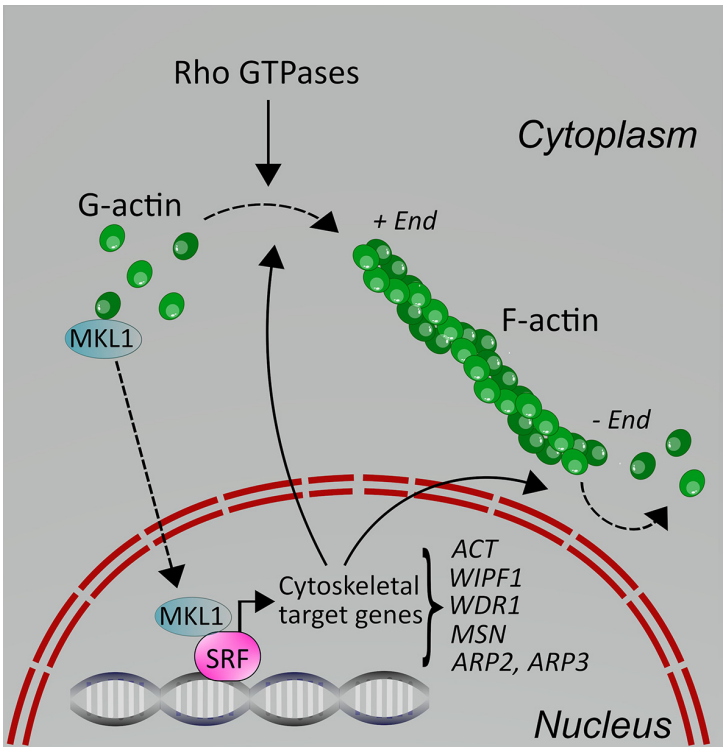

FIGURE 1 | Schematic depicting the molecular regulation of MKL1 in cells. In resting cells, MKL1 is sequestered in the cytoplasm due to binding to G-actin through its RPEL motifs. Upon cell activation through various stimuli, including serum, chemokines and integrins, Rho GTPases are activated, leading to F-actin polymerization. This releases MKL1, allowing it to translocate into the nucleus where it can activate gene transcription together with SRF. Some of the cytoskeletal target genes are shown here; for additional details, see text. Cytoskeletal target genes impact on the actin cytoskeleton in cells, leading to changes in processes such as cell adhesion and motility.

were reported in 2020 (11). The first sibling died after contracting pneumonia at an early age, the second one underwent a pre-emptive bone marrow transplant. MKL1 mutations result in primary immunodeficiency, with increased susceptibility to bacterial infections (*Pseudomonas* pneumonia). Lymphocyte counts and immunoglobulin levels were normal and no major defect in T- or B-cell function have been reported in these patients (14). Also no major platelet dysfunction has been reported (11, 14). Neutrophil numbers were also normal or elevated because of the concomitant infection. Phagocytosis in patient neutrophils has been reported both as normal – even of larger particles by neutrophils and macrophages (11) – and abnormal (14), while neutrophil reactive oxygen species (ROS) release and bacterial killing have been reported as being intact in MKL1 patients (11). Azurophilic granule release was found to be increased by MKL1-deficient neutrophils under suboptimal stimulation (11).

ROLE OF MKL/SRF SIGNALING IN LEUKOCYTE ADHESION AND MIGRATION

MKL1 deficiency in humans results in a severe migration defect of neutrophils (reduced chemotaxis towards chemotactic agents such as formyl-methionyl-leucyl phenylalanine (fMLF) and complement component 5a (C5a) (11, 14). Also spreading of both neutrophils and dendritic cells was reduced (11, 14). Interestingly, in neutrophils, firm adhesion, and transendothelial migration under shear flow conditions were severely affected, although cell adhesion under static conditions was not reduced (11). Total actin and F-actin levels in neutrophils were reduced (11, 14). Analysis of protein expression levels by high-resolution label-free quantitative mass spectrometry showed that indeed actin and actin regulators (WDR1, ARHGAP9, PFN1) were downregulated in MKL1-deficient neutrophils (11). The dramatic effect of the MKL1 mutation on neutrophil phenotype may be explained by the fact that these cells do not express MKL2, which may compensate for the lack of MKL1 in other cell types, such as fibroblasts. Indeed, primary fibroblasts with MKL1 deficiency display normal migration (11).

The role of MKL1 in dendritic cell adhesion and migration has also been investigated. Interestingly, the MKL1/SRF pathway is downstream of beta2-integrins and beta2-integrin-mediated RhoA activation and F-actin polymerization in murine leukocytes (12). 3D migration of MKL1 knockout dendritic cells in response to CCL19 was normal, while (static) adhesion to integrin ligands was slightly reduced. Integrins function as

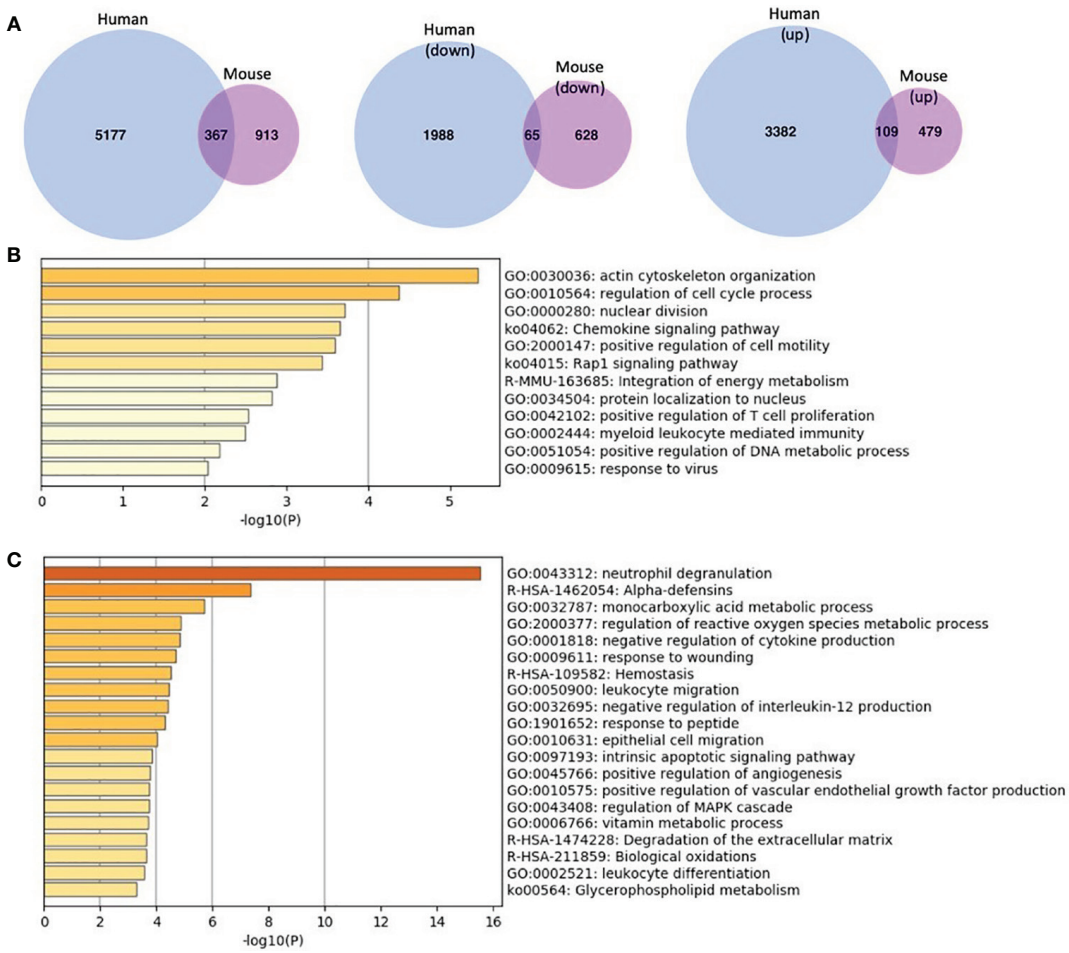

FIGURE 2 | Comparison of gene expression profiles for MKL1-deficient leukocytes (human and murine cells) **(A)** Differentially expressed genes (both up- and downregulated) in MKL1-deficient cells. There were 367 genes in common between them. The second and third Venn diagrams illustrate differentially expressed genes according to expression profile, i.e., upregulated and downregulated separately. There were 65 common downregulated genes and 109 upregulated genes between human and mouse MKL1 deficient cells. The mouse genes were converted to humans by Ensembl's BioMart package and then compared to the human genes. The Venn diagrams were created using venn.diagram package in R. **(B, C)** Pathway enrichment analysis using downregulated **(B)** and upregulated **(C)** common genes between human and mouse MKL1-deficient cells. This analysis was performed using Metascape.

mechanoreceptors in cells, transmitting mechanical force across the plasma membrane. In MKL1 knockout dendritic cells, integrin-mediated traction forces were reduced. Dendritic cells express both MKL1 and MKL2, which presumably underlies the fact that the adhesion/migration defect of MKL1 knockout dendritic cells was not as severe as in (human) neutrophils, which only express the one isoform.

MKL regulates transcription through interactions with SRF, which has been previously implicated in cell adhesion and migration in immune cells [reviewed in (15)]. SRF-deficient murine macrophages display reduced spreading, migration, and phagocytosis (9). In neutrophils, SRF plays an important role in cell trafficking *in vitro* and *in vivo*. SRF-deficient murine neutrophils failed to traffic into sites of inflammation *in vivo*, and displayed reduced binding to integrin ligands, and cell migration *in vitro* (16). Several actin regulators were downregulated in SRF knockout neutrophils, including *Actb*. Other SRF targets found to be downregulated also in murine MKL1 knockout dendritic cells include *Lima1, Actg1, Cnn2, Tpm4*, and *Wdr1* (12).

MKL1 (and presumably MKL1/SRF-dependent gene transcription of genes encoding cytoskeletal elements and additional proteins involved in signaling, etc) therefore appears to be most important for integrin-mediated cell adhesion under shear flow conditions, where cells have to withstand substantial mechanical forces to resist shear forces in blood vessels. In addition, its role in integrin-mediated traction force generation further implies that MKL1-regulated gene transcription is important for integrin mechanoreceptor function. However, certain other actin-dependent functions appear to function also without MKL1-signaling (ROS release, bacterial killing) and seem intact even in the presence of a strongly reduced G-actin level. It is possible that major actin polymerization is only required

for certain highly complex processes related to spreading and motility, whereas other actin-dependent activities such phagocytosis and (intraphagosomal) degranulation for the following killing process of the engulfed microbial particles may be less dependent on complex actin responses. In addition, although there are molecular similarities between the integrin/cytoskeletal processes involved in cell migration and phagocytosis, there are also differences [lack of myosin/contractility in phagocytosis, for example (17)] that may explain the differential dependence on MKL1/SRF of these processes.

Together, these human and murine data point towards an important role for MKL/SRF signaling for regulating myeloid immune cell integrin-mediated adhesion and migration *in vivo*, especially affecting neutrophils. Furthermore, mechanistically MKL1/SRF is probably involved in regulating neutrophil adhesion and migration through regulation of gene transcription of actin and actin-related factors/or other factors that regulate cell adhesion and migration.

ACTIN-RELATED PRIMARY IMMUNODEFICIENCIES INVOLVING MKL/SRF-TARGET GENES

Interestingly, several primary immunodeficiencies caused by cytoskeletal abnormalities ["actinopathies" (18–20)] are caused by mutations in genes which are recognized MKL(1/2)-dependent SRF-target genes, namely *ACTB*, *WIPF1*, *WDR1*, and *MSN* (13). Here, we summarize these actinopathies and their effect on immune cell function, in an attempt to dissect which MKL/SRF target genes may be important for leukocyte adhesion and trafficking.

Cytoplasmic Beta-Actin

Actin is one of the major targets of the MKL/SRF transcription axis (13) and, as observed in MKL1 deficiency (11, 14) important for homeostatic levels of actin. Actin has six isoforms, one of which is cytoplasmic beta-actin (encoded by *ACTB*) (21). Although loss-of-function mutations in *ACTB* have not been reported to cause a PID, it has been described that patients suffer from recurrent infections, which may imply an influence of these mutations on the immune system (22).

One report on immune cell function in a patient with a missense mutation in *ACTB* identified reduced chemotaxis and decreased ROS production of neutrophils (23). This mutation led to the expression of both normal and mutated cytoplasmic beta-actin in patient cells, including fibroblasts, leukocytes, Epstein–Barr virus-transformed B-cells, and platelets (23). Further investigation revealed that this mutated beta-actin had a slower polymerization rate than normal actin (24). More recently, a lack of beta-actin in mouse embryonic fibroblasts was shown to downregulate interferon-stimulated genes due to instability of the transcription factor interferon regulatory factor 3, thereby impairing the antiviral response (25). Likewise, SRF has been previously shown to be important for type I interferon signaling by induction of several interferon-stimulated genes (26).

Wiskott-Aldrich Syndrome Protein-Interacting Protein

Actin polymerization, i.e., the assembly of G-actin into F-actin, is mediated by the Wiskott-Aldrich syndrome protein (WASP), as it activates the actin-related proteins-2/3 (ARP2/3) complex. This complex, consisting of seven subunits, is an actin nucleator which initiates the branching of actin filaments (27). The Wiskott-Aldrich syndrome protein-interacting protein (WIP) interacts with WASP, thereby stabilizing its inactive state. When WASP gets activated, WIP is phosphorylated and dissociates from WASP (28, 29). The human gene encoding for WIP, *WIPF1*, has been identified to be a MKL-dependent SRF-target gene (13).

Only a handful of patients with WIP deficiency, caused by autosomal recessive mutations in *WIPF1*, have been identified thus far (30–32). As WIP regulates the stability and localization of WASP (33), loss of WIP will also lead to absence of WASP (30, 34). Therefore, patients with WIP deficiency have a similar clinical phenotype as WAS patients, i.e., recurrent infections, thrombocytopenia, and eczema (31, 35).

The adaptive immune cells, i.e., T-cells and B-cells, are most affected in WIP deficiency. Patients suffer from T-cell lymphopenia, where $CD8^+$ T-cells are more affected (35). Functionally, T-cells showed impaired proliferation, defective chemotaxis, defective exocytosis, and reduced target killing, most likely because there is a failure to assemble the immunological synapse (30, 32). Also, T-cells had an abnormal morphology, and failed to elongate and assemble a leading- and trailing edge upon stimulation (32). Natural killer (NK) cell–mediated cytotoxicity was reported to be reduced (30), and B-cells showed a chemotaxis defect, which could be rescued when WIP expression was restored in these cells (32). No defects in neutrophil function were reported in WIP patients, and monocyte-derived dendritic cells showed a normal phenotype (32). As the phenotype is quite different from that seen in MKL1 deficiency, *WIPF1* may not be the most important gene regulating neutrophil trafficking downstream of MKL/SRF.

WD Repeat-Containing Protein 1

Actin-interacting protein 1 (Aip1), encoded *WDR1*, is an actin-binding protein which enhances the disassembly and severing of actin filaments by cofilin (36, 37). *WDR1* is identified as an MKL/SRF target gene (13), and expression of *WDR1* is strongly reduced in murine cells lacking *Srf* (38), in murine dendritic cells lacking *Mkl1* (12), and on the protein level in neutrophils of MKL1-deficient patients (11). Mutations in *WDR1* have been reported to cause immunodeficiency (39–41), and *WDR1* is considered to be the main candidate gene to cause the "Lazy Leukocyte Syndrome", first described in 1971 (42). Patients with loss-of-function mutations in *WDR1* are reported to suffer from recurrent infections, mild neutropenia and impaired wound healing (39), but also a separate syndrome of auto-inflammation, periodic fever, and thrombocytopenia has been reported (40, 41).

WDR1-deficient patient cells, including neutrophils, monocytes, dendritic cells, and lymphocytes, are defective in actin depolymerization, resulting in increased F-actin levels (39–41). About 50% of the neutrophils had an altered herniation of their nuclear lobes, which co-localized with increased F-actin staining (39, 40). Neutrophils had a severe chemotaxis defect and showed abnormal spreading on glass, while *Staphylococcal aureus* killing and phagocytosis of opsonized *Escherichia coli* were reported to be normal. ROS production was found both normal and enhanced in WDR1-deficient neutrophils (39–41). Monocytes showed increased spreading over fibronectin, and $CD14^+$ peripheral blood mononuclear cells showed an increased caspase-1 activation, which corresponds with auto-inflammation (40, 41).

Furthermore, defects in the adaptive immune system were observed. T-cells had an increased spreading capacity and mildly impaired proliferation, but normal T-cell receptor internalization, normal migration, and normal killing of target cells by $CD8^+$ T-cells (40). B-cells showed increased apoptosis on B-cell receptor/Toll-like receptor stimulation and abnormal spreading, but normal migration. Also, patients suffered from peripheral B-cell lymphopenia and there was a lack of switched memory B-cells, reduced clonal diversity and paucity of B-cell progenitors in the bone marrow (40).

Moesin

Moesin (encoded by *MSN*) is a member of the ezrin, radixin, moesin (ERM) protein family, which link cortical actin filaments to the plasma membrane and membrane receptors. ERM proteins are important for structural stability and the integrity of the cell cortex (43). *MSN* is recognized to be a MKL-dependent SRF-target gene (13). As *MSN* is located on the X chromosome, the immunodeficiency was termed X-linked moesin associated immunodeficiency (44). Patients suffer from recurrent bacterial- and viral infections, persistent eczema and lymphopenia, as well as fluctuating neutropenia (44–46).

MSN-deficient T-cells showed impaired proliferation, impaired chemotaxis, and increased adhesion to vascular cell adhesion molecule 1 (44). While no functional analysis was done on neutrophils or monocytes from these patients, neutrophils from male moesin-deficient mice ($Msn^{-/Y}$) did display elevated rolling velocity in inflamed blood vessels, indicating that moesin has an important role in slow leukocyte rolling and subsequent trans-endothelial migration (47). Also, moesin-deficient mice showed a reduced neutrophil microbial killing ability towards *Pseudomonas aeruginosa* and reduced neutrophil-mediated vascular inflammation, while neutrophil adhesion was not affected (48).

Actin-Related Proteins 2/3 Complex Subunit 1B

The ARP2/3 complex, consisting of seven subunits, plays an essential role in the formation of branched actin networks by nucleating a daughter filament to the side of a pre-existing actin filament (27). These branched actin networks are especially of importance for generating a protrusive force that aids in cellular adhesion and motility (49). Almost all subunits of the ARP2/3 complex have been recognized to be MKL/SRF-target genes [ARP2, ARP3, ARP2/3 complex subunit 2 (ARPC2), ARPC4, and ARPC5] (13), suggesting that MKL1/SRF is involved in actin branching by regulating transcription of ARP2/3 complex family members. ARPC1 is present in two isoforms in humans, ARPC1A and ARPC1B, the latter being the dominant form in hematopoietic cells (50, 51). Although it is not (yet) identified as a MKL/SRF-target gene, patients with ARPC1B deficiency have a very similar neutrophil phenotype compared to MKL1-deficient patients.

Patients with ARPC1B deficiency suffer from a combined immunodeficiency including a neutrophil defect, resulting clinically in bacterial- and viral infections, bleeding tendency, eczema, allergy, and vasculitis (50–55). Similar to MKL1 deficiency, neutrophils have a severe migration defect due to an actin polymerization defect. Also, ROS production and the phagocytosis and killing of bacteria were found to be intact, and azurophilic granule release was increased under suboptimal stimulation (50).

Although no major defects in T- or B-cell function have been reported in MKL1 deficiency, ARPC1B deficiency does result in lymphocytes defects, including defective migration and proliferation, defective immunological synapse assembly by T-cells (53, 55), impaired regulatory T-cell suppressor activity and impaired NK-cell degranulation (52). Primary fibroblasts showed normal migration, most likely due to expression of ARPC1A in these cells (similar to possible MKL2 compensation in MKL1 deficiency) (50).

Thus, MKL1-deficient neutrophils have an almost identical phenotype as ARPC1B-deficient neutrophils, which could be explained by the involvement of MKL/SRF in transcription of ARP2/3 complex family members. However, ARPC1B deficiency also affects lymphocytes, which has not been observed in MKL1 deficiency. A possible explanation might be redundancy of MKL2 in lymphocytes, which express low levels of this isoform.

In conclusion, analysis of primary immunodeficiencies involving MKL/SRF targets and putative targets (**Table 1**) implicate ARP-subunits as possible targets downstream of MKL/SRF that could be involved in regulating leukocyte adhesion and migration *in vivo*.

TARGETING OF MKL/SRF IN IMMUNE CELL-MEDIATED INFLAMMATION—NEW POSSIBILITIES?

MKL/SRF signaling and MKL/SRF regulated gene expression of actin-related factors are important in leukocyte adhesion and migration, as shown by the actin-related primary immunodeficiencies described above. Interestingly, genetic syndromes also exist where actin dynamics is upregulated, eg X-linked neutropenia. In this disease, neutrophil levels in

TABLE 1 | List of MKL/SRF-related actinopathies with corresponding protein function, clinical symptoms, and reported functionally affected hematopoietic cells in these patients.

Actinopathy (gene)	Protein function	MKL/SRF target gene	Clinical symptoms	Hematopoietic cells functionally affected in patients	References
MKL1 deficiency (*MKL1*)	Coactivator of transcription factor SRF, thereby involved in regulating cytoskeletal gene transcription	Yes	Severe, recurrent bacterial infections	Neutrophils, dendritic cells	(11, 14)
Cytoplasmic beta-actin mutations (*ACTB*)	Non-muscle actin; one of the six isoforms of actin	Yes	Recurrent infections	Neutrophils	(23)
WIP deficiency (*WIPF1*)	Interacts with WASP, thereby stabilizing its inactive state	Yes	Recurrent infections, thrombocytopenia, eczema	NK-cells, T-cells, B-cells	(30–32, 35)
WDR1 deficiency (*WDR1*)	Actin-binding protein, enhances the disassembly and severing of actin filaments by cofilin	Yes	Recurrent infections, mild neutropenia, impaired wound healing, and auto-inflammation, periodic fever, thrombocytopenia	Neutrophils, monocytes, dendritic cells, T-cells, B-cells	(39–41)
Moesin deficiency (*MSN*)	Member of the ERM protein family; links cortical actin filaments to the plasma membrane and membrane receptors	Yes	Recurrent bacterial- and viral infections, eczema, lymphopenia, fluctuating neutropenia	T-cells	(44–46)
ARPC1B deficiency (*ARPC1B*)	Subunit of the ARP2/3 complex, thereby involved in branching of actin filaments	No, but most other ARP2/3 subunits are MKL/SRF-target genes	Recurrent bacterial- and viral infections, bleeding tendency, eczema, allergy, vasculitis	Neutrophils, NK-cells, T-cells, B-cells, platelets	(50–55)

A more elaborate table of immunological actinopathies, including those caused by mutations in non-MKL/SRF target genes, can be found in the review by Sprenkeler et al. (56). ARPC1B, actin-related protein complex 2/3 subunit 1B; ARP2/3 complex, actin-related protein complex 2/3; ERM, ezrin/radixin/moesin; MKL1, megakaryoblastic leukemia 1; NK-cells, natural killer cells; SRF, serum response factor; WASP, Wiskott-Aldrich syndrome protein; WDR1, WD repeat-containing domain 1; WIP, WASP-interacting protein.

blood are low, but there is increased migration of leukocytes into tissues. X-linked neutropenia is caused by gain-of-function mutations in *WASP*, leading to increased actin dynamics, and, as a consequence, to upregulation of actin-dependent neutrophil adhesion, migration, and recruitment into tissues (57).

As MKL/SRF signaling and downstream targets of this pathway are clearly associated with leukocyte migration, targeting this pathway in inflammatory diseases where leukocyte migration into tissues is upregulated may offer a new therapeutic strategy. MKL/SRF signaling is important in many cell types and tissues, and is also implicated in disease in different settings. Indeed, the MKL/SRF pathway has already been targeted in a number of different diseases, including several with an immune/inflammatory cell component, such as fibrosis and corneal wound healing. Interestingly, MKL/SRF inhibitors have been used to treat fibrosis in lung, skin, colon, liver, joints, and the eye (both the external cornea and the internal subretina) (**Table 2**). The target for these therapeutic approaches are myofibroblasts and the excess extracellular matrix (ECM) production by these cells, as MKL1 has been found to be important for myofibroblast differentiation (74). However, in scleroderma/systemic sclerosis patient skin samples, a disease associated with internal organ stiffening due to fibrosis, nuclear MKL1 has also been found in infiltrating macrophages (65), where it has been linked to proinflammatory gene expression (75, 76), indicating that also macrophages could be a target cell type for SRF inhibition in fibrosis.

Targeting the MKL/SRF pathway to improve corneal wound healing has emerged as an attractive strategy. The cornea is a transparent endothelial cell layer in the eye and its thinning as well as replacement with connective and corneal stroma is associated with blindness. In the cornea, stromal cells produce cytokines like interleukin-1 (IL-1) and transforming growth factor-β (TGF-β) to induce inflammatory and degradative processes or to induce ECM deposition (77). TGF-β1 activates the MKL/SRF pathway (67, 78), indicating MKL/SRF could play a role in corneal wound healing (79). Indeed, eye drops supplemented with the inhibitor Y-27632 to inhibit Rho Associated Kinase (ROCK) upstream of MKL/SRF, have been used to improve corneal endothelial wound healing *in vivo* (80).

SUMMARY AND FUTURE PROSPECTS

MKL1 deficiency is one of the most recently identified primary immunodeficiencies and is associated with globally impaired actin regulation, defective cell adhesion and abnormal trafficking of myeloid leukocytes. Studies in both mouse and man have provided novel insights into its complex role in immune cell migration and function in the context of infections. The main cell types affected by MKL1 deficiency and MKL/SRF signaling are neutrophils and macrophages. Neutrophils are involved in a wide variety of inflammatory processes including acute organ injury, cystic

TABLE 2 | MKL/SRF targeting in disease.

Disease	Inhibitor	Effect	Model	References
Fibrosis (Eye; subconjunctival scarring after glaucoma filtration surgery)	CCG-222740 and CCG-203971	Daily administration of 100 mg/kg CCG-222740 reduced and delayed subconjunctival scarring after glaucoma filtration surgery and improved surgery success by 67%. CCG-203971 delayed slightly less subconjunctival scarring and improved surgery success by 33% compared to a control. Inhibitors decreased cellularity and α-smooth muscle actin (α-SMA expression	Fibroblast mediated collagen contraction assay (inhibitor tests); *in vivo* scar tissue formation (subconjunctival scarring after glaucoma filtration surgery) model in rabbits	(58)
Subretinal fibrosis	CCG-1423	Inhibited cell migration; inhibition of TGF-β induced MKL1 shuttling, Paxilin and pro-MMP-2 expression; injection into vitreous cavity inhibited fibrosis development	Human retinal pigment epithelial cells (RPE-1 cells); murine *in vivo* CNV model	(59)
Dermal fibrosis	CCG-203971	Inhibited expression of connective tissue growth factor (CTGF), α-SMA, and collagen 1 (COL1A2) in fibroblasts. In mice CCG-203971 prevented bleomycin-induced skin thickening and collagen deposition	Systemic sclerosis fibroblasts, Bleomycin-Induced Injury Model	(60)
Dermal scarring	Fasudil (ROCK inhibitor)	Inhibited fibroblast contractility, and myofibroblast formation *in vitro*; inhibited wound contraction *in vivo*	Wistar-han rats	(61)
Colon fibrosis	CCG-1423, CCG-100602 and CCG-203971	Repressed matrix-stiffness and TGF-β mediated fibrogenesis	*In vitro* models using human colonic fibroblast CCD-18co cells (CRL-1459, derived from a female donor)	(62)
Liver fibrosis	CCG-1423	Alleviated c-Abl inhibition. c-Abl activation is associated with cellular fibrogenesis	Primary hepatic stellate cells, Immortalized human hepatic stellate cells (LX-2)	(63)
Osteoarthritis-related fibrosis	CCG-100602, Y-27632 (ROCK inhibitor)	Suppressed mRNA levels of α-SMA and type I collagen	Fibroblast-like synoviocytes cell line	(64)
Scleroderma/ systemic sclerosis (lung and skin in study)	CCG-1423	Decreases collagen, α-SMA, and CCN2 expression in SSc cells	Sclerodermal fibroblasts	(65)
Pulmonary fibrosis	CCG-203971 Fas-activating antibody	*In vitro:* inhibited MKL1 shuttling, blocked myofibroblast differentiation, inhibited TGF-β1-induced expression of fibronectin, X-linked inhibitor of apoptosis, and plasminogen activator inhibitor-1. *In vivo*: reduced lung collagen content, decreased alveolar plasminogen activator inhibitor-1 and promoted myofibroblast apoptosis	Adult primary human lung and primary human fetal lung fibroblast cell lines (CCL-210 IMR-90) Murine models of fibrosis induced by: •bleomycin •targeted type II alveolar epithelial injury	(66)
Pulmonary fibrosis	CCG-1423	Inhibited TGF-β–induced α-SMA expression	*In vitro* assay with primary isolated human lung fibroblasts	(67)
Cardiomyopathy	CCG-1423-8u (SRF targeting)	Inhibition of SRF in a disease mouse model increased survival from 98 days (ctrl) to 116	Murine CAP2 KO *in vivo* model	(68)
Vascular proliferative diseases	CCG-1423	Intraperitoneal treatment with 0.15 mg/kg for 3 weeks inhibited progression of vascular remodeling in arteries	Mice subjected to femoral artery wire injury	(69)
Aortic stiffening (cardivascular morbidity)	CCG-100602 SRF/ myocardin inhibitor	Reduced the stiffness of reconstituted tissue and changed LOX gene expression	Primary vascular smooth muscle cells (VSMCs) isolated from rats used for other experiments in the study, *in vitro* reconstituted tissue	(70)
Aortic stiffening	CCG-100602 1.5 mg/kg/ day for 2 weeks delivered by subcutaneous osmotic minipump	Reduced aortic stiffness and then blood pressure in SHR but not in WKY rats	Spontaneously hypertensive rats (SHR) Normotensive Wistar-Kyoto (WKY) rats	(71)
Hypertension	Y-27632 (Rock inhibitor), CCG-100602	Reduced aorta wall stiffness and blood pressure *in vivo*	*In vitro* VSMC models and SHR and WKY rats	(72)
Pulmonary Arterial Hypertension	CCG-1423	Attenuated pulmonary arterial hypertension and lung vascular remodeling	Sugen/hypoxia rats	(73)

Targeted diseases, inhibitors used, and the model systems used are described in this table.

fibrosis, ischemia reperfusion injury, atherosclerosis, and autoimmunity (such as rheumatoid arthritis). It is tempting to speculate that MKL/SRF, which is already being targeted in several diseases (**Table 2**) could also be used to reduce neutrophil and macrophage trafficking in these and other inflammatory disorders.

AUTHOR CONTRIBUTIONS

ES, CG, TK, and SF wrote the manuscript on behalf of the LADOMICS consortium. IF analyzed bioinformatics data and made **Figure 2**. 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.

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Monocyte Subset Recruitment Marker Profile Is Inversely Associated With Blood ApoA1 Levels

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Dyslipidemia promotes development of the atherosclerotic plaques that characterise cardiovascular disease. Plaque progression requires the influx of monocytes into the vessel wall, but whether dyslipidemia is associated with an increased potential of monocytes to extravasate is largely unknown. Here (using flow cytometry) we examined recruitment marker expression on monocytes from generally healthy individuals who differed in lipid profile. Comparisons were made between monocyte subsets, participants and relative to participants' lipid levels. Monocyte subsets differed significantly in their expression of recruitment markers, with highest expression being on either the classical or non-classical subsets. However, these inter-subset differences were largely overshadowed by considerable inter-participant differences with some participants having higher levels of recruitment markers on all three monocyte subsets. Furthermore, when the expression of one recruitment marker was high, so too was that of most of the other markers, with substantial correlations evident between the markers. The inter-participant differences were explained by lipid levels. Most notably, there was a significant inverse correlation for most markers with ApoA1 levels. Our results indicate that dyslipidemia, in particular low levels of ApoA1, is associated with an increased potential of all monocyte subsets to extravasate, and to do so using a wider repertoire of recruitment markers than currently appreciated.

Keywords: monocytes, atherosclerosis, dyslipidemia, adhesion, migration, cardiovascular disease

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INTRODUCTION

Monocyte recruitment into the blood vessel wall is an essential step for atherosclerotic plaque development which characterizes cardiovascular disease (CVD). Monocyte transformation into macrophages, particularly adoption of an inflammatory phenotype, promotes plaque progression including acquisition of an unstable plaque morphology which can lead to clinical events (1, 2). In murine models of atherosclerosis, monocyte accumulation is proportional to lesion size (3) and blocking monocyte recruitment reduces plaque progression (4). Similarly, inhibiting monocyte recruitment is

considered an attractive target for reducing human plaque development, inflammation and consequently, clinical events (5).

Monocyte recruitment is primarily mediated by two key steps: adhesion and migration (6, 7). Importantly, monocyte firm adhesion is mediated through a well-orchestrated arrangement of various adhesion molecules such as selectins and integrins (6–9) and their transmigration mediated by the chemokine receptors and their associated ligands (10–12). Which specific molecules monocytes use likely varies as they are a heterogenous population with three major subsets identified: Classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$), non-classical ($CD14^{+}$, $CD16^{++}$) (13). The expression of recruitment markers on human monocyte subsets differs (14, 15). In particular, classical monocytes express higher levels of adhesion molecules such as CD62L and chemokine receptors CCR2, CXCR2, whereas CD16+ (intermediate and non-classical) monocytes express higher levels of CCR5 and CX3CR1 (12, 14, 16–18). As such, classical monocytes are considered to have a greater potential to migrate into injured or inflamed tissue than the intermediate and non-classical subsets (19). Indeed, they do so in murine models of inflammation, including models of atherosclerosis (20); accordingly, a lack of classical, but not non-classical monocytes greatly reduce lesion size (21).

Within the circulation itself, monocyte numbers are increased in cardiovascular disease, with an increased proportion of intermediate monocytes associated with occurrence of major cardiovascular events (22, 23) and increased mortality (22, 24). Whether the distinct recruitment marker profile of monocyte subsets is also altered is unclear, but needs to be assessed if monocyte migration, particularly of a specific subset, is to be considered as a therapeutic target for reducing plaque progression.

The increased monocyte count in CVD is related to lipid levels (dyslipidemia), primarily, to low levels of HDL-C that promote monocytosis (25). HDL, through its major protein component apolipoprotein A1 (ApoA1), is also known to have anti-inflammatory properties (26–28), and in line with this, we previously found monocyte inflammatory status, such as production of IL-1β, is related to HDL-C and ApoA1 levels—even for generally healthy individuals (29). Whether dyslipidemia is also accompanied by increased potential for migration into the plaque is important to discern because with an estimated 2 in 3 adults having dyslipidemia (30), influx of inflammatory monocytes into the vessel wall may be silently promoting atherosclerotic plaque development in countless dyslipidemic individuals who are considered otherwise generally healthy. In this study, we assessed monocyte recruitment marker expression in individuals who were generally healthy but differed in lipid levels. The results were compared between monocyte subsets, between participants, and relative to participants' lipid levels.

MATERIALS AND METHODS

Study Population

This study was approved by the Western Sydney Local Health District (WSLHD) Human Research Ethics Committee. Informed signed consent was obtained from all participants. We recruited individuals (n = 30) who were in generally good health but differed in lipid levels – with a wide range of lipid levels achieved by including participants visiting the Westmead Lipid clinic. Exclusion criteria included: a documented history of CVD, diagnosed hypertension, diabetes mellitus (Type I or II), a current acute or chronic inflammatory disease (C-reactive protein (CRP) > 5.0 mg/L), being a current smoker, and/or taking lipid-lowering or anti-inflammatory medication.

Biochemical and Lipid Measurements

Peripheral blood samples were collected from overnight-fasted participants. Leukocyte counts, total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), triglyceride (TG), apolipoprotein A1 (ApoA1), apolipoprotein B (ApoB), glucose and CRP were measured using standard laboratory methods at the Institute for Clinical Pathology and Medical Research (ICPMR) at Westmead Hospital.

Assessment of Expression of Surface Adhesion Molecules and Chemokine Receptors on Monocyte Subsets

Surface marker assessment was performed by whole blood flow cytometry on K2EDTA-anti-coagulated blood. Whole blood aliquots (50 μL) were stained with anti-CD14-V450 (BD Pharmingen), anti-CD16-APC (Abcam) and anti-HLA-DR Per-CP (Biolegend) to identify monocyte subsets. PE-conjugated antibodies against surface adhesion molecules, selectins: CD44 and CD62L; integrins: CD11a, CD11b, CD11c, CD18, CD29, CD49d, and chemokine receptors: CD182, CD183, CD184, CD195, CD197, CCR2, CX3CR1 (all PE antibodies from BD Pharmingen) were also added. The tubes were then incubated for 30 min at 4°C in the dark. PE-conjugated matching isotype controls were used to determine relative degree of surface marker expression. The cells were fixed, and red blood cells lysed, by the addition of 250 μL Optilyse C (Beckman Coulter).

Flow Cytometry

Flow cytometry was used to detect monocyte surface marker expression. Data was collected on a BD FACS™ Canto II flow cytometer (BD) using FACSdiva software (v6.0, BD). At least 5,000 events—based on cells falling in a strong CD14 positive gate on SSC-A vs. CD14 density plot—were recorded.

CompBeads (BD), were used to generate a compensation matrix which was applied before data analysis, which was performed using FlowJo® software (v10.1r5, Tree Star, USA). The gating strategy for identifying the monocytes subsets, while excluding potential contaminating cell types including B cells, T cells, neutrophils and NK cells, was as previous (29). The relative level of expression was determined by the ratio of the geometric mean fluorescence intensity (MFI) of the marker of interest over the MFI of the isotype control as previously reported (31).

Statistical Analysis

SPSS software (v25, IBM Corporation) was used for statistical analysis. Data for adhesion molecule and chemokine receptor

expression are shown as mean ± SD unless otherwise stated. The data were log transformed to stabilise the variance prior to analysis. Comparisons between monocyte subsets were performed using ANOVA followed by *post-hoc* Tukey's test. The differences between the monocyte subsets were back transformed to provide fold changes and associated 95% confidence intervals (CIs). Comparisons between sexes were conducted using the Student's t-test for normally distributed data. Associations between monocyte subsets and monocyte subset adhesion and migration profile and participants' lipid levels were assessed using Spearman's rank correlations. All tests were two-tailed and a p value of <0.05 was considered statistically significant.

RESULTS

Monocyte Subsets Differentially Express Surface Adhesion Molecules and Chemokine Receptors

Characteristics of the participants are summarized in **Table 1**.

The expression of CD11b, CD62L, CCR2, and CXCR2 (CD182) was significantly higher on classical monocytes when compared to intermediate and non-classical monocytes, and higher on the intermediate compared to non-classical (**Figures 1A, B**: all $p<0.001$). Of note, there was no appreciable expression of CCR2 on the non-classical subset and low levels on the intermediate subset for most participants. The expression of CD11a, CD11c, and CD49d was highest on non-classical monocytes followed by the intermediate subset with significant differences between all the subsets (**Figure 1A**: for CD11a: NC/C and NC/I both $p<0.001$, I/C $p<0.01$; CD11c: NC/C and I/C: both $p<0.001$ and NC/I: $p<0.01$; CD49d: all $p<0.001$). The expression of CD18 was signficiantly higher on the intermediate and non-classical subsets compared to the classicals, with minimal difference between the intermediate and non-classicals (**Figure 1A**: I/C and NC/C: both $p<0.01$). The expression of CD29 was highest on non-classicals followed by classicals and then the intermediates (**Figure 1A**: NC/C and NC/I both $p<0.001$ and C/I $p<0.01$). All monocyte subsets expressed CD44 with minimal, but still significant, differences in expression between them (**Figure 1A**: C/I and NC/I $p<0.01$ and NC/C $p<0.05$). As a whole, no appreciable expression of CCR5 (CD195), CCR7 (CD197), CXCR3 (CD183), CXCR4 (CD184), or CX3CR1 was detected (data not shown) except for CCR5 for which low or minimal levels were detected on the intermediate and non-classical monocyte subsets of 8 individuals. As we had previously found that the expression of monocyte inflammatory markers is not distinct between the subsets, but rather a continuum from the classical, through the intermediate to the non-classical subset (29), we assessed whether the differences in expression of adhesion molecules or chemokine receptors seen between the monocyte subsets were distinct or occurred in a gradual manner. We viewed the expression of adhesion molecules (integrins: CD11b and CD11c) as well as the chemokine receptor, CCR2 within the subsets by heat map on the CD14/CD16 dot plot (**Figure 1C**). The change in expression of CD11b and CD11c between the subsets was independent of their subset divisions as evident by both low and high expression within the classical subset (**Figure 1C**). Interestingly, for CD11b its expression was relative to that of CD14. CCR2 expression followed the traditional classical to non-classical maturation understanding, decreasing through the intermediate to non-classical population (**Figure 1C**).

TABLE 1 | Characteristics of study participants.

Characteristics of study participants	*n = 30*
Age (years)	45 ± 12 (27–68)
Sex	
Male	16 (53)
Female	14 (47)
Blood pressure	
SBP (mmHg)	121 ± 17 (100–160)
DBP (mmHg)	77 ± 8.6 (64–95)
Lipid profile	
TC (mmol/L)	5.3 ± 1.5 (3.6–9.8)
HDL-C (mmol/L)	1.4 ± 0.48 (0.59–2.7)
LDL-C (mmol/L)	3.2 ± 1.4 (1.5–7.9)
Cholesterol/HDL-C Ratio	4.3 ± 2.2 (2.0–11)
Apo A1 (g/L)	1.4 ± 0.45 (0.53–2.5)
Apo B (g/L)	0.92 ± 0.38 (0.29–2.0)
Triglycerides (mmol/L)	1.2 ± 0.73 (0.27–3.6)
Glucose (mmol/L)	4.9 ± 0.87 (3.0–7.1)
Risk Factors	
Dyslipidemia	
TC (>5.5 mmol/L)	11 (36)
LDL-C (>3.5 mmol/L)	9 (30)
TG (>2 mmol/L)	3 (10)
HTN (SBP/DBP, mm Hg)	1 (3)
Glucose (> 5.4 mmol/L)	5 (16)

SBP, systolic blood pressure; DPB, diastolic blood pressure; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglycerides; HTN, hypertension and Apo, Apolipoprotein. Data for age, blood pressure and lipid profile are presented as mean ± SD and (range). Data for sex and risk factors are number of patients and percentage n (%).

The Expression of Recruitment Markers Varies Greatly Between Participants

There were considerable differences in the expression of recruitment markers between individuals (**Figure 2A**) with marker levels being higher—on all three subsets—for some participants compared to others.

With the increased recruitment marker levels occurring for all subsets, not just one, we looked to see if there was any relationship in the degree of marker expression between the subsets. This was indeed the case, the level of expression of recruitment markers for one subset correlated significantly with that of the next subset (**Figure 2B**: CD11a and CD44, all monocyte subsets—$p<0.001$ and CCR2, classical vs. intermediate—$p<0.001$). Note, CCR2 correlated only between the classical and intermediate subsets due to lack of appreciable expression on the non-classical subset.

With the expression of the most recruitment markers varying between participants, the question arises whether an increased level of one marker is associated with increased levels of others. This was

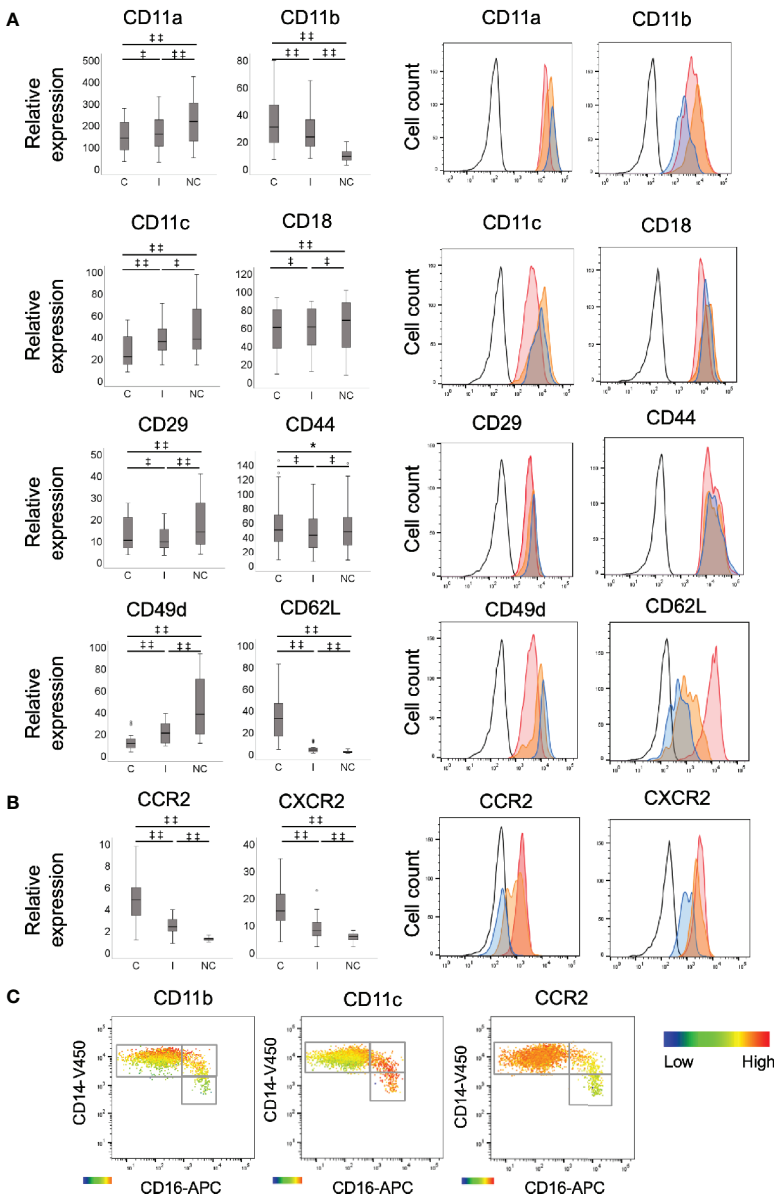

FIGURE 1 | Comparison of recruitment marker expression between monocyte subsets. Expression of **(A)** adhesion molecules, CD11a, CD11b, CD11c, CD18, CD29 (all integrins), CD44 (selectin), CD49d (integrin) and CD62L (selectin) and **(B)** chemokine receptors, CCR2 and CXCR2 by monocyte subsets, measured by flow cytometry. C: classical; I: intermediate; and NC: non-classical, n=30. Data presented as box and whisker plots, with outliers denoted by circles and representative histograms. Black lined histograms: isotype control, red histograms: classical monocytes, orange histograms: intermediate monocytes and blue histograms: non-classical monocytes. The data were log transformed to appropriate normality and in order to stabilise the variance prior to analysis. Statistical calculations of significance were performed using repeated measures ANOVA for significant differences in the relative expression levels of the markers between any 2 monocyte subsets within subject, $^{‡‡}p < 0.001$; $^{‡}p < 0.01$; $^{*}p < 0.05$. The differences between subsets were back transformed to provide fold changes (relative to the isotype control) and associated 95% confidence intervals (CIs). **(C)** Differential expression of recruitment markers on monocyte subsets. Heat map showing the degree of expression from high (red) to low (blue) of integrins, CD11b and CD11c and chemokine receptor, CCR2 on whole blood monocyte population based on CD16 and CD14 expression. The heat maps represent classical, intermediate and non-classical monocytes, respectively. The heat maps were created using Flow Jo software.

assessed (for each subset), and indeed, numerous significant moderate and strong correlations were observed (**Figure 2C**). Correlations were evident not just between the adhesion molecules (e.g. CD11a and all other adhesion markers for each subset) or between the chemokine receptors (CCR2 and CXCR2 for the classical and intermediate subsets), but also between adhesion molecules and chemokine receptors (for example CD11a and CXCR2 for the classical and intermediate subsets).

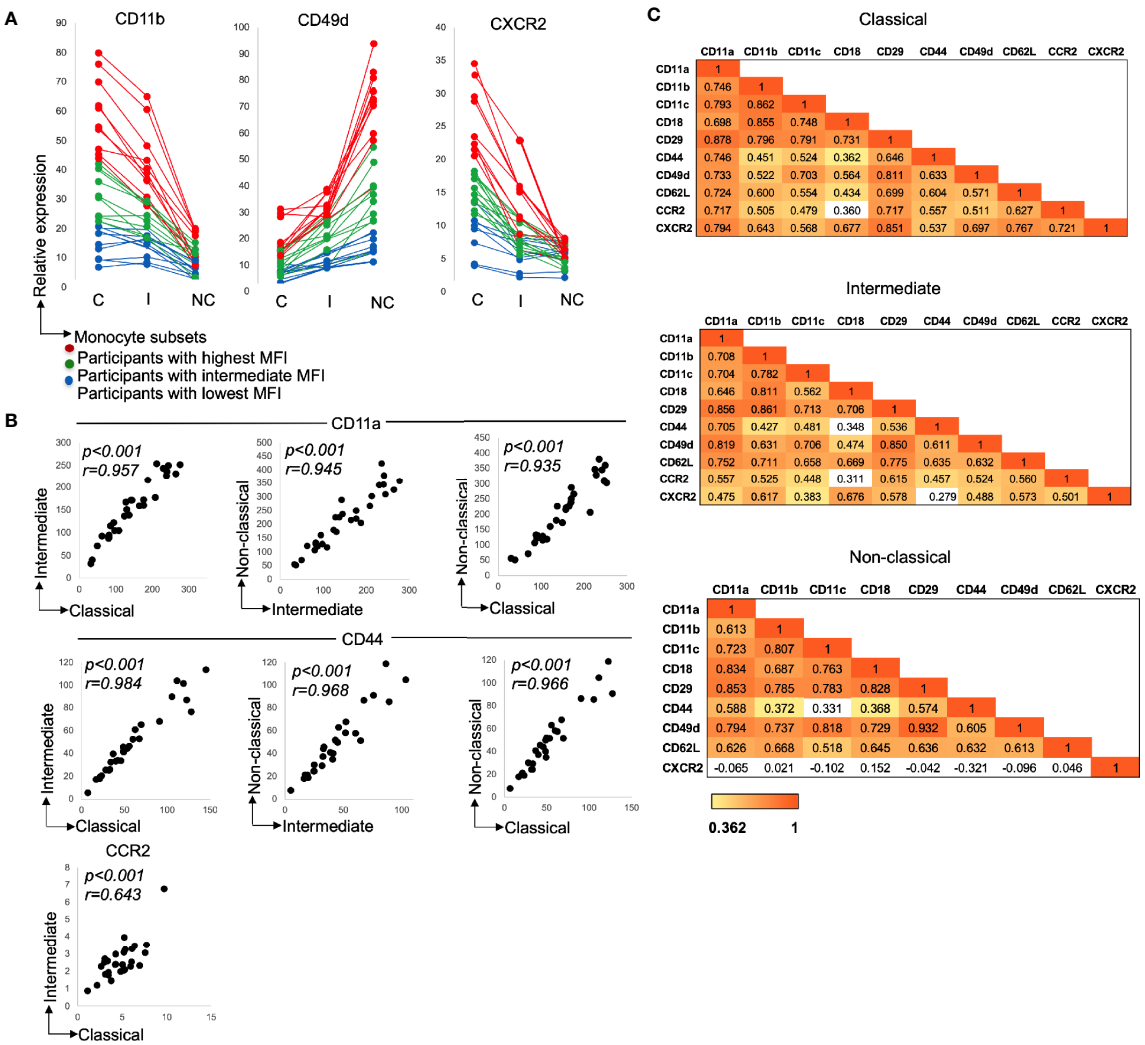

FIGURE 2 | **(A)** Variation in adhesion marker expression between the participants and monocyte subsets. Monocyte subset adhesion molecules, CD11b, CD49d (integrins) and chemokine receptor, CXCR2 expression. Each line on the graph is representative of relative adhesion marker expression from one participant. Participants with the highest marker expression were ranked based on monocyte subsets expressing the highest, CD11b, CD49d, and CXCR2. Red lines show participants with highest CD11b, CD49d, and CXCR2 expression (n = 10), green lines show participants with intermediate CD11b, CD49d, and CXCR2 expression (n=10) and blue lines show participants with lowest geometic mean fluoroscence intensity (MFI), CD11b, CD49d, and CXCR2 expression (n=10). C: Classical; I: intermediate and NC: non-classical. **(B)** Relationship between adhesion molecule expression by the monocyte subsets. Expression of CD11a (all $p < 0.001$; n=30), CD44 (all $p < 0.001$; n=30) and CCR2 (C- $p < 0.001$; n = 30). Data presented as scatter plots. The statistical significance of correlation was determined by Spearman's rank correlation. All tests were two tailed. r represents correlation coefficient and p represents the statistical significant value. **(C)** Heatmap with correlation matrix. Panel 1: Classical monocytes, 2: Intermediate monocytes and 3: Non-classical monocytes. For all heatmaps, values are Spearman's rank correlation coefficients. The color in the heatmap represents the degree of correlation. Red (value of 1) represents a stronger correlation coefficient and more significant p value. Yellow (value of 0.362) represents a moderate correlation coefficient and p value of *0.05* or less. White boxes represent non-significant correlations.

Altered Lipid Levels May be One Factor Promoting Monocyte Subset Recruitment

We then assessed whether the variation seen between the participants was explained by their lipid profile.

There were many correlations between the recruitment markers and ApoA1 levels—**Table 2**. Notably, for integrins, CD11b, CD11c, and CD29 expression inversely correlated with ApoA1 levels on all monocyte subsets (**Figures 3A, B**: CD11b: classical and intermediate, $p<0.001$ and non-classical, $p<0.01$; CD11c: classical, $p<0.01$, intermediate, $p=0.001$ and non-classical, $p<0.05$; and CD29: classical, $p<0.01$, intermediate and non-classical, $p=0.001$). Interestingly, of the integrins, CD29 inversely correlated with glucose levels on all monocyte subsets (**Table 2**, all $p<0.05$).

Correlations between chemokine receptors and lipid levels were evident for e.g. CCR2 and CXCR2—**Table 2**. Notably, the expression of CCR2 positively correlated with the LDL-C levels on classical monocytes only (**Table 2**: $p<0.05$) and inversely

TABLE 2 | Recruitment marker expression relative to lipid levels for each monocyte subset.

		TC (mM)		LDL-C(mM)		HDL-C(mM)		TC: HDL-C ratio		TG(mM)		Apo A1(g/L)		Apo B(g/L)		Apo A1: ApoB ratio		Glucose (mmol/L)	
		r	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*
C_CD44	***n=30***	0.045	0.811	0.080	0.674	−0.080	0.673	0.152	0.424	−0.266	0.156	−0.353	0.056	−0.092	0.630	−0.101	0.595	0.011	0.955
I_CD44		0.110	0.564	0.137	0.472	−0.115	0.546	0.197	0.296	−0.208	0.271	−0.361*	0.050	−0.044	0.819	−0.137	0.469	0.101	0.594
NC_CD44		0.104	0.583	0.112	0.555	−0.090	0.636	0.190	0.314	−0.219	0.245	−0.334	0.072	−0.080	0.672	−0.940	0.623	0.136	0.473
C_CD62L		0.028	0.883	0.190	0.314	−0.104	0.585	0.164	0.388	−0.104	0.583	−0.469**	0.009	−0.121	0.524	−0.208	0.269	−0.292	0.118
I_CD62L		0.227	0.228	0.256	0.172	0.029	0.878	0.156	0.411	−0.144	0.449	−0.362*	0.050	−0.023	0.902	−0.189	0.318	−0.150	0.429
NC_CD62L		0.234	0.214	0.248	0.186	−0.121	0.524	0.287	0.125	−0.248	0.186	−0.409*	0.025	0.010	0.956	−0.290	0.120	−0.140	0.461
C_CD11a		0.080	0.673	0.216	0.251	0.078	0.681	0.059	0.757	−0.238	0.205	−0.444*	0.014	−0.232	0.217	−0.050	0.793	−0.211	0.264
I_CD11a		0.116	0.541	0.290	0.120	0.031	0.869	0.130	0.493	−0.184	0.332	−0.414*	0.023	−0.157	0.408	−0.126	0.507	−0.233	0.216
NC_CD11a		0.107	0.574	0.307	0.099	0.138	0.468	0.065	0.732	−0.139	0.464	−0.345	0.062	−0.187	0.323	−0.050	0.795	−0.334	0.071
C_CD11b		0.224	0.235	0.339	0.067	−0.251	0.182	0.352	0.056	−0.002	0.993	−0.655**	0.000	−0.028	0.885	−0.415*	0.022	−0.321	0.084
I_CD11b		0.118	0.536	0.341	0.065	−0.200	0.290	0.276	0.140	−0.002	0.992	−0.618**	0.000	−0.093	0.623	−0.345	0.062	−0.307	0.098
NC_CD11b		0.263	0.160	0.279	0.136	−0.047	0.804	0.251	0.180	−0.208	0.270	−0.476**	0.008	0.027	0.889	−0.294	0.115	−0.335	0.070
C_CD11c		0.199	0.293	0.285	0.127	−0.105	0.580	0.260	0.166	−0.206	0.275	−0.490**	0.006	0.033	0.864	−0.297	0.112	−0.217	0.249
I_CD11c		0.147	0.438	0.203	0.283	−0.186	0.324	0.291	0.119	−0.269	0.151	−0.578**	0.001	−0.006	0.977	−0.325	0.080	−0.290	0.120
NC_CD11c		0.200	0.289	0.295	0.114	−0.049	0.795	0.237	0.207	−0.133	0.483	−0.399*	0.029	0.072	0.707	−0.275	0.141	−0.329	0.076
C_CD18		0.135	0.477	0.322	0.083	−0.032	0.868	0.139	0.463	0.035	0.853	−0.355	0.054	−0.070	0.713	−0.204	0.281	−0.206	0.275
I_CD18		0.186	0.326	0.410*	0.025	0.003	0.988	0.187	0.322	0.107	0.574	−0.234	0.213	0.065	0.733	−0.261	0.163	−0.298	0.109
NC_CD18		0.196	0.298	0.455*	0.012	−0.012	0.950	0.198	0.293	0.035	0.855	−0.433*	0.017	−0.097	0.608	−0.246	0.190	−0.340	0.066
C_CD29		0.010	0.958	0.267	0.154	−0.010	0.958	0.131	0.491	−0.100	0.599	−0.549**	0.002	−0.217	0.249	−0.168	0.376	−0.375*	0.041
I_CD29		0.108	0.571	0.348	0.060	−0.056	0.768	0.205	0.277	−0.067	0.723	−0.592**	0.001	−0.171	0.365	−0.254	0.176	−0.401*	0.028
NC_CD29		0.051	0.787	0.215	0.255	0.001	0.997	0.148	0.435	−0.151	0.426	−0.559**	0.001	−0.193	0.308	−0.164	0.387	−0.389*	0.034
C_CD49d		0.040	0.834	0.207	0.272	0.077	0.684	0.078	0.682	−0.236	0.208	−0.267	0.153	−0.061	0.748	−0.115	0.547	−0.142	0.454
I_CD49d		0.017	0.928	0.197	0.296	−0.008	0.966	0.122	0.522	−0.204	0.278	−0.518**	0.003	−0.168	0.374	−0.156	0.412	−0.331	0.074
NC_CD49d		0.022	0.908	0.156	0.410	0.010	0.956	0.115	0.545	−0.250	0.183	−0.479**	0.007	−0.135	0.476	−0.143	0.451	−0.300	0.108
C_CXCR2		−0.023	0.902	0.246	0.190	0.063	0.741	0.020	0.915	−0.023	0.904	−0.430*	0.018	−0.225	0.231	−0.055	0.773	−0.320	0.085
I_CXCR2		0.002	0.991	0.190	0.314	0.219	0.245	−0.115	0.545	−0.038	0.842	−0.201	0.286	−0.150	0.428	0.061	0.748	−0.226	0.229
NC_CXCR2		−0.159	0.401	−0.016	0.934	0.187	0.323	−0.276	0.140	−0.085	0.654	0.040	0.834	−0.224	0.233	0.233	0.215	0.063	0.740
C_CCR2		0.158	0.412	0.371*	0.048	0.060	0.757	0.141	0.464	−0.107	0.580	−0.418*	0.024	−0.070	0.719	−0.138	0.476	−0.437*	0.018
I_CCR2		0.070	0.719	0.304	0.109	0.056	0.774	0.011	0.953	−0.043	0.826	−0.429*	0.020	−0.097	0.616	−0.043	0.823	−0.163	0.399

*C, classical; I, intermediate; NC, non-classical; r, correlation coefficient; p, level of significance (2-tailed), **p ≤ 0.01; *p ≤ 0.05 (Spearman's rank correlations). Shading indicates the significant values shown in bold. Dark blue indicates the values significant at p ≤ 0.01 and light blue, at p ≤ 0.05.*

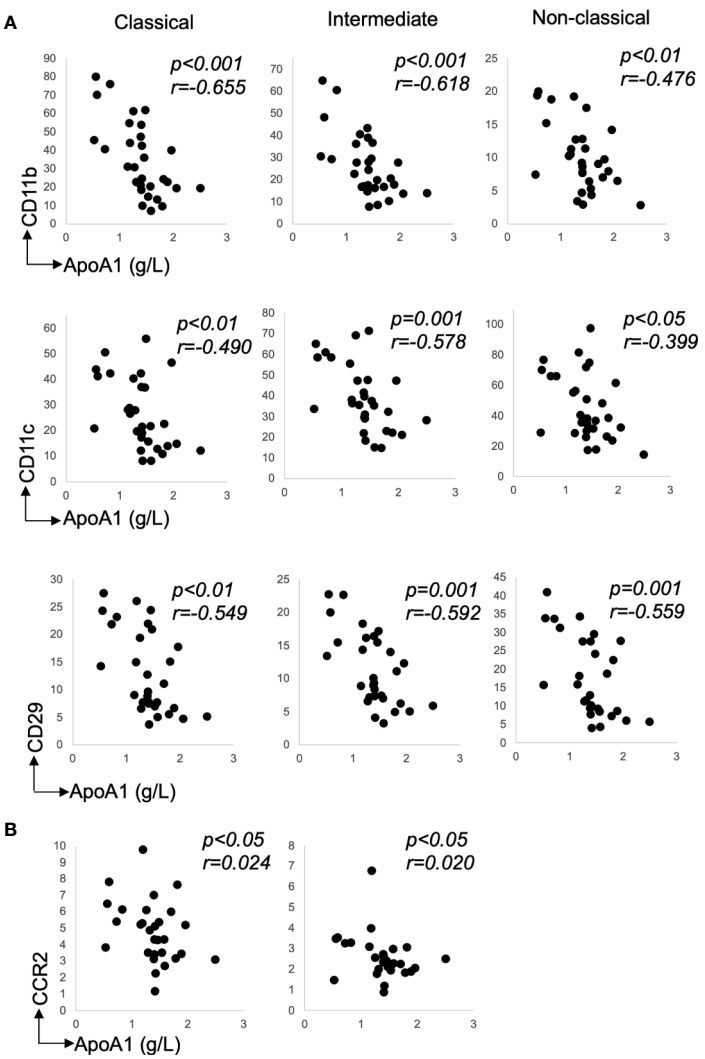

FIGURE 3 | Recruitment marker expression relative to lipid levels. **(A)** Classical, Intermediate and Non-classical correlations for chemokine receptor, CD11b, CD11c, and CD29 with ApoA1, n=30 **(B)** Classical and Intermediate correlations for chemokine receptor, CCR2 with ApoA1, n=30. Data presented as scatter plots. The statistical significance of correlation was determined by Spearman's rank correlation. All tests were two-tailed. r represents correlation coefficient; p represents level of significance and N.S. represents non-significant.

correlated with the ApoA1 levels on classical and intermediate subsets (**Table 2**: CCR2 - both $p<0.05$). CXCR2 expression inversely correlated with the ApoA1 levels but only on classical monocytes (**Table 2**: CXCR2—classical: $p<0.05$). The expression of chemokine markers, CCR2 (on the classicals) also inversely correlated with the glucose levels (**Table 2**—$p<0.05$). Note, while there was a spread in participants' ages—**Table 1**, there was no association between age or sex and monocyte recruitment marker profile (data not shown).

DISCUSSION

It is well recognized that classical and non-classical monocytes have distinct migratory patterns, and this is attributed to their specific expression of adhesion molecules and chemokine receptors. While significant differences in recruitment marker expression by the monocyte subsets were seen here, they were overshadowed by the considerable differences between participants which suggests that monocyte subset extravasation potential is specific for individuals. The relatively higher expression of recruitment markers on all monocytes of those with low ApoA1 would increase the cells capacity to extravasate and, given the wide range of markers this occurred for, suggests that the subsets could potentially do so using a wider repertoire of recruitment markers than currently appreciated.

The differences in monocyte subset recruitment marker expression is consistent with the understanding that monocyte subsets employ both common and distinct mechanisms in their recruitment to sites of injury (32), with classical monocytes

expressing higher levels of CD11b, CD62L, and CCR2 and non-classicals expressing higher levels of CD11a, CD11c, and CD49d. Consistent with other studies (16, 33), the intermediate subset expressed adhesion molecules such as CD11a, CD49d, and CD62L, as well as chemokine receptors, such as CCR2, at a level between that of the classical and non-classical subsets—which reflects their position as an intermediatory cell between the other two populations (34). The differences between subsets were substantial for markers such as CD62L, CD49d, and CCR2, and indeed, for this reason they are often used to define the subsets (14, 18, 35). Though significant differences between the subsets were seen for several other markers, such as CD44 and CD18, the actual differences were quite minimal, and not likely to be of biological significance. The subset differences reflect incremental changes as the cells mature from one subset to the next. Notably however, the subset expression of some makers did not align with the typical classical to non-classical transition pattern. A good example of this being CD11b, where its expression (as seen on a flow cytometry heat-map) was relative to that of CD14. This highlights the considerable heterogeneity within each subset and is consistent with the more recent understanding that there are actually a multitude of monocyte phenotypes (36), or perhaps there is more than one maturation pathway.

Interestingly, we could not detect clear expression of CX3CR1, CCR5, CXCR2, or CXCR4 on the subsets. This seems to contrast with some other studies (14, 16, 18, 37). Antibody-specific differences (such as clone and concentration) are likely to underlie the minimal expression of CX3CR1, which has shown clear expression in other studies (14, 16, 18). Using isolated PBMCs, both CXCR4 and CCR5 have been detected, with CXCR4 expressed by more than 80% of monocytes and CCR5 expressed by approximately 10–40% of monocytes; however, the relative level of expression was not reported in these studies (16). As isolation can lead to an incidental activation of monocytes (38), then the minimal manipulation with our experimental approach, not just choice of antibody, may explain why marker expression here did not mirror findings in isolated monocytes.

Overshadowing the differences between the subsets, was the large variation in recruitment marker expression between participants. Using 30 participants enabled us to see that although there was a consistent trend in the change in marker expression from one subset to the next, there were participants for whom all three of their monocyte subsets expressed a marker at higher levels than all three monocyte subsets of other participants. Thus though monocyte subsets are recognized to have distinct migratory potential (12), their extravasation potential is specific for individuals. Furthermore, the finding that recruitment marker expression on the classicals correlated with that of the intermediates, which then correlated with that of the non-classicals indicates that the migratory potential of the intermediates and non-classicals is determined by that of the classicals. We suggest that the migratory potential of the monocytes is likely to be pre-primed, beforehand, in the bone marrow as several studies show that alterations in the bone marrow are recapitulated as cells differentiate into the monocyte subsets (39–43). Of note, this potential is likely to vary with the presence of co-morbidities or in different disease states. For example, monocyte subset expression of CCR2 and CX3CR1 is altered in infection (44).

The inter-participant differences occuring for most markers raised the question as to whether there were correlations between the expression of the recruitment markers themselves. The considerable number, and strength, of the correlations seen would provide a synergistic increase in migration potential and the fact that the correlations were evident for all three monocyte subsets indicates that the monocyte subsets likely migrate using a wider repertoire of recruitment molecules than previously expected.

Consistent with our previous findings assessing the inflammatory profile of monocyte subsets in generally healthy individuals, we found that lipid levels explained the inter-participant variations (29). Interestingly, although there was a spread in participants' ages, there was no association between age or sex and monocyte recruitment profile. Most notably, low levels of ApoA1 were associated with higher levels of both adhesion molecules and chemokine receptors. Whether this is causal, in that ApoA1 impacts recruitment marker expression, or the reverse that marker expression alters ApoA1 levels, was not investigated. However, an increased potential to extravasate when ApoA is low is consistent with studies showing that incubating monocytes with ApoA1 can reduce monocyte diapedesis towards a range of chemokines under both acute and chronic conditions *in vitro* (26, 45). Because chemokines and chemokine receptors are critical in the development of atherosclerosis, modulation of their expression by ApoA1 suggest that low ApoA1 levels may allow increased monocyte recruitment. Though ApoA1 is a key protein associated with HDL, no associations were evident between markers examined and HDL-C. This may reflect the fact that ApoA1 mediates many of the inflammatory effects of HDL, including reduction of CD11b on monocytes (27). Some clinical studies indicate ApoA1 may be a better prognostic marker than LDL-C or HDL-C in prediction of severity of coronary artery disease (46, 47); its link with monocyte recruitment may factor into this.

The finding that the expression of CCR2 is associated with participants' LDL-C levels is consistent with previous findings of patients with hypercholesterolemia, including familial hypercholesterolemia (48–51).

As alterations in adhesion molecule and chemokine receptors impacts monocyte migration (49), our findings here suggests that changes in monocyte recruitment may be occurring, undetected, in individuals who are considered generally healthy even though they have dyslipidemia. This is important as a high proportion of adults [two thirds in Australia (52)] have dyslipidemia, many of whom would be untreated.

The expression of CD29 being associated with the participants' glucose levels suggests that adhesion potential of all monocyte subsets may be affected by elevated glucose levels. Indeed, various studies have shown that the levels of glucose impact on the expression of adhesion molecules (53–55). While increased monocyte migration into plaques has been seen in diabetes, whether this is due to increased levels of recruitment markers is unclear, as there is also increased myelopoiesis in

diabetes (56). Similarly, in other comorbidities, such as hypertension (HTN) increased monocyte adhesion occurs (57–59) and thus the level of expression of the markers examined here, may be increased further in established CVD and in particular in those with co-morbidities.

Overall, the finding that most of the associations between the monocyte adhesion molecule or chemokine receptor expression were with participants' ApoA1 levels suggest that therapies aimed to lower LDL-C or TC alone might not aid in full reversal of this acquired adhesive or migratory phenotype of monocytes, and thus therapies elevating ApoA1 levels should also be considered.

In summary, our results are consistent with the understanding that monocyte subsets differentially express adhesion molecules and chemokine receptors which would broadly dictate their recruitment potiential. However, these inter-subset differences are overshaddowed by differences between individuals, with all monocytes in some individuals having a greater potential to migrate and to do so using a wider repertoire of recruitment markers than currently appreciated. The findings in this study, combined with our previous observation that inflammatory markers, such as cytokines, are also raised on all monocytes relative to lipid levels indicates that all monocytes, not just one subset, can acquire an increased pro-atherogenic phenotype in the circulation itself i.e. even before they become macrophages. The impact of this on plaque development is yet unclear. However, monocyte adoption of pro-atherogenic phenotype would be expected to promote plaque development as they would enter the vessel wall primed to become pro-atherogenic macrophages. Together with other contributing risk factors, monocyte priming may exacerbate atherosclerosis development. That most associations were found with ApoA1 suggests that to effectively reduce monocyte migration into the plaques to slow/inhibit plaque progression, the lowering of LDL-C alone may not be sufficient.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because we have not sought consent from the participants for the release of the data.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Western Sydney Local Health District (WSLHD) Human Research Ethics Committee. The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

VP designed and performed the experiments, analyzed the results, performed statistical analysis, created the figures, and wrote the manuscript. HM planned the project, and with HW supervised the work and contributed to data interpretation and manuscript writing. SL identified suitable patients and with VP collected participant information. The project was conducted under JF. All authors contributed to the article and approved the submitted version.

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Lymphatic Endothelial Cell Activation and Dendritic Cell Transmigration Is Modified by Genetic Deletion of Clever-1

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Clever-1 also known as Stabilin-1 and FEEL-1 is a scavenger molecule expressed on a subpopulation of anti-inflammatory macrophages and lymphatic endothelial cells (LECs). However, its role in regulating dendritic cell (DC) trafficking and subsequent effects on immunity have remained unexplored. In this study, we demonstrate that DC trafficking from the skin into the draining lymph nodes is compromised in the absence of Clever-1. By adoptive transfer approaches we further show that the poor trafficking is due to the impaired entrance of DCs into afferent lymphatics. Despite this, injections of ovalbumin-loaded DCs into the footpads induced a stronger proliferative response of OT II T cells in the draining lymph nodes. This could be explained by the increased MHC II expression on DCs and a less tolerogenic phenotype of LECs in lymph nodes of Clever-1 knockout mice. Thus, although fewer DCs reach the nodes, they are more active in creating antigen-specific immune responses. This suggests that the DCs migrating to the draining lymph node within Clever-1 positive lymphatics experience immunosuppressive interactions with LECs. In conclusion, besides being a trafficking molecule on lymphatic vasculature Clever-1 is immunosuppressive towards migrating DCs and thus, regulates the magnitude of immune responses created by incoming DCs in the draining lymph nodes.

Keywords: dendritic cells, lymphatics, lymphatic vessels, traffic, migration, immunosuppression

INTRODUCTION

To initiate an effective adaptive immune response or tolerance, dendritic cells (DCs) among other leukocytes, migrate from the periphery to the draining lymph nodes (dLNs) *via* the afferent lymphatic vessels. In contrast to other cells, only lymphocytes can leave the nodes *via* the efferent lymphatics and exert their immune functions elsewhere in the body. DC trafficking from the periphery to the draining lymph nodes through lymphatic vessels and their ability to carry and present antigens in the node is an essential element in the induction of the immune response within the lymph nodes (1–4). It has been shown that chemokines and their receptors, adhesion molecules and integrins expressed on lymphatics are involved in DC transmigration into lymphatic vessels and their migration towards the dLNs. To date, a few molecules on lymphatic endothelial cells (LECs)

have been identified to be responsible for dendritic cell migration within the afferent lymphatics. They include sphingosine 1 phosphate, CD31, CD99, Semaphorin 3A, Podoplanin, LYVE-1, and chemokines such as CCL21 and CXCL12. In inflammatory conditions, also ICAM-1, VCAM-1, ALCAM, D6, and CX3CL1 on lymphatics contribute to the trafficking of dendritic cells (5–8). In addition to DCs, LECs of dLNs play an essential role in regulating both tolerance and immunity. Even though LECs do not express co-stimulatory molecules such as 4-1BBL, CD86, and CD80, they endocytose antigens, cross-present MHC I antigens and express PD-L1 and MHC II molecules (4).

Clever-1 is a scavenger receptor expressed on both afferent and efferent arms of the lymphatic vasculature and it has been demonstrated to be involved in lymphocyte and cancer cell trafficking within the lymphatics (9–11). Moreover, Clever-1 mediates trafficking of B cells and CD8 positive T cells into the splenic red pulp, but not significantly to other lymphatic organs *via* blood vasculature in normal conditions (12). However, it is also induced in the flat walled endothelium at sites of inflammation and in certain cancers (9, 12, 13). However, its role in DC trafficking from the periphery into the dLN *via* the lymphatic vessels as well as its role on lymphatics in modulating immune responses in the dLN have not been previously explored. In this work, we utilized Clever-1 KO mice and tested DCs trafficking *via* skin draining lymphatic vessels and compared that to the trafficking of DCs in wild type (WT) mice. Moreover, we studied antigen-specific immune responses in the dLNs in a delayed-type hypersensitivity (DTH) model. The Clever-1 KO mice showed impaired DC transmigration into the skin draining lymphatics that subsequently resulted in lower numbers of DCs in the dLNs. Despite impaired DC trafficking, the antigen-specific immune response was normal in KO mice. We also analyzed the characteristics of the lymphatics of dLNs in Clever-1 KO mice and their WT controls at steady-state and inflammation using RNA sequencing. LECs of LNs lacking Clever-1 has a more proinflammatory phenotype than their WT controls at steady state, explaining the normal level of the antigen-specific immune response, despite impaired DC trafficking into the dLNs.

MATERIALS AND METHODS

Study Design

This study examined the role of Clever-1 on lymphatic endothelium in regulating the traffic of DCs into the draining lymph nodes and the consequences of Clever-1-dependent interaction between DCs and lymphatics on the activity status of the migrating DCs. Both adoptively transferred and endogenous DCs and Kikume and CD11c^{+}-YFP^{+} reporter mice were used in the migration studies and entrance of DCs into the lymphatics was visualized using advanced microscopy. T cell responses against footpad injected ovalbumin were analyzed in the dLNs. To find the reason for the aberrant behavior of DCs with lymphatics, RNAseq analyses were performed for lymphatic endothelial cells of Clever-1 WT and KO mice. Moreover, the contribution of Clever-1 on lymphatics and blood endothelium was analyzed in OXA-induced skin inflammation (contact-induced hypersensitivity model).

Mice

Clever-1 KO mice with a mixed C57BL/6N and 129SvJ background were generated as previously described (10) and used with their WT controls (both sexes in a randomized fashion) at an age between 6 and 12 weeks. DsRed mice [B6.Cg-Tg(CAG-DsRed*MST)1Nagy/J] carrying a red fluorescent reporter protein as a transgene were from the Jackson Laboratory (stock number 006051). Balb/C mice were from Janvier and Charles River, CD11c^{+}-YFP^{+} mice from Jackson and Kikume mice (14) from Prof. Masayuki Miyasaka, Osaka University. All animal experiments were approved by The Finnish Act on Animal Experimentation (62/2006); animal license number 5762/04.10.07/2017 and 12537/2020).

Immunohistochemistry

To examine the cell populations of dLN and Clever-1 expression, frozen dLN sections were stained with antibodies specific for CD3 (BD Biosciences), B220 (eBioscience), MHCII (eBioscience), LYVE-1 (eBioscience), Clever-1 (9–11, recognizing both mouse and human Clever-1 (15). To evaluate the structure of lymphatic vessels as well as entry of DCs into the lymphatics, ear sheets were stained with antibodies specific for Podoplanin (BioLegend, 127410) and LYVE-1 (eBioscience). Thereafter, the entire sections were imaged using 3i spinning disk confocal microscopy (Carl Zeiss SAS, Jena, Germany), Zeiss LSM 780 or LSM 880 confocal microscope equipped with ZEN imaging software (both from Carl Zeiss SAS, Jena, Germany). Images were analyzed using ImageJ software (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, MD, USA) or Imaris 8 (Bitplane Inc).

Microlymphography

To evaluate the function of lymphatic vessels in WT and KO mice tetramethylrhodamine (TRITC)–dextran (molecular weight, 2 million Da; Sigma) was intradermally injected into the ear tip and its distribution was instantly imaged under fluorescence microscopy (Leica).

FITC Skin Painting

One percent FITC in 1:1 acetone: dibutyl phthalate was painted on the dorsal side of the ears of WT and KO mice. Ear-draining auricular LNs were collected for analysis 20 and 48 h after the painting. dLNs were digested for 30 min in 100 micrograms/ml DNase I and 0.5 mg/ml Collagenase P at 37°C. EDTA was added for the final 5 min incubation. The single-cell suspensions were stained for flow cytometry: CD45-BV650, CD103-PE, CD11c-PerCP-Cy5.5 or CD11C-VB421, CD11b-eFluor450 or CD11b-APC-Cy7, and MHC II-PE-Cy7 or MHC II- PerCP-eFluor 710 (all from BD Biosciences) for 20 min, washed and fixed with 4% PFA. Thereafter, the cells were washed with 0.5% saponin buffer and stained with Langerin-Alexa Fluor 647 (Dendritics, 929F3.01) for 20 min at RT. The samples were measured with LSR Fortessa flow cytometer (BD) and analyzed with FlowJo software (Treestar). Dendritic cell populations were defined among CD11c+MHCII^{hi}

events as follows: Langerin$^+$CD103$^-$ (Langerhans cells), Langerin$^+$CD103$^+$ (CD103$^+$ dermal dendritic cells, DDC), Langerin$^-$CD11b$^+$ (CD11b$^+$ DDC) and Langerin$^-$CD11b$^-$ (double negative DDC).

Dendritic Cell Injections

Bone marrow cells from CAG-DsRed reporter mice were cultured with recombinant murine GM-CSF (PEPROTECH) and matured with LPS (Sigma) as previously described (16). 1×10^5 fluorescent DCs in 25 µl PBS were injected subcutaneously into footpads of WT and KO mice. Mice were sacrificed 20 h after the injections and single-cell suspensions of the draining lymph nodes were stained for flow cytometry: CD45-PE, CD11c-FITC and MHCII-PE-Cy7 (all from BD Biosciences) for 20 min on ice. Samples were analyzed as described above. Adoptively transferred DCs were defined as follows: CD45$^+$CD11c$^+$MHCII^{hi}DsRed$^+$.

For visualizing the trafficking of adoptively transferred BMDCs in the ear dermis, 2.5×10^5 matured CFSE-labeled BMDCs were intradermally injected into the ear of WT and KO mice followed by application of 2% OXA. One day later, the mice were sacrificed, dorsal and ventral portions of the ear were separated, fixed with 4% PFA, permeabilized with 0.1% Triton and stained with conjugated primary antibodies against Lyve-1 (eBioscience) and Podoplanin (BioLegend).

Confocal Z-stack images of whole-mount ears were acquired using 3i spinning disk confocal microscopy (Carl Zeiss SAS, Jena, Germany) or LSM 880 confocal microscope equipped with ZEN imaging software (Carl Zeiss SAS). To quantify the number of luminal DCs, 3D images were constructed by using Imaris 8 (Bitplane Inc) and the number of FITC$^+$ DCs transmigrated inside the lumen versus those located in the vicinity of lymphatic vessels were counted.

Split Ear Assays

Dorsal and ventral portions of the ear were manually separated and dorsal portions were discarded. CD11c$^+$-YFP$^+$, DsRed$^+$ or WT LPS-matured BMDCs were generated as described and purified with the pan-dendritic cell isolation kit (Miltenyi). Matured BMDCs were incubated on the ventral portion of the ear for 20 min. Thereafter, the non-adherent cells were washed away and incubation was continued in fresh medium for 2 h at 37°C in 5% CO2. The ear sheets were fixed with 4% PFA and stained with conjugated primary antibodies against Podoplanin (BioLegend) and LYVE-1 (eBioscience). The whole-mount ear sections were imaged using 3i spinning disk confocal microscopy (Carl Zeiss SAS), Zeiss LSM 780 or LSM 880 confocal microscope equipped with ZEN imaging software (both from Carl Zeiss SAS). The length of lymphatic vessels, as well as the number of DCs colocalized with the lymphatic vessels, were counted using ImageJ software.

Ovalbumin-Specific T Cell Response Model

To evaluate OT II T cell response in the presence of a soluble antigen, 5.5×10^4 LPS activated WT BMDCs were loaded with 2.5 µg/ml of $OVA_{323-339}$ peptide (SSINFEKL) and after vigorous washes injected into the footpad of Clever-1 WT and KO mice. One day later, OT II splenocytes were first labeled with 5 µM Vybrant CFDA SE Cell Tracer (CFSE; Thermo Fisher Scientific) according to the manufacturer's instructions. 5×10^6 CFSE-labeled splenocytes in 200 µl of PBS were intravenously injected into the WT and KO mice. The popliteal draining LNs and inguinal distal LNs were collected 48 h later, single-cell suspensions were prepared and OT II T cell proliferation was evaluated by measuring CFSE dilution among the OT II CFSE labeled cells using flow cytometry. Single-cell suspensions were stained for flow cytometry: CD45-APC-Cy7, CD3-Alexa Fluor 647, B220-BV421, and CD4-APC-cy7 (all from BD Biosciences) for 20 min on ice. Samples were measured and analyzed as described above.

To analyze the uptake of OVA in the dLN, 5 mg/ml of DQ-OVA (Thermo Fisher Scientific) was subcutaneously injected into the hind footpad of WT and KO mice. The draining popliteal LNs were collected 90 min later and embedded in OCT blocks. Frozen sections of LNs were analyzed for the presence of digested DQ-OVA that emits in the green spectrum.

Contact Hypersensitivity Model

Mice were first sensitized on the shaved belly (50 µl) and each paw (5 µl) with 2% OXA (4-ethoxymethylene-2-phenyl-2-oxazoline-5-one; Sigma-Aldrich) in acetone/olive oil (4:1 volume/volume). Five days later, the mice were challenged by topical application of 10 µl of 1% OXA solution on each side of the ear. The ear measurements were performed from 1 to 8 days after the OXA challenge. Non-Inflamed control and inflamed ear-skin were collected and digested with whole skin dissociation kit (Miltenyi Biotec) according to the manufacturer's instructions. The single-cell suspensions were stained for flow cytometry: CD45-eFluor 700, CD3-FITC, CD4-APC-Cy7, CD8a-BV650, CD11c-BV421, MHC II-PerCP-eFluor 710, CD103-Bv510, CD25-APC, CD11b-PE (all from BD Biosciences), and CD25-APC (eBioscience) for 20 min on ice. For Langerin staining, samples were fixed with 4% PFA, washed with permeabilizing buffer (eBioscience) and finally stained with Langerin-Alexa Fluor 647 (Dendritics) for 20 min at RT. For Foxp3 staining, samples were fixed and permeabilized with Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer's instruction and stained with Foxp3-PE-CF594 (BD Biosciences). Samples were measured and analyzed as described above.

In another set of CHS experiments, mice were sensitized with 2% OXA as described above. Five days later, single-cell suspensions of skin-draining axillary and inguinal LNs (10 million cells in 160 µl of PBS) were injected *via* the tail vein of the recipients. Alternatively, T cells were isolated by T cell isolation kit (EasySep^{TM}) and 10 million isolated T cells in 160 µl of PBS were injected *via* the tail vein. One hour later, the mice were challenged by topical application of 10 µl of 1% OXA solution on each side of the ear. The ear measurement was performed 1 and 2 days after the OXA challenge.

Bone Marrow Transplantations

To generate bone marrow chimaeras, C57BL/6N Clever-1$^{-/-}$ and their WT control were irradiated twice with 5 Gy (Faxitron

Multirad 350) using 3-h intervals followed by injections of 10 X 10^6 bone marrow cells collected either from KikGR photoconvertible transgenic or $CD11c^+$-YFP^+ reporter mice. To track the photoconverted cells in Kikume chimaeras, the shaved belly was irradiated with UV light followed by application of 2% OXA (Sigma). One day later, the axillary draining LNs were stained with above-mentioned antibodies for flow cytometry analyses.

siRNA Silencing of Clever-1 on Human Dermal Lymphatic Endothelial Cells

HDLECs were obtained from Promocell and cultured in MV2 medium (C-22022, Promocell). For silencing, 30 000 cells/well were seeded on fibronectin-coated 12-well plates. One day later, the cells were silenced for Clever-1 by lipofection with Lipofectamine RNAiMAX (ThermoFisher Scientific) and using 15 nM siRNA (ON-TARGETplus siRNA, human STAB1, J-014103-08-0020, Dharmacon) or a control construct (ON-TARGETplus Non-targeting Control Pool, D-001810-10-20, Dharmacon).

qPCR

RNA from siRNA-silenced and control cells was extracted with the NucleoSpin RNA kit from Macherey-Nagel and the cDNA was generated with the SuperScript VILO cDNA Synthesis Kit (ThermoFisher Scientific). Clever-1 expression was determined by using the UPL system with probe #74 and primers (left: cac atg tgc caa gaa gat cc; right: cac agc gtg cca aag aaa c). For calculating gene expression, the $2^{(-ddCT)}$ method was used.

Generation of Human Monocyte-Derived DCs

moDCs were generated by extracting monocytes from buffy coats obtained from the Finnish Red Cross Blood Service (permit number 22/2018). This was done first by extracting peripheral blood mononuclear cells with gradient centrifugation (Ficoll-Paque PLUS, GE Healthcare), before extracting monocytes with CD14 MicroBeads from Miltenyi Biotec. The purified monocytes were then cultured for 6 days in RPMI 1640 medium (Sigma) that contained 10% FCS (Sigma), 4nM GlutaMax (Gibco, ThermoFisher Scientific), 100 U/ml penicillin, 100 µg/ml streptomycin, 500 U/ml GM-CSF and 350 U/ml IL-4 (both from Peprotech). On day 3, half the medium was replaced with fresh medium.

Co-culture of Human moDCs and HDLECs

In 12-well plates, 500 000 moDCs were co-cultured together with confluent Clever-1 silenced or control-treated HDLECs for 2 days in MV2 medium. After the co-culture, the activation-status of the moDCs was evaluated by staining them with antibodies against CD40, CD83 and MHC class II (all from BD) or respective control antibodies. The cells were run with LSR Fortessa flow cytometer (BD) and analyzed with FlowJo (v10, TreeStar).

Mixed Leukocyte Reaction Assay

T cells were extracted from the blood of healthy volunteers by using T cell isolation kit (EasySep™) and labeled with 1 µM Vybrant CFDA SE Cell Tracer (CFSE; Thermo Fisher Scientific) according to the manufacturer's instructions. To assess T cell proliferation, 100,000 T cells together with 10,000 moDCs were added to wells of a 96 well plate that contained either Clever-1 silenced HDLECs or their control-treated counterpart. The cells were cultured in a mix of 50% MV2 medium and 50% RPMI 1640 (containing 10% FCS, 4nM GlutaMax, 100 U/ml penicillin, and 100 µg/ml streptomycin). After 7 days of co-culture, the T cells were stained for flow cytometry: CD8-APC and CD4-PE (all from BD Biosciences) for 30 min on ice. Samples were measured and analyzed as described above.

RNA Sequencing

Popliteal and brachial LNs of the WT and KO mice at steady-state and 1 day after s.c injection of OVA (1 mg/ml) (1:1) (EndoGrade) emulsified in incomplete Freund's adjuvant (Sigma) into the footpads were collected and digested to obtain single-cell suspensions. Thereafter, the suspensions were depleted from hematopoietic cells using mouse anti-CD45 microbeads (Miltenyi Biotec). The enriched cells were stained with LIVE/DEAD fixable near-IR dead cell stain (Thermo Fisher Scientific) and conjugated primary antibodies against mouse Podoplanin (BioLegend) and CD31 (BioLegend). Live lymphatic endothelial cells ($CD45^-$ $CD31^+$ $Podoplanin^+$) were sorted into TRIsure (Bioline) using Sony cell sorter, equipped with a 100 µm tip.

Total RNA was extracted from the LECs with RNeasy Plus Micro kit (QIAGEN) and the libraries were prepared using SMART-Seq v4 Ultra Low Input RNA Kit (Takara) and Illumina Nextera XT DNA Library Preparation protocols (Illumina). Sequencing was performed with the Illumina HiSeq 3000 instrument using single-end sequencing chemistry with 50-bp read length at the Finnish Functional Genomics Centre, University of Turku and Åbo Akademi and Biocenter Finland.

Bioinformatics

The raw sequencing data were uploaded to the BaseSpace Sequence Hub (Illumina) as FASTQ files for further analysis. The quality control was performed using the FastQC application of BaseSpace and subsequently, the sequences were aligned against the mouse reference genome mm10 (UCSC, RefSeq gene annotation) with the RNA-Seq Alignment application, which uses the STAR aligner for read mapping and salmon for quantification of reference genes and transcripts. The differences in gene expression between the samples were identified with the RNA-Seq Differential Expression application using DESeq2. The genes exhibiting a fold change (FC) >2 (log2ratio ≥1 and ≤-1) and q-values (FDR) < 0.05 were selected as differentially expressed genes (DEG). The RNA sequencing data is in the Gene Expression Omnibus (GEO) database under the accession number GSE148730.

Further analyses of the data were performed using QIAGEN Ingenuity Pathway Analysis (QIAGEN IPA) and the Venn diagrams were calculated and drawn with the Venn diagram tool at http://bioinformatics.psb.ugent.be/webtools/Venn.

Multiplex Analyses

Skin ear and ear-draining auricular LNs from WT and KO mice at steady-state and 1-day OXA (Sigma) treated, 1- and 2-days CHS OXA-challenged mice were collected. Tissues were lysed with ReadyPrep™ Protein Extraction Kit (Bio-Rad) and stored at -70°C. Protein concentration was determined with the DC Protein Assay (Bio-Rad) and 12.5 µg of total protein was used for the multiplex. The multiplex analysis was performed with the Bio-Plex Mouse cytokines 23-plex assay (Bio-Rad), according to manufacturer's instructions. Bio-Plex 200 system (Bio-Rad) was used to analyze the samples.

Statistical Analyses

An unpaired two-tailed Student's t-test with Welch's correction (when variances were significantly different) and the Mann-Whitney U test were used for statistical analyses unless stated otherwise. For multiple comparisons, one-way ANOVA was used with Tukey's test. Data points determined to be significant outliers by Grubbs' test were not included in statistical analyses. $P < 0.05$ was considered statistically significant (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

RESULTS

Clever-1 Is Expressed in Mouse Skin Lymphatic Vessels

During the course of studies using Clever-1 KO mice, we have observed cell composition differences in their lymphoid organs compared to WT mice. To investigate the contribution of different immune cells to this phenomenon and the cause of this difference, we first performed a thorough assessment of the immune cell composition in the skin dLNs of Clever-1 KO mice. Flow cytometry analyses of skin dLNs revealed that the absolute number of leukocytes ($CD45^+$), total numbers of DCs ($CD11c^+MHCII^+$) and resident DCs ($CD11c^+MH\ II^{int}$) were comparable between the WT and KO mice, but significantly less migratory DCs ($CD11c^+MHCII^{high}$) were found in the dLNs of KO mice (**Figures 1A–D**). The KO mice also had a significantly higher number of $CD4^+$ T cells and lower number of B cells, while the numbers of $CD8^+$ T were similar (**Figures 1E–G**). Clever-1 is expressed by sub-capsular, lymphatic and medullary sinuses in the LNs but absent from DCs and lymphocytes (**Supplementary Figure 1A** and IMGEN; http://rstats.immgen.org/Skyline/skyline.html). It was, however, not clear to what extent Clever-1 is expressed in the most peripheral afferent lymphatic vessels residing in the skin that could explain the reduced migratory DC population in the dLNs. Indeed, we found a patchy staining pattern of Clever-1 on a subset of $LYVE\text{-}1^+Podoplanin^+$ lymphatics in the mouse skin (**Figure 1H**). To further evaluate, whether Clever-1 is expressed on the capillary or collecting lymphatic vessels, we stained the ear sections for Prox-1, Podoplanin, LYVE-1 and Clever-1. Clever-1 is expressed on both $LYVE\text{-}1^+$ capillary and $LYVE\text{-}1^-$ collecting lymphatic vessels (**Supplementary Figure 1B**). To directly evaluate the functionality of the skin lymphatic vessels in WT and KO mice, we performed microlymphography using TRITC-dextran and quantified the fluorescence intensity and the area of $TRITC^+$ lymphatic vessels. No major differences were found in the uptake and drainage of TRITC-dextran *via* the cutaneous lymphatics in KO mice indicating that the vessels were functionally normal (**Figures 1I–K**). To further test the influx of antigens into dLNs, we used DQ-OVA as a tracer molecule. DQ-OVA is a self-quenched fluorochrome, which only upon ingestion by phagocytes emits fluorescence in the green spectrum. We injected DQ-OVA into the footpad of WT and KO mice and 90 min later measured the distribution of the green fluorescence signal in the draining popliteal LN. The signal in the LN of KO mice and their WT controls was comparable, indicating an efficient and comparable antigen delivery system in both mice (**Figure 1L**). We further examined the morphology of lymphatic vessels in Clever-1 KO mice by quantifying the $LYVE\text{-}1^+$ area in whole-mount ear images. Lymphatic vessels in WT and KO mice appeared to be morphologically normal and no differences were found in the area that was covered by $LYVE\text{-}1^+Podoplanin^+$ lymphatics (**Supplementary Figures 1C, D**). To exclude the possibility that the number of DCs in the skin would be the reason for the lower number of migratory DCs in the dLNs of KO mice, we quantified the number of DCs and the total number of $CD45^+$ cells in the ear skin of WT and KO mice. The total number of DCs and the number of $CD45^+$ cells in the ear skin in WT and KO mice were comparable at steady state (**Supplementary Figures 1E, F**).

Dendritic Cell Trafficking *via* Lymphatics into the Skin dLNs Is Impaired in Clever-1 KO Mice

To explore the cause for the lower number of migratory DCs in the dLNs of Clever-1 KO mice, we first studied the migration of dermal DCs into dLNs 20-h after applying FITC on the ears of WT and KO mice. We observed an overall reduced number of FITC positive $CD11c^+MHCII^{high}$ cells in the dLNs of KO mice compared to WT controls (**Figure 2A**). Further analysis of the different DC subpopulations showed that the numbers of Langerhans cells ($CD11c^+MHCII^{high}CD207^+CD103^-$) and $CD103^+$ dermal DCs ($CD11c^+MHCII^{high}CD207^+CD103^+$) were significantly reduced while no statistically significant difference was seen in the migration of double negative dermal DCs ($CD11c^+MHCII^{high}CD207^-CD11b$; **Figure 2A**). The difference remained significant between the WT and KO mice both in Langerhans and $CD103^+$ dermal DC subpopulations 48 h after FITC application (**Figure 2A**).

We next took advantage of KikGR photoconvertible transgenic mice (i.e. so-called Kikume mice), in which the migration of endogenous green cells can be followed after they have been photoconverted to red by UV light. We produced Kikume chimaeras by lethally irradiating Clever-1 WT and KO mice and reconstituted their bone marrow with Kikume cells. The belly of the chimaeras was exposed to UV light followed by topical application of 2% oxazolone (OXA; **Figure 2B**). Eighteen hours after conversion significantly less skin-derived photoconverted cells (green-red) had migrated to the axillary dLNs of the KO mice, which were identified to consist mostly of migratory DCs ($CD11c^+MHCII^{high}$; **Figure 2C**). To further

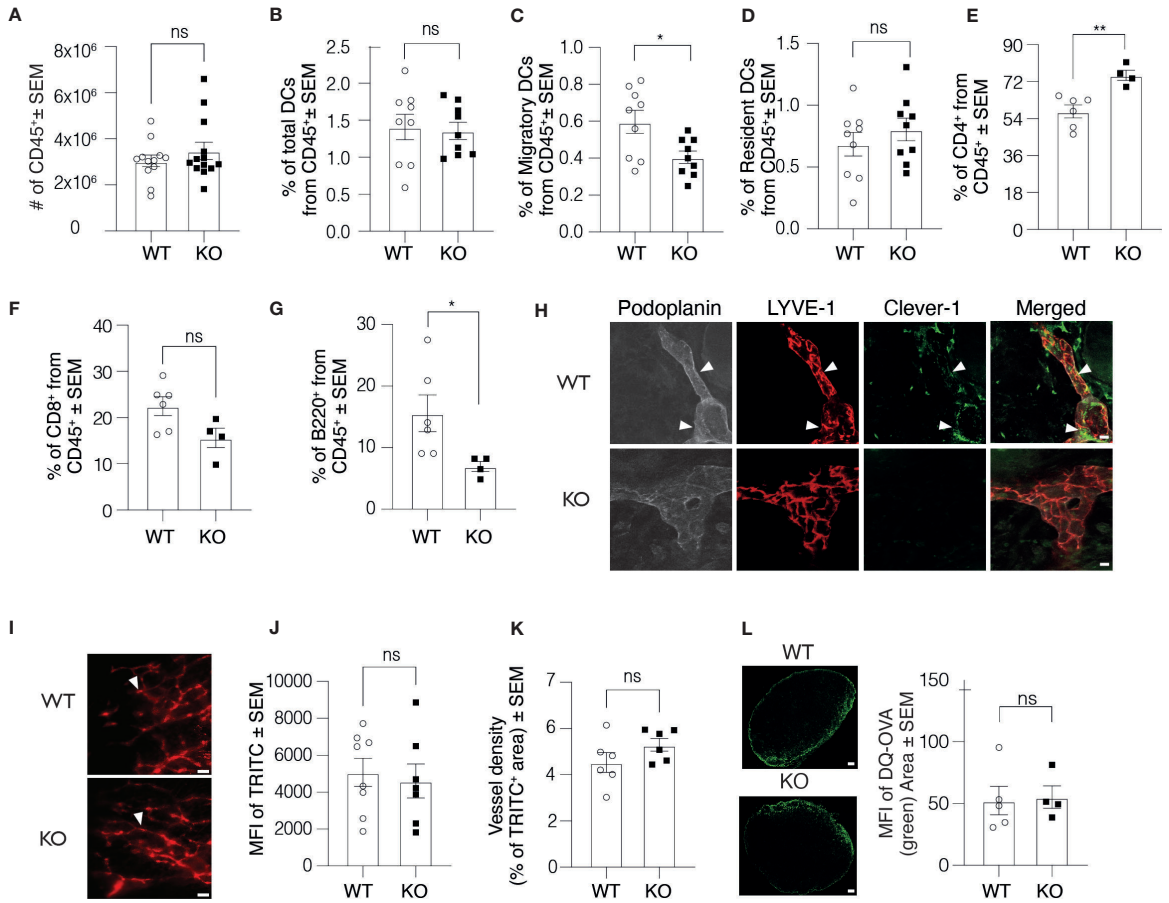

FIGURE 1 | Clever-1 KO mice have morphologically and functionally normal lymphatic vessels. **(A–G)** Flow cytometry analysis of digested auricular dLNs from WT and KO mice, showing the absolute number of leukocytes **(A)**, the percentage of the total number of DCs **(B)**, migratory DCs **(C)**, resident DCs **(D)**, $CD4^+$ T lymphocytes **(E)**, $CD8^+$ T lymphocytes **(F)**, and $B220^+$ B lymphocytes **(G)**; each dot represents one mouse. **(H)** Confocal microscopy images of whole-mount staining of the ear dermis of WT and KO mice stained with antibodies against Podoplanin (grey), LYVE-1 (red) and Clever-1 (green). Arrowheads point to the triple-positive lymphatics. **(I–J)** Fluorescence-microlymphography of the WT and KO ear after intradermal injection of 2 MDa TRITC-dextran **(I)**, and quantification of fluorescence intensity **(J)** and vessel density **(K)**. Arrowheads point to lymphatics; each dot represents one ear. **(L)** Confocal images and quantification of DQ-OVA distribution (green) in the dLNs of WT and KO mice 90 min after subcutaneous injections. Data are presented as mean ± SEM. *P < 0.05 and **P < 0.01, two-tailed Student's t-test. Scale bars **(H)** 10 µm, **(I)** 300 µm, and **(L)** 200 µm. ns, non-significant.

validate these findings, we injected $DsRed^+$ bone marrow-derived dendritic cells (BMDC) into the footpad of Clever-1 WT and KO mice and 20-h later harvested the popliteal LNs. In line with previous data, the migration of the transferred $DsRed^+$ BMDCs was reduced by 46±21% in KO mice compared to WT mice (**Figure 2D**). Thus, using these three different approaches we could confirm the impaired trafficking of migratory DCs to the dLNs in KO mice.

Clever-1 Regulates Transmigration of Dermal DCs Into Lymphatic Vessels

To further investigate at which level Clever-1 deficiency impairs the trafficking of DCs *via* lymphatics, we first employed the so-called split ear model where BMDCs are incubated on the exposed dermis of murine ear sheets to study DC adhesion and migration towards afferent lymphatics in the ear (17). The adherence of BMDCs on afferent lymphatics was comparable between WT and KO mice at 20 min and 2-h time points suggesting that this step was normal and not causing the impaired DC trafficking at steady state (**Figure 3A**). As inflammation affects the production and expression of different cytokines and adhesion molecules, we performed the split ear model on OXA inflamed ears. Against expectations, a greater number of BMDCs adhered on the ear lymphatics of KO mice after 2 h suggesting that the impairment in DC trafficking into the dLNs was not due to blockage of intra-lymphatic migration but rather in the entrance of DCs to lymphatic vessels (**Figure 3B**). We therefore performed whole-mount 3D confocal imaging of intradermally injected $CFSE^+$ BMDCs and found that DCs could not enter the lumen of the Clever-1 negative lymphatics as

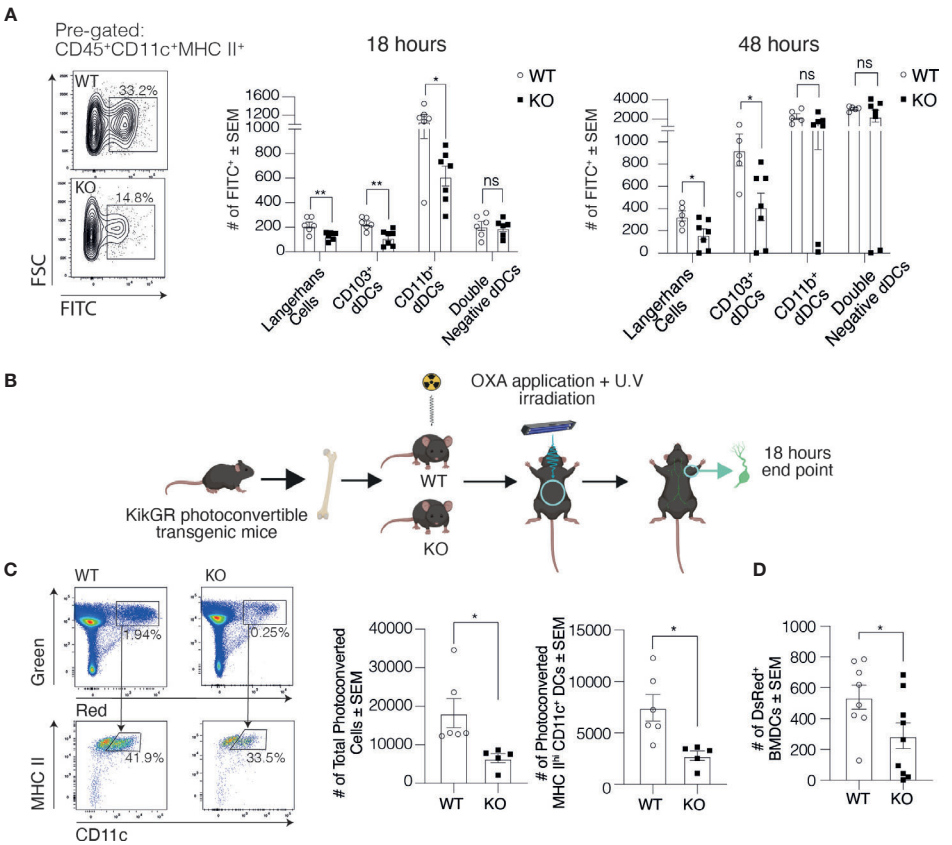

FIGURE 2 | Trafficking of DCs into the skin draining LNs is compromised in the absence of Clever-1. **(A)** Representative dot plots and quantification of FITC positive DCs in the ear-draining auricular LNs at 20 and 48 h after FITC painting. **(B)** Schematic presentation of the generation of KikGR (Kikume) photoconvertible transgenic bone marrow chimaeras. **(C)** Representative dot plots and quantification of flow cytometry analyses of the total number of photoconverted cells (green-red) and migratory DCs (green-red; $CD45^+CD11c^+MHCII^{high}$) in the axillary LNs of Kikume chimaeras 18 h after UV irradiation and OXA application. **(D)** Flow cytometry analysis of popliteal LNs 20 h after subcutaneous injection of $DsRed^+$ BMDCs into the footpad of Clever-1 WT and KO mice. Each data point represents one mouse. Data are presented as mean ± SEM. *P < 0.05 and **P < 0.01, two-tailed Student's t-test or two-tailed Student's t-test with Welch's correction. ns, non-significant.

efficiently as they did when Clever-1 was present (**Figures 3C, D**). Quantitative analyses of confocal Z-stack images showed that approximately 60±8,3% of the $CFSE^+$ BMDCs that aligned with $LYVE\text{-}1^+Podoplanin^+$ lymphatics were inside the vessel lumen in WT mice, whereas, in KO mice the BMDCs were accumulating at the basolateral surface of the lymphatics and only 30±7% of them were inside the lumen of the dermal lymphatics (**Figure 3D**). Thus, the entrance of DCs into the afferent lymphatics is impaired in the absence of Clever-1.

Lymphatic Clever-1 Regulates the Activity of DCs to Trigger Immune Response

Due to impaired DC trafficking, we next asked whether antigen-specific immune responses in the dLN were affected by the lack of Clever-1. In this set of studies, we first injected ovalbumin (OVA) peptide-loaded DCs into the footpad of WT and KO mice and 1 day later, OVA-specific CFSE labeled $CD4^+$ T cells from OT II mice were intravenously injected into the mice. The OVA-specific response was analyzed 2 days later by measuring T cell proliferation by CFSE dilution in the draining popliteal LNs (**Figure 4A**). Quantification of OT II cells showed that the total number of $CFSE^+$ $CD3^+$ $CD4^+$ OT II cells in the draining popliteal LNs of KO mice was 37.9 ± 8% higher than in WT mice, indicating a stronger antigen-specific immune response in the draining popliteal LNs of KO mice (**Figure 4A**). Further analysis of the proliferated OT II cells showed a 87.32 ± 22% increase in the percentage of divided OT II cells in the draining popliteal LNs of KO mice compared to their WT controls.

Engagement of MHC II expressed on DCs with T cell receptor on $CD4^+$ T cells results in T cell activation. To investigate the underlying mechanisms for the higher antigen-dependent immune response, we analyzed the expression level of MHC II on DCs 1 day after injecting OVA into the footpad of WT and KO mice. The DCs in KO mice expressed higher levels of MHC II but not CD40 in the draining popliteal LNs compared to WT mice (**Figure 4B** and **Supplementary Figure 1G**), indicating that DCs in the dLNs of KO mice could most likely activate more T cells on a per-cell basis. Comparable findings were observed in a non-manipulated situation where the MHC II signal was markedly higher within the LNs of KO mice (**Figure 4C**).

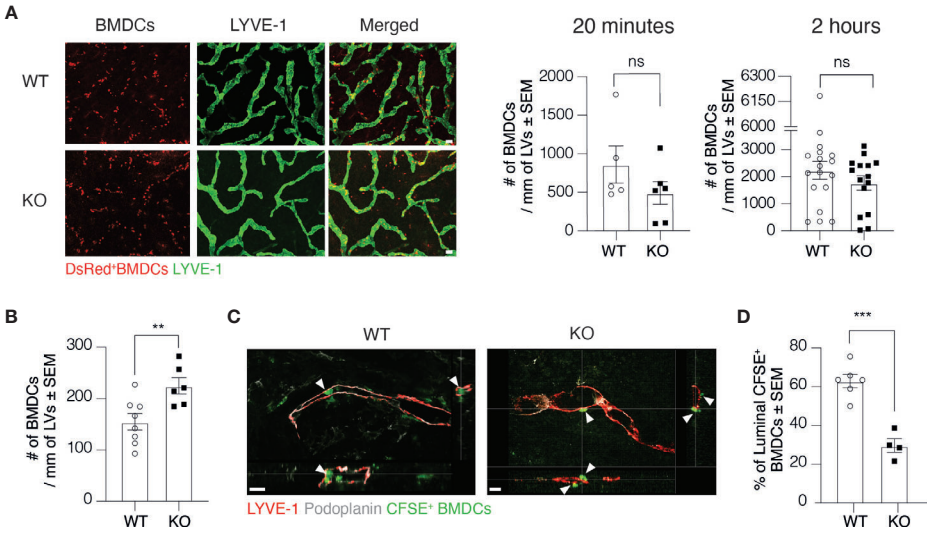

FIGURE 3 | Transmigration of DCs into lymphatic vessels is impaired in the absence of Clever-1. **(A)** Example images and quantification of DsRed$^+$ BMDCs on lymphatic vessels (LVs) in dermal ear explants of WT and KO mice at 20 min and 2-h time points. **(B)** Quantification of BMDC adherence on OXA-inflamed ear lymphatics in dermal ear explants of WT and KO at 2-h time point. **(C)** Orthogonal sections of whole-mount ear dermis of WT and KO mice demonstrating the spatial location of DCs to lymphatics (inside vs. outside of the lumen), and **(D)** quantification of the percentages of CFSE$^+$ BMDCs inside the lumen 20 **h** after their intradermal injection followed by topical application of 2% OXA. Each data point represents one ear. Data are presented as mean ± SEM. **P < 0.01 and ***P < 0.001, two-tailed Student's t-test. Scale bar **(A)** 100 µm and **(C)** 50 µm. ns, non-significant.

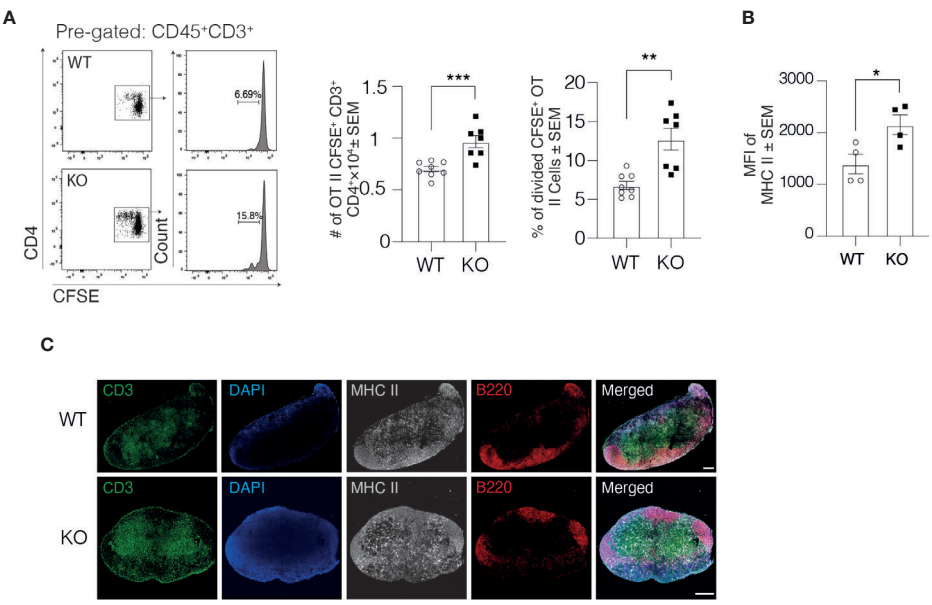

FIGURE 4 | DCs in Clever-1 KO mice have improved antigen presentation to support T cell activation. **(A)** Flow cytometry plots and quantification of the total number of OT II CFSE$^+$ CD45$^+$CD3$^+$CD4$^+$ T cells and the percentage of divided cells among donor OT II CFSE$^+$ CD45$^+$CD3$^+$CD4$^+$ T cells in the draining popliteal LN of WT and KO mice, 48 h after the i.v. injection of CFSE labeled OT II splenocytes. **(B)** MHC II expression by DCs recovered from the draining popliteal LNs 18 h after OVA (emulsified in incomplete Freund's adjuvant) injection and analyzed by flow cytometry; each dot represents 2 pooled animals. **(C)** Fluorescence staining of the dLNs of KO mice and their WT controls: B cells (anti-B220, red), T cells (anti-CD3, green), DCs and macrophages with anti-MHC II (grey) and nucleus with DAPI (blue). Data are presented as mean ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001, two-tailed Student's t-test or two-tailed Student's t-test with Welch's correction. Scale bars 200 µm.

These results together with the normal flux of antigen to the draining LNs in KO mice presented in **Figure 1** indicate that the delivery of free antigens to the dLNs is normal in KO mice but DCs that have migrated through Clever-1 negative lymphatics are more activated based on increased MHC II expression.

As mouse lymphatics do not retain Clever-1 expression in culture, we used human LECs to further study, whether the strong antigen-specific immune response is due to the lack of Clever-1 expressed by LECs. In this set of experiments, we first silenced Clever-1 HDLECs by siRNA. Clever-1 mRNA level reduced by 74% and protein level by 65% compared to control siRNA-transfected cells **(Supplementary Figure 4A)**. To further validate, whether the more activated phenotype of DCs is LEC-dependent, we cultured monocyte-derived DCs with Clever-1 or control siRNA transfected HDLECs for 2 days and analyzed the expression of MHCII, CD40 and CD83 by DCs. In line with our mouse data, MHCII expression was upregulated on DCs, when cultured with Clever-1 siRNA treated HDLECs. In addition to MHCII, expression of CD40 and CD83 were increased on DCs, when cultured with Celver-1 siRNA treated HDLECs. **(Supplementary Figure 4B)**. Next, we performed mixed leukocyte reaction (MLR) assays, in which monocyte-derived DCs were incubated with allogenic T cells in the presence of Clever-1 silenced HDLECs or siControl treated HDLECs for 7 days. In line with our *in vivo* mouse data, T cell proliferation in the presence of Clever-1 silenced HDLECs increased by approximately 17.65 ± 7% (**Supplementary Figure 4C**). These results suggest that the function of DCs is fine-tuned by Clever-1 on LECs.

CLEVER-1 NEGATIVE LYMPHATICS OVEREXPRESS GENES REGULATING IMMUNE RESPONSE AND DOWNREGULATE THOSE INVOLVED IN TRANSMIGRATION

To understand how the afferent lymphatics could regulate the phenotype of DCs arriving in LNs, we performed RNA-seq on $CD45^{-}CD31^{+}Podoplanin^{+}$ cells of the skin-draining LNs from Clever-1 KO mice and their WT controls. All the samples expressed high levels of LEC marker genes, including *Pdpn*, *Pecam-1*, *Prox-1*, and *Flt4* with a comparable expression between the WT and KO mice. The only exception was *Pdpn* that was slightly but statistically significantly lower in KO mice (**Supplementary Figure 2A**). *Stab1* was absent in the Clever-1 KO LECs as expected (**Supplementary Figure 2A**). At steady state, the Clever-1 KO LECs had 162 differently expressed genes (DEG) (FDR ≤0.05, log2ratio ≥1 and ≤-1) of which 75 were downregulated and 87 genes upregulated (**Figure 5A**). To analyze the biological functions of the observed DEGs Ingenuity Pathway Analysis (IPA) software was used. Disease and Bio-function analyses revealed an overrepresentation of genes comprising pathways of immune response regulation, such as inflammatory response and proliferation of T lymphocytes (**Figure 5B**). The most significant genes involved in these pathways were *Ifi202b, Il1b*, *Postn*, *Csf3*, *Fut7*, *Glycam1*, and *Madcam1*, among others (**Figure 5C**). Despite upregulation of pathways related to binding of professional phagocytes and adhesion of immune cells, a pathway related to the transmigration of leukocytes was significantly downregulated in Clever-1 KO LECs (**Figure 5B**). The upstream regulator analytic feature in IPA identified several possible upstream regulators that could explain the observed gene changes (**Figure 5D**). One of them is *Il10*, which is predicted to be significantly inhibited in KO LECs (Z-score -2.36, $p=5.8E^{-03}$). In contrast, *Il2* was predicted to be activated (Z-score 2.213; **Figure 5D**). This indicates that pro-inflammatory signaling cascades were a dominant feature in Clever-1 KO LECs at steady state. Protein analyses of whole LN lysates of WT and KO mice showed reduced levels of anti-inflammatory cytokines IL-4, -10, and -13 as well as CCL2 chemokine, which is in line with the pro-inflammatory gene dominance in the lymphatics of KO mice (**Figure 5E**).

To investigate how the Clever-1 KO lymphatics responded to an inflammatory stimulus, we analyzed the transcriptome of dLN LECs 1 day after injecting OVA emulsified in incomplete Freund's adjuvant into the footpad of WT and KO mice. WT LECs downregulated 274 and upregulated 400 genes by OVA-CFA administration. The Clever-1 KO LECs showed specific downregulation of an additional 899 and upregulation of 326 genes (**Figure 5F** and **Supplementary Figure 2B**). Interestingly, these changes in KO mice were associated with the downregulation of adaptive immune response and leukocyte migration/extravasation pathways (**Figure 5G** and **Supplementary Figure 2C**), which were opposite to the OVA-induced changes in WT LECs (**Supplementary Figure 2D**). However, the KO LECs upregulated *Ackr2*, *Tlr3* and *Csf2* (**Supplementary Figure 2D**), that is in line in the activation of a pathway related to "accumulation of dendritic cells" in LECs of KO mice (Activation Z-score: 1.406, $p=8.22E^{-05}$) (**Figure 5G**).

CLEVER-1 KO MICE HAVE LOW RESPONSE TO CONTACT HYPERSENSITIVITY REACTIONS

Besides being expressed on afferent lymphatics, Clever-1 is also present in efferent lymphatics and is induced to blood vasculature at sites of inflammation. Therefore, we wanted to investigate this whole cascade and chose the contact hypersensitivity (CHS) model for these analyses. In this model, we used OXA as the hapten for belly skin sensitizing and 5 days thereafter challenged the ear skin with OXA. OXA did not dramatically change the expression of Clever-1 on the ear lymphatics (**Supplementary Figure 1H**). Ear thickness measurements at different time points after the challenge showed that Clever-1 KO mice fail to develop a profound CHS response to OXA as their WT controls (**Figure 6A**). Flow cytometry analysis of CHS-inflamed ears at day 2 showed that the number of $CD8^{+}$ T cells in KO ears was 40.3 ± 6% lower than in those of WT mice, while the number of $CD4^{+}$ T cells remained comparable (**Figures 6B–D**). CD8+ T cells mediate skin inflammation by their cytotoxic activity and CD4+ T cells

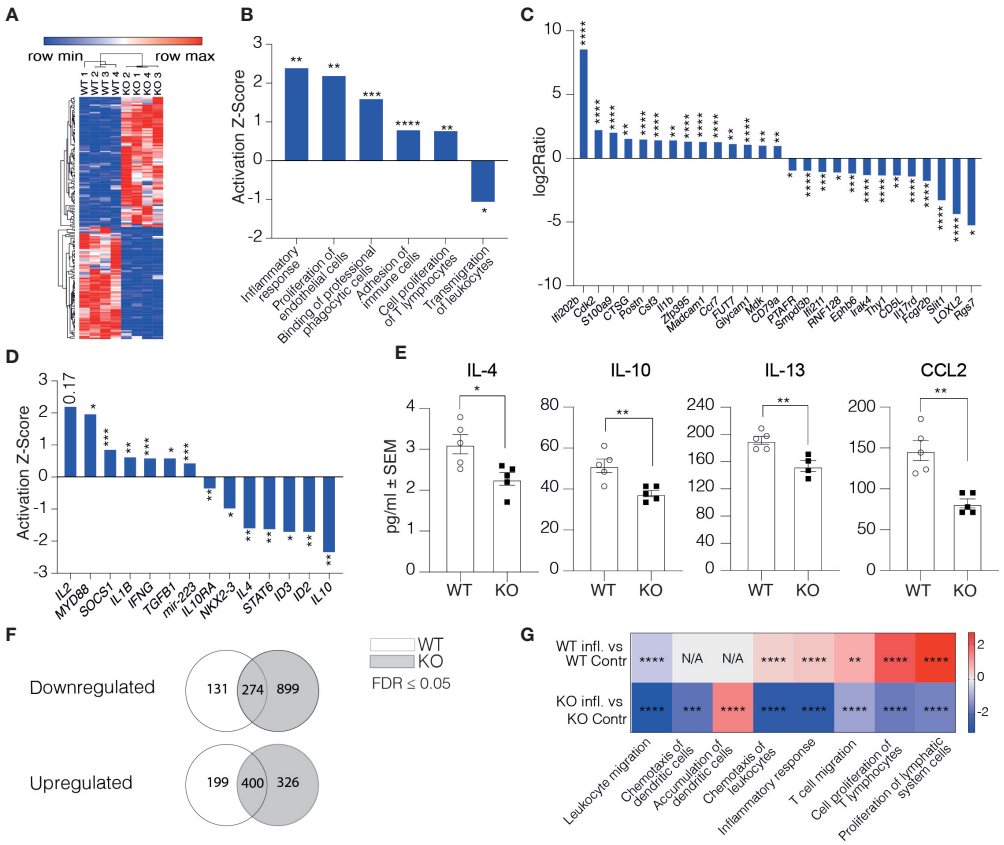

FIGURE 5 | Clever-1 supports lymphatic endothelial cell tolerance. **(A)** Heat map of the gene expression differences in LECs obtained from LNs of Clever-1 KO and their WT controls. Unsupervised hierarchical clustering was performed on the log 2 ratio of significantly (FDR < 0.05) up- (red) and down-regulated (blue) genes. **(B)** Altered pathways in KO LECs compared to WT LECs analyzed by using the disease and Bio-function in IPA. **(C)** Genes in KO LECs (Fold Change ≥ 2) belonging to the altered pathways shown in **(B)**. **(D)** IPA Upstream regulator analysis of DEGs between WT and Clever-1 KO LECs at steady state. **(E)** Cytokine levels in the dLNs at steady state. **(F)** Venn diagrams summarizing differentially expressed genes 1 day after footpad injection with OVA emulsified in incomplete Freund's adjuvant. **(G)** Most significantly altered pathways in inflamed and non-inflamed LECs of WT and KO mice analyzed by using the disease and Bio-function in IPA. Each dot represents one mouse. Data and statistical significance in **(B, D, G)** were analysed by using IPA, *P < 0.05, **P < 0.01 and ***P < 0.001 ****P < 0.0001. Data and statistical significance in **(C)** were analysed by using Illumina, * FDR < 0.05, **FDR < 0.01 and *** FDR < 0.001 **** FDR < 0.0001. Data in **(E)** is expressed as mean ± SEM. *P < 0.05 and **P < 0.01 , two-tailed Student's t-test.

suppress and resolve CHS in inflamed skin (18), providing a possible reason for the impaired OXA-induced CHS response in KO mice. In fact, FACS analyses of KO ears 2 days after OXA challenge showed that there tend to be more CD25 positive regulatory T cells, supposedly a more suppressive population among migratory T regs (19), in KO mice (**Figure 6D**). To analyze whether the reduced CHS response in KO mice was due to impaired lymphocyte egress from the dLNs after the sensitization phase or impaired lymphocyte entrance into the inflamed ear skin, we isolated OXA-primed lymphocytes from dLNs of DsRed$^+$ WT mice and adoptively transferred them into WT and KO mice before applying OXA on their ear skin. In line with our previous results, ear swelling was still reduced in KO mice although not that markedly as in the standard model (**Figure 6E**). Furthermore, whole-mount ear imaging showed that the number of DsRed$^+$ lymphocytes was significantly reduced in KO mice compared to WT mice (**Figures 6F, G**). These results indicate that both the exit of effector lymphocytes *via* efferent lymphatics from the sensitized lymph node and entrance into the challenged inflammatory ear *via* blood endothelial cells are affected by the absence of Clever-1.

We also assessed different cytokine levels in the ear skin of KO and WT mice at steady-state and inflammation. Intriguingly, pro-inflammatory cytokines, including IFNγ, IL-1α, and IL-17 were significantly higher at steady-state in KO mice compared to their WT control (**Figure 6H**, **Supplementary Figure 3A**). However, inflammation significantly decreased the levels of pro-inflammatory cytokines in KO mice, while the level of anti-inflammatory cytokine IL-4 was significantly higher in KO mice compared to their WT control, 1 day after the OXA challenge (**Figure 6H**, **Supplementary Figure 3A**). These results indicate that Clever-1 deficiency at steady-state causes a more pro-inflammatory microenvironment, while inflammation dampens an excessive pro-inflammatory state to help maintain homeostasis.

As macrophages play a crucial role in DC clustering and migration, and Clever-1 is also expressed on a subset of macrophages, we created bone marrow chimaeras using bone

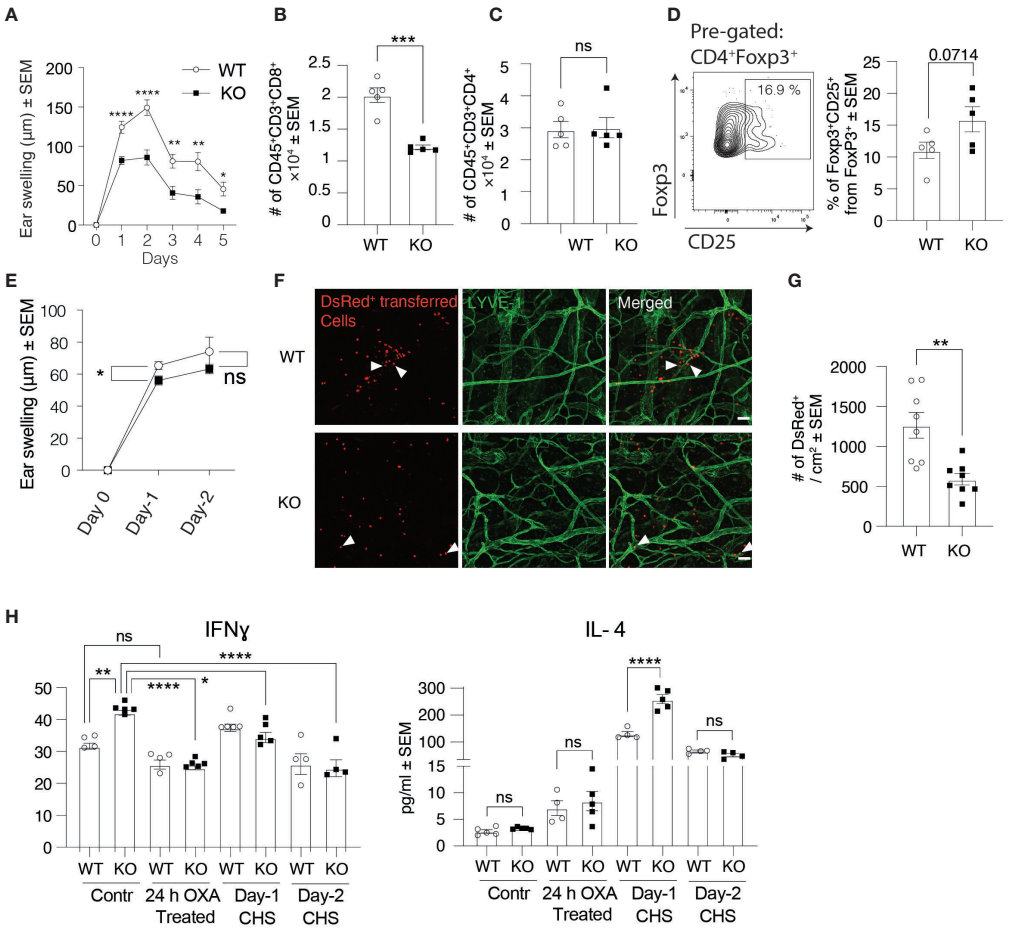

FIGURE 6 | Clever-1 KO mice develop milder inflammatory reactions than their WT controls. **(A)** Ear swelling at indicated time points after oxazolone (OXA) challenge in the CHS model (n≥7 in both KO and WT). **(B–D)** Flow cytometry analyses of digested ear tissue of WT and KO mice, 2 days after the OXA challenge, showing the percentages of endogenous $CD8^+$ **(B)**, $CD4^+$ **(C)**, and gating and quantification of $CD25^+$ Tregs **(D)**. **(E)** Ear swelling at indicated time points after applying OXA on the ears of WT and KO mice followed by adoptive transfer of $DsRed^+$ OXA-primed lymphocytes obtained from dLNs of WT mice. (n= 6-26). **(F, G)** Whole-mount confocal imaging **(F)** and quantification **(G)** of the number of $DsRed^+$ lymphocytes in WT and KO ear dermis 2 days after applying OXA on the ears. **(H)** Expression of cytokines in the ear skin of Clever-1 WT and KO mice at steady-state, 24 h–OXA treated ears (application of OXA onto the ears 24 h earlier), 1 and 2 days after OXA challenge (CHS). Each data point represents one mouse. Data are presented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001, two-tailed Student's t-test or two-tailed Student's t-test with Welch's correction. Additionally, **(H)** One-way ANOVA with Tukey's multiple comparison test. **(F)** Scale bar 50 µm and arrowheads point to adoptively transferred $DsRed^+$ lymphocytes. ns, non-significant.

marrow cells acquired from $CD11c^+$-YFP^+ reporter mice and transplanted them to lethally irradiated Clever-1 WT and KO mice (**Figure 7A**). This allowed us to exclude a possible contribution of Clever-1^+ macrophages in the CHS reaction. In line with previous data, the KO mice created milder inflammation as measured by ear swelling, confirming the role of the endothelium in the impaired CHS reaction of the KO mice (**Figure 7B**). This model also provided us with a possibility to further confirm the role of Clever-1 on afferent lymphatics in DC trafficking. To assess and further validate our results in **Figure 3D** regarding the transmigration of DCs into the lymphatic vessels, the entrance of migratory DCs into the lymphatics was visualized after applying 2% OXA onto the ear skin of the chimeric mice without previous belly sensitization followed by whole-mount confocal imaging of ears 20 h later. Quantification of the number of DCs migrated into the lumen confirmed the finding: although there are more $CD11c^+$-YFP^+ cells in the skin of KO recipients, they do not get access into the lumen of lymphatics as efficiently as in WT mice (**Figures 7C–E**). This was also reflected by the lower number of these cells in the ear draining auricular LNs (**Figures 7F, G**).

DISCUSSION

In this work, we show that the lymphatic endothelial Clever-1 is a crucial mediator of DC trafficking into the dLNs. Clever-1 displays immunosuppressive imprinting to the DCs during their journey to the LNs, as the DCs migrating *via* the lymphatics lacking Clever-1 trigger higher antigen-specific proliferative responses in the nodes

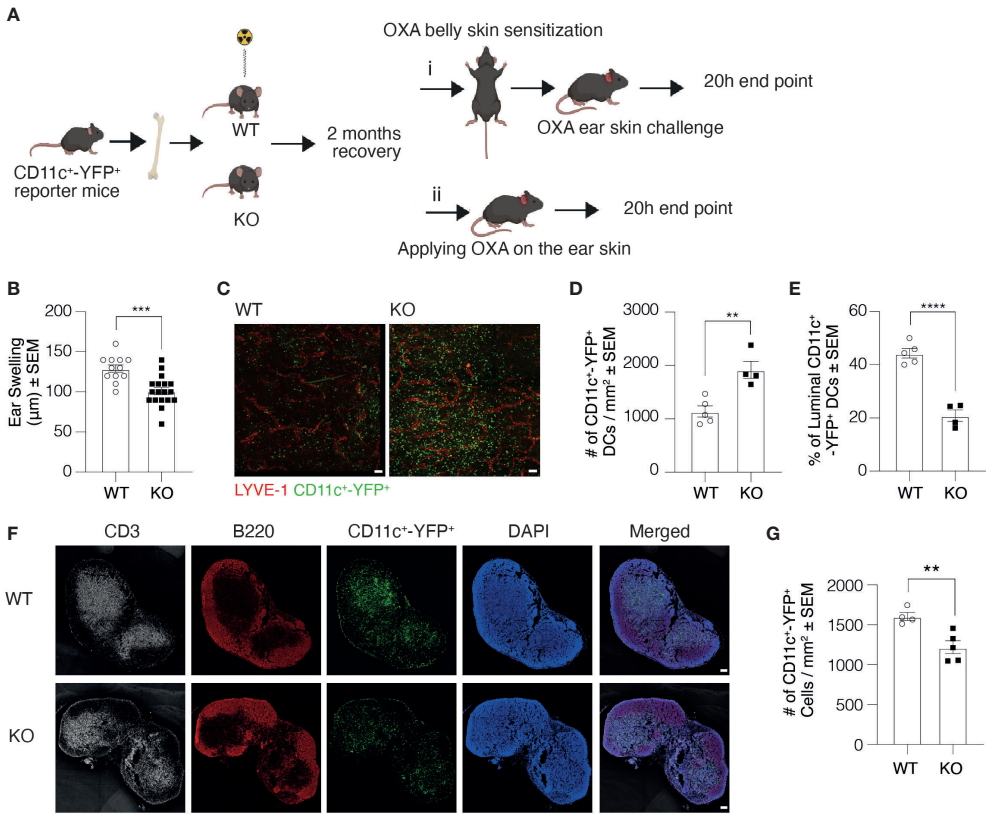

FIGURE 7 | Clever-1 KO macrophages do not contribute to the impaired transmigration of dermal DCs. **(A)** Schematic presentation of the study design illustrating the generation of bone marrow chimaeras and subsequent sensitization with OXA followed by i) challenging the ears with OXA or ii) without previous sensitization. **(B)** Ear thickness of CD11c$^+$-YFP$^+$ → WT and KO chimaeras 20 h after the OXA challenge (model i). **(C–E)** Whole-mount images of ear dermis **(C)**, and quantification of the total number **(D)**, and the percentage of CD11c$^+$-YFP$^+$ cells inside the lumen of ear lymphatics **(E)** of CD11c$^+$-YFP$^+$→WT and KO chimaeras 20 h after topical application of OXA (model ii). **(F, G)** Confocal images of auricular draining LNs **(F)**, and quantifications of the number of CD11c$^+$-YFP$^+$cells in the auricular LNs **(G)** of CD11c$^+$-YFP$^+$→WT and KO chimaeras 20 h after topical application of OXA. Each data point represents one mouse. Data are expressed as mean ± SEM. **P < 0.01, ***P < 0.001, and ****P < 0.0001, two-tailed Student's t-test. Scale bars 100 µm.

despite their lower numbers. This type of modulation may be central to avoid unnecessarily high immune responses and thus, to keep the immunity at an optimal level.

It has remained unclear how immune cell migration and transport of soluble components within the lymphatics determine the quality and quantity of the immune response (3). It is known that LN LECs can efficiently scavenge and present peripheral antigens on MHCI to induce tolerance by specific deletion of autoreactive CD8$^+$ T cells. Moreover, LN LECs can phagocytose, process exogenous antigen and cross-present it to naïve CD8 T cells. However, the dominant expression of co-inhibitory molecules and lack of co-stimulatory molecules by LECs can lead to specific deletion of reactive lymphocytes (20–22). The results of this work strongly suggest that Clever-1 on lymphatic vasculature is an important regulator of DC trafficking and simultaneously an immunomodulator of DC activity when evoking immune responses in the dLNs. Indeed our data showed that knockdown of Clever-1 from human lymphatic endothelial cells leads to a more activated phenotype of DCs in co-cultures. As Clever-1 is also a scavenger rapidly recycling between the plasma membrane and endosomal compartments and being able to scavenge both endogenous and exogenous antigens such as acetylated LDL, placental lactogen, apoptotic cells and bacteria (23–27), it may as well participate in antigen processing.

Clever-1 is expressed rather uniformly both on the afferent and efferent arms of the lymphatics and can play its effects at sites of its expression. In this study, we could demonstrate by both exogenous and endogenous DCs that in the absence of Clever-1 on lymphatics DCs do not enter the afferent lymphatics in a normal fashion. However, as DCs exert their function in the dLNs by presenting antigens and do not normally leave these nodes *via* the efferent lymphatic vessels (28), the contribution of Clever-1 in the efferent lymphatic arm seems mainly to be in the lymphocyte exit from the nodes (9).

Besides directly investigating DC traffic *via* the afferent lymphatics to the dLNs and consequences in the nodes, we also used a more complex model, namely a contact hypersensitivity model, that allowed us to analyze the summation of the lymphocyte exit from the nodes draining the belly area used for the sensitization and also the entrance *via* the blood vessels to the site of challenge in

the ears. Based on normal or even slightly accelerated immune response in the draining lymph nodes as shown in the OVA immunizations and reduced ear swelling at the site of challenge in the contact hypersensitivity experiments, the possible reasons for this phenomenon are: impairment either in the exit of OXA-specific T cells from the draining nodes *via* the efferent lymphatics, their entrance into the ear *via* the blood vessels or both. In this context, it is important to note that although Clever-1 is absent from normal flat walled endothelial cells, it is induced on this type of vessels at sites of inflammation (9, 29). Based on the transfer experiments presented in **Figure 6E** we suggest that both components are involved as exemplified in **Figure 8**. It should also be noted that macrophages deficient of Clever-1 have an increased pro-inflammatory phenotype (30) and could contribute to the increased inflammatory microenvironment that we observed in the LN and ear of Clever-1 KO mice at steady state. This most likely can additively stimulate the increased MHC II expression on migratory DCs.

Immunomodulatory roles for the lymphatic endothelium have been realized during recent years. For example, they can promote tolerance as well as archive and present antigens (31). Lymphatic endothelial cells are known to attenuate inflammation *via* suppression of the maturation of DCs (32). Also, it has been shown that lymphatic endothelial cells can archive, store and present antigens to DCs and prime naïve CD8 T cells into memory cells (33, 34). Moreover, it has been reported that DCs spend considerable time and closely interacting with endothelial cells of afferent lymphatics (35). Moreover, DCs form contacts with intra-lymphatic T cells during their journey indicating that modulation of adaptive immunity occurs already in afferent lymphatics before entering the LNs. In this scenario, Clever-1 deficient lymphatics are not able to down modulate the activity of DCs, which then effectively prepare T cells to provoke an accelerated immune reaction within the dLNs. Indeed, DCs co-cultured with Clever-1 silenced HDLECs, express higher levels of MHCII, and consequently, when used in MLR assay, the proliferation of naïve $CD4^+$ T cells significantly increases. These data are further supported by our RNAseq analyses of LECs from WT and KO mice. They unambiguously demonstrate that the LECs of the KO mice have higher expression of pro-inflammatory genes (*Il6, Ifi202b, S100A9, Csf3, Madcam1*) than their WT controls. Moreover, upon inflammation Clever-1 KO LECs up-regulated genes regulating migration, maturation, activation and tolerance (*Ackr2*, *Csf2, Tlr3*) whereas they downregulated several MHC I and II genes. *Ackr2* exerts its effects as a decoy receptor for quite a variety of chemokines regulating leukocyte trafficking, *Csf2* is important in DC homeostasis and activation and *Tlr3* is involved in tolerance (36–39). The downregulation of antigen-presenting molecules on the inflamed-LECs could possibly prevent the deletion of reactive T cells since they no longer would effectively present antigen improving DC-mediated T cell activation.

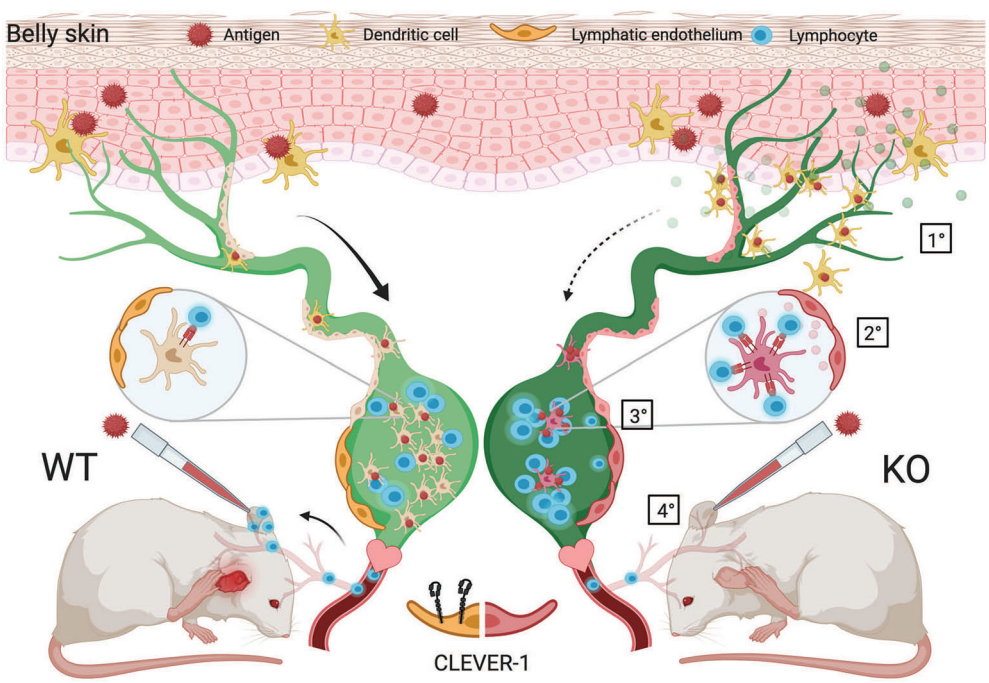

FIGURE 8 | Schematic presentation of the proposed action points of Clever-1. **1)** DC migration from the skin into the draining lymph nodes *via* lymphatic vessels is impaired in the absence of Clever-1 when mice are sensitized with an antigen on the skin. **2)** However, the DCs that end up in the LN show good capacity to activate T cells. This is due to a less tolerogenic interaction of the migrating DCs with Clever-1 deficient lymphatic vessels. **3)** When leaving the lymph node, the activated T cells cannot egress the Clever-1 KO LN as efficiently as in the WT situation and also **4)** the lack of Clever-1 expression on blood vessels at the site of inflammation impairs the homing of T cells into the tissue. This reduces the inflammatory reaction when mice are re-challenged in the ear with the same antigen.

Based on these results, we like to consider that while Clever-1 deficiency at steady-state increases the pro-inflammatory state, additional inflammatory insults lead to tissue resolution to sustain homeostasis and prevention of overt inflammatory reactions. Whether this is a compensatory mechanism created during the lifetime of Clever-1 KO mice remains unknown. We have earlier shown that Clever-1 can directly bind B cells and CD8 T cells (12), although the counter-receptor on those cells has not yet been identified. The current RNAseq results demonstrate that lack of Clever-1 causes marked changes to various genes encoding important regulators and therefore, its absence may indirectly affect a multitude of immune functions in the body. The exact mechanisms remain to be elucidated.

However, since Clever-1 has been shown to regulate the immunosuppressive activities of human monocytes (40) and mouse macrophages (30) our results suggest that lymphatic Clever-1 has similar properties besides its involvement in the trafficking of DCs. As Clever-1 is currently a therapeutic target in cancer trials, the treatment is expected to have consequences also on lymphatics. Although KO mice are not equivalent to patients receiving anti-Clever-1 antibody, based on the findings of this work we envision that the lymphatics contribute together with macrophages to the immune activation seen in the trial patients (41).

DATA AVAILABILITY STATEMENT

RNA sequencing data have been deposited in the Gene Expression Omnibus database under the accession no. GSE148730 and the unique reagents and mice are available at request from the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethical board of Turku University Hospital. The patients/participants provided their written informed consent to participate in this study. All animal experiments were approved by The Finnish Act on Animal Experimentation (62/2006; animal license number 5762/04.10.07/2017).

AUTHOR CONTRIBUTIONS

ST, JD, DE, and RV performed the experiments. AT and KE performed the RNA seq analyses. MH and SJ designed and supervised the work. SJ wrote the first draft. 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.2021.602122/full#supplementary-material

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Conflict of Interest: MH and SJ own stocks of Faron Pharmaceuticals.

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

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Released Myeloperoxidase Attenuates Neutrophil Migration and Accumulation in Inflamed Tissue

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Neutrophil (PMN) recruitment to sites of insult is critical for host defense, however excessive PMN activity and tissue accumulation can lead to exacerbated inflammation and injury. Myeloperoxidase (MPO) is a PMN azurophilic granule enzyme, which together with H_2O_2, forms a powerful antimicrobial system designed to kill ingested bacteria. Intriguingly, in addition to intracellular killing of invading microorganisms and extracellular tissue damage due generation of ROS, soluble MPO has been directly implicated in modulating cellular responses and tissue homeostasis. In the current work, we used several models of inflammation, murine and human PMNs and state-of-the-art intravital microscopy to examine the effect of MPO on PMN migration and tissue accumulation. We found that in the absence of functional MPO (MPO knockout, KO mice) inflammatory PMN tissue accumulation was significantly enhanced. We determined that the elevated numbers of PMNs in MPO knockout mice was not due to enhanced viability, but due to increased migratory ability. Acute PMN migration in models of zymosan-induced peritonitis or ligated intestinal loops induced by intraluminal administration of PMN-chemokine CXCL1 was increased over 2-fold in MPO KO compared to wild type (WT) mice. Using real-time intravital imaging of inflamed mouse cremaster muscle and *ex vivo* PMN co-culture with inflamed endothelial cells (ECs) we demonstrate that elevated migration of MPO KO mice was due to enhanced adhesive interactions. In contrast, addition of soluble recombinant MPO both *in vivo* and *ex vivo* diminished PMN adhesion and migration. Although MPO has been previously suggested to bind CD11b, we found no significant difference in CD11b expression in either resting or activated PMNs and further showed that the MPO binding to the PMN surface is not specific to CD11b. As such, our data identify MPO as a novel regulator of PMN trafficking in inflammation.

Keywords: neutrophils, adhesion, migration, inflammation, imaging, myeloperoxidase (MPO)

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INTRODUCTION

Inflammatory responses feature rapid mobilization of neutrophils (PMNs) towards the insult sites (1). While PMNs fulfill essential roles in combating invading pathogens, providing the first line of defense to the host, they can also promote exacerbated inflammation resulting in tissue damage. PMN-associated tissue damage occurs mainly due to the release of various proteinases, including

matrix metalloproteinases (MMPs), Elastase and Myeloperoxidase (MPO) into the surrounding tissue (2, 3)

MPO is the most abundant protein expressed by PMNs and perhaps one with the most damaging/cytotoxic potential to living cells. Together with H_2O_2, MPO forms a powerful antimicrobial system designed to kill ingested bacteria. MPO is stored in the azurophilic/primary granules and is released into the phagosomes, where it catalyzes the conversion of H_2O_2 to hypochlorous acid (HOCl) (4) Although it is safeguarded in the azurophilic/primary granules, which require stronger stimulation and are the last to be released during the hierarchical release of PMN granules (5), plenty of evidence suggests spilling of free MPO into the surrounding tissue during inflammation (6). As such, elevated plasma and tissue levels of MPO were detected in many inflammatory disorders, where the presence of MPO or its oxidative derivatives have been assigned pro-inflammatory or in some cases protective functions [summarized in (7)].

Importantly, in addition to the intracellular killing of invading microorganisms and extracellular generation of ROS, emerging evidence indicates that MPO can directly modulate cellular function and immune responses, making it both an attractive disease biomarker and a therapeutic target. Several new functions were identified for free MPO, which include modulation of endothelial and epithelial responses (8, 9) as well as macrophage and dendritic cell activation (10, 11). However, MPO paracrine modulation of PMN activity particularly stands out. Free MPO has been shown to bind CD11b at the PMN surface and *via* these interactions, trigger MAPK- and NFκB-dependent ROS production and degranulation by PMNs (12) or improve phagocytosis *via* the induction of FAK/ERK signaling (13). MPO has also been shown to provide adhesive support for PMNs (14) and modulate both Ca^{2+} levels and cytoskeleton organization (15), suggesting its potential role in PMN trafficking.

Since for many inflammatory disorders, resolution of inflammation is an important clinical endpoint and PMNs can both exacerbate inflammation and initiate reparative responses, understanding mechanisms that govern the trafficking and recruitment of these cells in tissue are of ongoing clinical interest. To mediate either the protective or pro-inflammatory action, PMNs must first exit the circulation by crossing the endothelial layer. Transendothelial migration (TEM) is a highly regulated process that is initiated with the capture of free-flowing PMNs at the vessel wall, followed by slow rolling and adhesion to ECs and terminated with migration across the EC monolayer [reviewed in (16)]. The PMN recruitment cascade is well-studied and the signaling and ligands mediating this process are well-recognized, however specific approaches to target PMN migration are still under investigation.

Given the implication of excessive PMN infiltration and activity in tissues in inflammatory disorders and the newly emerging evidence of MPO in modulating the PMN function, in the current work we thought to examine the impact of MPO on PMN trafficking and recruitment in inflammation. We utilized two murine models of sterile inflammation and murine and human PMNs to definitively show that in the absence of MPO the adhesive and migratory ability of PMNs is enhanced, resulting in elevated tissue accumulation. Furthermore, free MPO was found to significantly suppress PMN migration, independently of its binding to CD11b. As such, although additional studies are needed to define the specifics of MPO bindings to the PMN surface, our findings identify a new and physiologically relevant role of MPO in PMN trafficking in inflammation.

MATERIALS AND METHODS

Animals

C57BL6J mice, MPO knockout mice (B6.129X1-*Mpo*^{tm1Lus}/J) (Jackson Laboratories) and LysM-eGFP reporter mice (Lyz2tm1.1Graf, gift from Dr. Perlman, Northwestern University) ages 8–16 weeks, were maintained under specific pathogen-free conditions at Northwestern University, Feinberg School of Medicine animal facilities. At the end of all experimental procedures, animals were euthanized *via* rapid cervical dislocation. All experimental protocols were approved by the Institutional Animal Care and Use Committee.

Cells

Mouse microvascular brain endothelial cell (Bend-3) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin-streptomycin. Human umbilical endothelial cells (HUVECs) were grown in human endothelial SFM supplemented with 1% L-glutamine, endothelial cell growth supplement (0.0375 mg/mL), 10% FBS, 1% penicillin-streptomycin, and 1% non-essential amino acids. *Human PMNs* were isolated from blood obtained from healthy volunteers by density gradient centrifugation (17, 18) and handled according to protocols for the protection of human subjects, as approved by the Northwestern University Institutional Review Board. PMNs were used for experiments within 2 hours of isolation. *Murine Bone Marrow PMNs* were isolated from bone marrow and enriched to ~85-90% purity using Histopaque gradients (1077 and 1119, Sigma) as previously described (19).

Antibodies and Reagents

RPMI 1640, Dulbecco's modified Eagle's medium (DMEM) growth media, human endothelial SFM, endothelial cell growth supplement, L-glutamine, penicillin, streptomycin, nonessential amino acids, zymosan, collagen, Hanks balanced salt solution with/without Ca^{2+} and Mg^{2+} (HBSS+, HBSS-), Annexin V FITC, antiS100A8, and Propidium Iodide (PI) were obtained from Fisher Scientific (Waltham, MA). N-formyl-l-methionyl-leucyl-l-phenylalanine (fMLF) and Phorbol 12-myristate 13-acetate (PMA) were acquired from Sigma-Aldrich (St. Louis, MO). Human/murine interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) and murine PMN chemoattractant CXCL1 (KC) were obtained from PeproTech

(Rocky Hill, NJ). Bovine Serum Albumin (BSA) was obtained from Gemini Bio-Products (West Sacramento, CA). CD11b blocking antibody (clone M1/70), Pacific Blue™ anti-mouse CD45 antibody (clone 30-F11), APC anti-mouse CD144 (VE-cadherin, clone BV13), APC anti-mouse CXCR2 (clone SA044G4), PerCP cy5.5, anti-mouse CXCR4 (clone L276F12) and Alexa Flour 488 anti-His Tag (Clone J099B12) were from BioLegend (San Diego, CA). The rat IgG2b isotype was from BioXCell (Lebanon, NH). Recombinant mouse/human MPO protein, recombinant mouse interleukin 1-beta (IL-1β) and anti-h/m cleaved caspase Ab (Asp-135, clone 269518) were purchased from R&D systems (Minneapolis, MN). Anti-mouse PECAM-1 was purchased from Merck KGaA (Darmstadt, Germany) and labeled with DyLight 650 Fluorochrome purchased from Thermo Fisher Scientific (Waltham, MA). PE anti-CD11a (clone 2D7) and anti-Ly6G Abs (clone 1A8) were from BD Biosciences (San Jose, CA, USA). Anti-CD11b antibodies and rat monoclonal E-cadherin (DECMA-1) were from Abcam Inc. (Cambridge, MA). Horseradish peroxidase conjugated anti-mouse/rabbit secondary Ab was purchased from Jackson ImmunoResearch (West Grove, PA). Polyclonal anti-mouse p44/42 MAPK (ERK 1/2) and anti-mouse phospho-p44/42 MAPK (pERK 1/2; clone D13.14.4E) Abs were from Cell Signaling (Danvers, MA). Polymorphprep was purchased from Axis-Shield (Alere Technologies, Oslo, Norway). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Atlanta, GA). Ketamine and xylazine were obtained from Henry Schein (Dublin, OH) and Akorn (Decatur, IL), respectively.

Immunofluorescence Staining

For intestines, inflamed loops were OCT-frozen and 10μm-thick sections were blocked with 5% bovine serum albumin (BSA) in PBS, and incubation with the relevant primary antibody (10μg/ml, overnight, 4°C) followed by secondary antibodies (2hr, RT). *For cremaster muscle*, whole mount tissue was lightly permeabilized and blocked using PBS containing 0.3% Triton X-100, 2% BSA, and 1% goat serum overnight at 4°C. The samples were then incubated with primary antibodies (CD31 and S100A8) overnight at 4°C followed by secondary antibodies in permeabilization/blocking buffer (4h, RT). *For isolated PMNs*, BM-derived PMNs (3×10^5 cells per condition) were mounted on slides, fixed with 4% paraformaldehyde followed by permeabilization using 1% Triton X-100 and stained with the relevant primary antibody (10μg/ml, overnight, 4°C) followed by secondary antibodies (2hr, RT). All tissues were mounted on slides using FluorSave (EMD Millipore) for imaging. Imaging was performed using UltraVIEW VoX imaging system built on an Olympus BX-51WI Fixed Stage illuminator and equipped with a Yokogawa CSU-X1-A1 spinning disk, a Hamamatsu EMCCD C9100-50 camera, and a Modular Laser System with solid state diode lasers with DPPS modules for 488, 561, and 640 nm and the appropriate filters (all assembled by Perkin Elmer, Naperville, IL).

Intravital Imaging

For imaging of the cremaster muscle, which is a muscle surrounding the testis, male mice were used. Inflammation of the cremaster muscle was induced by intrascrotal injection of IL-1β (50ng, 4hr). When specified, murine recombinant MPO or denatured MPO protein (10 min, 95°C) was also administered immediately following stimulation (30μg/ml, iv). The ketamine and xylazine mixture (100mg/kg and 5mg/kg body weight, respectively) was used as initial anesthetic and to subsequently maintain appropriate anesthesia levels throughout the imaging procedures. The cremaster muscle was surgically exposed and prepared for imaging as previously described (20). Animal body temperature during imaging was maintained at 37°C and the tissue was continuously hydrated by superfusion of warmed perfusion buffer consisting of Tyrode's salts (Sigma Aldrich) and 1 g/l sodium bicarbonate (pH 7.4; Thermo Fisher Scientific, Waltham, MA). *Imaging* was performed using Olympus BX-51WI Fixed Stage illuminator and equipped with a Yokogawa CSU-X1-A1 spinning disk, a Hamamatsu EMCCD C9100-50 camera, and a Modular Laser System with solid state diode lasers with DPPS modules for 488, 561, and 640 nm and the appropriate filters (all assembled by Perkin Elmer, Naperville, IL) as previously described (21). Synchronization was managed by a Prosync 2 Synchronization Controller. Z-axis movement and objective positioning was controlled by Piezoelectric MIPOS100 System (Piezoystem Jena, Germany). Images were collected using a 20x water-immersion objective (1.00 numerical aperture). Volocity® software (Perkin Elmer) was used to drive the microscopy and acquire images, which were then analyzed using ImageJ.

Analyses of PMN-EC Interactions

For analyses of cell fluxes, PMN rolling and adhesion, 30sec recording of random fields containing a 30- to 50-μm postcapillary venule with steady flow were made using brightfield illumination and acquisition rate of 15 frames per second. Cell flux was defined as the number of cells that were visualized in the field of view, per 30 sec acquisition time. Rolling cells were defined as cells that have remained in continued contact with the vessel wall for greater than 5 seconds. Adherent cells were defined as cells that remained attached to the vessel wall for >20 sec. For analyses of transmigrated/tissue leukocytes, cells were quantified along 100μm vessel segments and within 50μm of the vessel of interest. Of note, it has been previously established that ~90% of all transmigrated leukocytes in IL-1β-stimulated cremaster muscles (4-5r hour stimulation) are neutrophils (22).

Sterile Intestinal Inflammation Model

Inflammation in the intestine was induced by intraperitoneal administration of TNFα and IFNγ (500ng each, 24hr) as has been previously described (23, 24). TNFα and IFNγ are the most dominant cytokines in intestinal inflammation and injury and such stimulation truthfully simulates human pathology as seen in Inflammatory Bowel Diseases.

Ligated Ileal Loop Model

Animals were anesthetized by ketamine and xylazine mixture (100 and 5 mg/kg, ip., respectively). A midline abdominal incision was made, and a 2-cm loop of small intestine was

exteriorized and clipped at proximal and distal ends. After luminal administration of PMN chemoattractant CXCL1 (KC, 1μM in 200μl HBSS+), excised loops were reinserted into the peritoneal cavity (21). After 1-2 hours incubation, luminal lavage samples were taken and analyzed by flow cytometry. PMNs were defined and gated as $CD45^+/CD11b^+/Ly6G^+$ cells.

Zymosan-Induced Peritonitis Model

Zymosan injection (2μg/ml in 500μl PBS, ip.) was used to induce peritoneal inflammation and PMN recruitment into the peritoneal cavity. Four hours following Zymosan administration, peritoneal content was lavaged using 3 mL of PBS supplemented with 10% FBS and the number of migrated PMNs was quantified using flow cytometry. PMNs were defined and gated as $CD45^+/CD11b^+/Ly6G^+$ cells.

Flow Cytometry

Single cell suspensions following the relevant treatment were analyzed using a BD LSR Fortessa X-20 (Becton Dickinson, Franklin Lakes, NJ) instrument and FlowJo 10.7 software (Becton Dickinson).

Transwell Chemotaxis Assay

Transwells (3.0μm pore size, CoStar Group, Washington DC) were coated overnight with 1μg/μl collagen in 0.2% acetic acid buffer. Human or murine bone marrow (BM)-derived PMNs were added to the upper chamber (1×10^6 per well) and induced to chemotax by the addition of an fMLF gradient to the lower chamber (200nM in 500μL HBSS+, 37°C). The number of migrated PMNs in the lower chamber following 30 minutes (for human PMNs) and 2hr (for murine PMNs) migration was quantified by counting of 7-10 randomly selected fields of view using an inverted phase-contrast microscope (Leica Microsystems; Wetzlar, Germany).

Adhesion Assay

BM-derived murine or human peripheral blood PMNs were labeled with CellTracker™ Green or Orange CMFDA (per manufacturer instructions) and added (3×10^5 cells) to ECs (grown to confluence in 96-well plates) in the presence of fMLF (200nM for human and 500nM for murine PMNs) stimulation with and without the relevant inhibitors. Following 30min incubation (37°C), EC monolayers were washed x3 times and the remaining adherent PMNs were quantified by counting of 7-10 randomly selected fields of view. For transwell and adhesion assays counts were performed equally by 2 investigators in a blinded fashion.

Viability Assays

For *ex vivo* assessment of WT and MPO KO PMN viability, freshly isolated BM-PMNs (5×10^5 cells per condition) were incubated in RPMI 1640 in low adhesion 24-well plates (CoStar Group, Washington DC) with/without fMLF pre-stimulation (500nM, 20 min). After 24 and 48h, PMNs were stained with Annexin V and Propidium Iodide and analyzed by flow cytometry. For *in vivo* assessment, BM-derived WT and MPO KO PMNs were stained with CellTracker™ Green and Orange CMFDA, respectively (yielding green and red PMNs) and injected intravenously into WT recipient mice. Blood samples were collected at 1 and 4hrs following PMN transfer and apoptosis of endogenous, WT and MPO KO PMNs were analyzed using Annexin V staining and flow cytometry. Endogenous and transferred PMNs were identified as $CD45^+/CD11b^+/Ly6G^+$ and red/green fluorescence negative or positive cells.

Differential Leukocyte Counts

Fresh blood samples were obtained from WT and MPO KO mice *via* tail bleed. Following red blood cell lysis using ACK buffer, leukocytes were spun onto slides (Cytospin 4 cytocentrifuge, Thermo Scientific; Waltham, MA), fixed and stained using a Diff-Quick™ Stain Set (Siemens; Munich, Germany). All cell counts were performed in a blinded fashion and included at 20 randomly selected fields of view per condition per independent experiment. Data are shown as percent of total counted cells.

Immunoblotting

Immunoblotting was performed as previously described (24). Briefly, BM-derived PMNs were lysed in Laemmli buffer. Proteins (10-30μg) were separated by SDS-PAGE gels (10-15%), transferred to nitrocellulose membrane and probed with relevant primary (overnight at 4°C) followed by HRP-conjugated secondary Abs (1h, RT). Peroxidase activity was detected using Immunobilon Crescendo Western HRP Substrate (Millipore).

Statistics

Statistical significance was assessed by two-tailed, unpaired Student t-test or by one-way ANOVA with Newman-Keuls Multiple Comparison Test using Graphpad Prism (V4.0). Statistical significance was set at $p<0.05$. For all experiments the data shown as ± SEM.

RESULTS

PMN Tissue Localization Is Enhanced in the Absence of Functional MPO

Following PMN activation and degranulation, MPO can associate with the cell membrane, where it can bind CD11b/CD18 (Mac1) integrin (12). Given the important role of CD11b in PMN trafficking and the potential of MPO binding to interfere with its function, recruitment of WT and MPO KO PMNs was compared in intestinal and skeletal muscle inflammation. Intraperitoneal administration of TNFα and IFNγ (500ng each, ip.) and intrascrotal injection of IL-1β (50ng) were used to stimulate inflammation in the intestinal mucosa and the cremaster muscle, respectively (23, 25). While PMNs are absent in unstimulated (control) WT and MPO KO mouse tissue, inflammatory stimulation resulted in ~2-fold increase in the number of tissue PMNs in both the intestinal lamina propria (**Figures 1A, B**) and the muscle tissue (**Figures 1C, D**) in MPO KO as compared to WT mice. These data suggest that the presence of MPO attenuates PMN tissue accumulation, whereas its removal relieves the inhibition.

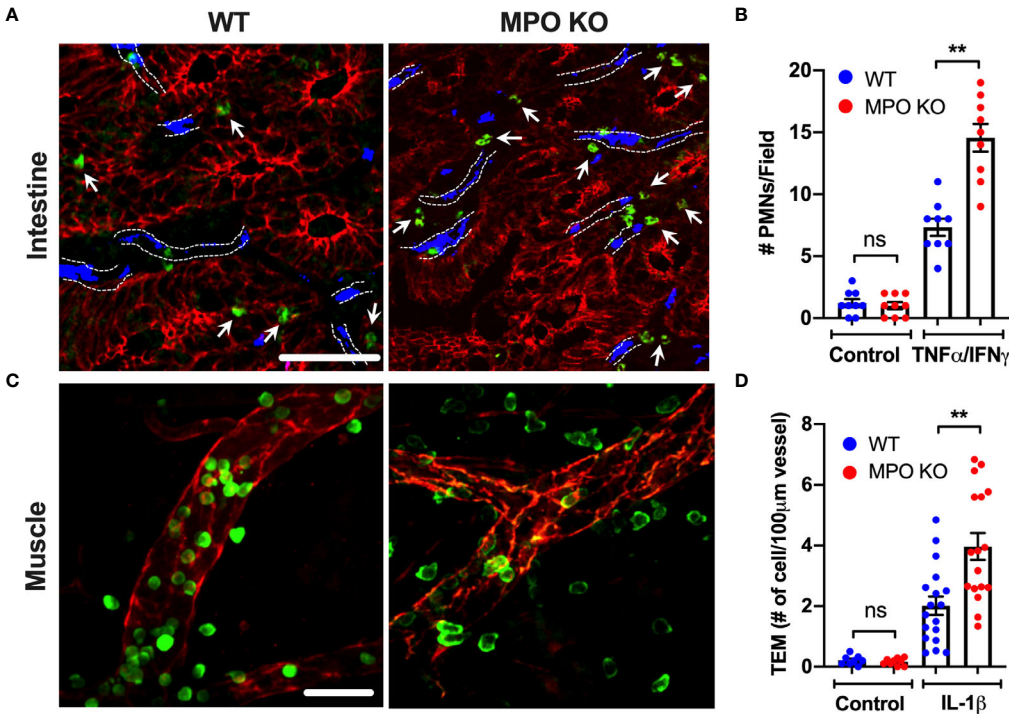

FIGURE 1 | PMN tissue localization is enhanced in the absence of functional MPO. **(A, B)** Inflammation of the intestinal mucosa was induced by intraperitoneal administration of TNFα and IFNγ (500ng, each 24hr). Immunofluorescence analyses were performed on OCT-frozen 10μm-sections. PMNs, intestinal epithelial cells and blood vessel were visualized by S100A8 (green), E-Cadherin (red) and PECAM (CD31, blue) staining respectively. **(A)** Representative images and **(B)** quantification show increased number of tissue-infiltrating PMNs in MPO KO mice. Blood vessel locations are highlighted by dotted lines. White arrow point to tissue localized PMNs. **(C, D)** Inflammation of the cremaster muscle was induced by intrascrotal administration of IL-1β (50ng, 4hr). PMNs and ECs were visualized in a whole mount muscle preparation by respective staining with S100A8 (green) and CD31 (red). Consistently, PMN numbers in tissue were significantly elevated in MPO KO animals. N=3 independent experiments with 3 mice per condition. **$p < 0.01$. ns, not significant.

MPO Expression Does Not Impact PMN Viability

Increased tissue accumulation can be driven by delayed cell apoptosis/increased survival or enhanced migratory ability. Indeed, exogenous MPO has been suggested to delay PMN apoptosis *via* activation of CD11b-dependent signaling (26). As such, we asked whether PMN viability was impacted by the lack of MPO expression. *Ex vivo* cultures of WT and MPO KO bone marrow-derived PMNs (BM-PMNs) with or without fMLF stimulation (500nM) showed no significant difference at 24hr (**Figure 2A**) to 48hr (at 48hr ~80 percent of PMNs were found to become apoptotic). Further assessment of cell apoptosis using cleaved Caspase-3 (Asp-175) by immunofluorescence similarly revealed no significant differences in fMLF-stimulated WT and MPO-KO PMNs (**Figures 2B, C**).

To test whether MPO impacts the lifespan of circulating PMNs under physiological shear conditions, WT and MPO KO BM-PMNs were stained with green and red fluorescence respectively and injected intravenously into WT-recipients ($2x10^6$ PMNs, 1:1 ratio), which were conditioned by IL-1β injection (50ng, ip. 1hr prior to PMN transfer) to induce inflammation. Apoptosis of transferred WT and MPO KO PMNs in inflamed circulation was examined by flow cytometry on whole blood sample 1- and 4- hours post-transfer as indicated in the schematic (**Figure 2D**). A substantial population of transferred WT (green) and KO (red) PMNs was detected at 1hr, however at 4hr, the majority (>80%) of transferred PMNs were similarly cleared from the circulation in WT and MPO KO mice (gating is shown in **Figure 2E**). Importantly, while the number of transferred Annexin V positive PMNs significantly increased over time, no significant difference was found between WT and MPO KO PMNs (**Figures 2F, G**). Although these data do not rule out the possibility that MPO may impact the lifespan of PMN in the tissue, they indicate that increased numbers of MPO KO PMNs in inflamed tissue could be driven by enhanced migratory ability of MPO KO PMNs and not due to increased survival in the circulation.

MPO KO PMNs Exhibit Enhanced Migratory Efficiency

To determine whether MPO expression impacts PMN migration, zymosan-induced peritonitis and ileal loop PMN migration assays were performed. Both assays allow for quantification of acute PMN mobilization from the blood

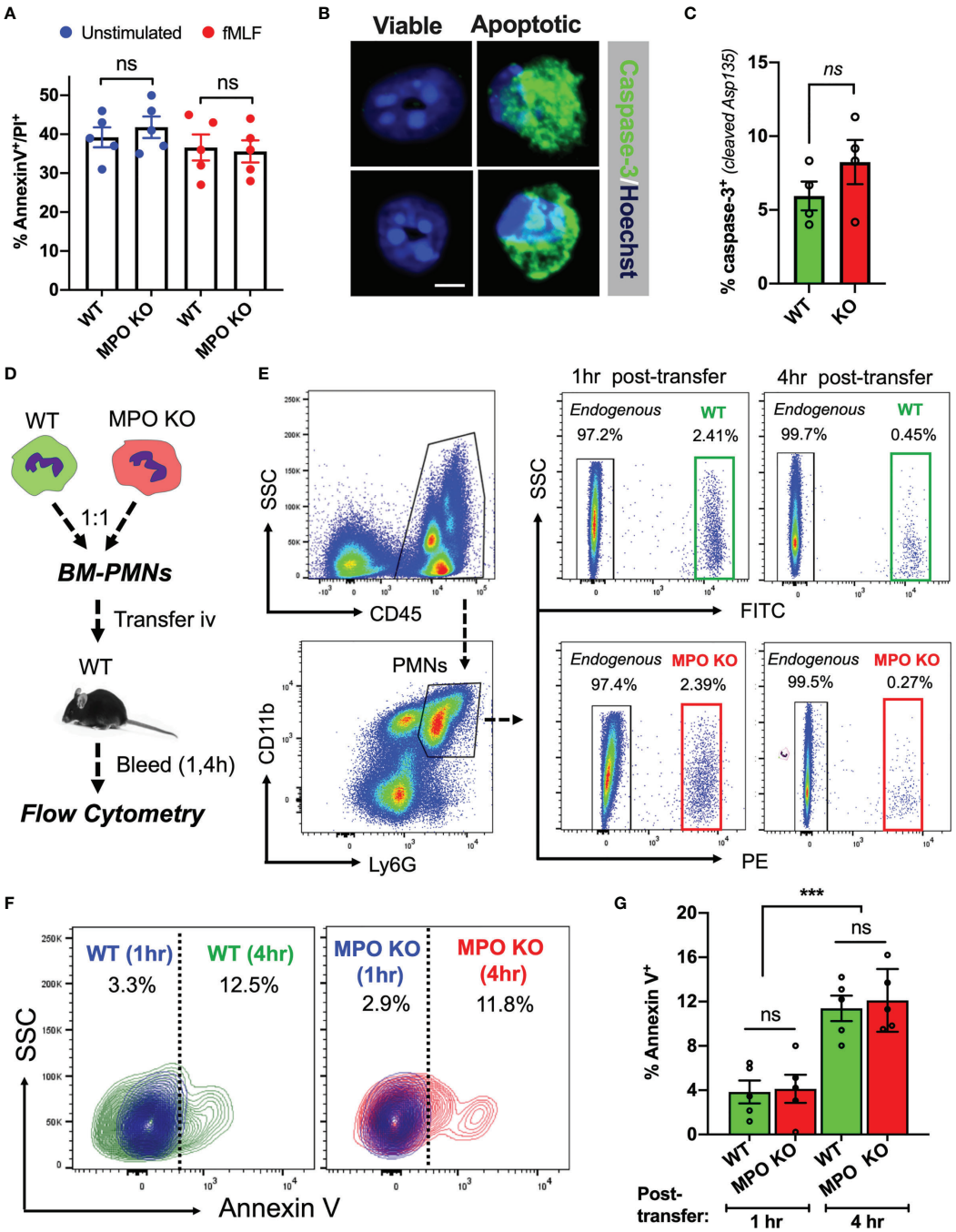

FIGURE 2 | MPO expression does not impact PMN viability. **(A)** PMN viability was assessed in *ex vivo* cultured BM-derived PMNs with/without fMLF-stimulation using Annexin V/PI staining and flow cytometry. Data shown as percent Annexin V/PI positive cells at 24hr in cultures. No significant differences between MPO KO and WT mice were observed. N=5 independent experiments. **(B, C)** Representative images and quantification of cleaved caspase-3 in fMLF-stimulated BM-derived WT and MPO-KO PMNs. No significant differences between MPO KO and WT mice were observed. N=4 independent experiments. The bar is 5μm. **(D–G)** PMN viability was examined *in vivo* in adoptively transferred PMNs. **(D)** Schematic depicting experimental timeline. Freshly isolated murine WT and MPO KO BM-PMNs were respectively labeled with green and red fluorescence (CellTracker) and injected intravenously at a 1:1 ratio into WT recipients that were pre-stimulated with IL-1β (50ng, 1hr, ip., to induce systemic inflammation). Annexin V staining and flow cytometry was used to gauge viability of transferred and endogenous PMNs at 1 and 4hr. **(E–G)** Representative flow diagrams of the gating strategy to identify transferred and apoptotic WT and MPO KO PMNs and quantification of apoptotic (AnnexinV-positive) PMNs in the circulation. No significant difference in viability between MPO KO and WT PMNs. N=5 mice per condition. ns, not significant. ***$p < 0.001$.

vessels into the tissue by flow cytometry and as such directly test the PMN migratory efficiency. Zymosan treatment (ip. 2µg/ml, 4h) as well as CXCL1 administration (a potent PMN chemoattractant, 1µg/ml, 2h) into the lumen of ligated ileal loops induced a rapid PMN mobilization into the peritoneal cavity (**Figures 3A, B**) and the intestinal lumen (**Figures 3C, D**), respectively. Importantly, in both models, PMN migration was significantly enhanced in the absence of functional MPO. Elevated tissue infiltration by MPO KO PMNs was further evident from whole-mount immunofluorescence staining and confocal microscopy imaging of the ileal loop tissue (**Figures 3E, F**).

MPO KO PMNs Exhibit Enhanced Adhesion to Inflamed Vascular Endothelial Cells

Rolling and firm adhesion of circulating PMNs are prerequisite steps for the crossing of the endothelial barrier and infiltration of the surrounding tissue. Thus, we performed high-speed intravital microscopy (IVM) using bright field illumination to assess in

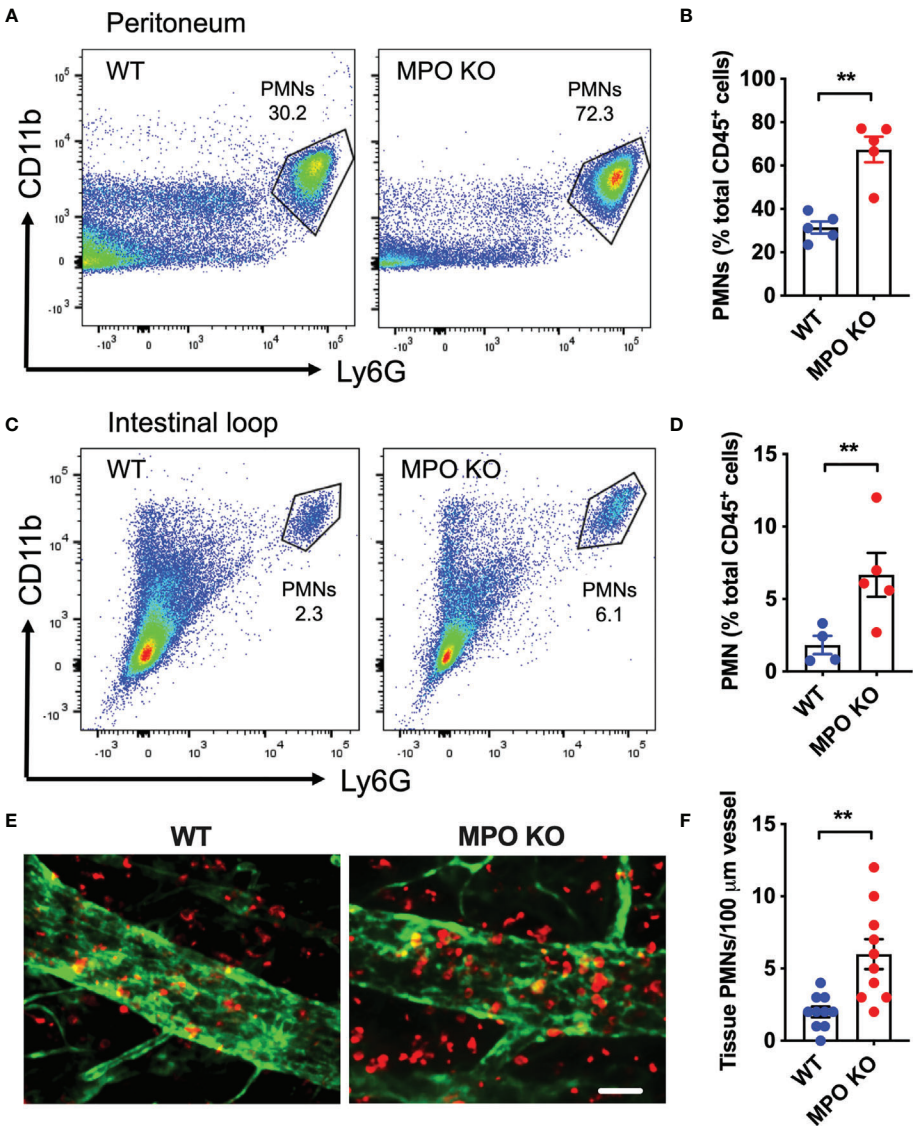

FIGURE 3 | MPO KO PMNs exhibit elevated migratory efficiency. Two *in vivo* PMN models were used to assess the migratory efficiency of WT and MPO KO PMNs. **(A, B)** Zymosan-induced peritonitis model was used. **(A)** Representative flow diagrams of lavaged PMNs that have migrated into the peritoneal cavity and **(B)** Quantification demonstrate enhanced PMN migration in the absence of MPO. **(C–F)** Intestinal loop model, where PMN migration into the lumen of ligated intestinal segments is induced by intraluminal CXCL1 administration was used to assess the impact of MPO on PMN migration. **(C)** Representative flow diagrams of lavaged luminal PMNs and **(D)** Quantification revealed consistently increased PMN tissue infiltration in MPO knockout mice. **(E)** Representative fluorescence images of intestinal whole mount tissue following lavage, stained for PMNs (red, S100A8) and ECs (green, CD31) and **(F)** Quantification similarly depict increased PMN infiltration in MPO KO mice. The bar is 50µm. N=5-7 mice per condition. $^{**}p < 0.01$.

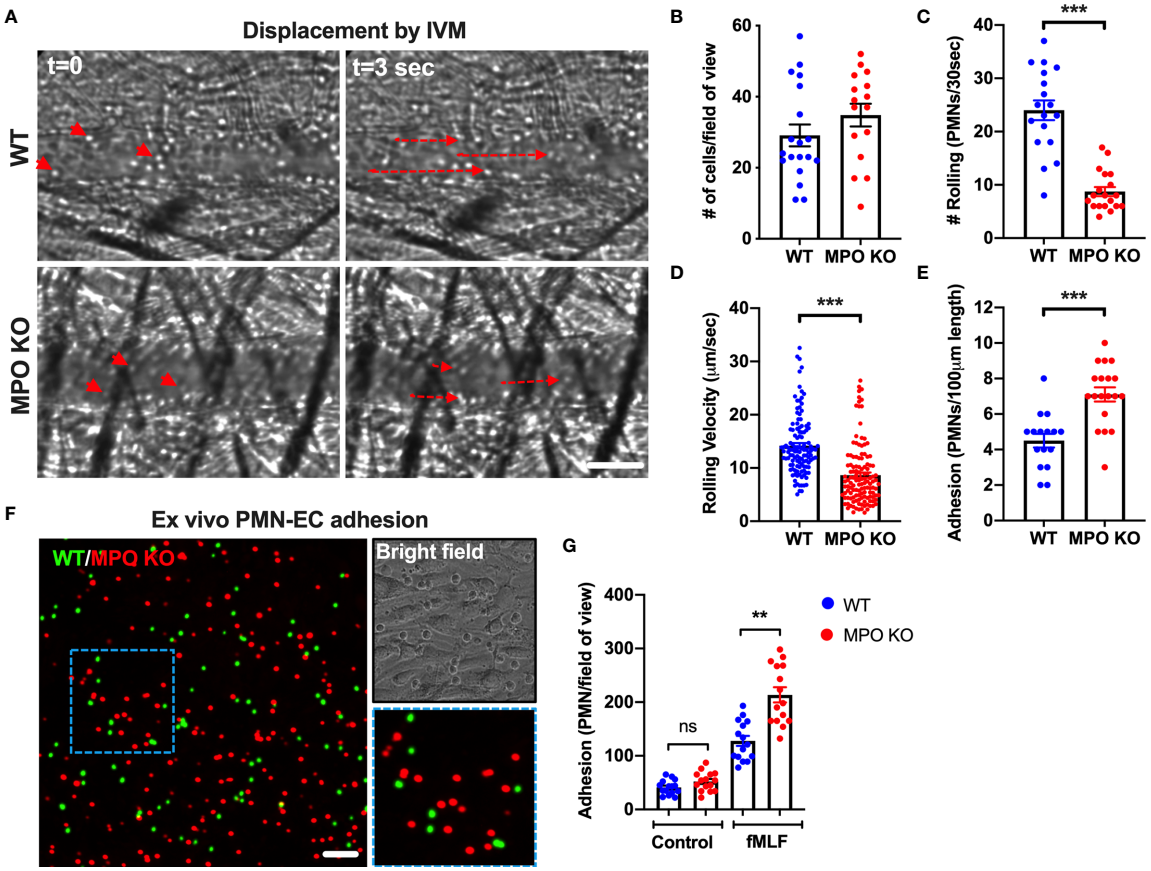

FIGURE 4 | MPO KO PMNs exhibit enhanced adhesion to inflamed vascular endothelial cells. **(A–E)** To assess the role of MPO in PMN-EC interactions intravital imaging (IVM) of the cremaster muscle was performed on WT and MPO KO mice. Inflammation of the cremaster muscle was induced by intrascrotal administration of IL-1β (50ng, 4hr). **(A)** Representative time-lapse images (based on a real-time acquisition) show decreased displacement of rolling PMNs in MPO KO animals. The bar is 50μm. **(B)** Analyses of total cell fluxes **(C)** Number of rolling cells per 30 seconds **(D)** Rolling velocities of individual PMNs and **(E)** PMN adhesion reveal a significant reduction in rolling and increases in adhesive interactions in MPO KO mice. N=5 mice with 5-6 fields of view per condition. **(F–G)** *Ex vivo* adhesion assay of BM-PMNs and murine ECs was performed. WT and MPO KO BM-PMNs were respectively stained with green and red fluorescence (CellTracker) and allowed to adhere to TNFα-stimulated (50ng, 24hr) Bend3 ECs in the presence of fMLF stimulation (500nM). **(F)** Representative fluorescent images and **(G)** Quantification reveal significant increases in MPO KO adhesion compared to WT. The bar is 50μm. N=3-5 independent experiments per condition. $^{**}p < 0.01$, $^{***}p < 0.001$. ns, not significant.

real-time the impact of MPO on PMN interactions with inflamed vasculature *in vivo* (**Figure 4A**). Localized inflammation of the cremaster muscle was induced by intrascrotal administration of IL-1β (50ng, 4hr). Intravital imaging revealed that the total number of circulating PMNs, was not different in WT vs MPO KO mice (**Figure 4B**). However, the number of rolling PMNs and the rolling velocity were significantly reduced in MPO KO mice (**Figures 4C, D**). Increased displacement of representative rolling WT compared to MPO KO PMNs are shown by time-lapse images, indicating higher rolling velocity (**Figure 4A** and **Supplemental Movies 1** and **2**). Consistent with reduced rolling fluxes, a significantly higher number of adhered PMNs (~2-fold) was observed in MPO KO as compared to WT mice (**Figure 4E**). Increased adhesion of MPO KO PMNs to vascular endothelium support the observed increases in tissue PMNs, confirming a role of MPO in PMN migration. Of note, blood differential analyses by Wright-Giemsa staining revealed no difference in the number of circulating lymphocytes and monocytes as well as in the number of circulating segmented (mature) and banded (immature) PMNs (**Supplemental Figure 1**). This indicates that production and release of PMNs into circulation was not impacted by the lack of MPO.

Supporting *in vivo* observations of circulating PMNs, MPO KO BM-PMNs exhibited increased adhesion to ECs in a complementary *ex vivo* static adhesion assay. Incubation of mixed WT and MPO KO BM-PMNs (respectively labeled with green and red fluorescence, at 1:1 ratio) with murine Bend3 ECs in the presence of fMLF stimulation (to enhance CD11b activation and PMN adhesion) revealed higher number of adhered MPO KO compared to WT PMNs (**Figures 4F, G**). The relatively low

baseline PMN adhesion without fMLF-stimulation was also not different between WT and MPO KO PMNs.

CD11b Surface Expression Is Not Altered by the Absence of MPO

CD11b is the most abundant and prominent adhesive receptor in PMNs, mediating both PMN adhesion and migration in inflammation (20, 27). As such, elevated levels of surface CD11b could account for the elevated adhesion and migration of MPO KO PMNs. To test this, we compared the CD11b surface expression (non-permeabilized conditions) on BM-derived and peripheral blood WT and MPO KO PMNs under resting and stimulated conditions. Interestingly, although PMN stimulation as expected elevated CD11b levels, no significant differences in expression patterns were noted (**Figures 5A–D**). To further examine this under physiological shear conditions, CD11b expression was tested in WT and MPO KO PMNs following their adoptive transfer into the circulation of inflamed recipient mice (as detailed in **Figure 2** and schematic **Figure 5E**). In this model too, under physiological shear conditions and inflammation, no significant differences in CD11b expression in transferred WT and MPO KO PMNs were noted (**Figures 5F, G**).

To further explore potential mechanisms of MPO-dependent increases in adhesion and migration of MPO KO PMNs, we compared the expression of CD11a, another important integrin receptor involved in PMN adhesive interactions as well as key PMN chemokine receptors CXCR2 and CXCR4 in WT and MPO KO PMNs. We found no significant differences in unstimulated or fMLF-stimulated PMNs (**Supplemental Figures 2A–C**). Similarly, immunoblotting analyses reveled no differences in extracellular signal-regulated kinase (ERK) activation (phosphorylation) between WT and MPO KO PMNs (**Supplemental Figure 2D**). ERK plays an important role in PMN chemotaxis (28) and can be activated downstream of CD11b (29).

Exogenous MPO Attenuates PMN Adhesion to Inflamed Endothelium

Since we found no difference in integrin/chemokine expression and signaling, we next tested the idea that released/exogenous MPO may competitively suppress PMN adhesion and migration by binding to the PMN surface. We first used flow cytometry and immunofluorescence/confocal microscopy to confirm that exogenous His-tagged recombinant MPO (rMPO) indeed robustly binds to the PMN surface (**Figures 6A, B**). Although rMPO has been shown to interact with CD11b and promote CD11b-dependent signaling (12) surprisingly, inhibitory Ab to CD11b did not significantly attenuate rMPO binding, suggesting that the MPO binding is not specific to CD11b.

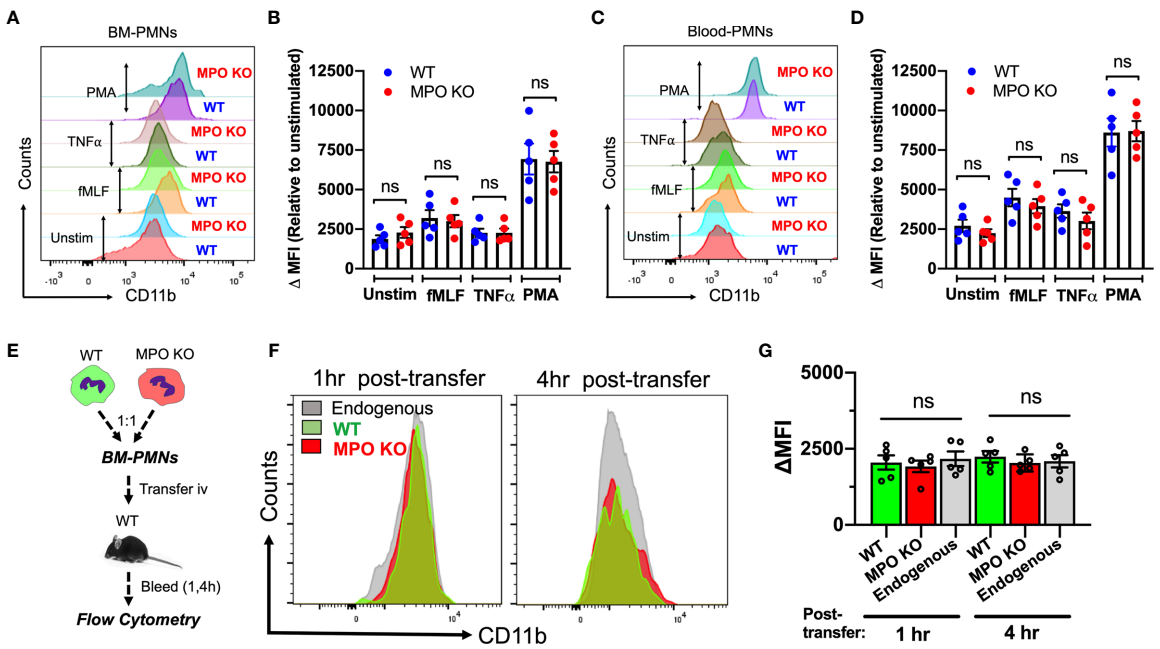

FIGURE 5 | CD11b surface expression is not altered by the genetic deletion of MPO. **(A–D)** CD11b expression was assessed by flow cytometry in BM-derived and circulating blood PMNs with/without *ex vivo* inflammatory stimulation. **(A, B)** Representative flow diagram and quantification of CD11b expression in BM-PMNs. **(C, D)** Representative flow diagram and quantification of CD11b expression in circulating PMNs. N=5 independent repeats per condition. **(E–G)** CD11b expression was assessed in fluorescently labeled WT (green) and MPO KO (red) PMNs at 1 and 4hr following adoptive intravenous transfer into IL-1β-stimulated (50ng, 1hr) WT recipient mice. **(E)** Schematic depicting experimental time line. **(F, G)** Representative flow diagrams and quantification reveal no significant differences in CD11b expression. N=5 mice per condition. ^{ns} not significant.

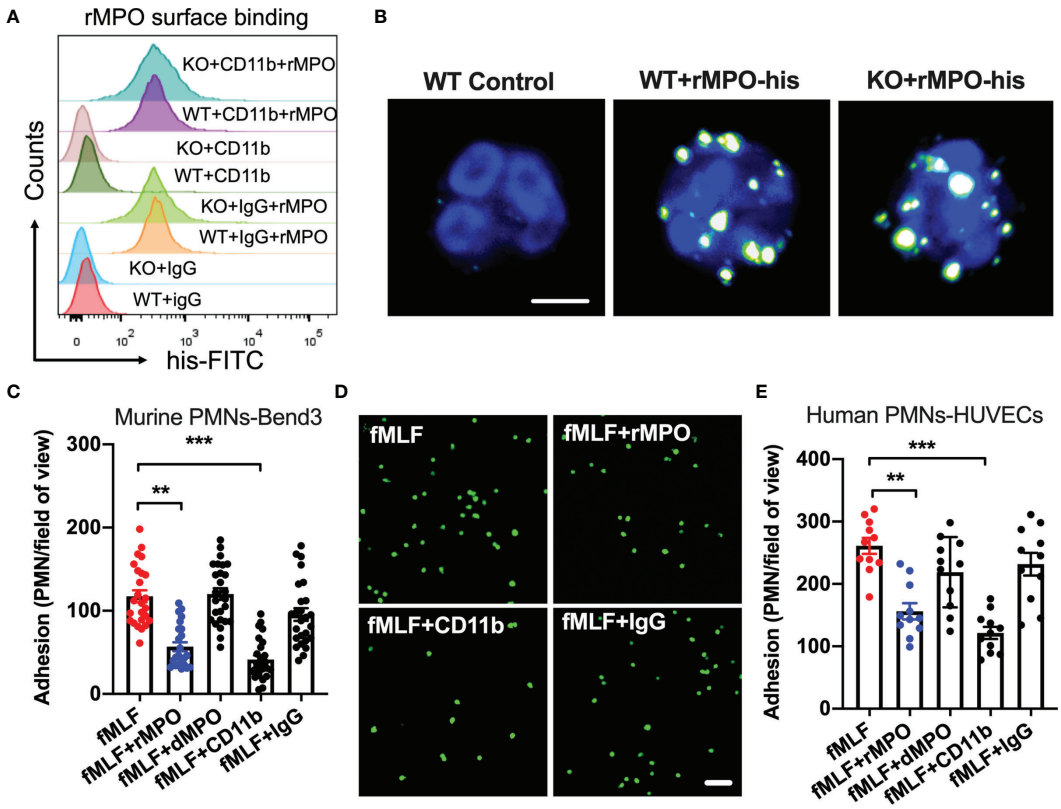

FIGURE 6 | Free MPO attenuates PMN adhesion to inflamed endothelium. **(A)** To assess MPO binding to the PMN surface and whether it is CD11b dependent, BM-PMNs were incubated with His-tagged recombinant MPO (rMPO, 10μg/ml 30min) in the presence of fMLF (500nM, to induce CD11b expression and activation) and with/without the addition of control IgG and anti-CD11b inhibitory Abs. MPO binding analyses were examined by flow cytometry on non-permeabilized PMNs and detection of His-tag. Robust MPO-His binding to PMNs was seen, however this was independent of CD11b expression. Representative of N=5 independent repeats. **(B)** The binding of His-tagged recombinant MPO to WT and MPO KO BM-PMNs was further confirmed by immunofluorescence. Surface-bound MPO was detected by anti-His antibody staining. The bar is 5μm. **(C, D)** To establish whether MPO impairs PMN adhesion, BM-PMNs were fluorescently stained (CellTracker Green) and incubated with TNFα-stimulated (50ng, 24hr) Bend3 ECs in the presence of fMLF stimulation (500nM) and with/without the addition of intact or control denatured (by boiling) rMPO. **(C)** Quantification and **(D)** Representative images reveal decreased adhesion in the presence of rMPO and CD11b inhibitory Ab but not the IgG control or denatured rMPO protein. The bar is 50μm. N=5 independent repeats in duplicates with at 3-5 randomly selected fields analyzed per condition. **(E)** Similar adhesion assays were performed using freshly isolated human PMNs and HUVECs, revealing consistent inhibition of PMN adhesion by rMPO. N=3 independent repeats in triplicates. $^{**}p < 0.01$, $^{***}p < 0.001$.

We next examined the direct impact of MPO on PMN adhesion to inflamed ECs. To this purpose, WT BM-PMNs ($5x10^5$ cells/condition) were incubated with TNFα-stimulated murine endothelial cells (ECs, Bend3) in the presence or absence of murine rMPO. PMN adhesion was significantly reduced (~2-fold) in the presence of rMPO (**Figure 6C** and representative images, **Figure 6D**). As expected, in control experiments, Ab-mediated inhibition of CD11b, which is an important contributor to PMN-EC adhesion, but not the addition of control IgG suppressed PMN adhesion to endothelial cells. Similar observations were made with freshly isolated human PMNs and ECs. The addition of human rMPO or an inhibitory anti-CD11b Ab but not denatured protein or IgG control Ab significantly inhibited PMN adhesion to HUVECs (**Figure 6E**). These observations support that idea that MPO binding sterically interferes with PMN adhesion to reduce PMN migration.

MPO Impairs *Ex Vivo* and *In Vivo* PMN Migration

Given the suppressive action of MPO on PMN adhesion, we postulated that this will lead to inhibition of PMN migration. To test this, chemotaxis assays [using a transwell setup (23)] were performed. In these experiments, murine WT and MPO KO PMNs or human PMNs were introduced to the upper chamber and were induced to chemotax across permeable supports by the addition of an fMLF gradient to the bottom chamber. Consistent with increased adhesion, chemotaxis of murine MPO KO PMNs was significantly elevated compared to WT PMNs (**Figure 7A**). Furthermore, supporting the idea that MPO binding to the PMN surface impairs PMN interactions with substratum, the addition of rMPO, but not a denatured protein, to either murine or human PMNs significantly reduced PMN chemotaxis (**Figures 7A, B**).

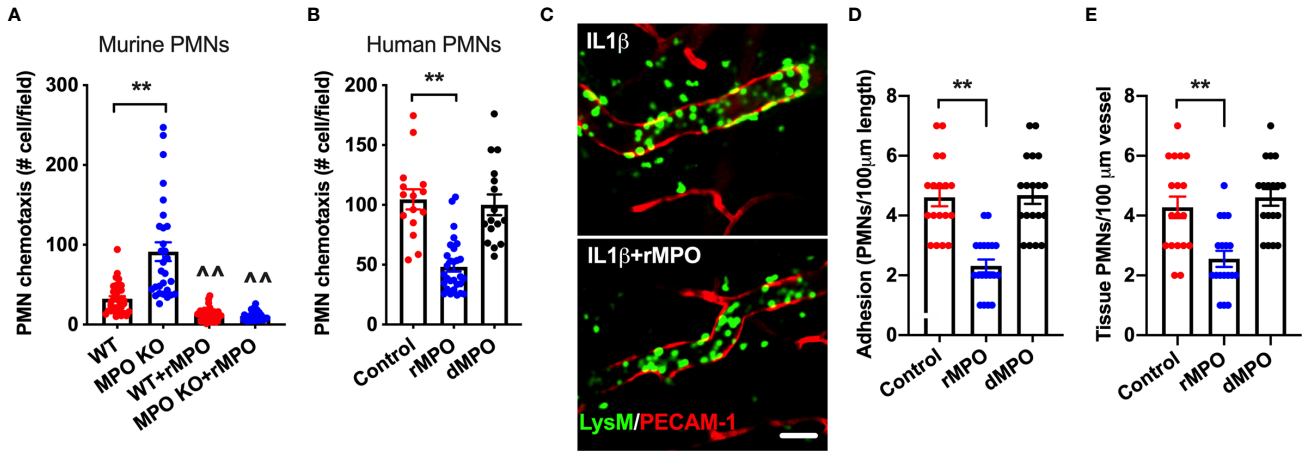

FIGURE 7 | MPO impairs *ex vivo* and *in vivo* PMN migration. **(A, B)** To test whether MPO-mediated impairment of PMN adhesion resulted in inhibition of PMN migration, *ex vivo* chemotaxis assays using transwells and murine BM-derived and human blood PMNs were performed. For all conditions PMNs chemotaxis was induced by the addition of fMLF to the bottom chamber. **(A)** Chemotaxis of MPO KO murine PMNs was significantly elevated compared to WT cells and was significantly suppressed with the addition of rMPO (20μg/ml). **(B)** Similarly, migration of human PMNs was inhibited by the addition of rMPO. N=5 independent repeats in triplicates. **(C, E)** IVM on the cremaster muscle in LysM-eGFP reporter mice (green PMNs) was performed to assess the impact of rMPO administration on PMN adhesion and TEM. Inflammation of the cremaster muscle was induced by intrascrotal administration of IL-1β (50ng, 4hr). rMPO (30μg in 100ml of sterile PBS) was administered retro-orbitally immediately following IL-1β stimulation. A significant reduction in **(D)** PMN adhesion and **(E)** TEM (extravasated PMNs in the tissue) was seen. The bar is 50μm. N=5 mice per condition with 3-5 randomly selected fields. **$p < 0.01$, ^^ significantly different from non-treated controls, $p < 0.01$.

Finally, as with *ex vivo* observations, the suppressive impact of MPO on PMN adhesion and TEM was confirmed *in vivo*, using cremaster muscle IVM. In these experiments, LysM-eGFP reporter mice (green PMNs and macrophages) were used and the vasculature was outlined by PECAM-1 fluorescence staining, allowing for clear separation of intravascular vs extravasated PMNs by fluorescence. Likewise, tissue macrophages are easily distinguishable from extravasated PMNs based on their size (significantly larger cells) and ramified morphology. Inflammation of the cremaster muscle was induced by IL-1β as in **Figure 4**. Intact or control denatured murine rMPO (30μg MPO, iv. 4hr) was administrated immediately following IL-1β stimulation. Consistent with the hypothesized role of MPO in impairment of PMN trafficking, significant reduction in the number of adherent and extravasated (tissue) PMN was observed with the addition of intact but not denatured rMPO (representative images **Figures 7C–E** and **Supplemental movies 3** and **4**). These experiments confirm the inhibitory role of MPO in the regulation of PMN migration in inflammation.

DISCUSSION

MPO is the most abundantly expressed protein in PMNs and its primary physiologic function is the killing of invading microorganisms. Together with H_2O_2, MPO forms a powerful antimicrobial system, that when released into the phagosomes, help kill the ingested bacteria *via* generation of hypochloric acid (HOCl) and other reactive oxidative radicals. While the anti-microbial function of MPO is mostly intracellular [with the exception of NETosis where MPO assists in catching and eliminating pathogens outside the cells (30, 31)], the pro-and-anti-inflammatory effects of MPO take place once it is released outside the cell. Indeed, in addition to the antimicrobial function of MPO other important roles of MPO in tissue homeostasis and cellular function have been identified.

In the current work, we demonstrate that MPO can directly bind to the PMN surface and attenuate PMN adhesion and migration in inflammation. Using *in vivo* models of inflammation and *ex vivo* murine and human PMN co-cultures with vascular ECs, we show that in the absence of functional MPO, PMN adhesion and migration is significantly enhanced. In contrast, in the presence of soluble MPO, PMN adhesion and migration were substantially suppressed. Thus, our findings suggest that MPO may play a protective role in inflammation by limiting PMN tissue accumulation.

There are plenty of data showing that under inflammatory conditions, MPO is spilled into the circulation, as elevated free MPO plasma levels were detected in many pathological conditions including sepsis and acute coronary syndromes (12).

Once released by PMNs, free MPO can bind vascular ECs and contribute to endothelial dysfunction by limiting NO bioavailability (32) or *via* HOCl-mediated modification of L-arginine (33). Interestingly, *ex vivo*, human PMNs were able to adhere to immobilized recombinant MPO (14) suggesting that MPO bound to vascular EC, could exhibit adhesive properties augmenting PMN-EC interactions. However, *in vivo*, MPO bound to ECs is rapidly transcytosed across the endothelium to modulate extracellular matrix proteins in inflammation (34). In our setting, administration of free MPO into inflamed

microcirculation reduced rather than augmented PMN adhesion, supporting an inhibitory role of MPO in PMN recruitment. PMN adhesion to the vascular endothelium is a prerequisite and necessary step for the subsequent PMN TEM. Adhesive events both guide PMNs to the specific location/portals where TEM can take place (35) as well as transduce essential signaling events in EC (via engagement of EC surface ligands such as ICAM-1 and PECAM-1) to accommodate PMN crossing (36, 37). As such, it is not surprising that impaired PMN adhesion in the presence of soluble MPO led to reduced tissue infiltration by PMNs. This novel regulatory role of MPO in PMN trafficking is of potential therapeutic relevance, given the interest in neutrophil trafficking as potential therapy approaches, and the ongoing debate on the pro-and anti-inflammatory functions of PMNs in inflammation.

Indeed, although MPO presence in tissues is mainly associated with inflammation and injury, protective functions of MPO were also identified. For example, MPO activity and the presence of its oxidative products have been implicated in glomerular injury (38, 39), atherosclerosis (40), lung damage in CF (41) and carcinogenesis (42). Free MPO or MPO released in extracellular vesicles has been shown to bind epithelial cells and impair intestinal wound healing (9). MPO internalized by alveolar and bronchial epithelial cells led to DNA strand breakage, however it also suppressed interleukin-8 production, potentially limiting PMN recruitment and retention in tissue (43). Finally, MPO has been reported to protect against experimental autoimmune encephalomyelitis, an animal model for multiple sclerosis, with MPO-knockout mice being more susceptible to these disease (44). Similarly, an unexpected protective role for MPO was found in murine atherosclerosis, where in the absence of MPO larger aortic legions and inflammation was observed (45).

Free MPO by binding to CD11b/CD18 has also been shown to induce neutrophil activation in an autocrine fashion promoting MAPK and NFκB signaling, ROS production and degranulation (12). Furthermore, MPO KO PMNs were suggested to better phagocyte zymosan particles due to slightly elevated levels of CD11b and the resulting induction of FAK/ERK signaling (13). However, by doing careful examination of CD11b expression, which included BM-PMNs, circulating blood PMNs with and without stimulation, as well as following adoptive competitive transfer of WT and MPO KO PMNs into inflamed recipient circulation we found no significant differences in CD11b expression. Consistently, although we confirmed that free MPO robustly binds to the surface of both murine and human PMNs, blockade of CD11b by inhibitory Ab did not reduce the MPO binding. These findings do not exclude the likelihood of MPO engagement with CD11b to induce signaling in PMNs, however also suggests that MPO binds other PMN surface ligands. There is also a possibility that in addition/alternatively to facilitating intracellular signaling to impact PMN adhesive interactions, MPO non-specifically coats the PMN surface to sterically hinder PMN binding to substratum and as such impeding PMN migratory ability. As such, our work reveals a new and important regulatory function of MPO in PMN migration, serving to "brake" excessive PMN accumulation in tissues. Our findings further provide basis for future studies of mechanisms and potential binding targets of MPO at the PMN surface.

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 Northwestern University Institutional Review Board. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by NORTHWESTERN UNIVERSITY Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

JR and RS conceived and designed experiments. JR, TB, CG-E, JU, XR, DP and RS conducted experiments and performed data analyses. JR and RS wrote the manuscript. TB, CG-E, JU, XR and DP 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.2021.654259/full#supplementary-material

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Circulating Adaptive Immune Cells Expressing the Gut Homing Marker α4β7 Integrin Are Decreased in COVID-19

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Background: Infection with the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causes a wide range of symptoms including gastrointestinal manifestations, and intestinal epithelial cells are a target of the virus. However, it is unknown how the intestinal immune system contributes to systemic immune responses in coronavirus disease 2019 (COVID-19).

Methods: We characterized peripheral blood lymphocytes from patients with active COVID-19 and convalescent patients as well as healthy controls by flow cytometry.

Results: The frequency and absolute number of circulating memory T and B cells expressing the gut homing integrin α4β7 integrin was reduced during COVID-19, whether gastrointestinal symptoms were present or not. While total IgA-expressing B cells were increased, gut-imprinted B cells with IgA expression were stable.

Conclusion: COVID-19 is associated with a decrease in circulating adaptive immune cells expressing the key gut homing marker α4β7 suggesting that these cells are preferentially recruited to extra-intestinal tissues independently of α4β7 or that the systemic immune response against SARS-CoV-2 is at least numerically dominated by extraintestinal, particularly pulmonary, immune cell priming.

Keywords: COVID-19, SARS-CoV-2 infection, T cell trafficking, integrins, gut homing

Abbreviations: CD, cluster of differentiation; COVID-19, coronavirus disease 2019; DCs, dendritic cells; GALT, gut-associated lymphoid tissue; MAdCAM-1, mucosal addressin vascular cell adhesion molecule 1; PD-1, programmed cell death protein 1; PBMCs, peripheral blood mononuclear cells; RALDH, retinaldehyde dehydrogenase; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SEM, standard error of the mean; VDZ, vedolizumab.

INTRODUCTION

The emergence of SARS-CoV-2 in late 2019 (1) has led to a global pandemic that is still far from being under control in many parts of the world. The disease caused by SARS-CoV-2 has been termed COVID-19, which is typically characterized by pneumonia potentially manifesting as fatal respiratory distress syndrome (2). However, it has already early been described that a subgroup of patients with COVID-19 also shows gastrointestinal symptoms like diarrhea or vomiting (2–6) and SARS-CoV-2 RNA is frequently detected in the feces of COVID-19 patients (7). Consistently, productive infection of enterocytes in the human gut has been demonstrated (8). Mechanistically, this observation could be linked to the expression of angiotensin-converting enzyme 2 (ACE2), which serves as the entry receptor for the spike protein of SARS-CoV-2 (9), not only in the lung, but also in the digestive tract (10, 11).

As the organ with the largest surface of the body, the gastrointestinal tract faces the challenge to allow absorption of nutrients, vitamins and minerals, while protecting against potentially harmful luminal microbiota or toxic products (12). Thus, a complex immune system has established in the gut including the secondary lymphoid organs of the gut-associated lymphoid tissue (GALT) as well as immune cells in the lamina propria of the intestinal mucosa (13). A hallmark and exclusive feature of immune cell priming in the GALT is that dendritic cells (DCs) expressing the enzyme retinaldehyde dehydrogenase (RALDH) metabolize nutritional vitamin A to retinoic acid. This induces the expression of the gut homing integrin α4β7 and the chemokine receptor CCR9 on T cells receiving antigenic stimulation from these DCs (14–16) and offers the opportunity to specifically track lymphocytes imprinted in the intestine. Moreover, GPR15 has been described to be specifically expressed on T lymphocytes homing to the large intestine (17, 18).

The α4β7 integrin specifically binds to mucosal vascular addressin cell adhesion molecule (MAdCAM)-1 (19, 20), which is virtually exclusively expressed on high endothelial venules in the gastrointestinal tract (21). Hence, upon recirculation, memory T cells primed within the GALT dispose of a particular signature to re-enter intestinal tissue or GALT. Moreover, retinoic acid generated by intestinal DCs has been shown to promote IgA-secreting B cells, at least partly by inducing an isotype switch towards IgA (22, 23). Thus, intestinal infection with SARS-CoV-2 might lead to the induction of virus-specific T cells with a gut-homing signature and IgA-producing B cells in the intestine. Importantly, IgA is present on surfaces and participates in upholding mucosal immunity. IgA produced by B cells shaped in the gut might therefore also lead to cross-protection of the mucosal surface of the respiratory tract and, consistently, oral vaccination strategies against SARS-CoV-2 have been advocated and are under development (24).

We therefore set out to investigate, whether infection with SARS-CoV-2 leads to systemic signs of virus-associated intestinal T and B cell immunity in the circulation. We show that in patients with COVID-19 α4β7-expressing memory T cells are reduced compared with healthy controls even in patients with intestinal symptoms, while IgA-producing B cells are stable. However, these do not seem to originate from the gut, suggesting that gut-imprinted immune cells are eliminated from the circulation or that immune responses generated at other sites of virus entry are dominating circulating immune cell profiles in COVID-19.

METHODS

Patient Cohort

Blood for PBMC isolation was collected following informed written consent at the University Hospital Erlangen. Hospitalized patients with active SARS-CoV-2 infection (n = 110), recovered patients presenting to the Department of Transfusion Medicine (n = 35) and healthy donors (n = 28) were included.

Clinical data of COVID-19 patients were retrieved from an internal database. Clinical data of COVID-19 patients and healthy donors are summarized in **Table 1**, clinical data for the recovered patients are not available. Blood collection was approved by the Ethics Committee of the Friedrich-Alexander University Erlangen-Nuremberg (174_20B).

PBMC Isolation and Flow Cytometry

Human peripheral blood mononuclear cells (PBMCs) were isolated by standard density gradient centrifugation with Ficoll Paque (GE) or Lymphocyte Separation Medium (Anprotec) and stained with the following antibodies:

CD3-APC (HIT3a, BioLegend), CD3-BV605™ (OKT3, BioLegend), CD4-APC-Vio770 (VIT4, Miltenyi Biotec), CD4-PerCP/Cy5.5 (OKT4, BioLegend), CD8a-PerCP/Cy5.5 (RPA-T8, BioLegend), CD19-VioBlue (LT19, Miltenyi Biotec), CD45RO-BV510™ (UCHL1, BioLegend), CD69-PE/DAZZLE™ 594 (FN50; BioLegend), CD154-FITC (24-31, BioLegend), CCR9-PE/Cy7 (L053E8, BioLegend) Integrin α4-PE/Cy7 (9F10, BioLegend), Integrin α4-VioBlue (MZ18-24A9, Miltenyi Biotec) Integrin β1-PE (TS2/16, BioLegend), Integrin β7-BV605™ (FIB504, BioLegend), GPR15-PE (SA302A10, BioLegend), IgA-FITC (ab97219, abcam), Vedolizumab (Takeda Pharmaceuticals) labeled with Alexa Fluor® 488 (Invitrogen) or Alexa Fluor® 647 (Invitrogen).

TABLE 1 | Baseline characteristics of patients with SARS-CoV-2 infection and healthy controls.

		Healthy controls	COVID-19 patients
Number		28	110
Male [%]		28	64
Female [%]		72	36
Ø Age in years		28.8	63.5
Diarrhea		/	7
Course of disease	Mild	/	17
	Severe	/	17
Pre-existing cardiovascular disease		/	17

PBMCs were washed for 5 min in PBS at 300x g and 4°C and fixed over night at 4°C using the Foxp3 transcription buffer staining kit (Thermo Fisher). Subsequently, PBMCs were washed, resuspended in 200µl FACS buffer (1% FBS, 2mM EDTA in PBS) and analyzed on a MacsQuant16 instrument (Miltenyi).

In some experiments, full blood was stained for flow cytometry as follows: 200µl peripheral whole blood was incubated for 15 minutes with 2ml Lysing Buffer (BD Pharm Lyse™, BD Biosciences) to lyse red blood cells. Samples were washed and subjected to antibody staining as described above. After fixation and washing of the samples exactly 200µl FACS buffer was added and the samples were analyzed on a MacsQuant16 instrument.

SARS-CoV-2 Specific T Cell Stimulation

SARS-CoV-2-specific T cell response was analyzed by stimulating PBMCs from COVID-19 patients and healthy controls with a pool of peptides covering the immunodominant sequence domains of the surface glycoprotein of SARS-CoV-2 (PepTivator® SARS-CoV-2 Prot_S-research grade, Miltenyi Biotec) for 5 hours according to the manufactures instructions. Subsequently, stimulated PBMCs were stained with antibodies as described above and analyzed on a MacsQuant16 instrument. SARS-CoV-2-specific T cells were detected with antibodies against CD69 and CD154.

Statistical Analysis

GraphPad Prism (GraphPad Software, Inc.) was used to perform statistical analyses.

Results are shown as scatter dot plots with individual data points. Center values and error bars represent mean and standard error of the mean (SEM).

Normal distribution was tested using Shapiro-Wilk test. When comparing two groups, statistical differences were tested using student's T test for data with normal distribution or Mann Whitney U test for data without a normal distribution. When comparing more than two groups, statistical differences were tested using one-way ANOVA with Tukey's multiple comparison for data with a normal distribution or Kruskal–Wallis test with Dunn's multiple comparison for data without a normal distribution. For correlation analyses, Spearman correlation was performed and a regression line is indicated. An α value of $p < 0.05$ was defined as statistically significant.

Significance levels are indicated by asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

RESULTS

α4β7 Integrin-Expressing Memory T Cells Are Decreased in COVID-19

We characterized T and B cells in the peripheral blood from a cohort of 80 patients with active COVID-19 and 35 patients recovered from COVID-19 as well as 18 healthy controls by flow cytometry of PBMCs. Clinical information on the patients is provided in **Table 1**. The overall frequency of $CD3^+$ and $CD3^+CD4^+$ T cells was similar in patients with active or previous COVID-19 and in controls (**Supplementary Figures 1A, B**). Peripheral $CD45RO^+$ memory $CD4^+$ T cell and $CD3^-CD19^+$ B cell frequencies in patients with active or after COVID-19 were numerically, but not significantly increased compared with healthy donors (**Supplementary Figures 1C, D**).

To explore the presence of T cells primed in the intestine during SARS-CoV-2 infection, we analyzed the expression of α4β7 integrin on T cells in the peripheral blood. Interestingly, the portion of $CD3^+CD4^+$ T cells expressing α4β7 was substantially reduced in previous COVID-19 patients compared to healthy controls (**Figure 1A**). On $CD3^+CD4^+CD45RO^+$ memory T cells, we observed a clear reduction of α4β7 expression in patients with active COVID-19. However, expression increased in the recovery phase, while still being lower than in controls (**Figure 1B**). Interestingly, there was no difference between patients with mild and severe course of COVID-19 as well as between patients with and without diarrhea (**Figure 1C** and **Supplementary Figure 2**). Even when relating the expression of α4β7 on $CD45RO^+$ memory T cells to total $CD3^+CD4^+$ T cells, frequencies were decreased in COVID-19 patients compared to healthy controls (**Figure 1D**).

Since these data were only indicating relative expression and not absolute cell numbers, and did not exclude the possibility of co-expression of α4 and β7 integrin without heterodimerization, we seeked to validate our findings in an additional patient cohort. Here, we stained full blood samples and used fluorescently labeled vedolizumab, a monoclonal antibody specific for the α4β7 heterodimer used for the therapy of inflammatory bowel diseases (25). In accordance with previous literature marked lymphopenia was present in COVID-19 patients (**Supplementary Figure 3**). Corroborating our previous observations, the fraction as well as the absolute number of $CD3^+CD4^+CD45RO^+$ T cells staining positive for vedolizumab was markedly reduced in patients with COVID-19 (**Figure 2A**).

To estimate, in how far expression of α4β7 integrin on overall memory $CD4^+$ T cells reflects α4β7 expression of SARS-CoV-2-induced memory T cells, we used a cocktail of viral epitopes to stimulate PBMCs from COVID-19 patients. Flow cytometry demonstrated that there was a high degree of correlation of α4β7 expression between these cell subsets (**Figure 2B**). Moreover, we compared the expression of α4β7 on SARS-CoV-2-specific $CD4^+$ memory T cells from COVID-19 patients with healthy donors without SARS-CoV-2 infection that also had SARS-CoV-2-specific T cells in their peripheral blood as previously reported (26). Again, we observed a striking decrease in the expression of α4β7 on these cells in COVID-19 patients compared with controls (**Figure 2C**), suggesting that circulating T cells primed in the gut are reduced in hospitalized patients with acute SARS-CoV-2 infection.

GPR15, CCR9 and α4β1 Are Not Specifically Reduced in COVID-19

Next, we aimed to determine, whether T cells expressing gut-homing chemokine receptors are similarly reduced. Accordingly, we quantified the expression of GPR15, a receptor specifically expressed on T cells homing to the large intestine (17, 18), in full blood samples by flow cytometry. We observed no significant

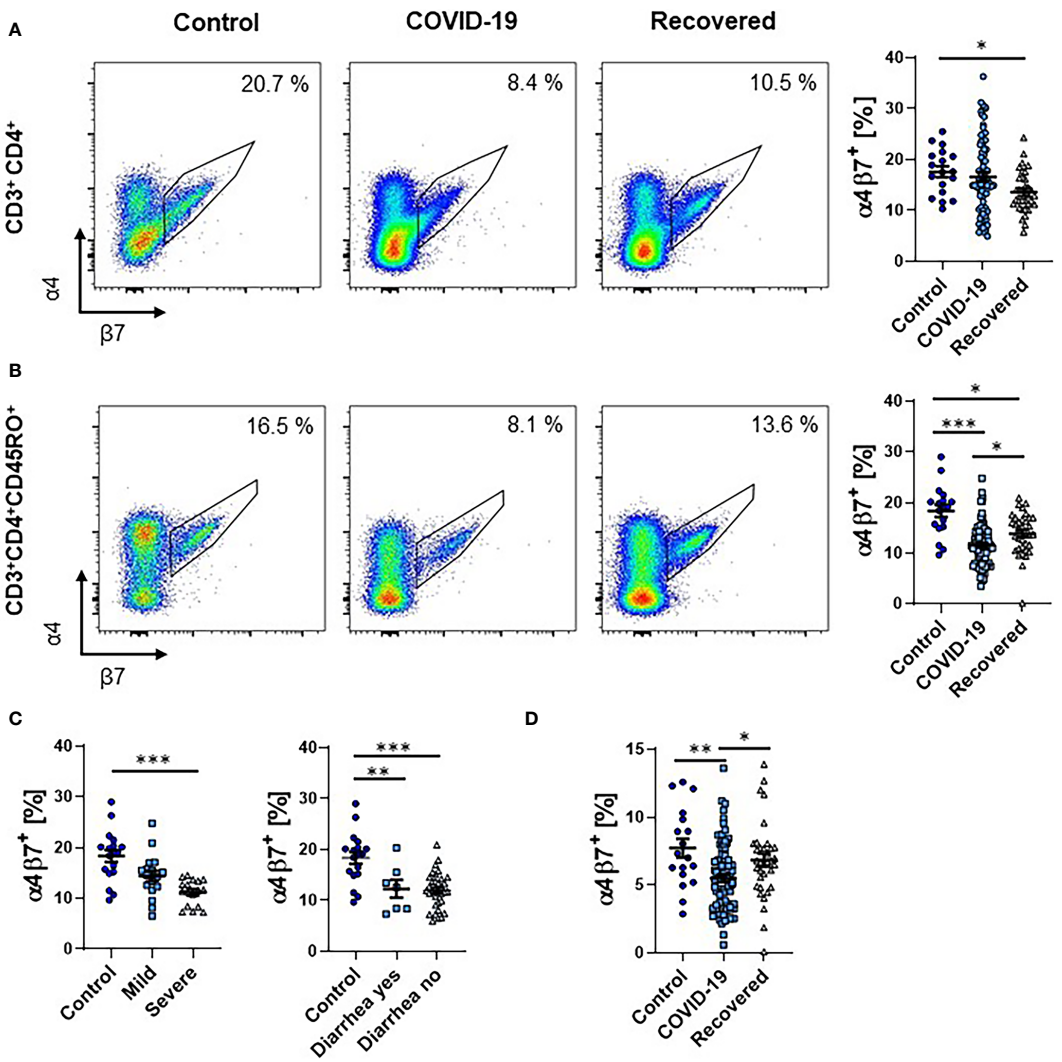

FIGURE 1 | Frequency of $\alpha4^+\beta7^+$ T cells during COVID-19. **(A, B)** Representative (left) and quantitative (right) flow cytometry of the frequency of α4β7 integrin-expressing $CD3^+CD4^+$ T cells **(A)** and $CD3^+CD4^+CD45RO^+$ memory T cells **(B)**. **(C)** Quantitative flow cytometry of the frequency of α4β7 integrin-expressing $CD3^+CD4^+CD45RO^+$ memory T cells in COVID-19 patients with mild or severe disease course (left) and with or without diarrhea (right) compared to healthy controls. **(D)** Quantitative flow cytometry of the frequency of α4β7 integrin-expressing $CD3^+CD4^+CD45RO^+$ memory T cells expressed as frequency of $CD3^+CD4^+$ T cells. Each symbol represents an individual subject, n = 7 - 80 per group. *p < 0.05, **p < 0.01, ***p < 0.001.

change of GPR15 expression in patients with active or after COVID-19 on $CD4^+$ memory T cells (**Figure 3A**).

While the fraction of $CD4^+$ memory T cells expressing the chemokine receptor CCR9, which is associated with trafficking to the small intestine (27), was similar between patients with COVID-19 and healthy controls, their absolute numbers in the circulation were significantly reduced (**Figure 3B**). We therefore decided to further investigate the abundance of memory T cells expressing the integrin α4β1, which has been implicated in homing to the small intestine (28). We observed a trend towards a reduction in the frequency of $CD4^+$ memory T cells expressing α4β1 and a significant reduction in the absolute number of these cells in the circulation (**Figure 3C**).

Together, these effects were largely driven by the lymphopenia present in COVID-19 patients and, thus, the data suggested that infection with SARS-CoV-2 does not affect the expression of other gut-homing markers to a similar extent as α4β7.

Reduction of Circulating α4β7-Expressing $CD8^+$ T Cells in COVID-19

Since $CD8^+$ T cells are crucially implicated in the immune response against viruses, we also explored the expression of gut-homing markers on $CD8^+$ T cells during COVID-19. The percentage as well as the absolute number of $CD3^+CD8^+$ T cells in the peripheral blood of patients with COVID-19 was clearly reduced (**Supplementary Figure 4**). As in the CD4 compartment, a smaller fraction of $CD8^+$ T

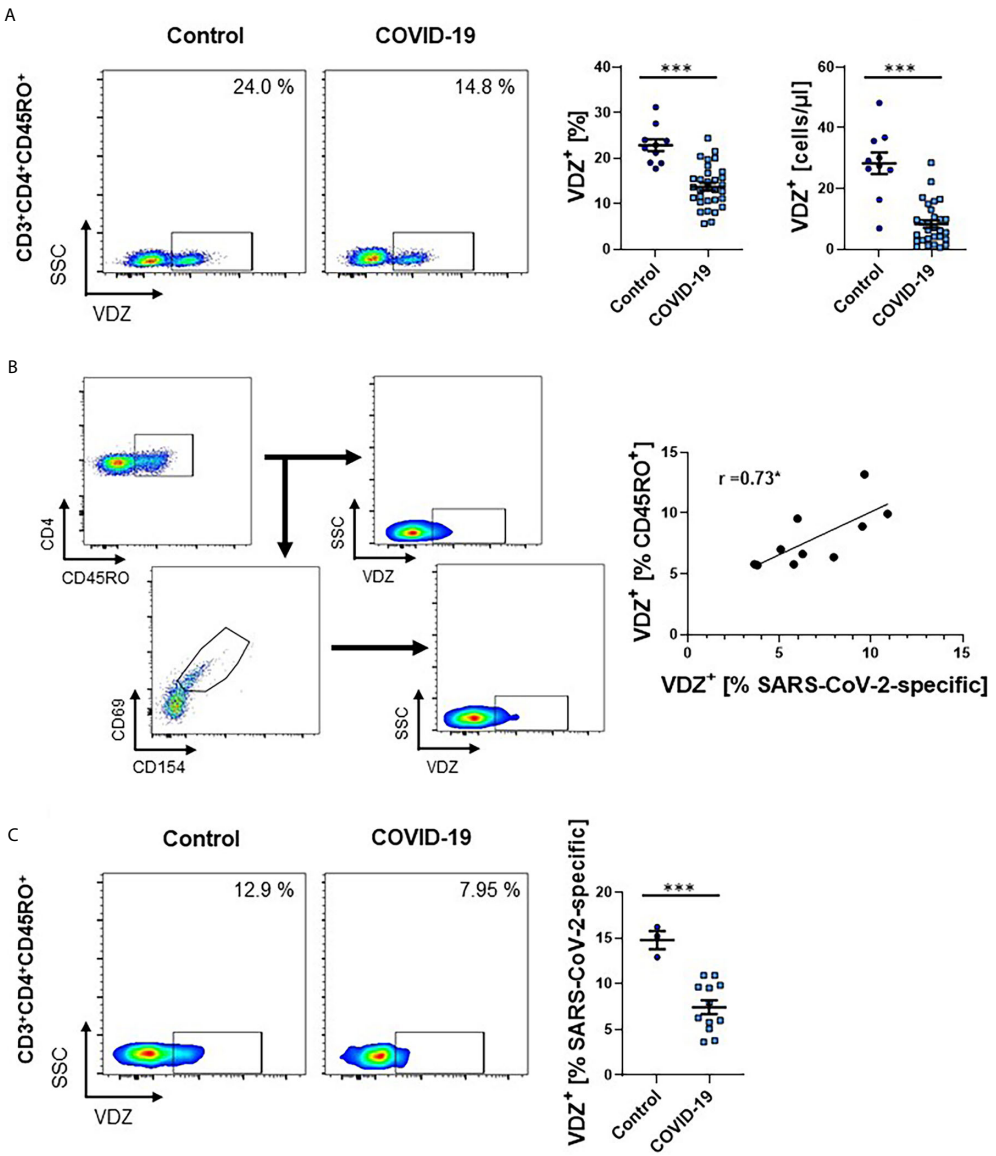

FIGURE 2 | Frequency of VDZ$^+$ CD4$^+$ memory T cells during COVID-19. **(A)** Representative (left) and quantitative (right) flow cytometry of whole blood samples of COVID-19 patients and healthy controls. Graphs indicate the frequency and absolute cell numbers of CD3$^+$CD4$^+$CD45RO$^+$ memory T cells staining positive for the anti-α4β7 integrin antibody VDZ. n = 10-31. **(B)** Exemplary gating strategy (left) and correlation (right) of α4β7 (VDZ) expression on SARS-CoV-2-specific CD3$^+$CD4$^+$CD45RO$^+$ memory T cells to α4β7 (VDZ) expression on overall CD3$^+$CD4$^+$CD45RO$^+$ memory T cells from patients with COVID-19. Spearman's r is indicated. n = 10. **(C)** Representative (left) and quantitative (right) flow cytometry of the frequency of α4β7 (VDZ) expression on SARS-CoV-2-specific CD3$^+$CD4$^+$CD45RO$^+$ memory T cells of COVID-19 patients and healthy controls. VDZ, vedolizumab. Each symbol represents an individual subject, n = 3 -12 per group. ***p < 0.001.

cells from COVID-19 patients expressed α4β7 integrin (as indicated by positive staining for vedolizumab) compared to healthy controls. Similarly, the absolute number of these cells was substantially reduced (**Figure 4A**). For CCR9, the relative expression was not significantly altered, but the absolute number of CCR9-expressing circulating CD8$^+$ T cells was reduced (**Figure 4B**). This was similar for α4β1, although, here, the increase in relative expression was significant, while the decrease in absolute cell numbers was not (**Figure 4C**).

Collectively, these observations largely recapitulated our findings for CD4$^+$ T cells.

IgA-Producing B Cells Without a Gut-Homing Phenotype Are Increased in Response to SARS-CoV-2

Finally, we quantified the frequency of circulating CD19$^+$ B cells expressing IgA. We observed a clear increase in patients with COVID-19 that persisted in the recovery phase (**Figure 5A**).

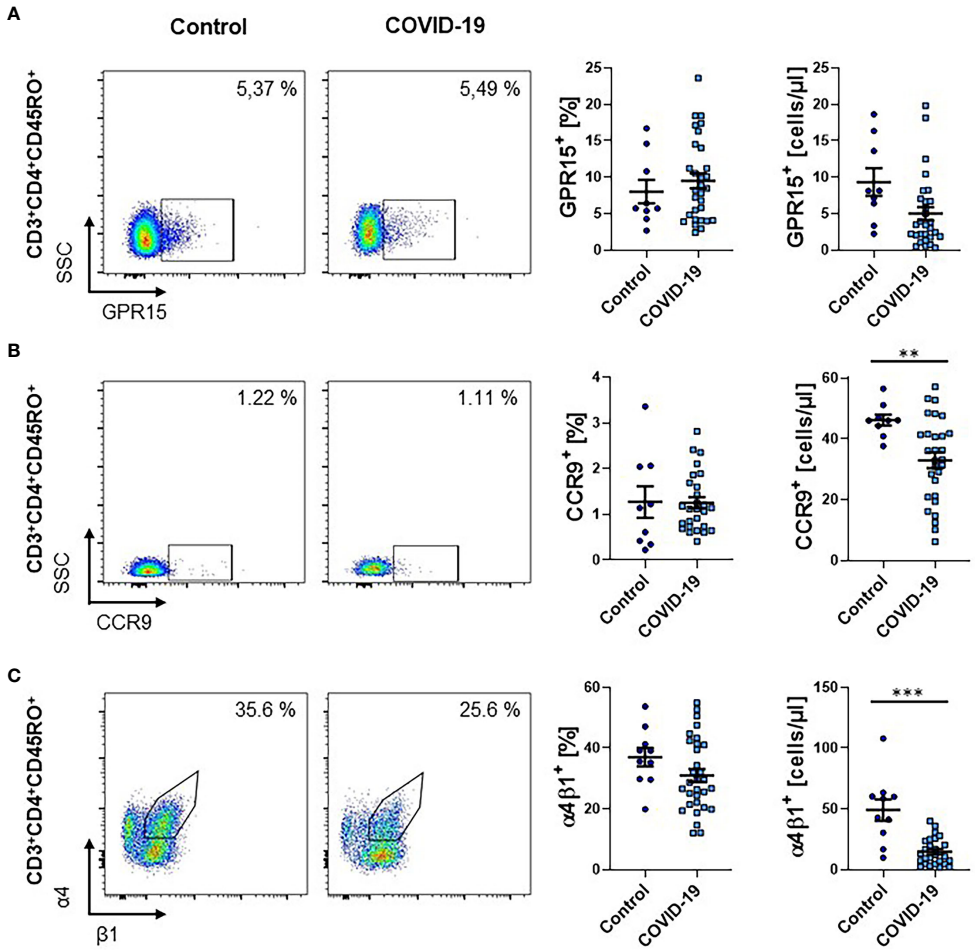

FIGURE 3 | Frequency of other gut homing markers on CD4$^+$ memory T cells during COVID-19. **(A–C)** Flow cytometry of whole blood samples from COVID-19 patients and healthy controls. Representative (left) and quantitative (right) flow cytometry of the frequency and absolute cell numbers of GPR15$^+$ **(A)**, CCR9$^+$ **(B)** and $\alpha4^+\beta1^+$ **(C)** CD3$^+$CD4$^+$CD45RO$^+$ memory T cells. VDZ, vedolizumab. Each symbol represents an individual subject, n = 10 – 31 per group. **p < 0.01, ***p < 0.001.

This was more pronounced in patients with severe disease course, but similar in patients with and without gastrointestinal symptoms (**Figure 5B**).

Since a gut-homing phenotype including the expression of α4β7 integrin is also induced in B cells in the gut (23), we subsequently assessed the expression of α4β7 on these B cells. Similar to T cells, the frequency of α4β7-expressing B cells in patients with COVID-19 decreased and slightly re-increased in the recovery phase (**Figure 5C**). Interestingly, the increase in IgA-expressing B cells was not significant in the $\alpha4^+\beta7^+$ subset (**Figure 5D**), supporting the notion that circulating IgA-expressing B cells during SARS-CoV-2 infection do not predominantly originate from the gut.

Again, we tried to reproduce our findings in a second patient cohort using full blood and staining with vedolizumab to detect α4β7 integrin. Similar to the analysis of PBMCs, α4β7-expressing B cells were significantly lower both on relative and absolute level (**Figure 5E**). Moreover, we again observed a numeric increase in the fraction of IgA-expressing B cells during COVID-19. Yet, this was not the case on absolute levels (**Figure 5F**). Notably, the expression of IgA on $\alpha4\beta7^+$ B cells was also lower in COVID-19 (**Figure 5G**).

Taken together, also in the B cell compartment, our data indicated that immune responses originating from the intestine in COVID-19 leave rather minor traits in the circulation.

DISCUSSION

The immune system is critical in the pathogenesis of COVID-19 (29, 30). Mediators released by infected cells initially recruit innate immune cells to the site of infection. This is the prerequisite for the subsequent generation of adaptive immune responses by presentation of SARS-CoV-2 epitopes to naïve T and B cells in local lymph nodes and the ensuing recruitment of antigen-experienced T cells to the site of inflammation as well as the production of specific antibodies by B cells (30). These processes are considered important for the clearance of the

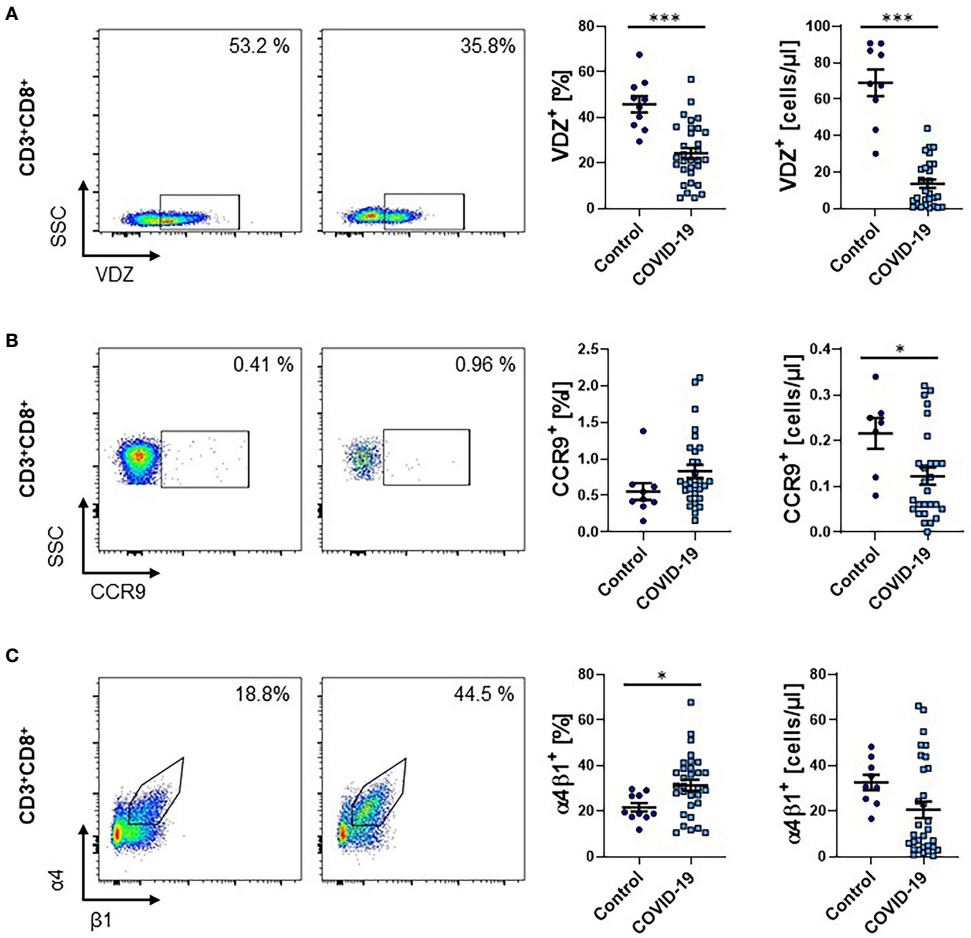

FIGURE 4 | Frequency of gut homing markers on $CD3^+CD8^+$ T cells during COVID-19. **(A–C)** Flow cytometry of whole blood samples from COVID-19 patients and healthy controls. Representative (left) and quantitative (right) flow cytometry of the frequency and absolute cell numbers of α4β7 integrin- **(A)**, CCR9- **(B)** and α4β1 integrin- **(C)** expressing $CD3^+CD8^+$ T cells. VDZ, vedolizumab. Each symbol represents an individual subject, n = 10 – 31 per group. *p < 0.05, ***p < 0.001.

infection and the resolution of inflammation, but insufficient control, to the contrary, is involved in the development of acute respiratory distress syndrome and hyperinflammation in patients with severe disease course (31). Consistently, dexamethasone is an effective treatment in patients with severe COVID-19 (32).

In particular, it has been shown that T cells recognizing multiple regions of the spike, M and N protein of SARS-CoV-2 develop during COVID-19 (26, 33, 34) and that recognition of multiple epitopes is associated with milder symptoms (35). SARS-CoV-2-induced T cell immunity is maintained for at least six months and symptomatic primary infection is associated with higher levels of the persisting T cell response (36).

The lung and the gut are considered the main portals of entry for SARS-CoV-2 (37, 38). However, pulmonary manifestation dominates the clinical disease phenotype and only a subset of hospitalized patients suffers from gastrointestinal symptoms (2, 4). It is currently unclear, how infection of the lung alveolar and the intestinal epithelium contribute to the generation of the systemic immune response against SARS-CoV-2. Importantly, memory T and B cells primed in the gut are characterized by the expression of the gut-homing marker α4β7 integrin (20, 23). We therefore decided to study circulating T and B cell responses in hospitalized patients with COVID-19 to determine the contribution of SARS-CoV-2-associated intestinal lymphocyte imprinting based on gut-homing phenotypes.

In conclusion, our data show that adaptive immune cells expressing the gut homing integrin α4β7 are reduced in the peripheral blood of patients with COVID-19. It is very likely that α4β7 expression on memory lymphocytes is reflecting priming following antigen contact in the GALT, where the exclusive production of retinoic acid by DCs induces α4β7 integrin expression and imprints a gut homing phenotype. Thus, our data suggest that cells having received intestinal antigenic cues in the GALT are reduced in the peripheral blood during COVID-19. It is essential to keep in mind that this does not indicate that the cells have been in the gut tissue itself or that they will later home to the gut tissue.

Two main interpretations for this observation are conceivable: (1) α4β7-expressing lymphocytes are preferentially recruited from the circulation to peripheral tissues or (2) predominant (re-)

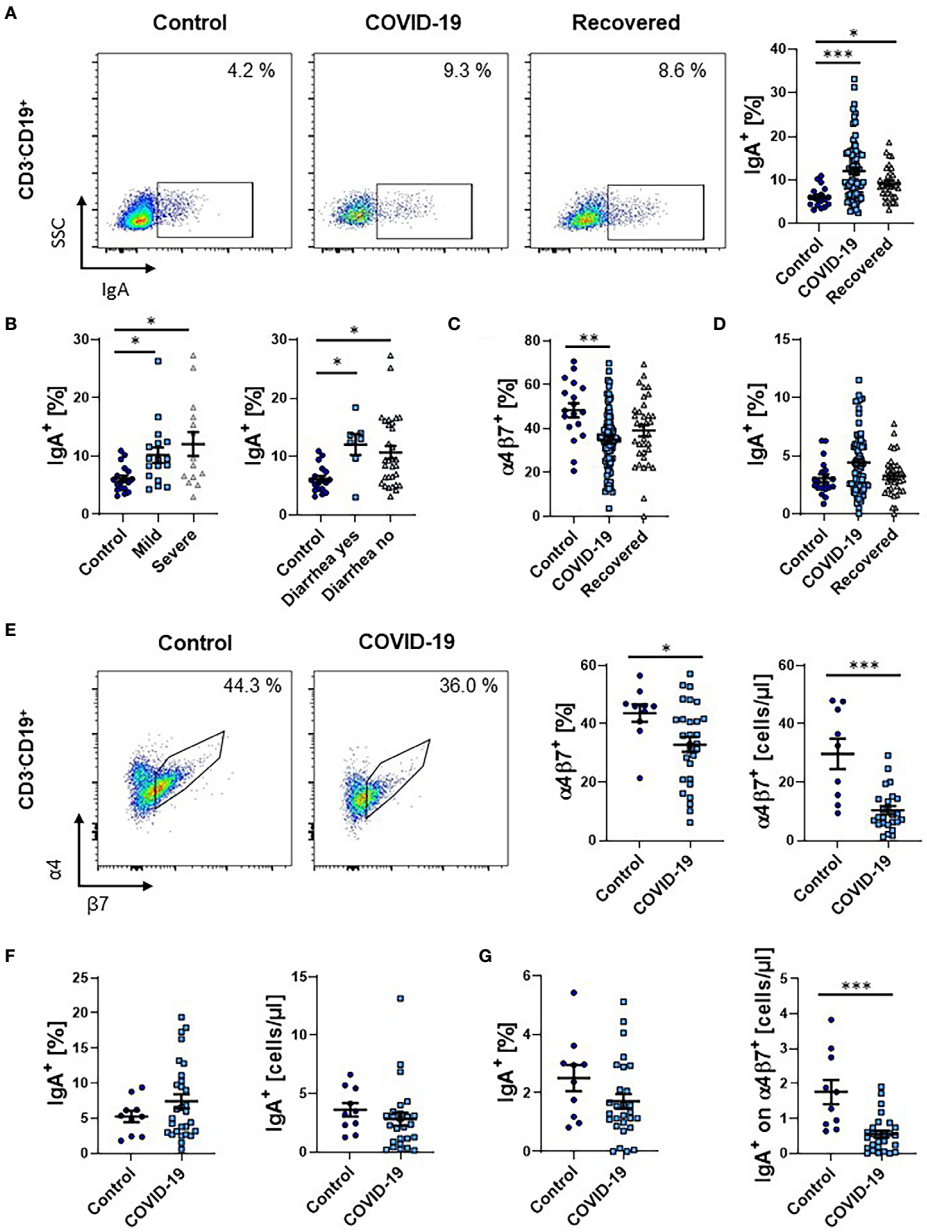

FIGURE 5 | Frequency of IgA$^+$ B cells during COVID-19. **(A)** Representative (left) and quantitative (right) flow cytometry of the frequency of IgA expressing CD3$^-$CD19$^+$ B cells from PBMCs. **(B)** Quantitative flow cytometry of the frequency of IgA-expressing CD3$^-$CD19$^+$ B cells in COVID-19 patients with mild or severe disease course (left) and with or without diarrhea (right) compared to healthy controls. **(C)** Quantitative flow cytometry of the frequency of α4β7 integrin-expressing CD3$^-$CD19$^+$ B cells. **(D)** Quantitative flow cytometry of the frequency of IgA$^+$ cells among CD3$^-$CD19$^+$α4$^+$β7$^+$ B cells expressed as frequency of CD3$^-$CD19$^+$ B cells. **(E–G)** Flow cytometry of whole blood samples from COVID-19 patients and healthy controls. **(E)** Representative (left) and quantitative (right) flow cytometry of the frequency and absolute cell numbers of α4β7 integrin-expressing CD3$^-$CD19$^+$ B cells. **(F)** Quantitative flow cytometry of the frequency (left) and absolute cell numbers (right) of IgA-expressing CD3$^-$CD19$^+$ B cells. **(G)** Quantitative flow cytometry of the frequency (left) and absolute cell numbers (right) of IgA$^+$ cells among CD3$^-$CD19$^+$α4$^+$β7$^+$ B cells expressed as frequency of CD3$^-$CD19$^+$ B cells. Each symbol represents an individual subject, n = 7 - 80 per group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

circulation of lymphocytes not expressing α4β7 leads to a "dilution" of gut-imprinted cells.

The former option goes along with the clinical hallmark of lymphopenia in COVID-19, which is thought to arise from lymphocyte recruitment to tissues (31). Since CD4$^+$ and CD8$^+$ T and B cell frequencies expressing α4β7 decreased regardless of the presence of diarrhea as a symptom indicative of gastrointestinal involvement and fecal detection of virus

RNA (39, 40), it is unlikely that such recruitment happens to the gut (30). However, it can still not be ruled out that $\alpha4\beta7^+$ cells might co-express other homing markers leading to their $\alpha4\beta7$-independent uptake in other tissues or that they are eliminated from the blood by other mechanisms under inflammatory conditions. In this case, $\alpha4\beta7$ expression might mark a lymphocyte population with particular pathogenetic relevance.

The second explanation would suggest that adaptive immune cells primed in the gut do at least numerically not play a major role for the systemic immune response against SARS-CoV-2 in COVID-19 even in patients, in which gastrointestinal involvement is likely. This would also be in line with the finding that IgA-expressing B cells with a gut homing phenotype did not contribute to the overall increase in IgA-expressing B cells observed in COVID-19 patients. One possible explanation might be that intestinal SARS-CoV-2 infection leads to the predominant generation of local immunity characterized by tissue-resident memory T cells (41) and, interestingly, a large part of intestinal T cells have a resident phenotype (42).

If this second interpretation is true, our findings might be interesting in the context of the ongoing efforts to develop efficient vaccines against SARS-CoV-2. While most candidate vaccines are designed as injections, some oral vaccines are also under development (e.g., VXA-CoV2-1). It has previously been shown for adenovirus subtypes and influenza that oral vaccines can also protect from pulmonary infections (43–45). Moreover, mucosal administration has been proposed to be more effective in inducing protective mucosal immunity (including the lung) than systemic application (46). It would then have to be doubted that this is similarly the case in COVID-19 in view of reduced gut-imprinted immunity in the circulation during active infection with the virus.

A limitation of our study is that most of our experiments did not assess SARS-CoV-2-specific T cells. However, our observations following virus-specific stimulation suggest that there is a clear correlation of $\alpha4\beta7$ expression on overall memory $CD4^+$ T cells with expression on SARS-CoV-2-specific memory $CD4^+$ T cells. Moreover, the consistent alterations we observed during active infection and recovery in different patients as well as in a second patient cohort, strongly suggest that the effects are COVID-19-associated. It should also be noted that a certain difference in the age of our control group compared with the COVID-19 patients exists and age-related effects on integrin expression cannot be completely ruled out.

While lymphopenia is a hallmark of COVID-19 and has repeatedly been reported, lymphocyte subset analyses in the peripheral blood of patients with COVID-19 have only rarely been performed. In a cohort of 44 patients, Qin et al. reported reduced memory T cell levels in patients with severe compared to mild COVID-19, which were, however, still in the normal range (47). Similarly, Sekine et al. also observed decreased frequencies of memory T cells in patients with COVID-19, more in severe than in mild disease, and described the upregulation of markers such as CD38, CD69 or PD-1 (48). However, data on the expression of gut-homing markers in the context of SARS-CoV-2 infection have so far been missing.

Taken together, our findings suggest that gut-imprinted adaptive immune cells are eliminated from the circulation during COVID-19 hinting at a potential central role in its pathogenesis or that – in line with clinical symptoms – not the intestinal immune system, but other sites such as the lung dominate the shaping of systemic immune responses to SARS-CoV-2.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Friedrich-Alexander University Erlangen-Nuremberg (174_20B). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

TM performed the experiments and analyzed the clinical data. TM, MN, and SZ designed the research. TM, EB, MW, LS, CV, SV, AK, MN, and SZ contributed samples or protocols and analyzed and interpreted the data. TM and SZ drafted 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.2021.639329/full#supplementary-material

Supplementary Figure 1 | Lymphocyte subsets in PBMCs from COVID-19 and recovered patients compared to healthy controls. Representative (left) and quantitative (right) flow cytometry of the frequency of $CD3^+$ **(A)** and $CD3^+CD4^+$ **(B)** T cells among lymphoid cells selected based on forward and sideward scatter, $CD45RO^+$ cells among $CD3^+CD4^+$ T cells **(C)** and $CD3^-CD19^+$ B cells **(D)** among lymphoid cells. Each symbol represents an individual subject, n = 18 – 80 per group.

Supplementary Figure 2 | Quantitative flow cytometry of the expression of the indicated markers stratified according to clinical parameters of COVID-19 patients. **(A)** Frequency of $\alpha4^+\beta7^+$ and $GPR15^+$ cells among $CD3^+CD4^+$ T cells according to oxygen saturation. **(B)** Frequency of $\alpha4\beta7$ integrin-expressing $CD3^+CD4^+CD45RO^+$ memory T cells according to respiratory rate and heart rate. **(C)** Frequency of IgA-expressing $CD3^-CD19^+$ B cells according to respiratory rate and heart rate. Each symbol represents an individual subject, n = 8 - 18 per group.

Supplementary Figure 3 | Lymphocyte subsets in whole blood samples from patients with COVID-19 and healthy controls. Quantitative flow cytometry of the frequency (left) and absolute cell numbers (right) of $CD3^+$ **(A)**, $CD3^+CD4^+$ **(B)** T cells among lymphoid cells selected based on forward and sideward scatter, $CD45RO^+$ cells among $CD3^+CD4^+$ T cells **(C)** and $CD3^-CD19^+$ B cells **(D)** among lymphoid cells. Each symbol represents an individual subject, n = 10 – 31 per group.

Supplementary Figure 4 | Lymphocyte subsets in whole blood samples from patients with COVID-19 and healthy controls. Representative (left) and quantitative (right) flow cytometry of the frequency and absolute cell numbers of $CD3^+CD8^+$ T cells among lymphoid cells selected based on forward and sideward scatter. Each symbol represents an individual subject, n = 10 – 31 per group.

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Targeting Immune Cell Trafficking – Insights From Research Models and Implications for Future IBD Therapy

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Inflammatory bowel diseases (IBDs), including Crohn's disease (CD) and ulcerative colitis (UC) are multifactorial diseases with still unknown aetiology and an increasing prevalence and incidence worldwide. Despite plentiful therapeutic options for IBDs, the lack or loss of response in certain patients demands the development of further treatments to tackle this unmet medical need. In recent years, the success of the anti-α4β7 antibody vedolizumab highlighted the potential of targeting the homing of immune cells, which is now an important pillar of IBD therapy. Due to its complexity, leukocyte trafficking and the involved molecules offer a largely untapped resource for a plethora of potential therapeutic interventions. In this review, we aim to summarise current and future directions of specifically interfering with immune cell trafficking. We will comment on concepts of homing, retention and recirculation and particularly focus on the role of tissue-derived chemokines. Moreover, we will give an overview of the mode of action of drugs currently in use or still in the pipeline, highlighting their mechanisms and potential to reduce disease burden.

Keywords: IBD, T cell, trafficking, homing, retention, therapy

INTRODUCTION

Trafficking of immune cells, including T lymphocytes, to the gut is a tightly regulated multistep process important for maintaining homeostasis and initiating immune responses (1–4). Naïve T cells circulate through secondary lymphoid organs until they encounter their cognate antigen presented by dendritic cells (DCs) in the gut-associated lymphoid tissue (GALT). This interaction leads to activation, proliferation and imprinting of T cells with a gut homing phenotype through upregulation of specific adhesion molecules. T cells imprinted for small intestinal homing express integrin α4β7, α4β1, β2 integrins and CCR9, while cells primed for migration to the colon show high levels of integrin α4β7 and GPR15 (5–8). Upon recirculation, these T cell subsets may subsequently migrate to the gut as their target tissue along chemotactic gradients, where they interact with the molecules expressed by endothelial cells to initiate the multistep extravasation

process of gut homing. Tethering and rolling mediated by low-affinity binding of selectins (predominantly L-selectin) and integrins (α4β7, α4β1) on T cells to their ligands expressed on endothelial cells (GlyCAM-1, mucosal addressin cell adhesion molecule-1 (MAdCAM-1), vascular cell adhesion molecule 1 (VCAM-1), respectively) slow the cells down to increase availability for activation by tissue-secreted chemokines (e.g. CCL25, CXCL10) (9). This leads to conformational changes of the integrins and, hence, to firm interaction of integrins with cell adhesion molecules and subsequent arrest of activated T cells, followed by transmigration through the endothelium into the tissue. Upon arrival at the site of action, T cells adapt their make-up of surface molecules to their environment (e.g., upregulation of integrin αEβ7) leading to retention in the tissue or, if not activated, recirculation to the blood and lymph (e.g., *via* S1PR/S1P) (10, 11).

T cell trafficking has emerged as one of the hallmarks of IBD pathogenesis and as a potential goldmine for a plethora of new treatment options for IBD by targeting the different steps of this process. This mini-review aims to provide a comprehensive overview of current and future therapeutics based on interference with T cell trafficking, highlighting their mechanisms and potential to reduce disease burden (**Figure 1**).

TARGETING CELL ADHESION MOLECULES – BLOCKADE ON THE SIDE OF THE EFFECTOR TISSUE

Cell adhesion molecules expressed by effector tissues are major mediators of T cell recruitment and intestinal inflammation and serve as promising targets for therapeutic anti-trafficking strategies.

Already in the 1990s, selectively blocking the interaction of β2 integrins with intercellular adhesion molecule 1 (ICAM-1) using antibodies against CD18/ICAM-1 or ICAM-1 antisense oligonucleotides showed promise by reducing inflammation and cell infiltration in 2,4,6-trinitrobenzenesulfonic acid (TNBS)-colitis in rats (12), dextran sodium sulfate (DSS) colitis in mice (13) or acetic acid-induced inflammation in rats (14). Expression of ICAM-1 is upregulated by endothelial cells under inflammatory conditions (13, 15), which leads to increased extravasation of leukocytes (e.g., neutrophils and T cells) expressing β2 integrins. In 1998, Yacyshyn and colleagues could demonstrate that the ICAM-1 antisense oligonucleotide ISIS 2302/alicaforsen administered intravenously was well tolerated and showed promising results for the treatment of CD (16). Treatment with alicaforsen reduced expression of

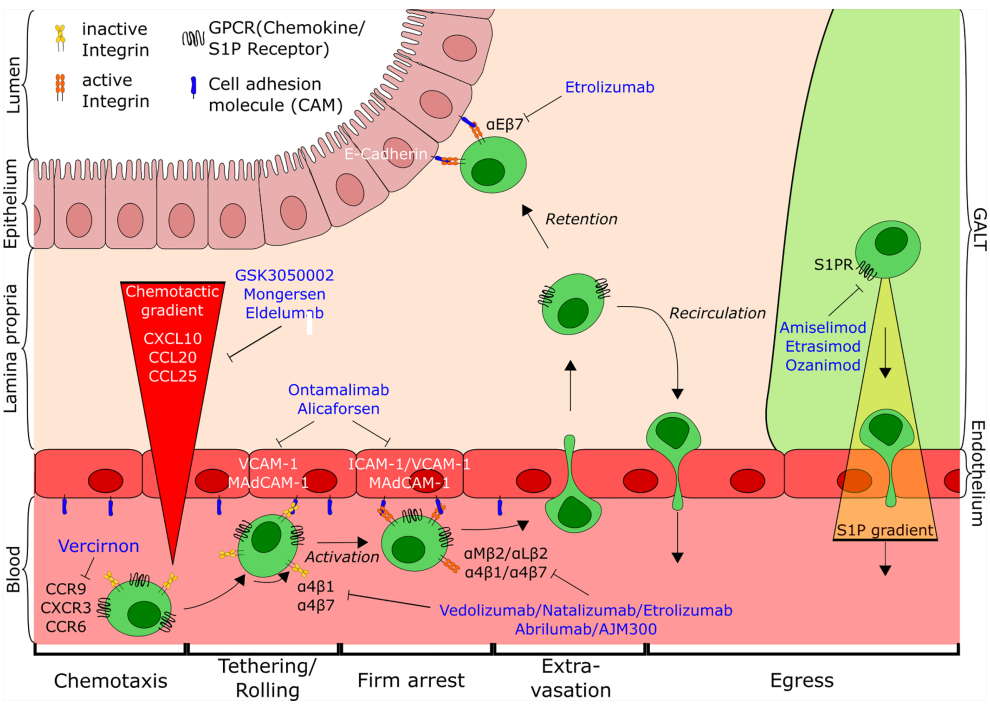

FIGURE 1 | Overview of T cell trafficking in the intestine indicating the points of action of current and potential future anti-trafficking agents for the treatment of IBD. Tethering and rolling of cells on the endothelial wall mediated by interaction of low-affinity integrins with their respective ligands (e.g. α4β7-MAdCAM-1) leads to the exposure to a chemokine gradient (CCL25, CXCL10, CCL20). Subsequent activation of cells causes conformational changes of the integrins, followed by firm arrest and extravasation of T cells to the gut. There, cells are either retained in the tissue through interaction with the epithelium (αEβ7-E-cadherin) or antagonism of egress, or recirculate into the blood from gut and GALT along the S1P-gradient. CD, cluster of differentiation; CCR, Chemokine receptor; CXCR, CXC-motif chemokine receptor; GPCR, G-protein coupled receptor; S1P, Sphingosine-1-phospate; S1PR, Sphingosine-1-phosphate receptor; ICAM-1, Intercellular adhesion molecule 1; VCAM-1, Vascular cell adhesion molecule 1; MAdCAM-1, Mucosal addressin cell adhesion molecule-1; GALT, Gut-associated lymphoid tissue.

ICAM-1 on high endothelial venules (HEV), thereby hindering leukocyte extravasation. However, two subsequent trials with alicaforsen in active CD could not demonstrate superiority over placebo (17, 18). Alicaforsen was also investigated as an enema for topical application in the treatment of UC and pouchitis. Initial clinical evaluations showed improved clinical scores for both diseases (19, 20). However, later studies in mild-to-moderate UC failed to reach their primary endpoints (21, 22). A phase III trial with alicaforsen enema for the treatment of pouchitis patients refractory to antibiotics was completed last year. The treatment with alicaforsen was safe and even though the primary endpoint of endoscopic remission at week 10 showed no difference between treatment with alicaforsen and placebo, the portion of patients reporting a reduction of stool frequency was higher in the alicaforsen compared with the placebo group (NCT02525523).

Another important cell adhesion molecule involved in gut homing and upregulated upon inflammation is VCAM-1. VCAM-1 antagonists proved superior to ICAM-1 and MAdCAM-1 blockade in the murine model of DSS colitis (23), and the monoclonal anti-α4 integrin antibody natalizumab has been successfully used for blockade of VCAM-1-dependent leukocyte trafficking in patients with active CD (24–27). However, due to the ubiquitous expression of VCAM-1, systemic blocking of the VCAM-1 homing cascade was associated with severe adverse events like progressive multifocal leukoencephalopathy (PML) (28, 29), underscoring the need for gut-selective targeting of T cell trafficking. Therefore, although VCAM-1–α4β1 is strongly involved in small intestinal T cell recruitment (30), it is questionable, whether targeting VCAM-1 is a promising target for the treatment of IBD.

Ontamalimab (formerly SHP647) is an antibody binding MAdCAM-1, the ligand of α4β7 integrin and L-selectin. MAdCAM-1 is predominantly expressed on HEVs of the gut and GALT (31) and its expression is strongly induced by TNF-α under inflammatory conditions and in IBD patients (32–34). Pre-clinical trials with the murine anti-MAdCAM-1 antibody MECA-367 demonstrated reduced lymphocyte recruitment to the gut and reduction of inflammation in the T cell transfer colitis model in Scid mice (35, 36). A first human phase I study could show safety of anti-MAdCAM-1 therapy in patients with active UC and a change of biomarkers compared to baseline (37), and efficacy in the treatment of UC was confirmed in a phase II trial (TURANDOT) (38). In a phase II trial for the treatment of moderate-to-severe CD (OPERA) clinical endpoints did not reach statistical significance in comparison to placebo (39, 40), but treatment led to a reduction of soluble MAdCAM-1 and to an increase of circulating $\beta7^+$ central memory T cells and elevated CCR9 gene expression (41). The phase III trials for ontamalimab in both UC and CD were discontinued following a take-over of the developing company (42). However, ontamalimab remains a promising therapeutic agent. Treatment did not lead to central nervous system complications and induced very low levels of anti-drug antibodies (43). With regard to L-selectin as an additional interaction partner of MAdCAM-1, ontamalimab might not just be an imitation of anti-α4β7 antibody therapy, but dispose over a unique mechanism of action (44, 45). Furthermore, expression of MAdCAM-1 in other mucosal tissues as well as in joints, eyes, skin and liver (46–48) make it a potential treatment option for extraintestinal manifestations of IBD and other inflammatory diseases (49).

Taken together, these data show the potential of targeting cell adhesion molecules in the treatment of IBD, especially in selected subgroups of patients, and suggest that, despite some deceptions and obstacles, it seems worth further developing respective compounds.

BLOCKADE OF CHEMOKINES – REDUCING LEUKOCYTE ATTRACTION

In addition to cell adhesion molecules, chemokines play a pivotal role in T cell recruitment to the gut and offer another approach for therapeutic targeting.

CCL25 is a chemokine expressed in the small intestine under homeostatic conditions and strongly upregulated in the ileum and also the colon upon inflammation (50–52). Its receptor CCR9 is found on T cells imprinted for gut homing (53–56). Even though CCR9 is highly expressed on regulatory T cells (Treg) and plays a leading role in establishing self-tolerance in the thymus (57), the CCR9-CCL25 axis has been implicated in inflammation, especially of the small intestine (52). Isolated $CCR9^+$ T cells from CD patients show markedly higher expression of IL17 and IFNγ upon stimulation compared to controls (58), and stimulation of T cells through CCR9 leads to activation of α4β1 and α4β7 integrins and, hence, increased extravasation (59, 60).

Blocking either CCR9 or CCL25 in mice treated with TNFα or in the SAMP1/YitFc model of ileitis demonstrated reduction of leukocyte migration to the small intestine and strong inhibition of inflammation (61, 62). The oral CCR9 antagonist CCX282-B/vercirnon was successfully used in the $TNF^{\Delta ARE}$ ileitis mouse model (63) as well as for the treatment of moderate-to-severe CD in a phase II study (64). A subsequent phase III study failed to demonstrate efficacy of vercirnon as induction therapy (65, 66). Data from animal models and patients show a strong homeostatic role for the CCR9-CCL25 axis in the small intestine, but a clear association with inflammation in the colon (66, 67) and a study depleting $CCR9^+$ cells through leukapheresis (68) showed promising results, suggesting that blocking CCR9-CCL25 interaction might be an option for the treatment of UC.

Another chemotactic stimulus for gut infiltration of T cells is CXCL10. CXCL10 expression is induced by IFNγ (69) and markedly upregulated in colitis (70, 71). Its receptor CXCR3 is found on effector T cells, $\beta7^+$ peripheral blood mononuclear cells (PBMCs), lamina propria mononuclear cells (LPMCs) and intraepithelial lymphocytes (IELs) and a high number of $CXCR3^+$ cells can be found in biopsies from UC and CD patients (72). Treatment with anti-CXCL10 antibodies attenuated colitis in IL10-deficient mice and in DSS colitis and reduced cell infiltration to the lamina propria (73–76). In clinical studies, treatment with a monoclonal antibody against CXCL10 was efficient for the treatment of rheumatoid arthritis (MDX-1100) (77). However, blocking CXCL10 with the antibody

eldelumab failed to induce remission in patients with moderate-to-severe UC or CD (78–80). Still, in subgroups of anti-TNFα naïve patients, CXCL10 blockade ameliorated mucosal response, suggesting that this treatment could be effective in selected patients (66).

CCL20 is a chemokine implicated in both inflammation and homeostasis and is predominantly expressed by mucosal epithelial cells (81). CCL20 expression is induced through TNFα and elevated in CD patients (82). In pre-clinical studies, neutralization of CCL20 reduced T cell infiltration and attenuated colitis in the murine TNBS-model (83). Bouma and colleagues reported a dose-dependent decrease of cells bearing CCR6, the receptor for CCL20, in healthy human volunteers after treatment with the humanized antibody GSK3050002 against CCL20 (84). However, to our knowledge, no CCL20 antagonist has been used in clinical trials of IBD so far. Yet, mongersen, an oral Smad7 anti-sense oligonucleotide indirectly regulates CCL20 expression. Smad7 is highly expressed in the mucosa of IBD patients and acts as an inhibitor of TGFβ1 signalling, an important negative regulator of TNFα signalling. Consistently, blocking Smad7 expression through the administration of an anti-sense nucleotide restored TFGβ1 signalling (85). In the TNBS and oxazolone colitis mouse models, treatment with a Smad7 anti-sense oligonucleotide led to reduction of inflammation (86). Treatment of CD organ explants with mongersen reduced Smad7 and CCL20 expression and serum levels of CCL20 in patients responding to mongersen were significantly reduced (82). In 2015, Monteleone and colleagues reported significantly higher response and remission rates after treatment with mongersen compared to placebo in patients with active CD (87). However, a subsequent phase III study published last year failed to demonstrate efficacy for the treatment of CD (88). Data to interpret these results with regard to the indirect effect on CCL20 are lacking.

GPR15 is a recently deorphanized receptor expressed on a large subset of colon-homing T cells. GPR15 is found on $Foxp3^+$ Treg cells in mice and important for the maintenance of large intestinal homeostasis, while data from humans suggest higher expression on effector T cells (7, 89, 90). The ligand for GPR15 (GPR15L) is expressed by epithelial cells in the colon and the skin and chemotactic abilities have been reported (91), suggesting that the GPR15-GPR15L axis might be a potential target for modulating intestinal inflammation.

Collectively, these studies show the large potential of treating IBD by targeting chemokines and their receptors, but also indicate that interfering with chemokine signalling seems to be a complex approach that has not resulted in the approval of therapeutic agents so far.

TARGETING TRAFFICKING ON THE T CELL SIDE – A STORY OF SUCCESS

The prime example for successful treatment of IBDs by targeting leukocyte trafficking is the anti-α4β7 integrin antibody vedolizumab (92–97). Binding to its target leads to the internalization of the α4β7 integrin, inhibiting the interaction with its ligand MAdCAM-1, which is virtually exclusively expressed on the endothelium of the gut and GALT (31, 98, 99). This very selective and highly gut-specific mode of action leads to reduced intestinal lymphocyte counts and inflammation, while retaining an excellent safety profile with few side effects (100–102). The gut specificity is also thought to account for the safety profile advantage over broader α4 integrin blockade by the antibody natalizumab, which, while being effective for the treatment of preclinical cotton-top tamarin colitis (103) and active CD (24, 25), was withdrawn from widespread use after several cases of PML (28, 29).

The example of vedolizumab paved the way for the current development of additional drugs with a similar mode of action. Abrilumab, another anti-α4β7 integrin antibody that is subcutaneously administered, successfully completed phase II trials for moderate-to-severe UC (104–106). Moreover, the oral small molecule α4 integrin inhibitor AJM300 successfully attenuated inflammation and cell infiltration in the adoptive T cell transfer colitis model (107) and currently undergoes phase III testing in UC. While no cases of PML were observed in phase II trials, it will be important to thoroughly investigate the safety profile of AJM300 in further studies, since it is likely that it affects central nervous immune surveillance similar to natalizumab, although it might have a favorable pharmacological profile (108, 109).

INTEGRIN BLOCKADE BEYOND A4B7-BLOCKADE – INTERFERING WITH RETENTION

Aiming to expand the clinically successful anti-α4β7 strategy, the humanized monoclonal antibody etrolizumab was developed to target the β7-subunit of α4β7 as well as αE (CD103) β7 integrin hetereodimers (110). CD103 expression on T cells is induced by T cell receptor signalling and TGFβ, which is released by several cellular sources in the intestine (111, 112). αEβ7-expressing cells are able to interact with epithelial (E-) cadherin expressed by intestinal epithelial cells (IECs) and may thereby be retained in the tissue (113). Furthermore, evidence from cancer and gastritis research suggests that this interaction serves as a costimulatory factor for T cell receptor activation in $CD8^+$ and $CD4^+$ T cells, respectively (114–117). Despite CD103 expression being associated with a Treg phenotype in mice (118–120), recent evidence suggests a pro-inflammatory Th1, Th17 and Th1/17 phenotype for $\alpha E\beta7^+$ $CD4^+$ T cells with reduced expression of Treg markers in the large intestine of UC patients, proposing a role for these cells in disease pathobiology (121). Furthermore, the role of $CD4^+$ tissue resident memory T cells (Trm), which can also express CD103, in human IBD and murine models of colitis has recently been highlighted (122). The data suggested involvement of these cells in the development of IBD flares and as a switch-point for experimental colitis further substantiating the potential of αEβ7 as a therapeutic target (122). With promising results from the adoptive transfer colitis mouse

model (123) and phase II trials, large-scale phase III programs were launched for etrolizumab in active UC and CD. In recently presented data from the UC trials, etrolizumab, while being well tolerated, only met the primary endpoint in two out of three induction studies and in none of the two maintenance studies (124–126). Despite these discouraging results, several key secondary endpoints were met and, strikingly, numerically similar clinical and endoscopic outcomes were reported for etrolizumab and the anti-TNFα antibodies infliximab and adalimumab (127, 128), thus supporting biological activity of etrolizumab. Further analyses, including the previously suggested ability of CD103 to predict response to therapy (129) are eagerly awaited. Moreover, the pivotal CD phase III trial program is nearing its completion and experimental evidence indicates that αEβ7 integrin might be even more important in that context. Specifically, in line with previous reports, Ichikawa, Lamb and colleagues demonstrated an increased abundance of $CD103^+$ cells in the ileum compared to the colon (130), suggesting that ileal CD might be a particularly promising entity to treat with etrolizumab. This is further supported by the observation that α4β7 blockade alone did not sufficiently reduce homing of CD patient-derived effector T cells to the ileum in an adoptive transfer model (131).

Taken together, in spite of disappointing results in the UC phase III trials, books should not be closed prematurely over etrolizumab, especially regarding subsets of patients with increased CD103 expression or ileal disease location.

SEQUESTRATION OF CELLS IN LYMPH NODES BY S1PR MODULATORS

Cellular retention cannot only be modulated by interfering with tissue anchorage, but also by modifying exit cues. This is the principle of the emerging field of S1P modulation. Physiologically, recirculation of T cells from the tissue to the blood is mediated by a constantly generated S1P gradient, with high concentrations in the blood. Low concentrations in tissues are upheld by enzymatic degradation of S1P by the S1P-lyase (132–134). S1P is sensed by S1P receptors (S1PR)1-5, which internalize on ligand binding, thereby inducing transient tissue retention and providing the opportunity for activation and antigen sensing of retained cells (135–137). Furthermore, it has been demonstrated that activation-induced CD69 directly interacts with S1PR1 and leads to its removal from the cell surface, thereby contributing to tissue retention of activated T cells (138). S1P modulation for IBD therapy aims to sequester naïve and central memory T cells in lymphoid tissues, inducing circulatory lymphopenia and thereby cutting off the supply of potentially pathogenic T cells migrating to the site of inflammation (11). Generally, S1PR modulators for IBD treatment are agonists, which can be distinguished by differential selectivity for S1PRs.

After showing promising result in preclinical models (TNBS colitis in rats and adoptive transfer colitis in Scid mice) (139) and after successfully completing a phase II study in moderate-to-severe UC (140), first results from phase III trials of the oral S1PR1- and S1PR5-selective agonist ozanimod for the treatment of moderate-to-severe UC were presented recently. Ozanimod demonstrated statistically significant improvements in clinical remission and response, endoscopic and mucosal healing without raising safety issues and can therefore be considered to enter the treatment algorithm for UC in the future (141, 142). In the adoptive transfer colitis model, etrasimod, a S1PR1, 4, 5 agonist, was able to reduce inflammation (143). After successful completion of phase II trials, etrasimod recently entered phase III testing for moderate-to-severe UC (144). In contrast, a clinical trial of the selective S1PR1 modulator amiselimod as a treatment for CD could not demonstrate an impact on clinical or biochemical disease activity, despite promising results in a preclinical study employing the adoptive T cell transfer model of chronic colitis (145, 146).

Taken together, S1PR modulation proved to be a potent tool for the treatment of IBD, but different results observed with agonists with differential selectivity highlight the complexity of this approach and therefore warrant further research.

DISCREPANCIES BETWEEN MOUSE AND MAN – CHALLENGES IN DRUG DEVELOPMENT

As referenced at several points of this review, promissing preclinical observations have promted clinical trials (**Table 1**). However, not all insights from preclinical studies have been reproducable in humans. Reasons for late stage failures in drug development despite preclinical efficacy and often clear indications of biological effects in humans are manifold. Animal models are a valuable and indispensable tool to uncover disease pathogenesis and mechanisms underlying therapeutic effects (3, 134) and advances in medical research today would not be on the same level without insights from such model systems. However, mouse models of intestinal inflammation cannot fully reproduce the complexity of IBD as a multifactorial disease in certain aspects. In recent years, the importance of the microbiome in IBD was highlighted by several studies (147–150). The controlled environments in animal facilities limit microbiome diversity in experimental animals, but can differ between facilities and therefore can influence results (151). Specific-pathogen-free (SPF) environments in preclinical setups limit the predictability of adverse events related to infections (e.g. PML). These apparent limitations can also be considered as a strength, as comparable housing and nutrition enables researchers to uncover the influence of microbiota on disease pathology (152). Furthermore, IBD pathomechanisms are portrayed only partially, e.g. DSS-induced colitis is driven by the innate immune system and is induced even in the absence of lymphocytes (153–155), whereas pathology in the T cell transfer colitis model is induced by the transfer of naïve T cells to immunodeficient hosts (156, 157). Therefore, different models can produce contradicting results for the same treatment, as it is, for instance, the case for anti-α4β7

TABLE 1 | Anti-trafficking agents used in the clinic or still in development with details on their specific target, mode of action and preclinical and clinical efficacy.

Target	Origin	Drug	Mode of action	Preclinical Data			Clinical Data		Observed effects
				Model	Effective?	Disease	Administration	Primary endpoint (s) met?	
ICAM-1	Endothelium	Alicaforsen	Anti-sense oligonucleotide	DSS colitis mouse model (13)	Yes	CD (Phase II) (16)	IV	No	Steroid withdrawal
						CD (Phase II) (17)	IV	No	Steroid withdrawal
						CD (Phase II) (18)	IV	No	N/A
						UC (20)	Enema	Yes	Clinical response
						Pouchitis (NCT02525523)	Enema	Not yet published	Reduced stool frequency
MAdCAM-1	Endothelium	Ontamalimab	Monoclonal antibody	Adoptive T cell transfer colitis mouse model (35,36)	Yes	UC (Phase I) (37)	IV/SC	No	Changes in biomarkers compared to baseline
						UC (Phase II) (38)	SC	Yes	Clinical remission
						CD (Phase II) (39,40)	SC	No	Clinical remission in patients with higher endoscopic activity
						UC and CD (Phase III)	SC	Discontinued	
CCR9	Lymphocytes	Vercirnon	Small molecule antagonist	$TNF^{\Delta ARE}$ ileitis mouse model (62)	Yes	CD (Phase II) (64)	PO	Yes	Clinical response, remission and steroid-free remission
						CD (Phase III) (65)	PO	No	N/A
CXCL10	Epithelium	Eldelumab	Monoclonal antibody	$IL\text{-}10^{-/-}$ and piroxicam colitis mouse model (74)	Yes	UC (Phase II) (78)	IV	No	Improvement of IBDQ score
				DSS colitis mouse model (75)	Yes	UC (Phase II) (80)	IV	No	N/A
				$IL\text{-}10^{-/-}$ colitis mouse model (76)	Yes	CD (Phase II) (79)	IV	No	Numerically higher remission and response rates
Smad7	Epithelium	Mongersen	Anti-sense oligonucleotide	TNBS and oxazolone colitis mouse model (86)	Yes	CD (Phase II) (87)	PO	Yes	Clinical remission and response
						CD (Phase III) (88)	PO	No	N/A
α4 integrin	Lymphocytes	Natalizumab	Monoclonal antibody	Cotton-top tamarin colitis model (103)	Yes	CD (24)	IV	Yes	Clinical remission and response
						CD (25)	IV	Yes	Clinical remission and response
						CD (Phase III) (26)	IV	Yes	Clinical response
						CD (27)	IV	Yes	Clinical response
		AJM300	Small molecule antagonist	Adoptive T cell transfer colitis mouse model (107)	Yes	UC (Phase II) (109)	PO	Yes	Clinical remission and mucosal healing
α4β7 integrin	Lymphocytes	Vedolizumab	Monoclonal antibody	Cotton-top tamarin colitis model (96)	Yes	UC (Phase III) (94)	IV	Yes	Clinical response, remission and mucosal healing
						CD (Phase III) (97)	IV	Yes	Clinical response, remission and steroid-free remission
		Abrilumab	Monoclonal antibody	N/A		UC (Phase II) (104)	SC	Yes	Clinical response, remission and mucosal healing
						UC (Phase II) (105)	SC	Yes	Numerically higher remission, response

(Continued)

TABLE 1 | Continued

Target	Origin	Drug	Mode of action	Preclinical Data		Disease	Clinical Data		Observed effects
				Model	Effective?		Administration	Primary endpoint (s) met?	
									and mucosal healing rates
β7 integrin	Lymphocytes	Etrolizumab	Monoclonal antibody	Adoptive T cell transfer colitis mouse model (123)	Yes	UC (Phase II) (110)	SC	Yes	Clinical remission
						UC (Phase III) (124, 125, 127)	SC	Yes/No	Clinical response and endoscopic improvement
						CD (Phase III)	SC	Ongoing	
S1PR	Lymphocytes	Ozanimod (S1PR1/5)	Small molecule agonist	TNBS colitis rat model and adoptive T cell transfer colitis mouse model (139)	Yes	UC (Phase II) (140)	PO	Yes	Clinical response and mucosal healing
						UC (Phase III) (141, 142)	PO	Yes	Clinical remission, response and mucosal healing
		Etrasimod (S1PR1/4/5)	Small molecule agonist	Adoptive T cell transfer colitis mouse model (143)	Yes	UC (Phase II) (144)	PO	Yes	Clinical remission, response and histological improvement
		Amiselimod (S1PR1)	Small molecule agonist	Adoptive T cell transfer colitis mouse model (146)	Yes	CD (145)	PO	No	Reduced lymphocyte counts

IV, intravenous; SC, subcutaneous; PO, per os; UC, ulcerative colitis; CD, Crohn's disease; N/A, not available.

blockade in acute DSS-induced and T cell transfer colitis (158). On the other hand, this cell specific mode of action can help to unravel the contribution of different cell types to disease processes and lead to a better understanding of therapeutic mechanism (122).

Moreover, GPR15 expression was previously reported to direct Treg cells to the large intestine and defects in GPR15 led to increased susceptibility to colitis in a *Citrobacter rodentium* infection model and reduced suppression or rescue of inflammation in anti-CD40 and T cell transfer colitis models (7). In contrast, Nguyen and colleagues demonstrated GPR15 expression on murine Th1 and Th17 cells in addition to Treg cells and a GPR15 dependency in the induction of colitis in the T cell transfer model, thus further highlighting the potential of differential outcomes even when working with the same receptor in different setups (159). The same study also highlighted another reason for potential species discrepancies: in contrast to GPR15 expression on murine Th1, Th17 and Treg cells, expression of GPR15 was associated with a Th2 phenotype in the large intestinal lamina propria of UC patients. This observation was attributed to species-specific enhancer sites binding GATA3, the Th2 lineage defining transcription factor, in the human GPR15 gene, which are absent in the mouse genome. Species differences between mouse and man have also been reported for other potential targets of investigated drug candidates, including CD103 (118–121). Inadequate experimental design can further be the cause for limited reproducibility. Therefore, many groups have developed concepts to improve the quality of animal studies (e.g., by using completely randomised experimental designs or by conducting experiments at a similar time of day) (160–162). Finally, several of the compounds reviewed here showed promising results in phase II trials but failed to reach primary endpoints in phase III studies (see **Table 1**). Possible explanations could be the stricter definition of primary endpoints in phase III trials [e.g. mongersen (87, 88)], or differences in patient cohorts or study design [e.g. vercirnon (64, 65)].

Taken together, these aspects demonstrate the complexity and importance of preclinical testing in IBD anti-trafficking agent development underscoring the need for careful evaluation of different model systems as well as systematic analysis of potential species differences for successful translation of preclinical findings to the clinic.

CONCLUDING REMARKS

The implication of intestinal T cell trafficking in the pathogenesis of IBD is undisputed. Targeting associated either on endothelial/epithelial cells or on the circulating T cells has proven to hinder cell infiltration effectively. However, the important role of T cell recruitment for tissue homeostasis and pathogen defence underscores the need for selective inhibition strategies to ensure the safety of the therapeutic agent. Discrepancies between human and murine physiology (e.g. GPR15, CD103 expression) need to be carefully evaluated, when translating preclinical findings into clinical treatment options. Despite being outside the scope of this mini-review, the therapeutic options discussed may also affect trafficking of other immune cells that need to be taken into account. And, finally, development of further and a more detailed understanding of approved therapeutic options can only be the first step. Regarding the substantial portion of patients showing primary or secondary non-response, individualized treatment strategies to predict and optimize therapeutic outcomes are an important

unmet need. However, advances in the field of T cell trafficking might also contribute to solutions to that problem.

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Changes in Circadian Rhythms Dysregulate Inflammation in Ageing: Focus on Leukocyte Trafficking

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Leukocyte trafficking shows strong diurnal rhythmicity and is tightly regulated by circadian rhythms. As we age, leukocyte trafficking becomes dysregulated, contributing to the increased systemic, low-grade, chronic inflammation observed in older adults. Ageing is also associated with diminished circadian outputs and a dysregulation of the circadian rhythm. Despite this, there is little evidence to show the direct impact of age-associated dampening of circadian rhythms on the dysregulation of leukocyte trafficking. Here, we review the core mammalian circadian clock machinery and discuss the changes that occur in this biological system in ageing. In particular, we focus on the changes that occur to leukocyte trafficking rhythmicity with increasing age and consider how this impacts inflammation and the development of immune-mediated inflammatory disorders (IMIDs). We aim to encourage future ageing biology research to include a circadian approach in order to fully elucidate whether age-related circadian changes occur as a by-product of healthy ageing, or if they play a significant role in the development of IMIDs.

Keywords: inflammation, circadian rhythm, leukocytes, trafficking, chronotherapy

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INTRODUCTION

Under homeostatic conditions, leukocytes migrate between the vasculature and different tissues for immune surveillance. In response to infections or injuries, leukocytes are recruited to the site of inflammation and play key roles in pathogen clearance and tissue repair. Temporal expression of adhesion molecules on the leukocyte surface and on endothelial cells (ECs) mediates leukocyte trafficking, in a process known as the leukocyte adhesion cascade (1). Critically, it is essential that leukocyte trafficking is tightly regulated as aberrant leukocyte recruitment into tissues contributes to the development of most immune-mediated chronic inflammatory diseases (IMIDs).

Leukocyte trafficking was first identified to be under circadian control over 50 years ago, when it was observed that circulating lymphocyte numbers oscillate according to the time of day (2). Since then, core circadian machinery has been identified in almost all immune cells and a strong reciprocal relationship between immunity and circadian clocks has been well established (3–5). Disruption of circadian rhythms due to genetic manipulation or lifestyle (for example, shift work) dysregulates the immune response and increases the susceptibility to cancers, cardiovascular disease, and metabolic disease (6). Several IMIDs show daily patterns of symptom intensity and

responsiveness to treatment which has created a new avenue of chronotherapy that involves optimal timing of drug delivery (4). In older adults, leukocyte trafficking becomes dysregulated, contributing to age-related low-grade, chronic inflammation (inflammageing) that predispose the older population to IMIDs (7). Although it is known there is a dampening of circadian rhythms with increasing age, little work has been done to investigate the effects of this circadian disruption on the increase in IMIDs observed in older adults.

In this review, we explore the current knowledge regarding circadian control of leukocyte trafficking and the circadian oscillations of inflammatory conditions. We discuss the changes that occur to the circadian clock with increasing age and investigate whether this may contribute the age-related increase in inflammation and diseases.

CIRCADIAN RHYTHM AND AGEING

The Master Pacemaker

The circadian rhythm refers to the endogenous cycles seen in nearly all organisms that correlate with the Earth's 24-hour day-night cycle. Numerous biological processes are regulated by circadian clocks including behaviour, sleep, metabolism and body temperature (8, 9). In mammals, the master circadian pacemaker is found within the suprachiasmatic nucleus (SCN) which is entrained by the external environment and synchronises peripheral oscillators (10). Light enters the eye and sends input to the SCN *via* the retinohypothalamic tract enabling the central clock to entrain to external light/dark cues (11). SCN neurons send rhythmic outputs to peripheral organs and other brain areas, allowing global synchronisation with the external environment (12). Additionally, SCN neurons are able to generate autonomous circadian outputs allowing for circadian rhythms to exist even under constant darkness (13).

Molecular Mechanisms

In mammalian cells, the intracellular circadian clock is made up of an autoregulatory negative feedback loop. Transcriptional activators CLOCK (Circadian Locomotor Output Cycles Kaput) and BMAL1 (Brain and Muscle ARNT-Like 1) dimerise and form a complex. The CLOCK/BMAL1 complex then translocates to the nucleus where they bind to E-Box elements in promoter sequences of clock-controlled genes to positively regulate their own transcription (14). CLOCK/BMAL1 also promotes transcription of the clock regulators, Cryptochrome (CRY) and Period (PER), which in turn dimerise and undergo nuclear translocation where they inhibit CLOCK/BMAL1, repressing their own transcription (15). In addition to this feedback loop of core clock genes, nuclear receptor subfamilies Rev-erb and ROR (retinoic acid-related orphan receptor) compete for binding to ROR responsive elements (ROREs) in *Bmal1* promoter sequences to repress and promote expression of BMAL1, respectively (16). Post-translational modifications of clock transcription factors further regulate this feedback loop. Phosphorylation of PER and CRY proteins by casein kinase Iε/δ and AMP kinase promotes ubiquitination by E3 ligases resulting in their degradation (8). Recently, another circadian repressor gene has been identified which is under control of the circadian clock (17). CHRONO (ChIP-derived repressor of network oscillator) inhibits CLOCK/BMAL1 transcription activation in a histone deacetylase (HDAC)–dependent manner, adding an epigenetic arm to the mammalian circadian clock (18). Overall, this negative feedback loop takes about 24 hours and results in the circadian oscillation seen in a multitude of physiological processes.

Ageing and the Circadian Rhythm

It is well-established that the circadian system influences ageing and longevity, and vice versa. Circadian outputs are diminished in older animals (19); transplantation of foetal SCN tissue into aged hamsters led to increases in longevity and restored the age-associated loss of behavioural rhythmicity seen in control animals (20). BMAL1 knockout mice (KO) have significantly shorter lifespans than wild type (WT) controls, and display a premature ageing phenotype (21). Reactive oxygen species (ROS) accumulate in the kidney, spleen and heart of BMAL1 KO animals which all show an age-related decrease in size, suggesting a role of oxidative damage in age-associated degeneration. Inhibition of endogenous BMAL1 by siRNAs in murine fibroblast cell lines also increases ROS levels (6). Oxidative damage caused by increasing ROS production could drive the progression of cellular senescence of local cells, promoting a senescence-associated secretory phenotype, and subsequent dysregulation of the inflammatory response. CLOCK KO mice also have significantly reduced lifespans than WT controls, but show a milder ageing phenotype than BMAL1 KO mice, whereas PERIOD-deficient mice only have reduced lifespans after challenge with irradiation (22). The severe ageing phenotype limited to BMAL1 KO mice may be due to systemic effects independent of the circadian role of BMAL1, or functional redundancy seen by other core clock proteins. Importantly, BMAL1 KO mice also lose all time-of-day dependent leukocyte trafficking when housed in constant darkness, in contrast to WT littermates (23) indicating the pivotal role of the circadian clock in regulation of leukocyte trafficking.

In humans, ageing is associated with a reduced sleep quality and disrupted sleep cycles (9), which in turn further dysregulates the robustness of the circadian rhythm. Importantly, circadian rhythm dysregulation is associated with the development of age-related disorders, including inflammatory and metabolic disorders, and neurodegenerative diseases such as Alzheimer's Disease and Parkinson's (24). Several changes occur to the circadian clock with increasing age and identifying which of these are natural processes of healthy ageing and which of these are pathological will increase our understanding of age-associated aberrant inflammation.

CIRCADIAN REGULATION OF LEUKOCYTE TRAFFICKING IN AGEING

It is well established that leukocyte trafficking follows a circadian oscillation [reviewed extensively in (3, 4, 25)]. The expression of circadian clock genes is ubiquitous to nearly all immune cells, and clocks can directly regulate immune cell trafficking.

Leukocyte trafficking becomes dysregulated in ageing as expression of adhesion molecules, chemokines and integrins change and senescent cells accumulate (26), contributing to inflammageing and increasing susceptibility to IMIDs. Despite the abundant research focusing on circadian changes with increasing age, very little work has been done on how this affects circadian control of leukocyte trafficking. Future research should concentrate on this interaction to identify which changes to the immune system and to the circadian machinery occur as a natural by-product of ageing and which are signs of pathology.

Neutrophils

Neutrophils are the first innate immune cell recruited to sites of inflammation, where they phagocytose pathogens and secrete anti-microbial agents (27). Under steady state conditions, neutrophils are retained in the bone marrow by the key retention signal, CXCL12, acting through its receptor, CXCR4. Diurnal adrenergic signals inhibit CXCL12 expression in the bone marrow, resulting in daily variations in chemokine expression which regulates the circadian egress of neutrophils (and haematopoietic stem cells) from the bone marrow (28, 29). Following LPS challenge, neutrophils show a circadian-regulated recruitment to the lungs (30). Interestingly, this is regulated by the circadian clocks in lung epithelial cells, and not the clocks within neutrophils themselves. Local lung epithelial cells regulate the diurnal expression of the chemokine, CXCL5, in a glucocorticoid-dependent mechanism, which attracts neutrophils to the lungs (30). This identifies the complexity of circadian regulation of leukocyte trafficking, as immune cells both contain intrinsic clock machinery and are regulated by chemokine expression, which can be under the control of circadian clocks in other cells.

Recently, it has been reported that human and murine neutrophils possess an intrinsic, cell-autonomous diurnal ageing programme that acts to regulate trafficking of neutrophils to infections, whilst promoting their removal from the bloodstream, thus protecting vessels from inflammation (31). In young (6-12 weeks old) WT mice, neutrophils lose CD62L expression and gain CXCR4 as they age, promoting their recruitment to the bone marrow for elimination (32). This diurnal change is mediated by BMAL1, which upregulates expression of CXCL2, enables autocrine surface CXCL2-CXCR2 interactions and in turn promotes CD62L expression (31). Neutrophil-specific CXCR4 KO mice showed constitutive ageing as seen by low levels of CD62L (31). Persistence of aged neutrophils in the vasculature of these mice increased thrombo-inflammation in a model of ischemia-reperfusion, and depletion of these neutrophils prevented thrombus formation and improved survival after infarction (31). This suggests that the importance of this diurnal neutrophil ageing process is to prevent senescent neutrophils accumulating in the vasculature and to prevent thrombo-inflammation. Healthy aged mice have an accumulation of CD11b^{high}/ICAM-1^{high} neutrophils in lymphoid organs (33) which may be in response to the increased levels of inflammation in aged mice. However, no research has been done on the diurnal neutrophil ageing process in aged mice and future work should aim to identify if this accumulation of neutrophils in aged lymphoid organs is a result of dysregulated neutrophil ageing due to reduced circadian outputs.

Monocytes/Macrophages

Macrophages are key regulators of the innate immune response and show strong circadian oscillations in genes involved in cytokine secretion, which are essential mediators of leukocyte trafficking (34). REV-ERBα has been highlighted as a direct link between the circadian clock and the macrophage inflammatory response (35) prompting further investigation of this nuclear receptor as a therapeutic target. The role of REV-ERBα in immune responses is well established [reviewed in (36)], and recently synthetic REV-ERBα agonists are being used *in vivo* to investigate a direct circadian modulation in IMIDs such as in Rheumatoid Arthritis (RA) and colitis. RA shows strong symptom rhythmicity, underpinned by daily fluctuations in serum IL-6 concentrations (37). Synthetic REV-ERB ligands have been shown to control the release of IL-6 from macrophages and can alleviate disease symptoms (38). REV-ERBa also has a protective effect against colitis *via* down-regulation of Nlrp3 inflammasome activity (39). Activation of REV-ERBα ameliorates ulcerative colitis in WT mice (39) suggesting it may be a promising target for colitis treatment.

Several aged-related changes occur in macrophages, including polarization towards an alternate M2 phenotype and reduction in phagocytosis (40). Despite M2 macrophages originally considered to display an 'anti-inflammatory' phenotype, these age-associated M2 like macrophages secrete several pro-inflammatory mediators including TNFα, IL-1 and IL-6. In mice, Ly6C^{high} (inflammatory) monocytes but not Ly6C^{low} (patrolling) monocytes exhibit diurnal oscillations in trafficking under both homeostatic conditions and in a model of sterile peritonitis (41). A recent study discovered BMAL1 is induced following stimulation of M1, but not M2, macrophages by inflammatory stimuli (42). It appears that the classically activated, pro-inflammatory 'M1' phenotype is more tightly regulated by the circadian clock. Very little research has been done on the effect of ageing-associated circadian dysregulation and macrophage function, and it would be interesting to understand how the clock alters within ageing macrophages and whether circadian control of transcriptional programmes is affected. As discussed above, it appears that circadian regulation can have varying effects on different macrophage subsets, which highlights the complexity of the relationship between inflammation and circadian clocks. Targeting circadian mechanisms may be important to maintain homeostasis and responses to inflammation.

Lymphocytes

Numbers of T-cells in the circulation follow daily oscillations, with the highest numbers during the behavioural rest phase and decreasing up to 40% at the peak of the active phase (43). However, individual T-cell subsets show varying migration patterns throughout the day, which is regulated by varying changes in serum concentrations of glucocorticoids and

catecholamines (43). Cortisol levels peak in the blood at the beginning of the active phase and up-regulates IL-7 receptor (IL-7R) and the chemokine receptor, CXCR4, on the surface of naïve and central memory T-cells, mediating their extravasation into the bone marrow. Conversely, numbers of circulating effector $CD8^+$ T-cells peak during the active phase at the same time as epinephrine. Effector $CD8^+$ T-cells have increased intrinsic expression of beta-2-adrenergic receptors and CXCR1, which is proposed to be the reason for the effector $CD8^+$ T-cell response to epinephrine. This subset-specific variation in trafficking is thought to provide increased immune defence during the active phase, when injury or infection is most likely to occur (43, 44).

Numbers of lymphocytes in the lymph nodes fluctuate in the opposite manner than those in the circulation. In young WT mice, migration of lymphocytes to lymph nodes peaked at the start of the active phase roughly 8 hours after peak blood lymphocyte concentrations (45, 46). BMAL1 regulates rhythmic expression of CCR7 and the sphingosine-1-phosphate receptor (SIPR1) on lymphocytes, which mediates their homing to and egress from lymph nodes respectively (45). Lymphocyte egress from lymph nodes is also regulated by adrenergic innervation through B2-adrenergic receptors (AR) (46). B2-AR-deficient mice lost the daily fluctuations of lymphocyte numbers in blood and lymph nodes due to reduced levels of norepinephrine in peripheral lymph nodes (46). It is thought that circadian oscillations in immune cells prime the immune system for stronger responses in the active phase when interaction with pathogens are most likely to occur. Retention of lymphocytes within the lymph nodes during the active phase is thought to increase the chance to encounter T-cells with their cognate antigen. These studies highlight the importance of both cell-intrinsic clocks and cell-extrinsic rhythmic signals for driving daily trafficking of lymphocytes.

T-cell recruitment is impaired in older adults, leading to a compromised adaptive immune response, increased vulnerability to infections, and weakened responses to vaccinations (reviewed in (26). Age-related dysregulation of lymphocyte recruitment has mostly been attributed to changes in expression of chemokines and adhesion molecules, and accumulation of senescent immune cells. However, very little research has investigated the contribution of diminished circadian outputs on lymphocyte trafficking in ageing.

Endothelial Cells

Leukocyte recruitment is also regulated by oscillating expression of adhesion molecules on the EC surface. Autonomic innervation *via* β-adrenoreceptors differentially regulates adhesion molecule expression in different tissues (23), resulting in a highly-tissue specific temporal expression of EC adhesion molecules. A screen of adhesion molecule expression in multiple murine organs revealed a general peak in expression of adhesion molecules on ECs at the start of the active phase, parallel to the increased leukocyte emigration from blood (47). Adoptive transfer of cells into EC-specific Bmal1-deficient mice lost the time-of-day dependent leukocyte migration out of the circulation seen in WT controls (47). Therefore, leukocyte recruitment is regulated by rhythmic expression of adhesion molecules on both the EC surface and the leukocyte surface, increasing efficacy of the leukocyte-endothelium interaction required for leukocyte rolling, adhesion, and transmigration across the endothelial barrier (**Figure 1**).

CIRCADIAN RHYTHMS AND THE AGEING IMMUNE RESPONSE

Vaccination

Older adults (>65 years) often have weaker responses to primary vaccination than younger adults, in terms of titre and immunity to infection (48, 49). Older adults are particularly susceptible to infections and are at increased risk for serious complications due to ageing-related comorbidities and increased immunosenescence (50). Therefore, vaccine optimisation is essential to limit hospitalisation and deaths due to vaccine-preventable infections in the older population. Interestingly, Suzuki et al. (46) showed that in young (8-12 week) WT mice immunisation *via* intradermal injection of a soluble antigen conjugated with chicken γ-globulin (NP-CGG) resulted in an elevated humoral response when administered at peak lymph node lymphocyte cellularity (46). Recently, this has been confirmed in humans by administration of BCG vaccines. Early morning vaccination produced a stronger adaptive immune phenotype and increased cytokine production compared with later morning and evening administration (51). The immune microenvironment present during the initiation of an adaptive response is therefore an important regulator of the overall strength of the response, and timing of vaccine administration needs to be considered when developing and researching novel vaccines (52).

Circadian Misalignment

The importance of a robust circadian rhythm for maintaining health span with increasing age is evident as chronic circadian misalignment caused by night-shift work is associated with several age-related disorders (53). Adult, WT mice subjected to chronic jet-lag by shifting light-dark conditions by an 8-hour phase advance every 4 days had significantly shorter lifespans than control mice, increased levels of senescent immune cells, and increased inflammatory cell infiltration to the liver indicating chronic inflammation (54). Another jet-lag model revealed significantly shorter lifespans of aged (27-31 months old), but not young (8-12 weeks old) C57BL/6 male mice, suggesting circadian misalignment has more severe consequences in aged animals (55). The exact mechanisms responsible for premature ageing seen in human shift-workers are multifaceted and not fully understood.

A recent study found night-shift workers had increased plasma levels of C-reactive protein compared to day workers, indicating increased systemic inflammation (56). Importantly, night-workers had slightly decreased levels of long pentraxin 3 (PTX3), a pattern recognition receptor, which positively correlates with leukocyte telomere length, a marker of biological ageing. This suggests night-workers are more susceptible to premature ageing through increased systemic inflammation and loss of protective PTX3. Therefore, people

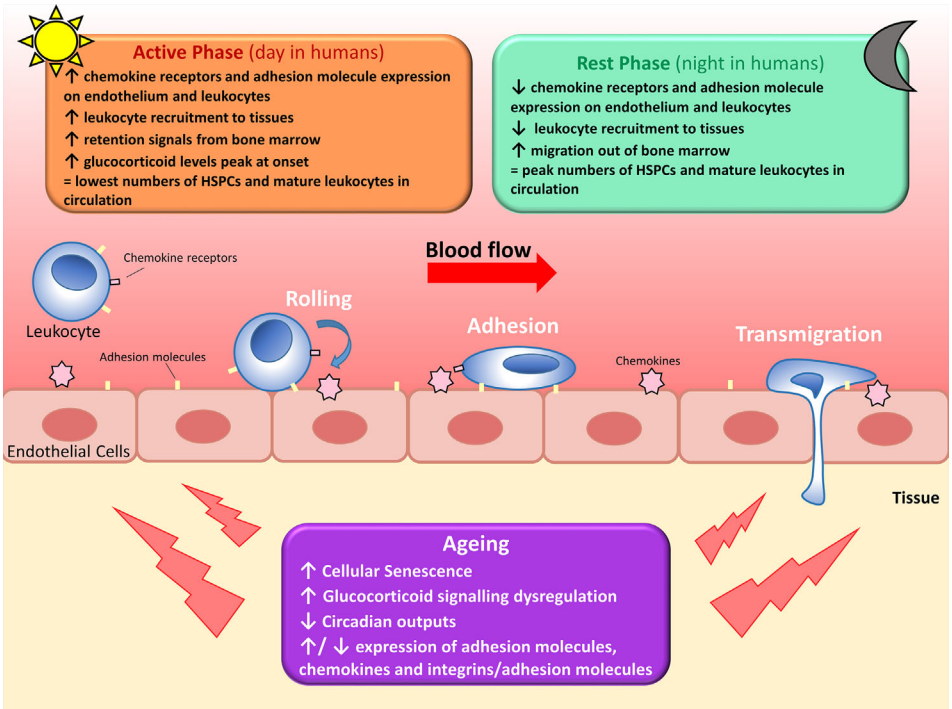

FIGURE 1 | Circadian regulation of leukocyte trafficking. Migration of leukocytes such as lymphocytes, neutrophils and monocytes, out of circulation and into surrounding tissues is regulated by circadian clocks. Hematopoietic stem cells (HSPCs) and mature leukocytes (except $CD8^+$ T cells) peak in the circulation during the rest phase as there's less migration out of the blood and increased migration of leukocytes and haematopoietic stem cells out of the bone marrow. Conversely, circulating HPSCs and mature leukocytes (except $CD8^+$ T cells) are at their lowest during the active phase due to increased leukocyte recruitment to tissues and reduced migration out of the bone marrow. Leukocyte migration is regulated by diurnal changes in expression of chemokines and adhesion molecules, and fluctuating glucocorticoid levels and adrenergic signalling. With increasing age, leukocyte trafficking becomes dysregulated due to a multitude of factors. Senescent cells accumulate and increase secretion of pro-inflammatory cytokines, glucocorticoid levels decrease and signalling becomes dysregulated, several age-associated changes occur to cytokine and adhesion molecule expression, and circadian outputs diminish. All of these contribute to dysregulated leukocyte trafficking seen in older adults.

who experience chronic circadian misalignment should consider the impact this may have on their health, and restoration of immune homeostasis may be a therapeutic target against age-related disorders in these people.

Inflammation and Rhythmicity: A Reciprocal Relationship

Inflammation itself can directly affect circadian rhythmicity. TNFα inhibits the CLOCK/BMAL1-induced activation of E-box regulatory elements in clock-controlled genes in fibroblasts *in vitro*, and in livers of mice *in vivo* (5). Other recent studies in rheumatoid synovial cells have shown TNFα suppresses PER2, while inducing expression of BMAL1 by upregulating RORα (57). Additionally, long term treatment with IFN-γ reduced the amplitude of the circadian rhythm of Per1-luc expression in individual cultured rat SCN neurons (58), and LPS injection caused transient suppression of core clock genes in male rats *in vivo*. These studies highlight the complex, reciprocal relationship between inflammation and clock genes, and support the idea increased inflammation seen in older adults may result in dysregulation of the circadian rhythm.

FUTURE DIRECTIONS AND CONCLUSIONS

Circadian rhythms play an essential role in immune homeostasis and regulate the diurnal rhythmicity seen in leukocyte trafficking under both steady state and inflammatory conditions. Inflammation itself can inhibit clock gene expression, demonstrating a complex and reciprocal relationship between the two biological systems. In older adults, there is a parallel increase in systemic inflammation and dysregulated leukocyte trafficking, and also a reduction of circadian outputs, both of which can enhance the other, therefore increasing vulnerability to disease. Despite a multitude of research into circadian systems and leukocyte trafficking, there is a need for more research into chronotherapy to optimise timing of drugs and vaccine delivery in order to improve drug efficacy, reduce side effects, and target chronic inflammation, particularly in aged individuals. Similarly, current work on vaccination has focussed on either how responses to vaccines vary with increasing age, or on how responses vary with the time of administration, but not on the two angles combined. Circadian rhythm research can also be

beneficial to the development of non-pharmacological treatment strategies. For example, diminished circadian output leads to reduced sleep quality in older adults, which subsequently dysregulates global circadian rhythmicity. Entrainment of peripheral clocks *via* regulating food intake and light exposure may help alleviate the effects of dampened circadian outputs seen in older adults, and help prevent one of the contributing factors for increased inflammation. Importantly, ageing research struggles to characterise changes that occur as a natural result of healthy ageing, versus those that are a condition of age-associated pathology, or changes in the circadian circuitry. More research into the circadian clock and inflammageing could determine if circadian rhythmicity can be used a sign of pathological ageing.

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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.

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Vascular Endothelial Galectins in Leukocyte Trafficking

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Leukocyte recruitment to the site of injury is a crucial event in the regulation of an inflammatory response. Tight regulation of interactions between the endothelium and circulating leukocytes is necessary to ensure a protective response to injury does not result in inflammatory disease. Rising interest in the broad immunoregulatory roles displayed by members of the glycan-binding galectin family suggests that these proteins could be an attractive target for therapeutic intervention, since their expression is significantly altered in disease. The focus of this review is to summarize current knowledge on the role of galectins in leukocyte trafficking during inflammation and the clinical approaches being taken to target these interactions for treatment of inflammatory disease.

Keywords: galectins, leukocyte trafficking, glycan-binding protein, endothelial cell, vascular biology

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INTRODUCTION

Glycans are one of the four major components that constitute cells, accompanied by nucleic acids, proteins and lipids. Recognition of specific glycan motifs by glycan-binding proteins (lectins) is crucial for facilitating highly sophisticated cross-communication between leukocytes in the bloodstream and endothelial cells (ECs) lining the blood vessels (1). Several key lectins (E-, L- and P-selectins and Cluster of differentiation [CD] 44) have already been identified as mediators of leukocyte recruitment and trafficking at the site of injury in the initial stages of the immune response (1). β-galactoside-binding galectins have emerged as an interesting family of glycan-binding proteins involved in the initiation and resolution stages of the inflammatory response (2, 3). Our understanding of the roles of endothelial-expressed galectins in the leukocyte trafficking cascade is relatively poor when compared to other known glycan-binding proteins involved in this process. As such, this review will provide an up-to-date overview on the role of endothelial derived galectins in leukocyte trafficking; the factors that regulate their expression and function; and discusses the therapeutic potential of targeting these interactions to treat immune-mediated chronic inflammatory disease (IMID).

Upon injury or infection, tissue resident immune cells release pro-inflammatory factors such as reactive oxygen species and proteases that partially degrade glycoprotein components of the glycocalyx; an EC-expressed matrix that serves to shield the vascular walls from direct exposure to blood components and flow (4, 5). Glycosaminoglycans (GAGs) in the reduced layer of glycocalyx bind and immobilize chemokines, accompanied by the activation of ECs and upregulation of surface expressed adhesion molecules (6). Hemodynamic forces facilitate the outward movement of leukocytes towards the venular endothelium in a process known as

margination, enabling interactions between leukocytes and newly exposed molecules displayed on the endothelium (6). Tight regulatory mechanisms are required to ensure leukocyte recruitment occurs only when appropriate, as such ECs are able to detect and respond to environmental changes such as the hemodynamic forces of blood flow (7, 8). Continuous pulsatile and laminar flow activates mechanosensitive channels and suppresses nuclear-factor kappa-B (NF-κB) *via* the MEK5/ERK5 (mitogen-activated protein kinase 5-extracellular signal-regulated kinase 5) pathway. Consequently, this prevents initiation of the inflammatory response through the downregulation of adhesion molecules and thus reduced leukocyte recruitment to the endothelium (9).

Adhesion molecules displayed on the surface of activated ECs, particularly selectins (E- and P-selectins), reduce the velocity of leukocytes rolling along the endothelium through binding to heavily glycosylated counter-receptors [PSGL-1, P-selectin glycoprotein ligand-1 (CD162)] involved in the capture and subsequent rolling events of the leukocyte adhesion cascade (**Figure 1A**) (10). This interaction induces conformational changes in integrins [MAC-1, Macrophage antigen-1 (CD11b/CD18); VLA-4, very late antigen-4 (CD49d/CD29); and LFA-1, lymphocyte function-associated antigen-1 (CD11a/CD18)] and facilitates firm adhesion of leukocytes to the endothelium by binding EC-expressed immunoglobulin superfamily adhesion molecules [ICAM-1, Intercellular Adhesion Molecule 1 (CD54) and VCAM-1, Vascular cell adhesion protein 1 (CD106)] (11). Additionally, the interaction between integrins and adhesion molecules facilitates the intraluminal crawling of leukocytes across ECs (12). Migration of leukocytes through the endothelium occurs either at endothelial junctions between ECs (paracellular) or through the body of the EC itself (transcellular). This migration of leukocytes into the surrounding tissue is facilitated by leukocyte-integrin binding to junctional adhesion molecules (JAMs) and PECAM-1 [CD31] expressed on the EC surface (**Figure 1A**) (12).

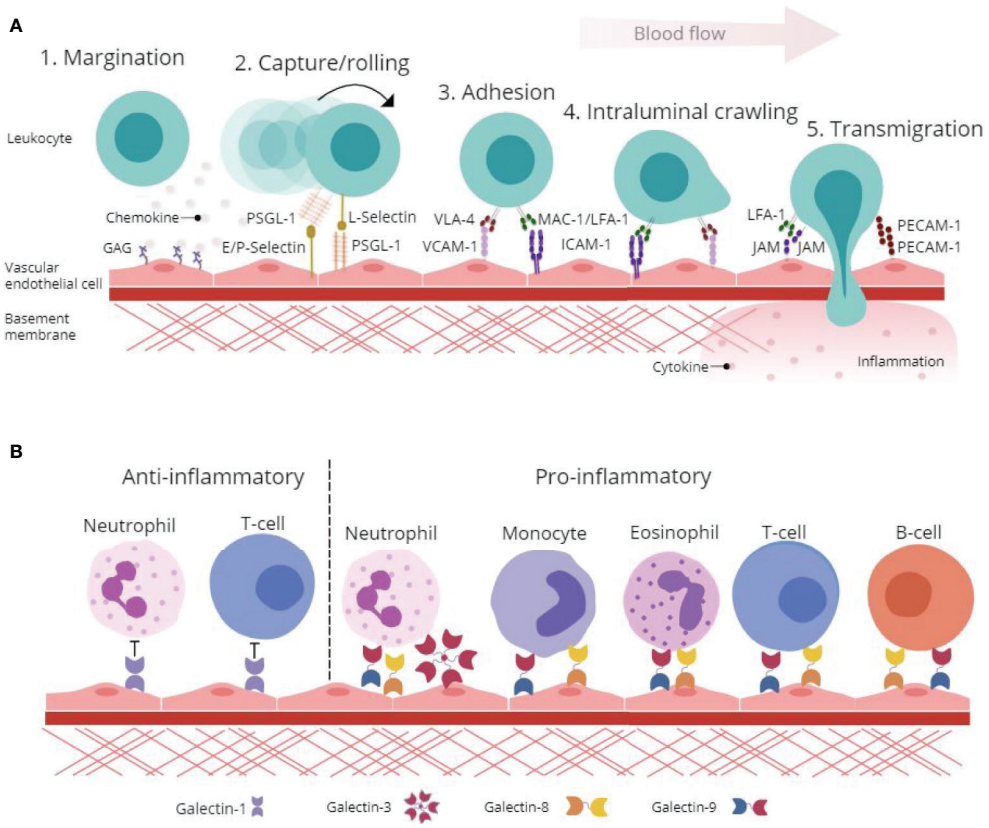

FIGURE 1 | Glycan-binding proteins regulate endothelial-leukocyte interactions in inflammation. **(A)** Protein-protein and protein-glycan interactions are crucial regulators of leukocyte-endothelial interactions involved in the leukocyte adhesion cascade. Firstly, glycosaminoglycan (GAG)-presented chemokines promote leukocyte margination to the vessel wall. Capture and rolling of leukocytes along the endothelium is facilitated by interactions between selectins and their ligands such as P-selectin glycoprotein ligand (PSGL)-1. This interaction leads to cell activation and conformational changes in leukocyte integrins including Very Late Antigen (VLA)-4, Macrophage (Mac)-1 antigen and lymphocyte function-associated antigen (LFA)-1 that then interact with adhesion molecules such as Vascular Cell Adhesion Molecule (VCAM)-1 and Intracellular Cell Adhesion Molecule (ICAM)-1 to facilitate adhesion and intraluminal crawling. Finally, transmigration through the endothelial cell (EC) layer is mediated by homophilic interactions between platelet EC adhesion molecule (PECAM)-1 and junctional adhesion molecule (JAM). **(B)** β-galactoside binding galectins facilitate and inhibit leukocyte trafficking to the endothelium to promote pro- and anti-inflammatory responses. Galectin-1 inhibits leukocyte-endothelial interactions to promote an anti-inflammatory phenotype, whilst galectin-3, -8 and -9 facilitate pro-inflammatory leukocyte trafficking.

Whilst many aspects of the leukocyte trafficking cascade have been studied in detail, a lot still remains unknown. Specifically, the galectin family of glycan-binding proteins have recently come to light as functionally important immunoregulatory proteins with involvement in leukocyte activation, apoptosis and in mediating leukocyte adhesion and migration (**Figure 1B**) (13, 14). Enhancing our understanding of the regulators of galectin expression and their roles in mediating leukocyte trafficking could uncover novel mechanisms of inflammation and highlight distinctive opportunities for the treatment of IMIDs.

GALECTIN STRUCTURE AND SIGNALING

Galectins recognize β-galactoside-containing glycan side chains on proteins and lipids *via* their unique carbohydrate recognition domains (CRDs) (15). At present, 15 members of galectins have been identified in mammals, of which 12 are associated with genes found in humans (https://www.ncbi.nlm.nih.gov/genbank/) Galectins-1, -3, -8 and -9 are amongst the most studied members since many of their functions have been closely linked to inflammation and disease (2). Importantly, murine and human galectins share approximately ~79% protein sequence homology (16), allowing their function to be studied in pre-clinical murine models. Galectins can be classified into three distinct groups; prototype, tandem repeat, and chimeric as determined by their structural properties (**Figure 2**). Oligomerization of galectin monomers is typically required for the formation of functional galectin-glycan lattices on the surface of cells (17). Additionally, and despite the absence of a classical secretory signal, galectins are released into the extracellular compartment and mediates extracellular matrix (ECM) assembly and remodeling through binding to ECM components (laminin, fibronectin and vitronectin) (18).

Key conserved amino acids in the CRDs across different galectins are responsible for the specific binding to β-galactoside-containing glycans. Despite these similarities, each galectin type reserves specificity for particular branched glycan's and glycan motifs attached to proteins and lipids alike (**Figure 2**)

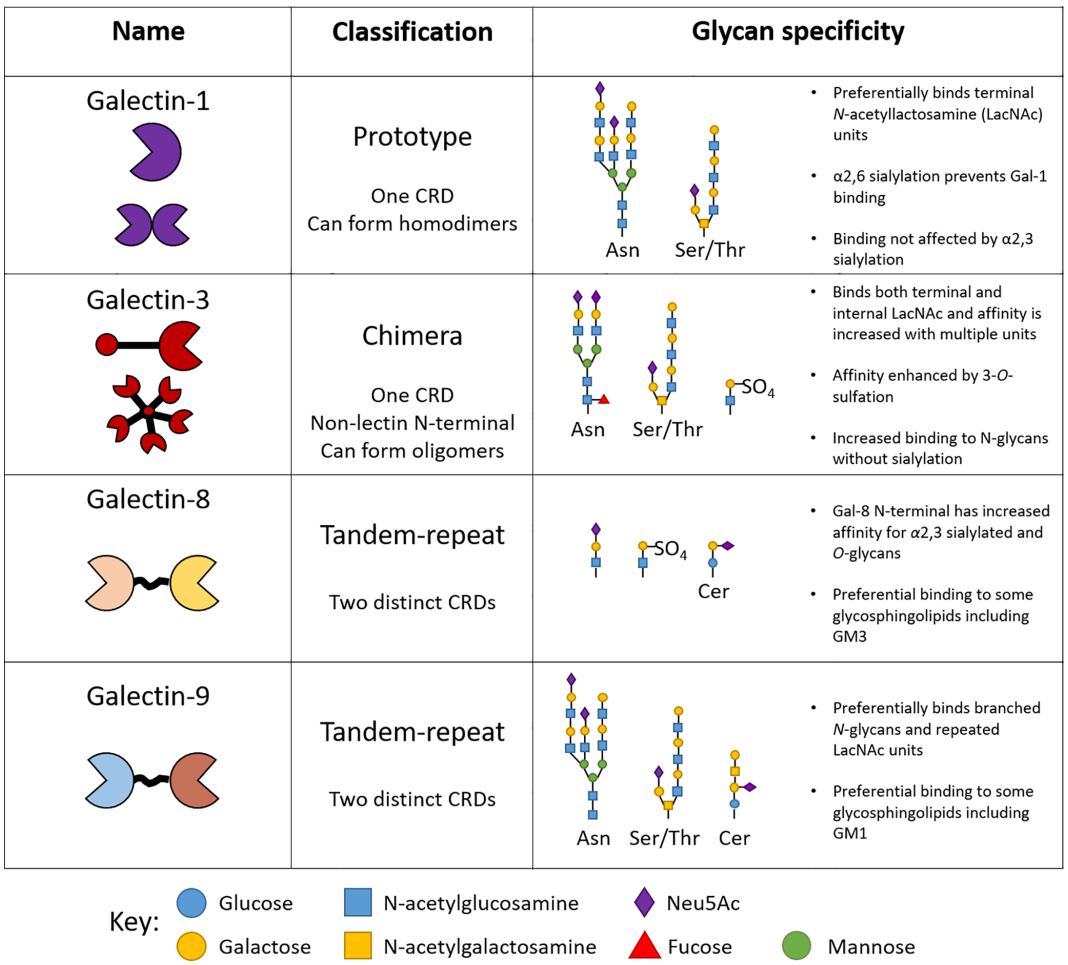

FIGURE 2 | Structure and specificity of human endothelial-expressed β-galactoside binding galectins. The classification of galectins into prototype, tandem-repeat and chimera types is based on the number of carbohydrate recognition domains (CRDs) they contain. Each galectin type reserves high specificity for certain glycan motifs.

(19). For example, the presence of α2,6-linked sialic acid prevents binding of galectin-1 to glycan chains, whilst the same motif enhances the affinity of galectin-8 for its ligand, determining important interactions regulating inflammation (**Figure 2**) (17). The structural determinants of galectin-glycan recognition have recently been reviewed in (20). The effects of glycan modification on galectin–glycan interactions has been reviewed elsewhere and are summarized in **Figure 2** (17, 21). Whether or not structural differences contribute to distinct roles for individual galectin members is yet to be fully elucidated and since galectins are expressed by many cell types it is important to consider both the exogenous and endogenous function of galectins in the context of specific cell types and their physiological interactions.

REGULATION OF GALECTIN EXPRESSION IN INFLAMMATION

To play a role in leukocyte trafficking, galectins must be present at the site of inflammation where endothelial-leukocyte interactions occur. Expression of galectin-1, -3, -8 and -9 mRNA and protein have been detected in *in vitro* cultured human macro and microvascular ECs, with expression largely intracellular (22). The release of soluble galectin-1 and -8 from human macro- or microvascular ECs respectively, has also been reported (23, 24). Immunohistochemistry (IHC) on sections of human placenta, liver, kidney and colon tissue revealed that low levels of galectin-1 were consistently detected, whilst galectin-3, -8 and -9 expression was detected at variable levels in some but not all of the tissue sections (22). Interestingly, the cellular localization of endothelial galectin expression is highly variable between the different vascular tissue beds (22, 25). Specialized high endothelial venules (HEV) present in sections of human lymph node and tonsil tissue showed higher levels of galectin-9 expression than ECs in other vascular beds (26). Conversely, galectin-3 was not detected and galectin-1 expression was relatively low in HEV from healthy human lymph node sections (25, 27). The variable expression of galectins reported in lymphoid tissues could be indicative of distinct immunoregulatory roles between the galectin types, since the HEV reside within close proximity to leukocytes within the tissue.

An additional consideration when studying ECs is that they possess a complex system of mechanosensitive proteins that respond to hemodynamic forces (28). In response to physiological blood flow, ECs maintain an atheroprotective environment by suppressing gene expression of inflammatory, pro-apoptotic and proliferative pathways *via* mechanotransduction pathways (29). The expression of integrins and adhesion molecules, including PECAM, are modulated *via* this mechanism. Perturbations in shear stress can disturb EC mechanosensitive protein signaling, contributing to the pathophysiological angiogenesis in tumor vasculature where low shear stress is experienced, and driving inflammation in atherosclerosis-prone vascular niches and flow-obstructing pathologies where shear stress is disturbed (30, 31). Deep sequencing of the transcriptome of human umbilical vein EC (HUVEC) and human aortic EC (HAEC) in response to patterns of shear stress has identified changes in expression of galectins not only in response to specific flow patterns, but also across EC type (32). Collectively, these findings suggest that expression of individual galectins is highly dependent on the tissue microenvironment and that a more in-depth comparison of the patterns of galectin expression across different vascular beds could be insightful for understanding galectin regulation and function in this context.

As is the case with many immunoregulatory proteins, galectin expression is modulated with EC activation in response to inflammatory mediators, pathogen exposure, and injury (22, 33–35). A concentration-dependent increase in expression of galectin-9 mRNA and protein was observed *in vitro* following treatment of primary HUVEC with double-stranded RNA viral mimetic, polyinosinic-polycytidylic acid [poly(I:C)], a Toll-like receptor (TLR) 3 ligand (33, 34). Similarly, through the TLR4 pathway, lipopolysaccharide (LPS) stimulation up-regulated surface expression of galectin-8 in human microsvascular EC (HMVEC), suggesting a specific role for galectins in viral and bacterial related infections (24). More basic chemical mediators involved in acute and chronic immune responses have also been shown to differentially regulate endothelial galectin expression. Surface expression of galectin-3 on HUVEC is increased in response to treatment with IL-1β (36). Interestingly, stimulation of HUVEC with IFN-β/γ did not increase expression of galectin-3 (or galectin-1), though a significant increase in galectin-9 surface expression was observed (26). More complex cocktails of soluble disease mediators, such as those released in the conditioned culture medium from colon carcinoma cell lines and in mixtures of oxidized low-density lipoproteins, a known driver of atherosclerosis and cardiovascular disease, stimulated galectin-1 translocation to the cell surface in HUVEC and HAEC (22, 25). The link between increased galectin expression and inflammation is well established, and elevated levels of galectin-3 in the serum and tissue of IMID patients is now recognized as a biomarker for detecting early stages of autoimmune and chronic inflammatory disease (37). Similarly, elevated serum levels of galectin-3 are detected in various cancers and even more so in metastatic disease, correlating with increased levels of metastasis-promoting cytokines released from vascular endothelial cells (38). The galectin expression profile within the tumor vasculature itself highlights a role for endothelial galectins in disease pathogenesis, where soluble factors secreted from tumor cells have been shown to induce galectin-1 expression and translocation to the EC surface to promote tumor angiogenesis and inhibit T-cell migration across the endothelium (22, 23, 39). The link between galectins and tumor angiogenesis, metastasis, and immune suppression could make for an attractive immunotherapy target in combination with currently available cancer therapeutics. Blockade of vascular endothelial growth factor (VEGF) is the current standard of targeted anti-angiogenic therapy and offers variable treatment efficacy dependent on the cancer type (40). Perhaps galectin-1 could be a more appropriate and effective target, even more so if used in combination with targeted therapy such as CAR-T cells to increase tumor infiltration. As has been confirmed with *in vitro* studies, galectin expression in leukocytes is also modulated in

response to stimulation with various inflammatory mediators and could be a major influence on the differential expression observed in inflamed tissue and patient serum samples (41). As such, soluble galectins released from immune cells can contribute to the differential levels of galectins observed in inflamed tissue and circulation, potentially counteracting endothelial cell-bound and expressed galectin functions. Distinguishing the specific effects of endothelial galectins on leukocyte trafficking and pathogenesis from the roles of alternative sources of galectins in the inflamed microenvironment is necessary to fully explore the potential for therapeutic intervention.

LEUKOCYTE TRAFFICKING REGULATION BY ENDOTHELIAL GALECTINS

The interaction between glycans and glycan-binding proteins is crucial at all stages of leukocyte trafficking (1) and remains an active area of research. A large proportion of the literature investigating galectins in inflammation report pro- and anti-inflammatory functions of exogenous galectins. In the absence of inflammatory stimuli, galectin-1 has been reported to promote neutrophil migration *in vitro* (42). Conversely, in the presence of acute inflammatory mediators IL-8 and TNFα, neutrophil chemotaxis was inhibited by galectin-1 (43, 44). Similarly, the transmigration of modified T-lymphocyte cell lines across stimulated HUVEC was significantly inhibited by galectin-1 compared to unstimulated HUVEC (23). Local injection of galectin-3 into the dorsal air pouch was shown to increase monocyte infiltration (45). More recently, galectin-3 was shown to directly dimerize with the chemokine CXCL-12 *via* an interface containing the GAG-binding motif, inducing anti-chemokine effects and inhibiting CXCL-12-induced migration of monocytic THP-1 cells and neutrophils *in vitro* (46). This latter finding could indicate an endothelial-specific galectin function whereby soluble galectin-3 released from ECs binds to the cell surface to mediate the interaction between leukocytes and CXCL-12. This concept is supported by the study from Yamamoto et al., which reported enhanced binding of T-cells, B-cells, neutrophils, eosinophils and monocytes to HUVEC following pre-incubation with increasing concentrations of both galectin-8 and -9 (47). Interestingly enhanced binding was not observed with galectin-1 or -3, although others have shown that oligomerization of galectin-3 at the EC surface was observed by fluorescence resonance energy transfer (FRET) and facilitated leukocyte clustering at the tricellular corners of HUVEC monolayers where leukocytes preferentially transmigrate (48). Galectin-9 was originally identified as a potent chemoattractant and activator of eosinophils (49). It has since also been shown to bind to protein disulfide isomerases on T-helper 2 cells (Th2), influencing their migration through recombinant galectin-9 coated matrigel by increasing the reduction of disulfide bonds on integrins (50). In the absence of inflammatory stimuli, galectin-9 has been shown to significantly induce monocyte chemotaxis *in vitro* compared to control (51). This finding was also supported *in vivo*, with increased monocyte and macrophage infiltrate in isolated knee tissue from mice receiving galectin-9 *via* intra-articular knee injection (51). The earlier reports strongly suggest a positive role for galectin-9 in driving Th2-type immune responses that could be contributing to the development of diseases such as allergic asthma. However, the substantial increase in galectin-9 expression in ECs following stimulation with Poly : IC and IFNγ, and the increased monocyte and macrophage infiltrate observed in response to galectin-9 injection, suggests that galectin-9 mediated immune responses may be broader than Th2-type only. Together, these findings highlight a potential role for galectins as both pro- and anti-inflammatory molecules affecting leukocyte trafficking dependent on the mediators and cell types in the local microenvironment. It is also worth noting that the glycosylation patterns of leukocyte surface molecules change upon cell activation, and as such, are worth considering when studying the interactions between glycans and glycan-binding proteins to elucidate context specific roles and functions. With only a limited amount of research on the secretion of galectins from ECs, we cannot predict that the results observed in response to exogenous galectins would translate to such function *in vivo*. Thus, further investigation is required to understand the regulators of endothelial galectin secretion and the impact of this on leukocyte migration *in vivo*.

Galectin knockout mice and knock-down studies have been invaluable for studying the endogenous functions of galectins in a more physiological context. Galectin-1 appears to exhibit anti-inflammatory functions since extravasation to inflamed cremaster tissue was enhanced in galectin-1 knockout mice compared to wildtype (44). This mirrored *in vitro* results showing neutrophil recruitment and rolling along TNF-α stimulated HUVEC was enhanced following galectin-1 knockdown in ECs (44). Similarly, endogenous galectin-1 appeared to inhibit T-lymphocyte capture, rolling and adhesion to stimulated HUVEC under physiological flow (52). Interestingly in an acute model of edema, we found that the absence of galectin-1 did not exacerbate the inflammatory response and recruitment of leukocytes as predicted. This in part was due to redundancy amongst galectins, as we demonstrated galectin-9 levels were significantly elevated in these mice and could therefore potentially compensate for the absence of galectin-1 and promote resolution (53). Galectin-3 on the other hand, has been shown to promote leukocyte recruitment *in vivo*, as impaired slow rolling and emigration of leukocytes to IL-1β stimulated cremasteric postcapillary venules of galectin-3 knockout mouse was observed by intravital microscopy (54). Interestingly, these galectin-3 null mouse ECs showed reduced surface expression of ICAM-1 and E-selectin following treatment with IL-1β and vehicle compared to wildtype, suggesting endothelial function might be impaired in the absence of endogenous galectin-3 (54). There is limited research on the roles of galectin-8 and -9 on leukocyte trafficking, especially *in vivo*. The correlation between the results from *in vivo* studies using galectin knockout mouse models and *in vitro* studies using galectin-knockdown ECs support the endogenous galectin function on leukocyte trafficking and inflammation. Despite these convincing reports, an endothelial-specific galectin knockout mouse model is necessary to explore and understand mechanistic pathways and regulators of galectin function in the endothelium. Several endothelial-specific Cre and Cre/ERT2 mouse models have successfully been used in the

vascular biology field and should be exploited to study endothelial galectin function.

THERAPEUTIC POTENTIAL OF TARGETING ENDOTHELIAL GALECTINS

The therapeutic potential of targeting galectins for the treatment of inflammatory and autoimmune diseases is currently being explored and showing promising signs of success in early clinical trials (55). Whilst the current and most developed approach is focused on inhibiting galectin-3 with complex carbohydrate mimetics, the potential benefits of administering galectin-1 to mimic its anti-inflammatory effects are also being considered as treatment options in acute myocardial infarction, ischemic stroke and autoimmune diseases (56, 57). Whilst both approaches offer a seemingly promising route to treating chronic inflammation, the systemic approach to treatment might lead to non-specific effects and reduced treatment efficacy. The use of targeted delivery to affected tissues and cells might optimize the potential benefits of drug delivery mimicking or modulating the effects of galectins *in vivo*. Whilst more remains to be understood about the regulation and contribution of endothelial galectins to leukocyte trafficking in acute and chronic inflammation, the interaction between EC-galectins and leukocytes observed *in vivo* and *in vitro* suggests specific pro- and anti- inflammatory, or even pro-resolution roles that could be manipulated for patient benefit.

CONCLUSIONS AND PERSPECTIVES

Over the last two decades, there has been a steady rise in the number of publications detailing the involvement of galectins in widespread physiological functions, including inflammation, immune responses, apoptosis, autophagy and angiogenesis. Despite a clear link between galectins and disease there still remains ambiguities around the mechanisms by which galectins contribute to pathology, particularly in a cell-specific context since galectin expression and function is diverse and complex. Thus, the challenge remains to gain mechanistic insight, particularly with regards to endothelial galectins in leukocyte trafficking and inflammation to uncover the most appropriate routes for clinical intervention in treatment of IMIDs.

The conditions under which cells and their surface expressed molecules are glycosylated is complex and differs depending on cell state of differentiation, activation and disease (58). As such, attention to the physiological context under which the function of sugar-binding proteins are studied is crucial. A recent study from the Huang group examined the glycosylation-dependent interactions of galectins with intracellular and surface expressed ligands through proximal labeling of cells with galectin-fusion proteins (59). The application of this technique to ECs exposed to inflammatory mediators and/or culture under fluid shear stress could be extremely valuable for understanding the mechanisms and regulators of galectin-glycan interactions in context.

Critically, *in vitro* culture of ECs still remains problematic. The distinct expressional changes between human EC types in response to different shear stress patterns emphasizes the importance of choosing the right models, with consideration for cell type and contextual interactions, to study endothelial-specific responses *in vitro* (32). Whilst many research groups have attempted to mimic physiological flow conditions using expensive and/or specialist equipment, the problem still remains that the complex physiological EC environment cannot be mimicked *ex vivo*. One such example of this is that cultured ECs display deficiencies in the glycocalyx, casting doubts on the reliability of using ECs *in vitro* as a method to study vascular function and role in pathology (60). With major developments in organ on chip technology, we may have greater success in elucidating the role and mechanisms of endothelial galectins in a tissue specific context (61). Finally, the imminent prospect of galectin-targeted therapeutics is encouraging, and whilst interest in galectins remains high and spread across multidisciplinary subjects our understanding of galectin function will only develop further in the coming years.

AUTHOR CONTRIBUTIONS

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REVIEW
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New Pharmacological Tools to Target Leukocyte Trafficking in Lung Disease

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Infection and inflammation of the lung results in the recruitment of non-resident immune cells, including neutrophils, eosinophils and monocytes. This swift response should ensure clearance of the threat and resolution of stimuli which drive inflammation. However, once the threat is subdued this influx of immune cells should be followed by clearance of recruited cells through apoptosis and subsequent efferocytosis, expectoration or retrograde migration back into the circulation. This cycle of cell recruitment, containment of threat and then clearance of immune cells and repair is held in exquisite balance to limit host damage. Advanced age is often associated with detrimental changes to the balance described above. Cellular functions are altered including a reduced ability to traffic accurately towards inflammation, a reduced ability to clear pathogens and sustained inflammation. These changes, seen with age, are heightened in lung disease, and most chronic and acute lung diseases are associated with an exaggerated influx of immune cells, such as neutrophils, to the airways as well as considerable inflammation. Indeed, across many lung diseases, pathogenesis and progression has been associated with the sustained presence of trafficking cells, with examples including chronic diseases such as Chronic Obstructive Pulmonary Disease and Idiopathic Pulmonary Fibrosis and acute infections such as Pneumonia and Pneumonitis. In these instances, there is evidence that dysfunctional and sustained recruitment of cells to the airways not only increases host damage but impairs the hosts ability to effectively respond to microbial invasion. Targeting leukocyte migration in these instances, to normalise cellular responses, has therapeutic promise. In this review we discuss the current evidence to support the trafficking cell as an immunotherapeutic target in lung disease, and which potential mechanisms or pathways have shown promise in early drug trials, with a focus on the neutrophil, as the quintessential trafficking immune cell.

Keywords: neutrophil (PMN), respiratory, ageing, proteinase, chemotaxis, monocyte

INTRODUCTION

The lungs, especially the alveolar network, are the area of the body where the external environment is in closest proximity to the circulating blood. The average diameter of the alveolar membrane is 0.2μm, and each minute, approximately 5L of blood and 5-8L of air (and the pollutants and microbes contained therein) pass through these organs, which have an internal surface area of 50-75 square metres. The lungs serve to enable gaseous exchange, but also need to preserve health by preventing damage caused by infections or inflammation. In health, the lungs maintain homeostasis through complex interactions between the lung microbiome (defined recently as the characteristic microbial community occupying the lungs, prone to change in time and scale and thought crucial for host function and health) (1), resident immune cells and defences and the trafficking of non-resident immune cells from the systemic circulation to the lung in the presence of more challenging inflammation, infection or injury.

Non-resident immune cells include neutrophils, eosinophils and monocytes, all of which are involved in the inflammatory process. The exact make up of both the trafficking cell type and cellular phenotype within cell types depends on the nature of the challenge, but an optimal response includes a swift and accurate recruitment of cells to the location of the injury or infection, clearance of the threat (be that pathogens or inflamed/necrotic tissue) through phagocytosis, and then resolution of inflammation *via* programmed cell death and clearance by efferocytosis or expectoration (within sputum) or retrograde migration back into the circulation (2). Phagocytosis of pathogens should lead to pathogen-killing through exposure to proteinases (especially in the case of neutrophils), bactericidal proteins or reactive oxygen species, combined and contained within phagolysosomes. This intracellular process limits host tissue exposure to injurious enzymes, but extracellular release does occur (as part of degranulation, so called 'sloppy eating' or during NETosis) and here, local tissue damage is unavoidable, although limited by the presence of anti-oxidants and anti-proteinases (3).

Pro and anti-inflammatory signals leading to immune cell recruitment and immune cell clearance are held in exquisite balance by cross talk between resident tissue and the migratory cells as the inflammatory challenge is overcome. When these processes go awry, through excessive, sustained cell recruitment, inaccurate migration, or impaired clearance; unresolved inflammation can lead to lung damage and contribute to the development of chronic lung disease. This can lead to a vicious cycle of lung damage, described first in Cole's theory of bronchiectasis [a suppurative lung disease (4)], where tissue damage leads to an increased susceptibility to infection, which leads to immune cell recruitment and degranulation, with proteinases capable of digesting all components of the extracellular matrix, which leads to increased inflammation and subsequent on-going tissue damage. There is significant interest in therapeutically breaking this cycle, potentially limiting subsequent lung damage and maintaining lung health.

Initially it was assumed that excessive immune cell recruitment to the lung was a normal, physiological response to a pathological stimulus. In this model, only the recruiting stimuli (the lung inflammation or the microbe) could be targeted to reduce cell infiltration. It was thought that targeting the trafficking immune cell would lead to immunoparesis and impair the ability to respond to subsequent infections, placing the host at risk. However, there is increasing evidence of altered and dysfunctional migrating cell behaviour in chronic and acute lung disease (5, 6), and emerging evidence that targeting leukocyte trafficking may improve these cells responses to infection while reducing absolute numbers of cells in the lungs, thus reducing the inflammatory burden. See **Figure 1** for an overview of this.

This review will discuss the current evidence to support the trafficking cell as an immunotherapeutic target in lung disease, and which potential mechanisms or pathways have shown promise in early drug trials, with a focus on the neutrophil, as the quintessential trafficking immune cell.

LEUKOCYTE TRAFFICKING FROM THE BLOOD

Pro-Migratory Signals

Inflammation within the lung parenchyma leads to the release of a milieu of cytokines and chemokines from damaged epithelial cells, as well as activated alveolar macrophages and other resident or recruited immune cells such as neutrophils and T cells. Chemokines attract leukocytes with varying affinity and capacity. They are divided into groups based on the position of their conserved cysteine residues, with the CXC and CC families the most important for inflammatory disease (7). CXCL-8 and CXCL2 are important neutrophil chemokines, acting *via* tCXCR-1 and CXCR-2 receptors (8), alongside monocyte chemoattractant protein (MCP-1) for monocytes, which acts on the CCL2 receptor, resulting in monocyte recruitment and macrophage activation (9). These topics have been extensively reviewed elsewhere (10).

Initially it was thought that there was a simple relationship between the release of Damage-associated molecular patterns (DAMPs) and leukocyte recruitment. However, the complexity of signalling cascades from inflamed tissues is increasingly recognised. As vital components of the host defence, leukocytes must sense, prioritize and integrate all of the chemotactic cues from the environment into a migration response towards damaged tissues (11). To achieve this, neutrophils express more than 30 different receptors able to sense pro-inflammatory mediators and modulate neutrophil migration (12), whereas monocytes express various receptors depending on their subset (13).

The migration of neutrophils to inflamed tissues is thought to occur in phases. Early neutrophil recruitment ("scouting" cells) respond to tissue DAMPs through the SRC family kinase LYN. DAMPs induce the production of CXC-chemokines and

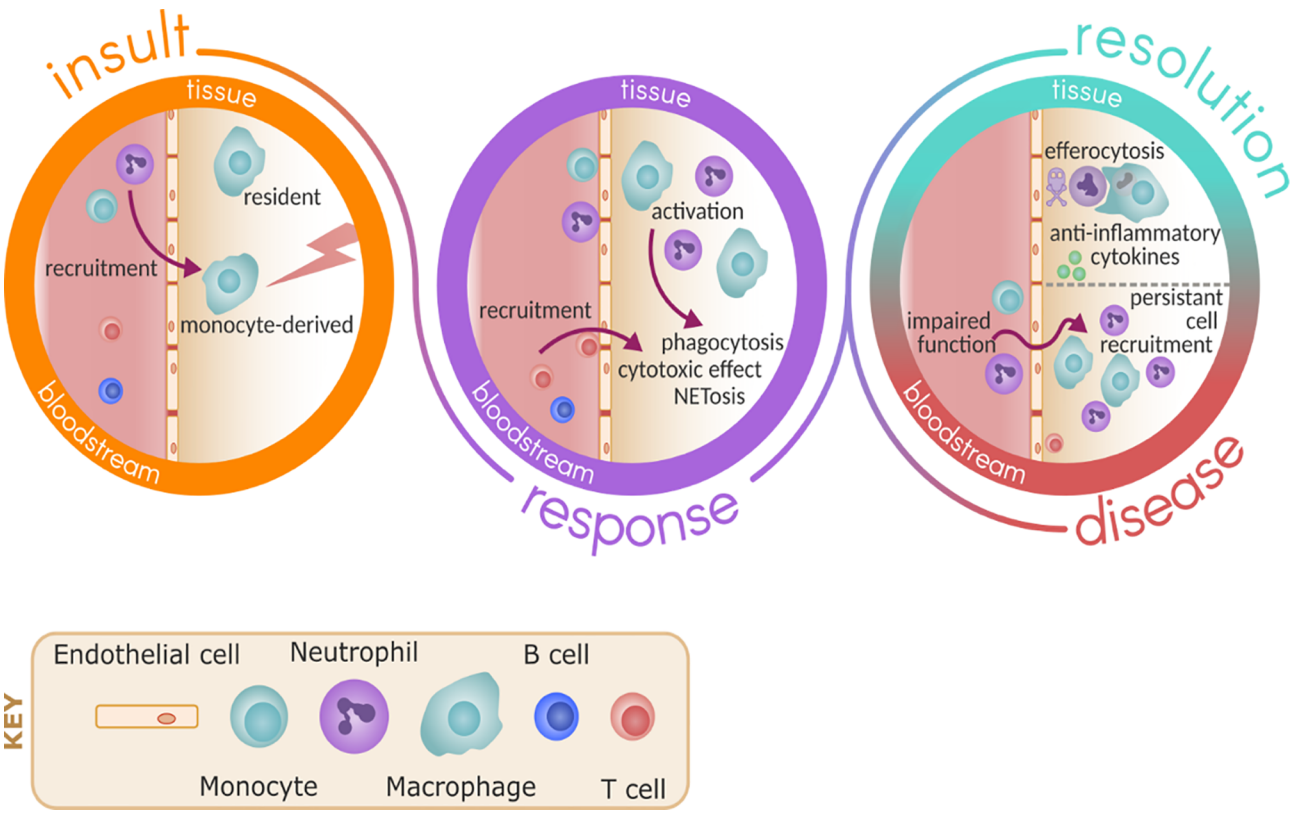

FIGURE 1 | Immune response to inflammation and infection. Upon insult, either due to pathogen or sterile injury, resident immune cells such as macrophage are ready to respond and promote the recruitment of monocytes and neutrophils *via* activation of the endothelium. As part of the response, monocytes differentiate in the tissue to macrophage and these cells become activated to respond to the insult, promoting further recruitment of other immune cells such as T cells and carrying out effector functions including phagocytosis and NETosis. In health, resolution follows by death of neutrophils and clearance by efferocytosis, promoting the release of anti-inflammatory cytokines and repair. In disease, the persistent recruitment of immune cells and potential impaired effector functions of these cells perpetuate inflammation and damage.

leukotrienes from surrounding tissues (14). Early-arriving neutrophils add to this inflammatory cocktail, as, through activation, neutrophils directly and indirectly promote further secretion of CXCL8 and leukotriene B4 (LTB4) to induce further neutrophil recruitment from the circulation. The fine control of neutrophil extravasation is seen with CXCL1 promoting crawling of neutrophils along blood endothelial cells and then CXCL2 promoting unidirectional movement across the endothelium (15). In infection, the release of pathogen-associated molecular patterns (PAMPs) and the presence of other recruited immune cells prolongs and amplifies neutrophil infiltration. There appears to be a signal hierarchy with DAMPS and cytokines such as CXCL8 forming a migratory "start" signal which can be superseded or ignored in the presence of PAMPs such as fMLP (16, 17).

Monocytes exert many of their functions outside the vascular compartment, thus requiring trafficking to tissues. Monocytes in the tissues respond to chemokines and cytokines, differentiating into macrophages or dendritic cells during infection, as well as wound-associated macrophages or tumour induced myeloid suppressor cells (18). However, monocytes can also remain undifferentiated, at least in the resting state.

The Components of Leukocyte Trafficking From the Systemic Circulation

In health, neutrophils and monocytes are released from the bone marrow in a quiescent state and maintain this in the circulation during homeostasis. They become primed in response to an initial activation signal *via* a plethora of agents, including bacterial products, cytokines and metabolic cues (19) and can then become activated whereby effector responses are deployed (20). Originally, the focus on neutrophil priming was on enhancing the ROS response (19), but is now known to also control other aspects of cell function including adhesion (21) and chemotaxis (22). Primed neutrophils also show a slower transit time through the lung vasculature, shown in patients that have inflammatory lung diseases such as COPD (23) or even low-grade inflammation (24). Priming of other immune cells such as macrophage has also been described, whereby metabolic signals such as exogenous heme or apoptotic bodies induce changes

allowing the macrophage to respond to a later pathogenic and activating signal (25).

Leukocytes are recruited to the lung from the pulmonary and bronchial circulation, including alveolar capillaries and post-capillary venules. Recruitment of leukocytes to the lungs is a complex process, tightly regulated by both leukocytes and the vascular endothelium. In the bronchial circulation, or in larger vessels, the leukocyte trafficking cascade can be broadly split into three stages: rolling, firm adhesion, and extravasation or transmigration (26). Each of these stages has distinct groups of adhesion molecules that govern the interaction of leukocytes with the endothelium (27).

Rolling

Endothelial cells within the blood vessels detect chemokines and are able to rapidly increase the expression of P-selectin (CD62P) (28, 29) and E-selectin (CD62E) (30). These two selectins can be bound by P-selectin glycoprotein ligand-1 (PSGL-1) which, despite its name, can bind all three main selectins (CD62P, E and L) (31). PSGL-1 is expressed on the surface of neutrophils and monocytes (32, 33) allowing for increased interaction between activated endothelium and passing leukocytes (31). CD62L is the only selectin expressed by neutrophils (34), but also by monocytes (35) and is maintained on the plasma membrane (36). Human neutrophils are also able to directly bind CD62E with CD62L (37). PSGL-1 is also expressed by activated endothelium (38) and is, therefore, capable of also binding to CD62L on the surface of the leukocyte. Together, the expression of selectins and PSGL-1 results in rolling – a process that occurs under shear stress in the circulation, and indeed requires shear stress to function correctly (39). These interactions provide multiple points for pharmacological intervention to either block or enhance leukocyte recruitment to sites of activated endothelium.

Extravasation/Transmigration

At the point of firm adhesion and rolling arrest, two processes can occur: crawling along the vascular lumen or transmigration into the tissue. Intraluminal crawling has been visualised *in vivo* in mice using intravital microscopy, identifying the reliance on LFA-1 for initial adhesion and Mac-1 for efficient crawling (40). Transmigration predominantly occurs paracellularly (between endothelial cell junctions) (41), however, movement through the endothelial cytoplasm, known as transcellular migration (42), has also been described *in vitro* (43).

Two major signalling pathways have been identified as of central importance neutrophil chemotaxis: PI3K and MAPK (16). Responses to intermediate chemoattractants are heavily reliant on the dual action of phosphoinositide 3-kinase (PI3K), specifically the gamma and delta isoforms in human leukocytes, at the leading edge and phosphatase and tensin homolog (PTEN) at the lagging edge (44, 45) – two enzymes that control the phosphorylation of phosphatidylinositol. In contrast, p38 MAPK co-ordinates neutrophil chemotaxis to end-point chemoattractants (46).

The process of cellular recruitment through the pulmonary vasculature is thought to occur *via* slightly different processes, dependant on adhesion receptor expression (42). At their smallest diameter, tight and tortuous pulmonary capillaries have an internal diameter of less than 2μm, significantly smaller than a neutrophil, which, in an unpolarised form, has a diameter of approximately 7μm. Despite this, *in vivo* studies have demonstrated that in health, human neutrophils are able to pass through the pulmonary capillaries with a similar speed to red blood cells (47). Furthermore, the deformation of the neutrophil in passing through these capillaries may actually provide an innate mechanical mechanism to 'de-prime' neutrophils in the circulation: neutrophils that were artificially primed ex-vivo and reintroduced to the host circulation initially increased their transit time through the lungs, but this effect was slowly lost (47). Complementing these findings, forced mechanical deformation ex-vivo of neutrophils also reversed the changes observed in primed neutrophils, suggesting a mechanism of de-priming (48). Neutrophil transit though narrow capillaries, such as those in the pulmonary vasculature, might, therefore, have important functions for immunomodulation, allowing primed neutrophils to return to the quiescent state.

Response Within the Parenchyma

Neutrophils are the first wave of leukocytes to arrive in the lungs upon infection, followed by monocytes (10, 49). To migrate through the dense and elastic extracellular matrix of the lungs, it has been suggested that neutrophils release small amounts of proteinases and then reactive oxygen species sequentially (50). Inflamed tissue tends to be hypoxic and lactataemic, conditions that promote neutrophil survival *via* a distinct signalling pathway involving hypoxia-inducible factor 1α (HIF-1) (51). In the lungs, neutrophils actively kill invading pathogens by a number of processes, including phagocytosis and by the release of antimicrobial molecules including reactive oxygen species (ROS) and neutrophil extracellular traps (NETs) (52). Once in the lungs and in response to inflammatory stimuli, monocytes differentiate into monocyte-derived macrophages (MDM) or monocyte-derived dendritic cells (MoDC) dependant on the microenvironment (53).

On resolution of inflammation, a proportion of neutrophils die by apoptosis, and many are cleared by macrophages through a process called efferocytosis (54). Apoptosis is triggered either by intrinsic loss of mitochondrial membrane integrity, causing release of cytochrome c into the cytoplasm and promoting activation of caspase 3; or by extrinsic signalling through death receptors to drive caspase 8-dependant activation of caspase 3 (55). Apoptosis triggers the externalisation of phosphatidylserine (PS), an 'eat me' signal, as well as downregulation of 'don't eat me' signals CD47 and CD31. This process can be regulated by the cell, suggesting modulation of the pathways and receptors involved may be a mechanism by which efferocytosis of excessively trafficked neutrophils could be enhanced in lung disease (54).

Other mechanisms of clearance of neutrophils and other dying immune cells from the lung include *via* the mucociliary escalator (56), whereby ciliated epithelial cells covered with a mucus layer beat synchronously to move entrapped particulates, including cells, up to the throat for removal by expectoration. In lung diseases such as COPD and IPF, there are both increases in

mucus production and expectoration and increases in the number of trafficking cells within these secretions (57, 58). Neutrophils may also leave the site of inflammation through a process known as reverse transmigration, whereby neutrophils migrate across the endothelium and re-enter the vasculature (59). Although not yet fully characterised this, and integrins which are needed for this activity, may be another therapeutic target.

Neutrophil phagocytosis occurs through direct interactions between bacteria and immune cells ("unopsonised phagocytosis") but is more efficient when bacteria are coated with immunoglobulins and complement ("opsonised phagocytosis"). Optimal opsonisation requires both immunoglobulins and complement (60). However, unopposed neutrophil elastase can impede both immunoglobulin and complement activity, cleaving the hinge region of IgA and complement C3bi, forming a functional opsonin mismatch (61, 62) which may be important in predisposing the host to secondary lung infections in chronic illness.

Neutrophils are not the only cells implicated in these processes. Monocytes are able to phagocytosis to a small extent, but they are also key modulators of the immune response through inflammatory mediator release. In response to inflammatory stimuli, monocytes are induced to differentiate into MDM through high levels of GM-CSF in the lungs, which is elevated during inflammation (63). MDM add to the pool of local alveolar macrophages, and contribute to high levels of phagocytosis of bacteria, inflammatory mediator release and, on resolution of inflammation, efferocytosis of dying neutrophils and epithelial cells, to ensure safe clearance of these dying cells (10, 54). MoDC also supplement the local pool of dendritic cells, to take up infectious agents, process and present antigen on the cell surface, followed by migration to the lymph nodes to activate T cells and the adaptive immune response (64).

The containment of the inflammatory signal to where it is needed, for only as long as needed is especially important in lung tissue. The lungs rely on their elastic properties to maintain adequate ventilation. Elastic fibres are highly complex matrix structures because of their size, molecular complexity, and the requirement for numerous helper proteins to facilitate fibre assembly (65). Previous studies have conclusively shown that elastin degradation caused by neutrophil proteinases is a key step in the pathogenesis of many chronic lung diseases and that lung cells are unable to repair damaged elastic fibres, leading to permanently compromised lung function and ongoing degenerative disease (66).

CHANGES WITH AGE AND IN LUNG DISEASE

Alterations in the innate immune response have been identified in lung diseases including COPD and IPF, but it is important to note that most lung diseases are more common with advancing age, and there are changes to both the structure and function of the lung and immune cells (including neutrophil) responses with age, which might influence cellular trafficking. This has identified a number of processes that could be targeted for treatment.

Ageing

Increased age is associated with both elevated rates of infections and chronic lung disease, as well as worse outcomes after illness or injury. In the UK, 95% deaths from pneumonia (67) and 86% of deaths from influenzae (68) were in those over 65 years of age. During the COVID-19 pandemic, 73% of deaths recorded so far occurred in those aged 75 or over (69). Over 90% of those with COPD (70), 75% of those with IPF (71) and 90% of those with bronchiectasis (67) are aged over 65 years of age. This elevated risk is likely due to a number of factors. There are age-associated changes to the lung structure and function. These include a less compliant thoracic cage; a weaker diaphragm; less elastic lung parenchyma leading to senile emphysema; reduced efficiency of the muco-ciliary escalator reducing the clearance of bacteria and microparticles from the lung as well as a reduced ability to maintain homeostasis (including reduced responsiveness to hypoxia and hypercapnia) (72). Ageing is associated with chronic low grade inflammation characterised by increased basal levels of cytokines including Interleukin (IL)-1, IL-6 and tumour necrosis factor (TNF)-α (73). The function of the immune system can also alter with age, termed immunosenesence, with impaired innate and adaptive immune responses to infections and inflammation, and this includes alterations in most neutrophil cellular functions.

Neutrophils show a gradual decline in the accuracy of migration (chemotaxis) with increasing age, although chemokinesis, or the ability to move in any direction, appears unaltered (74, 75). Imprecise migration is thought to have significant consequences, leading to both a delay in reaching the site of inflammation, but also contributing to inflammation, as these cells appear to release both proteinases and reactive oxygen species during their convoluted migratory pathways. The deficit is associated with frailty, with adults displaying more pronounced frailty having the most impaired neutrophil responses (76). *In vitro* studies have suggested impaired migration can be restored to levels which reflect those of a younger adult by inhibiting PI3K, especially gamma and delta isoforms, indicating involvement of this pathway (77, 78). As well as alteration to migration, neutrophils that are recruited to the aged lungs show suboptimal superoxide generation and degranulation, and reduced phagocytosis (79, 80). The cause of these changes is unclear, but *in vitro* work suggests that merely exposing neutrophils to the inflammation found with age (by incubating cells from young adults with plasma or serum from older adults) is insufficient to reproduce the cellular phenotype, suggesting the altered functions are not merely a consequence of the inflammatory environment (76, 77).

Once again, these age-related changes are not only seen in neutrophils. Monocytes also show changes during ageing, with levels of intermediate (CD14++CD16+) and non-classical (CD14+CD16++) monocytes increased compared to younger adults. These cells also show impaired phagocytosis, altered cytokine release and elevated expression of migration marker CD11b (81, 82). On stimulation, aged monocytes produce less inflammatory cytokines including IL-1β, TNFα, IL-6 and IFNα (83–85) which may contribute to susceptibility to respiratory infection. As with neutrophils, there is a link with frailty and increased monocyte number, however it is as of yet unclear how

this relates to cell function (86). Mitochondria in aged classical monocytes have reduced membrane potential compared to young monocytes, which may impair cell function due to impaired energy generation (87). Further analysis of metabolic effects of aging on monocyte function may reveal novel insights about the role of these cells in normal aging.

Neutrophil Trafficking in Lung Disease

There is evidence that the detrimental changes to cellular function seen with age are heightened in lung disease and indeed most chronic and acute lung diseases are associated with an influx of neutrophils to the airways as well as neutrophilic inflammation. Hypoxia is another feature of lung disease, with the potential to alter neutrophil responses further, as described above. Indeed, across many lung diseases, pathogenesis and progression has been associated with an exaggerated and sustained presence of trafficking cells, with specific examples discussed below.

Chronic Obstructive Pulmonary Disease

COPD is a common, debilitating and chronic disease thought to affect 10% of the adult population. It is currently the fourth leading cause of death globally and defined by persistent respiratory symptoms and airflow limitation which is associated with airways inflammation (88). The disease is often complicated by acute worsening of symptoms, termed exacerbations and commonly caused by viral or bacterial infections (89). Innate immune cells are considered to be key drivers of COPD. COPD is associated with greatly increased numbers of neutrophils in lung secretions, having been recruited from the systemic circulation into the airways due to epithelial damage, inflammation and infection (2). Both the lung tissue and secretions contain elevated numbers of macrophages (20x) as a result of elevated influx of monocytes which differentiate into monocyte derived macrophages (90–92).

Alveolar macrophages are likely to be a key driver of elevated leukocyte recruitment to the lungs during COPD, with COPD sputum and bronchi-alveolar lavage (BAL) containing elevated levels of CXCL-8 (93), LTB4, growth-related oncogene (GRO) alpha (94, 95), and MCP-1 (96), amongst other inflammatory agents.

Despite this high number of neutrophils and macrophages in the lungs or airways of patients with COPD, patients suffer with recurrent infections which suggests these cells are dysfunctional (53). In keeping with this, neutrophils from patients with COPD from mild to severe disease have been shown to have an increased speed of migration but also a reduced accuracy of migration towards single chemokines, bacterial products and sputum, associated with reduced pseudopod extension but correctable with PI3K inhibition (97). Of note, similar characteristics were seen in smokers aged between 30 and 40 years of age with respiratory symptoms including chronic bronchitis but no airflow obstruction (98), suggesting altered cellular functions are an early manifestation of disease. Once established the airways inflammation and altered cellular functions appear to persist even after smoking cessation, with heighted cell trafficking to the lungs seen even many years after the patients have stopped smoking (99).

Monocytes from COPD patients show enhanced migration to chemoattractant, which may contribute to enhanced levels of MDM in the COPD lung (100). These monocytes display a heightened pro-inflammatory phenotype, including elevated IL-6 and MCP-1 release (101), but do not show impaired phagocytosis compared to AMC (102). Monocyte-derived macrophage phagocytosis is impaired in COPD (102, 103), alongside impaired mitochondrial function (104) which implicates defective monocytes as pre-cursers to these cells.

Idiopathic Pulmonary Fibrosis

Idiopathic pulmonary fibrosis (IPF) is a progressive condition believed to arise in genetically susceptible individuals as a consequence of an aberrant wound-healing response following repetitive alveolar injury. It is characterised by progressive deposition of extracellular matrix and collagen within the interstitium of the lung, leading to impaired gas exchange, breathlessness and eventually death. The involvement of leukocytes is acknowledged but remains unclear. CXCL-8 levels are elevated in IPF, with BAL neutrophilia a risk factor for early death. Neutrophil elastase damages epithelial cells, and it has been hypothesised that this damage and the subsequent release of DAMPS drives ECM component turnover. Indeed, NE deficient mice are resistant to bleomycin induced PF, however the role in humans is still unclear (105) just as it is unclear what drives the neutrophil recruitment to the lungs in the first instance.

Recently, a role for N-formyl peptide (fMLF) receptors (FPRs) has been described, which might be specific for lung fibrosis. FPR-1–deficient (fpr1−/−) mice are protected from bleomycin-induced pulmonary fibrosis but can develop renal and hepatic fibrosis as normal with the model utilised (106). It is known that infections can drive IPF progression, so potentially the neutrophils may have initially been recruited in response to an infective event, with subsequent recruitment reflecting the abnormal response to wound repair (107). However, the initiating driver of recruitment might reflect other stimuli, as an increasing number of alternative, non–formyl peptide ligands for FPR-1 are being uncovered. Monocytes and macrophages may play a role in disease pathogenesis, although this is yet to be fully determined. Depletion of murine LyC6 monocytes reduces both alveolar macrophages and fibrosis in mice (108), while in humans, an association has been described between monocyte numbers and survival in IPF, with a high monocyte count linked to poorer outcomes (109).

Community Acquired Pneumonia

It is not just chronic disease where altered leukocyte functions, including neutrophil migration, are associated with poor outcomes. During Community Acquired Pneumonia (CAP), neutrophils are recruited to the airways in high numbers, with the alveolar spaces becoming filled with an exudate made up of inflammatory cytokines, immune cells and systemic proteins leading to hypoxia and ventilation/perfusion mismatch. Neutrophil functions have been shown to be impaired in CAP, with reduced migratory accuracy but increased degranulation and NETosis (110). Of note, in older adults, the defect appears

sustained and to worsen with the severity of the infectious event, with less dysfunction in simple lower respiratory tract infections and most dysfunction when CAP is associated with sepsis (111). In this instance, dysfunction could be replicated by exposure to plasma from septic patients (110), suggesting the inflammatory systemic environment adds to the cellular dysfunction. This CAP neutrophil phenotype again appears correctable, with correction associated with improved patient outcomes (112), highlighting the potential benefit of targeting these cells.

CURRENT STRATEGIES TO TARGET LEUKOCYTE TRAFFICKING IN LUNG DISEASES

The wealth of evidence describing the negative associations with lung disease and excessive or sustained leukocyte influx into the lungs highlights the need to target these processes, without compromising the hosts' ability to respond to infections. A plethora of drugs targeting leukocyte trafficking have been developed, however, many to date have failed to make it to market for respiratory diseases. **Table 1** describes the potential targets and therapies developed, but key examples are provided below and **Figure 2** provides an overview of potential mechanisms.

Targeting Priming Agents, Chemokines or Their Receptors

CXCR2 Inhibitors

CXCR2 is a major neutrophil and monocyte chemokine receptor, responsible for controlling migration towards ligands such as CXCL8. Inhibition of CXCR2 signalling is, therefore, an attractive target to dampen recruitment to CXCL8-rich tissue. The first report of a selective CXCR2 antagonist demonstrated reduced neutrophil migration to CXCL8 both *in vitro* using human neutrophils and *in vivo* blockade of neutrophil margination within rabbits (120). Several studies confirmed that blockade of CXCR2 reduced neutrophilic inflammation including in cigarette smoke-exposed rats (121); in an acute lung injury model in mice (122) and in an LPS airway challenge model in guinea pigs (123). Despite broad evidence from *in vivo* and *in vitro* models, clinical trials using CXCR2 antagonists provided a mixed picture.

The CXCR2 antagonist MK-7123 was used at various doses in a small phase 2 study including 616 patients with COPD, reporting that the highest dose of MK-7123 was able to improve FEV_1 and increase the time to exacerbation, indicating a clinical benefit to patients. However, reductions in absolute neutrophil counts led to withdrawal of 18% of patients for safety reasons and there was also a significant increase in the inflammatory marker, C-Reactive protein (CRP) (124).

In a clinical trial of danirixin, another CXCR2 inhibitor, initial studies suggested clinical benefit in COPD (116). A subsequent larger trial (including 614 COPD patients) (116) found no significant clinical benefit in respiratory symptoms but significantly exacerbations and pneumonia events in the highest dose group, suggesting impairments in host responses to infection.

Targeting Other Chemokines and Their Receptors

LTB_4 is a potent and proinflammatory chemoattractant, synthesised by neutrophils following the enzymatic conversion of arachidonic acid and facilitated by 5-lipoxygenase activating protein (FLAP). A study by Crooks et al. showed an increased concentration of LTB_4 at presentation of infective exacerbation, compared to resolution of exacerbation, in a cohort of chronic bronchitis patients. Moreover, this finding coincided with an increase in sputum chemotactic and MPO activity, suggesting the role of LTB_4 in bronchial inflammation (125).

Blockade of LTB_4 was investigated in a phase II, randomised and placebo-controlled trial in a small cohort (n=17) of stable COPD patients (115). Participants were randomised to receive BAYx1005, an antagonist against FLAP, or placebo, for 14 days. Follow-up spontaneous sputum collection (day 14) revealed significant reduction of LTB_4, compared to baseline, in the treated group. Although this reduction did not show complete suppression of LTB_4, the observed change was similar to that observed at resolution of an exacerbation of chronic bronchitis. Hence, this trial suggested

TABLE 1 | Therapeutic agents that target leukocyte function and their clinical trial results.

Category	Target (therapeutic agent)	Cohort	Outcome	Reference
Priming agent	TNF-α (Infliximab)	n=234 stable COPD, randomised	No therapeutic benefit	Rennard et al. (113)
	TNF-α (Etanercept)	n=81 AECOPD, randomised	No therapeutic benefit vs prednisone	Aaron et al. (114)
Migratory stimuli	LTB_4 (BAYx1005)	n=17 stable COPD	Non-significant reduction in bronchial inflammation	Gompertz and Stockley (115)
Migratory receptors (PMNs)	CXCR2 (Danirixin)	N=614 symptomatic COPD, randomised	No therapeutic benefit, increased exacerbations in treated groups	Lazaar et al. (116)
Proteinases	Neutrophil elastase (Alvestat)	N=615 stable COPD, randomised	No clinical benefit	Kuna et al. (117)
	Alvestat	N=38 bronchiectasis, randomised	Improved FEV1	Stockley et al. (118)
Migratory pathways	PI3K (Idelalisib)	N=5 lymphoma/leukaemia patients	Impaired neutrophil functionality	Alflen et al. (119)
	Statins	N=62 CAP+S	Improved neutrophil chemotaxis	Sapey et al. (112)

A non-exhaustive list of the targets identified for leukocyte trafficking in lung disease, and the initial results of clinical studies.

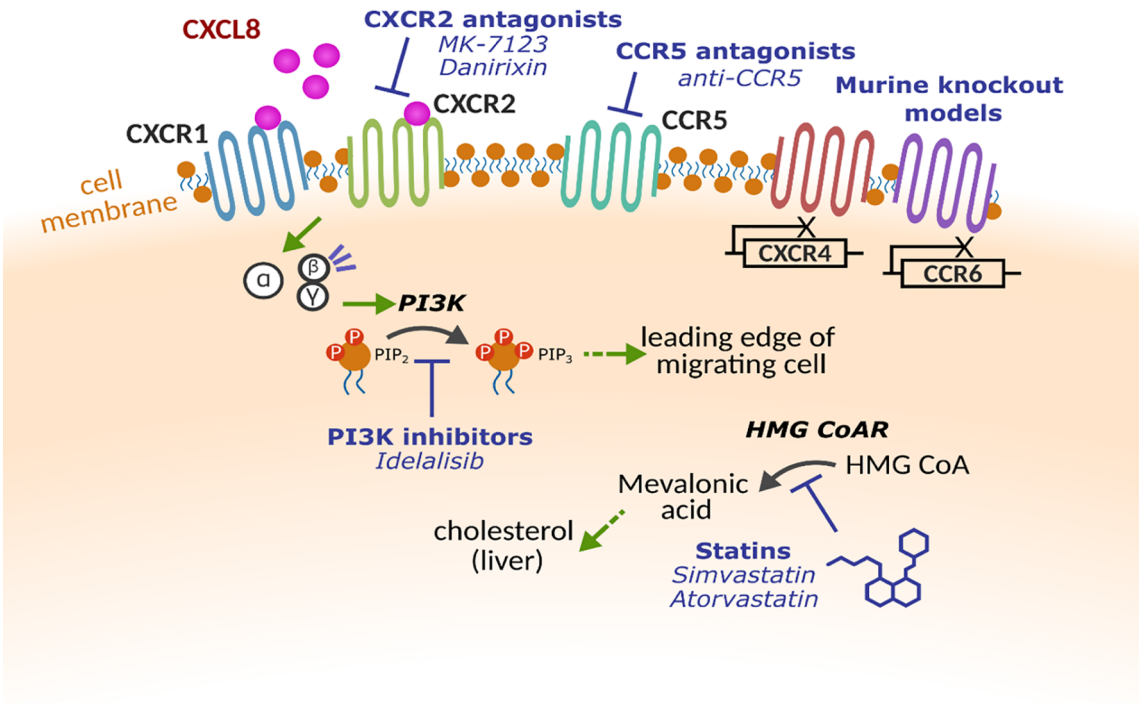

FIGURE 2 | Molecular targets for altering leukocyte trafficking. Multiple receptors and proteins have been targeted to alter leukocyte trafficking. Chemokine receptors CXCR1, CXCR2, CXCR4 CCR5 and CCR6 have all been investigated either using pharmacological intervention or in early studies with gene knockout models. Within the cell, key enzymes such as phosphoinositide 3-kinase (PI3K) and β-Hydroxy β-methylglutaryl-CoA reductase (HMG CoAR) that have been implicated in cell motility.

the efficacy of LTB_4-blockade for the reduction of neutrophil-associated bronchial inflammation in patients with chronic lung disease.

Animal models of COPD have begun testing other chemokine receptor antagonists to identify promising candidates. *CCR6* and *CXCR3* knockout mice displayed reduced lung inflammation and evidence of protection against emphysema, when exposed to cigarette smoke (126, 127). Treatment with anti-CCR5 in an emphysema mouse model resulted in the reduction of apoptosis, DNA injury and alveolar remodelling with a subsequent reduction in lung inflammation (128). Whether there are any benefits of targeting these receptors in human disease remains to be tested.

Priming Agents

TNF-α is a priming agent for neutrophils, inducing their expression of β_2 integrins and augmenting cell migration (129). TNF-α-induced degranulation, release of reactive oxygen intermediates and phagocytosis gives rise to local and systemic inflammatory responses (130). Given its pro-inflammatory consequences, studies suggest its role as a primary mediator of inflammation in COPD disease pathogenesis (131). Inhibition of TNF-α in COPD was investigated by Rennard and colleagues who conducted a randomised, placebo-controlled trial to assess the efficacy of TNF-α antagonism in moderate to severe COPD patients (113). A total of 157 patients were randomised to receive Infliximab, an anti-TNF- α antibody. No benefit was observed in the treated groups compared to placebo, in terms of changes in health status, lung function or exacerbation frequency. A later trial, using an alternative TNF-α-antagonist, sought to determine the efficacy of anti-TNF-α for the reduction of inflammation in a cohort of exacerbating COPD patients (114) with no clinical benefits.

Targeting Intracellular Processes

PI3K

The PI3K pathway, activated by binding of ligands to G-protein coupled receptors, or tyrosine kinase receptors on the cell surface, is implicated in numerous leukocyte functions (132). Downstream effectors of PI3K activation include protein kinases that regulate cell motility and membrane trafficking, scaffolding proteins and other signalling processes (133). Neutrophils, monocytes, macrophages and T cells have all been show to require PI3K for chemotaxis, but also for phagocytosis through similar mechanisms of actin remodelling (134). *In vitro* experiments from neutrophils from older adults and COPD patients showed a relationship between inaccurate neutrophil migration and increased PI3K signalling, and that inhibition of PI3Kγ or δ restored accuracy (77). Further *in vitro* experiments using idelalisib, a PI3K inhibitor used for non-Hodgkin lymphoma, showed that after TREM-1 ligation, idelalisib reduced L-selectin shedding, oxidative burst, degranulation and

cytokine release in neutrophils (119). The reduction in all key neutrophil functions has led to some concerns about the potential safety of this therapy, with both the potential to normalise and neutralise neutrophil responses. In recognition of this, studies in COPD have used inhaled PI3K inhibitor therapies in the first instance, limiting systemic exposure. First reports suggest signals of clinical benefit (135), but wider trials are needed across all chronic lung diseases.

Repurposing Statins

Statins, or 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG CoA) Reductase Inhibitors are primarily used to treat dyslipidaemia. However, their association with reductions in all-cause mortality has led to further exploration of their anti-inflammatory and immunomodulatory properties. Several randomised controlled trials have demonstrated the reduction of systemic inflammation in cohorts treated with statins; a reduction in inflammatory biomarkers, namely high-sensitivity CRP (hsCRP) and IL-6, were observed in those treated with atorvastatin (136). Similarly, a downregulation of IL-8 and immune cell activation was seen in HIV patients treated with pitavastatin (137). Observational studies suggested that statins were associated with a reduction in mortality from pneumonia and influenza, despite patients taking these therapies being older and having more co-morbidities (138). Clinical trials have explored this further (139) and older patients with community acquired pneumonia and sepsis receiving 80mg simvastatin demonstrated improved neutrophil chemotaxis and reduced systemic neutrophil proteinase burden, as well as improving hospitalisation-free survival compared to placebo (140). Unfortunately, this benefit was not replicated in trials of statins in Acute Respiratory Distress Syndrome (ARDS) (for example (141),) leading to statins falling out of favour as an adjunct treatment. However, when patients were sub-stratified into those with the highest burden of inflammation, those with the most inflammation gained most benefit from a statin intervention (142), suggesting the need for careful patient selection.

In COPD the burden of inflammation rarely meets that seen in pneumonia or ARDS, however it was recently shown that neutrophils isolated from COPD patients, when incubated with simvastatin, improved their migratory dynamics towards CXCL8 and fMLP, to levels similar to aged matched healthy controls (143), indicating a potential benefit of statins directly on leukocyte migration. Meta-analyses have suggested that statins reduce not only cardiovascular risk in COPD, but also acute exacerbations and CRP (144) although this finding has not been universally (145), suggesting further studies are needed.

Targeting Neutrophil Proteinases

Neutrophil proteinases represent a promising target in chronic respiratory diseases, including COPD, AATD and IPF, as proteinases have been shown to be important in trafficking processes. There are a number of neutrophil elastase inhibitors under development (146).

In AATD, the clear association between neutrophil proteinases and lung disease has led to the use of augmentation therapy of infused AAT. This therapy is already licensed for use in some countries within Europe and the USA, but only for limited indications in the UK. Studies such as the RAPID trial (167 patients, placebo controlled) have demonstrated a reduction in the decline of lung function (147) and smaller studies have highlighted the positive impact of augmentation on neutrophilic inflammation (148). However, not all patients respond, and there is now interest in determining who gains the most benefit, for example by identifying and focusing on those with the fastest decline in lung function (149).

Alvestat (AZD9668) is a selective NE inhibitor with oral availability. In randomised control trials of COPD patients, 12 weeks of treatment with AZD9668 showed no positive effect on exacerbation frequency, symptoms, lung function or inflammatory biomarkers, but with 300 participants on active treatment, the study was likely underpowered for these heterogeneous outcome measures (117). Alvestat has also been studied in bronchiectasis, where 4 weeks of treatment improved FEV_1, highlighting a potential signal of benefit (118). More recently, a trial of Brensocatib (an oral reversible inhibitor of dipeptidyl peptidase 1 (DPP-1), an enzyme responsible for the activation of neutrophil serine proteases) showed a reduction sputum neutrophil proteinases and improvements in clinical outcomes in bronchiectasis (150). This has renewed interest in anti-proteinase therapies, with many more trials in development or actively recruiting.

THE CHALLENGES OF TREATMENT EFFICACY

Despite a strong rationale for targeting recruited immune cells, results of many trials have been negative. This might reflect the heterogeneity of the disease or population under study, a lack of stratification of the patient population, the wrong dose, modality or timing of the intervention.

Inflammation is very heterogeneous both within individuals and between individuals (95) and some studies may be underpowered to see changes in the biomarker they are assessing. Disease heterogeneity is also considerable. For example, COPD is an umbrella term for multiple pathologies and the resulting patient population can be very diverse. Attempting to treat all patients with the same therapy may hide the positive impact the treatment is having on some, due to a lack of effect in others. An example of both of these processes is that studies have highlighted a proportion of COPD patients with a polymorphism in the TNFα receptor, who experience an increased decline in FEV_1, low body weight and altered sputum neutrophil recruitment which could be reduced with TNFα antibody (151). Potentially a lack of efficacy of TNFα in COPD trials might reflect a recruited population which has not been enriched for patients with this polymorphism. Other patient characteristics may also influence trial effectiveness. These include smoking status (as smoking retains the pro-inflammatory insult that triggered the disease initially), frequency of exacerbation, and the rate of lung function decline. The biology behind these differences in patient phenotype needs to be understood to allow new targets to be developed or repurposed therapies to be focused.

There have also been inconsistencies in the drug, dose and modality of therapies used in clinical trials. For example, the variable

results from clinical trials of statins in chronic and acute lung disease might reflect differences in doses (with the greatest effects on cellular function seen at high dose), the population chosen (with most beneficial effects seen in older adults) and the timing of the intervention (with trials focusing earlier in the inflammatory journey having greater impact than those based within the Intensive Care Unit) (152). A more developed understanding will be needed across all these variables before the full impact of immunomodulatory targets can be harnessed for patient benefit.

CONCLUSION

Leukocyte trafficking represents a promising target for the treatment of acute and chronic respiratory disease. These novel treatments could target the pathophysiology of disease, and so may provide significant impact for patients. However, often the complexity of immune cell trafficking and function and the heterogeneity of both patients and the respiratory disease have been poorly considered, with a "one size fits all" approach deployed in clinical trials. Our increased understanding of physiological and pathological immune cell responses provides an opportunity to rethink clinical trials in this space. Recent studies have shown more promise when targeting trafficking cells, and the learning from these studies have led to the expectation of a raft of new immunomodulatory therapies for lung disease in the near future.

AUTHOR CONTRIBUTIONS

KB, MH, DS, EW, and ES wrote the first version of the manuscript. ES finalised content. All authors contributed to the article and approved the submitted version.

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REVIEW
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PI3K in T Cell Adhesion and Trafficking

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PI3K signalling is required for activation, differentiation, and trafficking of T cells. PI3Kδ, the dominant PI3K isoform in T cells, has been extensively characterised using PI3Kδ mutant mouse models and PI3K inhibitors. Furthermore, characterisation of patients with Activated PI3K Delta Syndrome (APDS) and mouse models with hyperactive PI3Kδ have shed light on how increased PI3Kδ activity affects T cell functions. An important function of PI3Kδ is that it acts downstream of TCR stimulation to activate the major T cell integrin, LFA-1, which controls transendothelial migration of T cells as well as their interaction with antigen-presenting cells. PI3Kδ also suppresses the cell surface expression of CD62L and CCR7 which controls the migration of T cells across high endothelial venules in the lymph nodes and S1PR1 which controls lymph node egress. Therefore, PI3Kδ can control both entry and exit of T cells from lymph nodes as well as the recruitment to and retention of T cells within inflamed tissues. This review will focus on the regulation of adhesion receptors by PI3Kδ and how this contributes to T cell trafficking and localisation. These findings are relevant for our understanding of how PI3Kδ inhibitors may affect T cell redistribution and function.

Keywords: PI3K, integrin, LFA-1, CD62L, CCR7, adhesion, trafficking

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INTRODUCTION

PI3K signalling controls numerous pathways that are involved in regulating trafficking and localisation of T cells between lymphoid system and organs, and tissues through the circulatory and lymphatic systems. During the process of T cell migration, integrins are crucial mediators of adhesion and are extended to an open high-affinity conformation following stimulation of chemokine receptors and/or T cell receptor stimulation. The major integrin expressed on T cells is Leukocyte Function-associated Antigen 1 (LFA-1), which is expressed on all subsets of T cells as well as other leukocytes, including B cells and neutrophils. LFA-1 mediates T cell transendothelial migration as well as formation of a stable immunological synapse with antigen presenting cells (APC). The mechanistic regulation of LFA-1 affinity has been extensively studied since its discovery in 1981 as a target for monoclonal antibodies inhibiting cytotoxic T cell-mediated killing (1–3), yet many questions remain about its precise regulation and function. Besides LFA-1, several other adhesion molecules are involved in T cell migration, including L-selectin (CD62L) found on naïve T cell subsets and on central memory T (T_{CM}) cells. CD62L binds ligands such as GlyCAM-1 and

CD34 expressed on endothelial cells and is required for efficient naïve T cell homing to LNs through high endothelial venules (HEV) [reviewed in (4)].

In this article we will review how PI3K signalling regulates T cell adhesion, migration and localisation by regulating CD62L and LFA-1 affinity, as well as how this can be targeted by PI3K inhibition. Cytokines and chemokines are also essential for coordinating the trafficking of lymphocytes. Of these, the expression of CCR7 and IL7Rα (CD127) are negatively controlled by PI3Kδ signalling in a FOXO1-dependent manner and will also be considered.

PI3K SIGNALLING IN T CELLS

Class I PI3Ks phosphorylate the D3-position of the inositol ring of PtdIns (4,5)P_2 (PIP_2) to generate PtdIns (3,4,5) P_3 (PIP_3). PIP_3 is bound by a subset of pleckstrin homology (PH) and other PIP_3-binding domains. Proteins with PIP_3-binding properties are hence recruited to the membrane resulting in initiation of downstream signal transduction. The class I PI3K subfamily is comprised of class IA PI3Ks (PI3Kα, PI3Kβ, and PI3Kδ) and class the IB PI3K (PI3Kγ). The class I PI3Ks are heterodimeric proteins consisting of a regulatory domain (class IA PI3Ks; p85, class IB PI3K; p101) and a catalytic domain [p110α (PI3Kα), p110β (PI3Kβ), or p110δ (PI3Kδ), or p110γ (PI3Kγ)] (5). Class II and class III PI3Ks use PtdIns or PtdIns (4)P as a substrate and are involved in intracellular membrane trafficking, these will not be considered here [reviewed in (6)].

In T cells PI3Kδ is the dominant class I PI3K isoform. PI3Kδ is activated downstream of the TCR as well by costimulatory and cytokine receptors, that stimulate the phosphorylation of tyrosines within YXXM motifs that bind to the SH2 domains of the p85 subunit (7) (**Figure 1**). Indeed, LFA-1 can also activate PI3Kδ *via* so called outside-in signalling (8). PI3Kγ is also expressed in T cells and predominantly mediates signals downstream of G protein-coupled receptors such as chemokine receptors (5) (**Figure 1**).

PI3K in T Cell Development

Signalling through class I PI3K plays important roles at multiple stages of T cell development. Loss of both p110δ and p110γ results in near complete ablation of thymocyte β-selection, while individual loss of p110δ and p110γ individually only causes minor perturbations of T cell development (9–11). These findings revealed an unexpected redundancy between p110δ and p110γ in developing T cells and was explained by cooperative signalling from the chemokine receptor CXCR4 *via* p110γ and pre-TCR signalling *via* p110δ, either of which is sufficient to generate PIP_3 required during thymocyte β-selection (12). Consistent with this, deleting the PIP_3 phosphatase PTEN bypasses the requirement for pre-TCR stimulation during thymic β-selection, presumably by enabling sustained CXCR4-dependent PIP_3 levels (13). Beyond thymocyte β-selection, mice lacking PTEN show impaired thymocyte negative selection and evidence of autoimmunity, suggesting a role for PI3K activity in maintaining central tolerance (14). In addition, mice expressing a kinase-dead p110δ show increased numbers of Treg within the

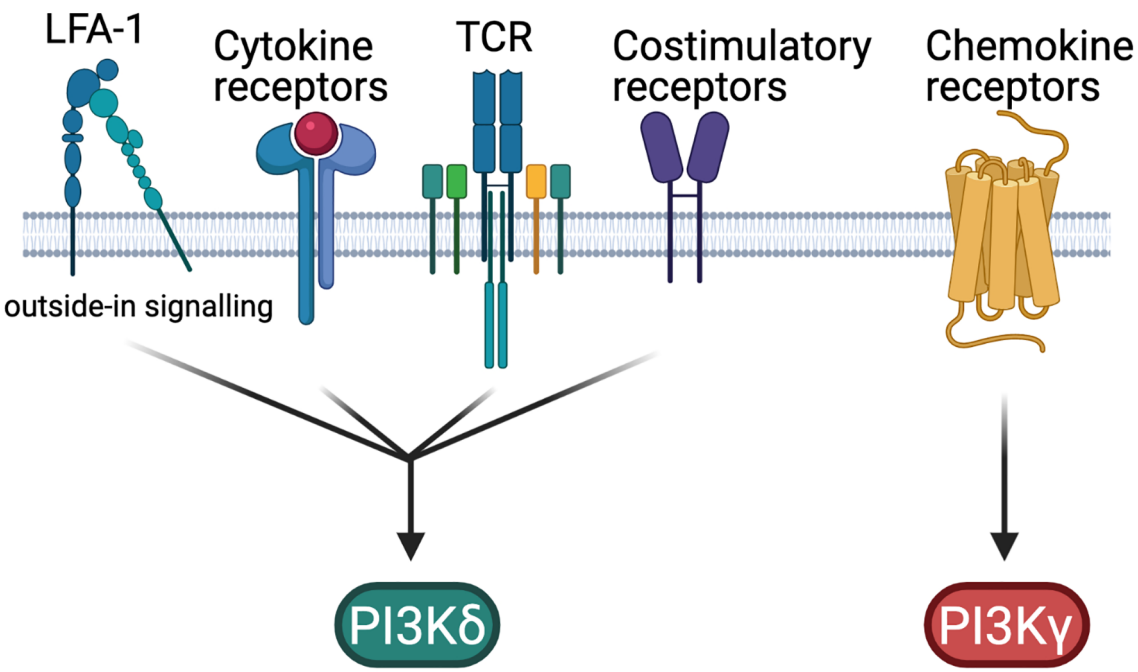

FIGURE 1 | PI3K activation in T cells. Simplified schematic of the differential regulation of PI3Kδ and PI3Kγ in T cells. Figure made in BioRender.

thymus, highlighting a role for p110δ in thymic T_{reg} selection (15). Together these findings underpin an important role for PI3K signalling in the development of T cells.

Following thymic development, class I PI3K signalling is also crucial for the peripheral development of multiple subsets of T cells; T follicular helper cells (T_{FH} cells) require ICOS signalling during development, and ICOS mutant mice that do not activate downstream PI3Kδ, as well as mice lacking p110δ in T cells, lack T_{FH} cells (16, 17). The loss of T_{FH} cells is the main reason underpinning the lack of germinal centres and immunoglobulin class switching after immunisation of PI3Kδ-deficient mice (16). PI3Kδ also regulates the differentiation of other T_H subsets, including T_H1, T_H2, T_H17, and T_{reg}, as well as production of cytokines and granzymes in both $CD4^+$ and $CD8^+$ T cells (15, 18–26). Human patients with loss of function mutations in PI3Kδ have also been identified (27–30). These have a more profound defect in B cell development than observed in mice. By contrast, profound defects in T cell development have not been observed so far in patients lacking the p85α or p110δ subunits of PI3Kδ; but this has been difficult to evaluate systematically in such immune-deficient patients who also suffer from inflammatory disease.

Activated PI3Kδ Syndrome

More recently, gain-of-function mutations that lead to increased PI3Kδ activity have also shown to be the cause of a novel immunodeficiency syndrome called Activated PI3K delta Syndrome (APDS) (31–35). APDS patients show increased susceptibility to airway infections (e.g. with *Streptococcus Pneumoniae*), chronic virus infections (CMV and EBV) and pertinent to this review, have enlarged LNs and spleens as well as signs of autoimmunity, mainly manifested as cytopenia (34). Remarkably after a 12-week trial of the PI3Kδ inhibitor Leniolisib, the LNs and spleens of these patients reduced in size by up to 50% (36). This may reflect in part the potential of PI3Kδ inhibitors to cause redistribution of lymphocytes in addition to the inhibitory effect on lymphocyte proliferation. Several groups have generated mouse models of APDS which recapitulate many of the features of the patients, including increased susceptibility to airway infections, enlarged LNs and spleen and production of autoantibodies (37–41). Altogether, these studies shine light on the paradox that both loss-of-function and gain-of-function of PI3Kδ leads to immunodeficiency, and highlight how this pathway needs to be dynamically regulated for optimal lymphocyte development and function (42, 43). This, as we will see, is also key for the control of lymphocyte trafficking.

Currently four different PI3Kδ inhibitors are approved for the treatment of B cell malignancies (44). A detailed description of these is beyond the remit of this review, however two concepts learned from the treatment of these patients are worth noting. Chronic lymphocytic leukaemia (CLL) patients treated with PI3Kδ inhibitors such as Idelalisib initially experience a dramatic redistribution of the malignant B cells from the LNs (45). This phenomenon is referred to as lymphocytosis and is now recognised as a beneficial clinical feature of this class of drugs. Lymphocytosis is thought to be secondary to the interference with BCR-dependent integrin activation and chemokine responsiveness (46). CLL cells that are purged from their protective LN environment are more susceptible to undergo apoptotic cell death which can be accelerated with chemotherapy or drugs such as rituximab (anti-CD20) (45). Immune-mediated colitis and hepatitis are common adverse effects of PI3Kδ inhibitors, but skin inflammation is also seen in some studies (47). These are thought to be caused by the selective depletion or inactivation of Tregs, especially from tissues with high exposure to microbial antigens, such as the gut, liver and skin (44, 48). In this context, by targeting Treg, PI3Kδ inhibitors can unleash potent antitumour immune responses (49). Recent evidence suggests that PI3Kδ inhibitors can purge Treg from the tumour microenvironment and into the circulation (50). Hence the capacity of PI3Kδ inhibitors to not only affect lymphocyte function, but also to cause redistribution out of lymphoid tissues may underpin the therapeutic effects of PI3Kδ inhibitors.

INTEGRINS IN T CELL LOCALISATION, MIGRATION, AND ADHESION

Integrins are transmembrane, heterodimeric proteins that are involved in cell-cell and cell-extracellular matrix interactions as well as binding of soluble ligands. In mammals the heterodimeric transmembrane structure of integrins is composed of one of 18 α subunits and one of eight β subunits, that can form up 24 combinations. Integrins are involved in T cell migration and localisation within tissues, where conformational priming (activation) of the integrins by intracellular signalling events ("Inside-out" signalling) results in high affinity binding of their ligands. Further, integrins mediate signal transduction, where binding of their ligands stimulates intracellular signalling pathways ("outside-in" signalling).

T cells are known to express at least 15 of the 24 known integrins depending on their differentiation and activation state (51, 52) (**Figure 2A**). LFA-1 (αLβ2) is expressed by all T cell subsets and specifically binds Intercellular Adhesion Molecules (ICAMs) and Junctional Adhesion Molecules (JAMs) (53, 54). Under steady state, LFA-1 is found in a closed conformation which has low affinity for its ligands. However, following inside-out mediated activation by chemokines, cytokines, or TCR-stimulation, LFA-1 rapidly changes conformation from its low affinity closed/bent conformation to an intermediate affinity extended conformation, where the extracellular domain is partly open, but the cytosolic domain remains closed. This intermediate affinity extended conformation allows for binding to ICAM-1, which can further increase affinity through outside-in signalling resulting in the high affinity open-extended conformation (**Figure 2B**) reviewed in (51, 55). Multiple other integrins are expressed in subsets of T cells, including Very Late Antigen 4 (VLA-4) (α4β1) which binds VCAM-1, however in this review we will focus on the roles of LFA-1.

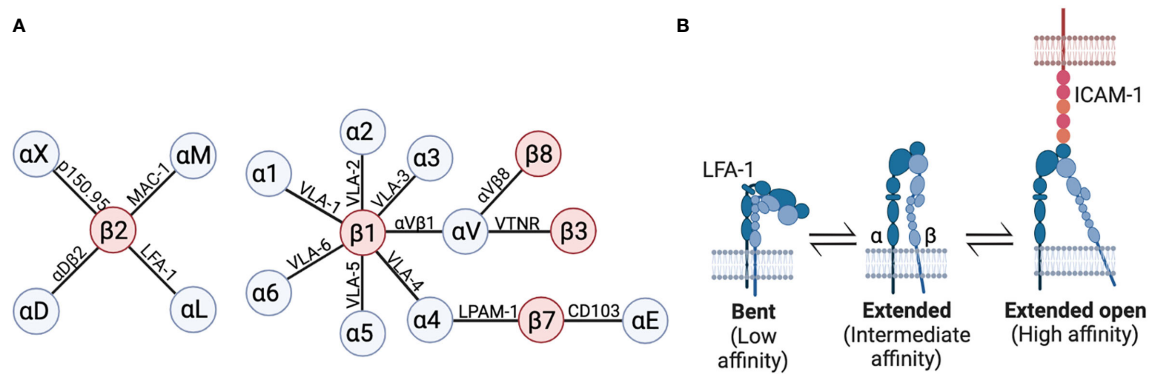

FIGURE 2 | Integrins in T cells. **(A)** Schematic of integrin chains expressed in T cells with α integrin chains in blue, and β integrin chains in red. Lines indicate which integrin chains form heterodimeric integrins, and names over lines are commonly used names of the resulting integrin. **(B)** LFA-1 (αLβ2) integrin in a bent/closed conformation with low affinity, extended/closed conformation with intermediate affinity, and extended open conformation with high affinity. Figure made in BioRender.

Integrins in T Cell Migration

T cells recirculate between LNs through the blood, probing antigen-presenting cells for their cognate antigen. To exit blood vessels, selectins, integrins, and chemokines are required to halt the T cells at the right place and resist the shear stress in the blood (1-70 dyn/cm^2) (56). This is a tightly regulated process. Selectin-mediated binding of their ligands facilitates rolling along the endothelial membrane which slows down the T cells. This allows the T cells to respond to chemokines secreted from the endothelia and immobilised on glycosaminoglycans (GAGs) on the surface of the endothelial cells Reviewed in (57). As a result, integrins (such as LFA-1 and VLA-4) are activated. LFA-1-mediated binding of endothelial ICAMs (ICAM-1 and -2) leads to firm adhesion to the endothelial barrier. This allows the T cells to crawl against the flow towards chemotactic gradients until the cell will undergo transendothelial migration (TEM, also termed diapedesis) through the endothelial barrier into the underlying tissue.

Recirculation and homing of naïve T cells to secondary lymphoid organs (SLOs), including LNs, requires expression of the chemokine receptor CCR7 and CD62L, both which are downregulated following PI3K activation as discussed later. CD62L interacts with peripheral node addressins (PNAds) expressed on high endothelial venules (HEVs) which are formed by specialised endothelial cells lining post-capillary venules associated with lymph nodes (LNs). The interaction between CD62L and PNAd causes T cells to start rolling along the HEVs. After slowing down, CCR7 on the T cells binds CCL21 presented by HEVs (58, 59), which rapidly induces LFA-1 activation, leading to arrest and transendothelial migration (60, 61). The process of LN entry is highly dependent on LFA1; LFA-1-deficient mice have greatly reduced adhesion to HEVs, particularly in peripheral LNs (pLNs) and therefore elicit limited to no migration to the LNs (62, 63). Similarly, LFA-1-blocking antibodies block adhesion to HEVs and prevent repopulation of LNs (64). Together, HEVs thus function as a selective gateway to the LNs, attracting naïve and resting memory T cells, but largely blocking entry of other leukocytes such as neutrophils under steady state (65). Migration to gut-associated lymphoid tissues, spleen and inflamed lymphoid tissues are governed by other mechanisms and molecules, such as α4β7/MAdCAM-1 interactions, and this integrin seems to be regulated by different pathways than LFA-1 and VLA-4 (66). After entering LNs, the role of LFA-1 is less clear; studies of LFA-1-deficient T cells indicate that LFA-1 is required for retention of T cells in the parenchyma (67). However, other studies using LFA-1-deficient T cells (68) or dendritic cells lacking integrins altogether (69), suggest that interstitial and intranodal motility of T cells and DCs in the absence of antigen is much less dependent on integrins than is the entry into and egress out of the LNs.

Following screening of antigen within the LNs, T cells will egress through the efferent lymphatics in a process regulated by LFA-1/ICAM-1-interactions (67). Egress is guided by the lipid sphingosine-1-phosphate (S1P). S1P is found in low concentrations inside the LNs, but high concentrations in lymph and blood, creating a gradient which attracts the T cells through binding of the GPCR S1P receptor 1 (S1PR1) expressed by naïve T cells (70). Following egress through efferent lymphatics, the lymphatics connect LNs in series, but eventually merge with the thoracic duct allowing the T cells to recirculate through the blood. Interestingly, S1P-mediated egress from inflamed tissues is also partially dependent on interactions between LFA-1/ICAM-1 and VLA-4/VCAM-1. Here LFA-1/ICAM-1 are required for effective migration of memory T cells through afferent lymphatics into LNs (71–73).

Antigen-stimulated T cells downregulate L-selectin and glycosylate P-selectin glycoprotein ligand-1 (PSGL-1) resulting in functional PSGL-1 ligand which allows for binding of L-, P-, and E-selectins that are upregulated on inflamed endothelial tissues reviewed in (4). Other integrins are also involved in migration along, and adhesion to vascular endothelium as well as transendothelial migration of antigen-stimulated T cells.

Of particular importance are VLA-4/VCAM-1, α4β7/MAdCAM-1, and αV integrin-mediated (74) interactions that facilitate migration to distinct inflammatory sites reviewed in (75, 76).

Integrins in the Immunological Synapse

Within the T cell follicles of the LNs, T cells recognise their cognate antigen-MHC complex on the surface of antigen presenting cells (APCs) or target cells. This induces TCR signalling and triggers the formation of an immunological synapse (IS) at the contact area between the T cell and the APC/target cell (77). The IS is a highly specialised and dynamic cell-cell interface that allows for fine-tuning of signalling events leading to T cell activation (78, 79). Integrins and especially LFA-1 are key components in IS formation. In the immature IS, PI3K-dependent chemokine-mediated LFA-1 activation initiates the adhesive contact between T cells and APCs/target cells allowing the T cell to scan its interaction partner for cognate antigens (80). Concomitantly, LFA-1 triggers recruitment of organelles such as mitochondria to the IS thereby preparing the T cell for optimal TCR-induced activation and Ca^{2+} signalling during later activation stages (80).

In the immature IS, LFA-1 is found at the centre of the synapse with TCRs and downstream kinases clustered at the periphery (81, 82). During maturation of the IS, the synapse is reorganised into annular supramolecular clusters (SMACs) allowing for spatiotemporal clustering of receptors, adhesion molecules, and signalling effector proteins (**Figure 3A**) (83). In the mature IS, centripetal movement relocates TCR/pMHC complexes to the central SMAC (cSMAC) together with their co-stimulatory molecules, intracellular kinases, and adaptor proteins. Simultaneously, LFA-1/ICAM-1 complexes are redistributed to the integrin-enriched peripheral SMAC (pSMAC) surrounding the cSMAC (81). In the pSMAC, LFA-1 both stimulates T cell activation by increasing the accumulation of TCR/pMHC complexes in the cSMAC, and recruiting signalling molecules to the pSMAC; LFA-1 may also help segregate the phosphatase CD45 to the distal SMAC (dSMAC) (84, 85). In addition to LFA-1, the α4β1 integrin, VLA-4, is also enriched in the pSMAC and is involved in T cell activation by regulating the mobility of SLP-76, an essential adaptor protein functioning downstream the TCR (86). It is thought that VLA-4 can restrain SLP-76 in the pSMAC, so that SLP-76 both remains in closer contact with its upstream activators and avoids the cSMAC, where signalling complexes will eventually be internalised and degraded to terminate TCR signalling (86, 87). Collectively, an important function of integrins in the IS appears to be regulating the localisation of both inhibitory and stimulatory signalling molecules. The phosphatidylinositol (PIP) composition of the IS might also contribute to this spatial regulation of signalling proteins within the IS (**Figure 3B**). Early studies confirmed the accumulation of PIP_3 inside and outside the IS between APCs and T cells (88–90). However, PIP_3 seemed less concentrated at the cSMAC, and later studies using transgenic $CD8^+$ T cells have found that PI3K-generated PIP_3 seems specifically accumulated in the periphery of the IS (91–93). Further, both PIP_2 and PIP_3 are cleared from the cSMAC during conjugate formation, and sustained PI3K activity is necessary for proper T cell activation possibly through the regulation of PIP_3 binding proteins (91–93). It has further been suggested that PI3K-dependent actin remodelling in the periphery of the IS can mediate synaptic force on the target cell thereby potentiating target cell killing by $CD8^+$ T cells (94).

Integrin Affinity Regulation in T Cells

Inside-out signalling in T cells is a complex process mediated by a range of proteins that collectively result in increased LFA-1 affinity and avidity. Following TCR stimulation, multiple proteins are recruited to phosphorylated CD3 ITAMs, including the tyrosine kinase ZAP-70. ZAP-70 consequently phosphorylates tyrosine residues of the scaffolding protein LAT. These residues act as anchors for a range of T cell signalling proteins, including SLP76, which is also phosphorylated by ZAP-70. The pSLP76/LAT-complex functions as a scaffold for downstream effector

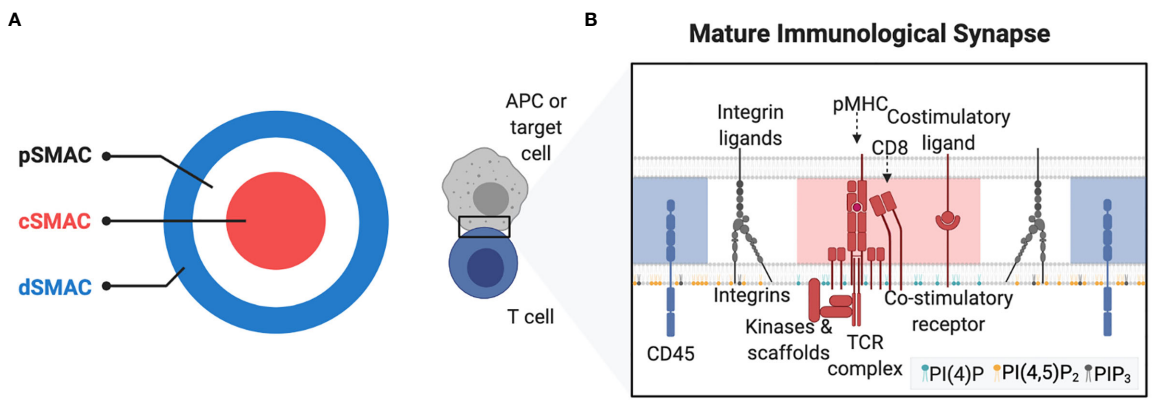

FIGURE 3 | Phospholipids in the immunological synapse. **(A)** shows the bulls eye of the synapse in the Z-plane. **(B)** Schematic of the immunological synapse showing approximate location of critical receptors in the distal SMAC (dSMAC), peripheral SMAC (pSMAC), and central SMAC (cSMAC) as well as approximate composition of the phosphoinositols PI (4)P, PI (4,5)P_2 and PI (3,4,5)P_3 in the inner leaflet of the T cell. Figure made in BioRender.

proteins, including PLCγ (95). PLCs catalyse the hydrolysis of PIP_2 to generate the second messenger signalling molecules diacylglycerol (DAG) and inositol 3-phosphate (IP_3). In turn, IP_3 stimulates the release of Ca^{2+} from the endoplasmic reticulum (ER). Ca^{2+} and DAG stimulate activation of the RAP1 guanine exchange factor (GEF), CalDAG-GEFI (also known as RASGRP2) which activates RAP proteins by exchanging GDP for GTP (96). In T cells, RAP1 is a dominant isoform, with both Rap1a and Rap1b being expressed. Of note however, CalDAG-GEFI is not expressed in mouse lymphocytes, suggesting other RAP GEFs are involved in the regulation. Another pathway leading to RAP1 activation is recruitment of the CRKL-C3G complex by the WAVE2-Arp2/3-Abl complex (97, 98). This results in activation of the RAP-GEF, C3G (Also known as RAPGEFI), thus further activating RAP1. Active GTP-bound RAP1 is critical for the process of LFA-1 activation (99–103).

Chemokine receptors are GPCRs that following chemokine binding induce a multitude of signals, some which converge in activation of the small GTPase RAP1 *via* the activation of phospholipase Cβ (PLCβ) which also hydrolyses PIP_2 to DAG and IP_3 (104–106). Besides activating the PLC-dependent signalling-cascade, chemokine receptors also induce activation of PI3Kγ resulting in initiation of PI3K-mediated signals discussed further below.

GTP-bound RAP1 interacts with RIAM (107–109) and RAPL (100). In turn, this complex mediates activation (109), and plasma-membrane binding of TALIN1 (108). The FERM3 (F3) domain of TALIN1 in turn binds the β chain of LFA-1 thereby mediating conformational activation of LFA-1 from low to intermediate affinity, as well as mediating downstream cytoskeletal remodelling (110, 111). RIAM has a PH domain that preferentially interacts with PIP_2. By binding PIP_2 RIAM is thought to act as a proximity detector mediating binding of activated RAP1 and TALIN1 to the membrane (112). Another PH-domain containing protein involved in the process is SKAP1 (also known as SKAP55). SKAP1 is constitutively associated with ADAP (also known as FYB) and has been shown to also mediate binding of RAP1 to the plasma membrane through its PH domain (113, 114). Together SKAP1/ADAP integrates with the RIAM-RAPL-RAP1 complex during TCR-induced LFA-1 activation, and likely stabilises the complex (115). In parallel during LFA-1 activation, KINDLIN-3 binds the cytoplasmic tail of β integrins and is required for stabilisation of the high affinity conformation of LFA-1 (116–118). TALIN1 thus mediates conformational maturation to an intermediate affinity of LFA-1, whereas binding of both KINDLIN-3 and TALIN1 to the β-chain results in the high affinity conformation of LFA-1 (116).

Several negative regulators of LFA-1 activation exist. RhoH is required to keep LFA-1 in a non-adhesive state (119). RhoH also contributes to TCR signalling by interacting with ZAP70 and LCK (120, 121). Interestingly, chemokine-induced LFA1 activation is suppressed by RhoH, whereas RhoH enhances TCR-induced LFA-1 activation, suggesting that RhoH can divert T cells from chemotactic towards antigen-dependent response (122). CBL-B is an E3 is ubiquitinase that can suppress LFA-1 activation by interfering with the capacity of CRK-L to recruit and activate C3G (123).

Together these intricate regulatory mechanisms integrate migratory signals, such as chemokines, and TCR engagement with integrin activation. Consequently, LFA-1 affinity is turned on and off in a highly regulated manner by multiple microenvironmental cues.

PI3K-MEDIATED ACTIVATION OF INTEGRINS – A GATEKEEPER OF ANTIGEN-DEPENDENT ADHESION

Early studies of PI3Ks roles in $CD4^+$ T cell activation found that broad inhibition of PI3Ks with Wortmannin reduced antigen-specific interactions between DO11.10 $CD4^+$ T cells and OVA-pulsed B cells, as well as T cell adhesion to immobilised ICAM-1 (ICAM-1-coated plastic) (124, 125). Further, Wortmannin was found to inhibit CD28-induced activation (126), and Wortmannin and LY294002 (class I PI3K inhibitor) inhibit CD7-induced activation of β1-integrin-mediated adhesion (VLA1-6) to immobilised fibronectin (127). In accordance, overexpression of a hyperactive p110-CAAX mutant increased ICAM-1-binding in response to PDBu/Ionomycin (95). These early findings all supported a role for PI3K in activation of integrins downstream of TCR-engagement, although caution must be taken with some of these inhibitor studies, as that Wortmannin can affect multiple kinases. Further, caution must be taken when evaluating affinity vs avidity in these studies (**Box 1**).

Further supporting a role for PI3K in integrin activation, kinase-dead $p110\delta^{D910A}$ $CD4^+$ T cells had reduced affinity towards ICAM-1 after stimulation with anti-CD3, as measured by binding of soluble recombinant ICAM-1 by flow cytometry. As a consequence, OT-II transgenic $p110\delta^{D910A}$ $CD4^+$ T cells did not form conjugates with $OVA_{323\text{-}339}$-pulsed B cells as well as WT OT-II T cells. $p110\delta^{D910A}$ mutant T cells had reduced RAP1-GTP, indicating a role for PI3Kδ in RAP1-GTP activation.

BOX 1 | Affinity Contra Avidity of Integrins – Note of Caution.

When studying integrins such as LFA-1, regulation can either be modulated by direct changes to affinity by inside-out signalling and outside-in signalling, changes in avidity through surface clustering of the integrins, and lastly by levels of expression or presence at the surface. Studies investigating LFA-1-mediated adhesion often do not clearly distinguish these mechanisms and it is often unclear whether particular mechanisms affect LFA-1 affinity or avidity.

In human T cells antibodies specific to the intermediate or high affinity conformation of LFA-1 can be used to measure affinity, however similar affinity-specific antibodies are not as well established for mouse T cells. Binding of ICAM-1 can be used as a proxy for LFA-1 activity and here binding to single ICAM-1 molecules is more dependent on affinity changes and LFA-1 surface expression, whereas binding of ICAM-1-coated surfaces (immobilised ICAM-1) or conjugate-formation is dependent both on changes to overall avidity as a result of increased affinity, surface expression, and clustering.

Interestingly, the activation of LFA-1 was less dependent on AKT suggesting other PIP_3-binding proteins were responsible for the PI3K-mediated activation of LFA-1 (128).

Treatment of lymphocytes with Wortmannin or LY294002 decreases SDF1α, CCL19, and CCL21-mediated adhesion to ICAM-1. However, this decrease seemed to rather be a consequence of decreased avidity than affinity as a result of decreased chemokine-induced LFA-1 mobility following PI3K inhibition (60). Indeed, chemokine-dependent migration of T cells was largely PI3Kγ-independent and instead mediated by DOCK2 (129). Similarly, interstitial migration and S1P-mediated egress was independent of PI3Kγ (130). PI3Kδ signalling was also not required for chemokine-induced LFA-1 activation (128). Therefore, PI3Kδ activity downstream of TCR-stimulation increases LFA-1 affinity, whereas PI3Kγ-signalling seems dispensable for chemokine-induced LFA-1 affinity regulation.

Regulation of LFA-1 by PH-Domain Containing Proteins

Multiple proteins involved in the process of LFA-1 activation, including CYTOHESIN-1, SKAP1, and KINDLIN-3, have PH domains that bind PIP_3 and may hence regulate LFA-1 affinity in a PI3K dependent manner (**Figures 4A, B**).

CYTOHESINs

The intracellular ARF-GEF protein, CYTOHESIN-1, was described early on to bind β2 integrins (e.g. LFA-1, MAC-1) and activate LFA-1-mediated adhesion to immobilised ICAM-1. The PI3K-mediated membrane recruitment of the PH domain of CYTOHESIN-1 was found to partially facilitate the CYTOHESIN-1-mediated activation of LFA-1 (132–134). CYTOHESIN-1 binds directly to the cytoplasmic tail of β2 integrin, and this interaction as well as the ARF-GEF functionality of its SEC7 homology domain have been shown to regulate the activation of LFA-1 in T cells and LFA-1 mediated transendothelial migration (135, 136). Moreover, CYTOHESIN-1 further regulates activation of RhoA and integrin activation in dendritic cells (137). Surprisingly, CYTOHESIN-1 seems to have opposing roles in regulation of MAC-1 (αMβ2) integrin-mediated adhesion to fibrinogen by neutrophils, suggesting a more complex involvement of CYTOHESIN-1 in regulation of integrin activation (138). This potentially hints a differential role of PI3K-signaling in regulating integrins in different immune subsets depending on their integrin expression. Other CYTOHESIN molecules have also been implicated in integrin regulation, but rather seem to rather be involved in the recycling of integrins from the surface. Whereas CYTOHESIN-2 (ARNO) seems to increase β1 integrin-mediated adhesion and recycling, CYTOHESIN-3 (GRP1) results in decreased adhesion (139), and these opposing effects of CYTOHESIN-2 and -3 were dependent on phosphoinositide specificity (140). How the CYTOHESINs divergently regulate integrins, and further, the mechanism by which PI3K regulates CYTOHESIN-1-mediated LFA-1 activity is still unclear, but it is likely due to dominant negative effects between the different

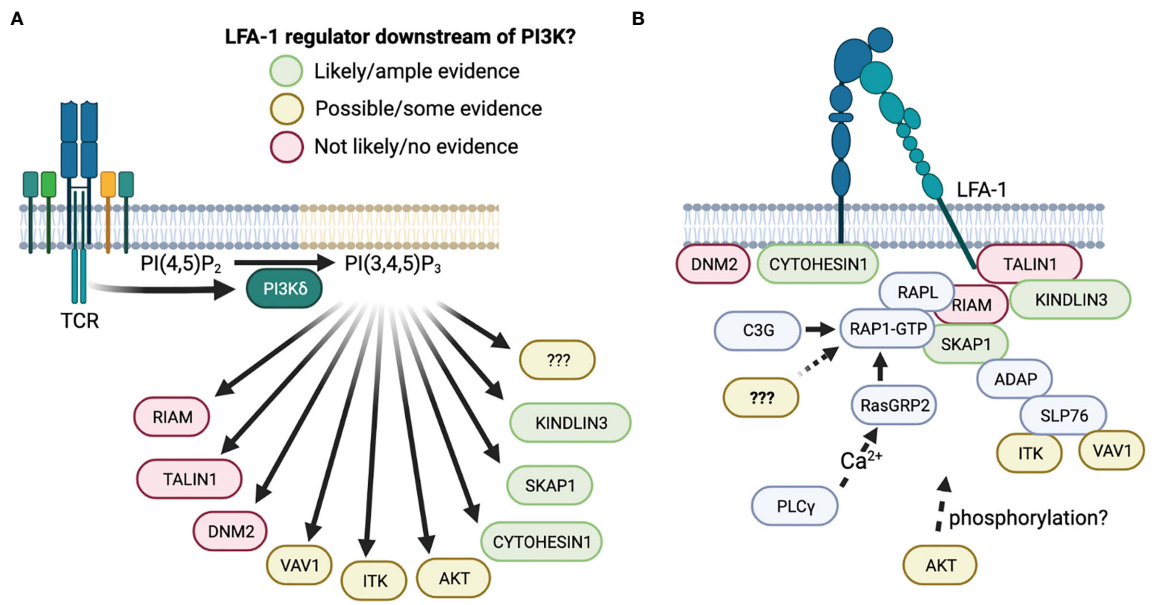

FIGURE 4 | LFA-1 regulators downstream of PI3Kδ. **(A)** Schematic of proteins that have been implicated in LFA-1 regulation, and the likelihood that they are regulated by PI3K based on the literature. Green proteins have ample evidence that they are regulated by PI3K and have been implicated in LFA-1 regulation in multiple studies. Orange proteins have some evidence suggesting that PI3K regulates their functions and are to some extent involved in LFA-1 regulation. Red proteins have PH domains, but are unlikely to be regulated by PI3K due to low PIP_3 affinity. It is important to note that PIP_2 is up to 100X more abundant on the plasma membrane than PIP_3 (131). Therefore, a given protein needs to have high selectivity for PIP_3 over PIP_2 in order to be directly regulated by PI3Ks. **(B)** Simplified schematic of how the proteins in **(A)** are involved in regulation of LFA-1, showing interaction partners and approximate location. Figure made in BioRender.

homologs, and should highlight the importance of not treating all integrin signalling pathways equally.

SKAP1

SKAP1 is recruited to SLP-76 *via* the adaptor protein ADAP. ADAP/SKAP1 then binds the Rap1-interacting protein, RIAM and contributes to TCR-induced inside-out LFA-1 activation and clustering by supporting formation of RAP1/RAPL complexes as well as membrane recruitment of these essential proteins involved in LFA-1 activation (113, 115, 141, 142). The SKAP1 PH domain was found to be required for membrane recruitment, and this in turn was necessary for the recruitment of RAPL to the membrane (114). A SKAP1 mutant that was constitutively associated with the membrane by addition of a myristoylation site disrupted the requirement for PI3K signalling in binding immobilised ICAM-1 suggesting that PI3K-mediated activation of LFA-1 is dependent on ADAP/SKAP1/RIAM signalling. Indeed, although RIAM contains a PH domain with high affinity for PtdIns monophosphates *in vitro*, SKAP1 is required for recruitment of Rap1/RIAM to the membrane during LFA-1 activation (142). In accordance, K152E mutation of SKAP1 eliminated PIP_3-binding *in vitro* and as a result impaired immobilised ICAM-1-binding (143). Unexpectedly, this mutant did not abolish SKAP1/ADAP/RIAM/RAPL binding to the membrane, suggesting redundancy in the pathways resulting in membrane-recruitment of these proteins. Surprisingly, SKAP1 mutants lacking the PH domain do not significantly alter its role in integrin-mediated adhesion, suggesting the mechanism by which PI3K regulates SKAP1 is still incompletely understood (113, 143, 144).

KINDLIN3

Mutations of the crucial LFA-1 regulator, KINDLIN-3, are the cause of leukocyte adhesion deficiency III (LAD-III), a rare autosomal disorder, resulting in severe bleeding and life-threatening infections as a result of defective β1- and β2-integrin-mediated adhesion (145, 146). Studies by Hart et al. suggest that KINDLIN-3 has higher affinity to PIP_3 than PIP_2 (147). They also found that the PIP_3-binding was necessary for the function of KINDLIN-3, as KINDLIN-3 mutants that specifically did not bind PIP_3 failed to rescue adhesion of LAD-III cells to ICAM-1. KINDLIN-3 was also found in structural studies to bind PIP^3 with higher affinity than PIP_2 (148) and the PH domain was found to regulate the translocation of KINDLIN-3 to the surface membrane in neutrophils (149). These studies therefore suggests that KINDLIN-3 is at least partially regulated by PI3K, though this has yet to be confirmed in lymphocytes.

Thus, several studies indicate a key role for PI3K effector molecules in regulating integrin affinity/avidity, and multiple other proteins have been implicated in PI3K mediated integrin regulation indirectly (**Figure 4B**).

Regulation of the RHO Family of GTPases

The RHO family of GTPases, which include RAC, RhoA and CDC42 are both positively and negatively regulated by PH domain-containing GEFs and GAPs that have affinity for PIP_3 and have been implicated in regulation of LFA-1. RhoA and RAC1 have been implicated in positively regulating LFA-1 avidity by controlling the affinity and clustering of LFA-1 (150, 151). By contrast, CDC42 and RhoH negatively regulate LFA-1 suggesting a complex integrated role of these proteins in LFA-1 regulation (119, 151). TEC kinases regulate cytoskeletal remodelling and LFA-1-mediated adhesion through activation of RHO-family proteins (152–154). In T cells the highest expressed TEC kinases are ITK and RLK, and *Itk* KO cells have decreased adhesion to ICAM-1 (154). RLK does not contain a PH domain, whereas ITK contains a PH domain that binds selectively to PIP_3 (155), but the role of this in T cell integrin-mediated adhesion is unclear. Similar results have been described for the RHO GEF, VAV1 implicating it in clustering of LFA-1, but it is not known if this effect is PI3K-dependent although VAV1 can be regulated in part by PI3K (156–158). Similarly the RAC-GEF, P-REX1, which also contains a PIP_3-binding PH domain, has been implicated in LFA-1 affinity and avidity regulation (159). It is likely that TEC kinases, VAV1, and P-REX1 are more important for LFA-1 clustering, and thereby increased avidity, than for affinity regulation as RHO family proteins are known to be important for cytoskeletal remodelling, and recruitment of proteins to the synapse (154). PI3K activity is not sufficient to activate all RHO family proteins (160) and has in some studies been shown to inhibit RAC activity in T cells (128), suggesting a complex interplay of this network of regulators in RHO regulation and downstream regulation of LFA-1.

Intriguingly, DOCK proteins which do not contain PH domains have been suggested to have affinity to PIP_3 *via* so-called Dock Homology Domains (DHR1) (161). However, the extent of direct PIP_3 affinity, and whether the affinity is a result of DOCK-proteins interacting with the PH-domain-containing ELMO proteins is still debated (91, 162). DOCK2 was described earlier in the review in the context of chemokine-stimulated LFA-1 activation but does not seem to affect TCR-induced LFA-1 activation, as it seems to be involved in TCR-induced RAC-dependent TCR clustering, without affecting LFA-1 translocation to the IS (91, 163). However, it is possible that this is context-specific, and some subsets thus might be more or less dependent on DOCK2 for efficient LFA-1 activation.

Additional PH-Domain Containing Regulators of LFA-1

DNM2 which is known for its role in regulating vesicular traffic, has been suggested to also regulate integrin affinity directly *via* FAK/PYK2- and C3G-mediated RAP1 activation (164). DNM2 has a PH domain, however it does not appear to have affinity for PIP_3 in screens of PIP_3-binding (165, 166), and it is therefore unlikely that it is regulated by PI3K.

Interestingly, some RAP GTPase activating proteins (GAPs) have PH domains, including the GAP1-family members RASA3 and RASAL (167–169). In platelets, RASA3 inhibits the affinity of the integrin αIIbβ3 in a PIP_3-dependent manner (169). How the function of PIP_3-dependent inhibitors of integrins is

coordinated with PH-domain containing proteins that activate integrins is an area of active investigation.

The fact that such a high proportion of LFA-1 regulators contain PH domains suggests key roles for PI3K-mediated signalling in regulating LFA-1, though these may be cell, receptor, and context dependent. Multiple possible mechanisms of PI3K-mediated regulation are plausible; firstly, it is possible that PI3K activity directly activates the PIP_3-binding LFA-1 regulators by inducing a conformational change in the proteins as is suggested for KINDLIN3 (147). Secondly, PIP_3 could colocalise proteins that interact and activate each other (As observed during activation of AKT by PDK1). Thirdly, it is possible that microclusters of PIP_3 colocalise with LFA-1 spatiotemporally during LFA-1 activation. Similarly, it is possible that PIP_3 inactivates negative regulators of LFA-1 as has been suggested for RASA3-mediated regulation of platelet integrins (169) by similar mechanisms, i.e. conformational inactivation, colocalization of negative regulators with other proteins that inhibit them, or by sequestering the negative regulators from LFA-1 during activation.

PI3K-MEDIATED REGULATION OF NAÏVE T CELL MIGRATION AND HOMEOSTASIS

The expression of homing molecules CD62L and CCR7 on the surface of naïve T cells is critical for orchestrating naïve T cell trafficking to LNs, where these cells may become activated following antigen encounter and differentiate into effector cells. The maintenance of CD62L and CCR7 expression on naïve T cells is regulated by PI3Kδ signalling and transcription factors of the Forkhead Box protein family, with FOXO1 being a particularly important player. FOXO1 is inhibited by AKT downstream of PI3K (**Figure 5**) (170–172). Once phosphorylated by AKT, FOXO1 is excluded from the nucleus

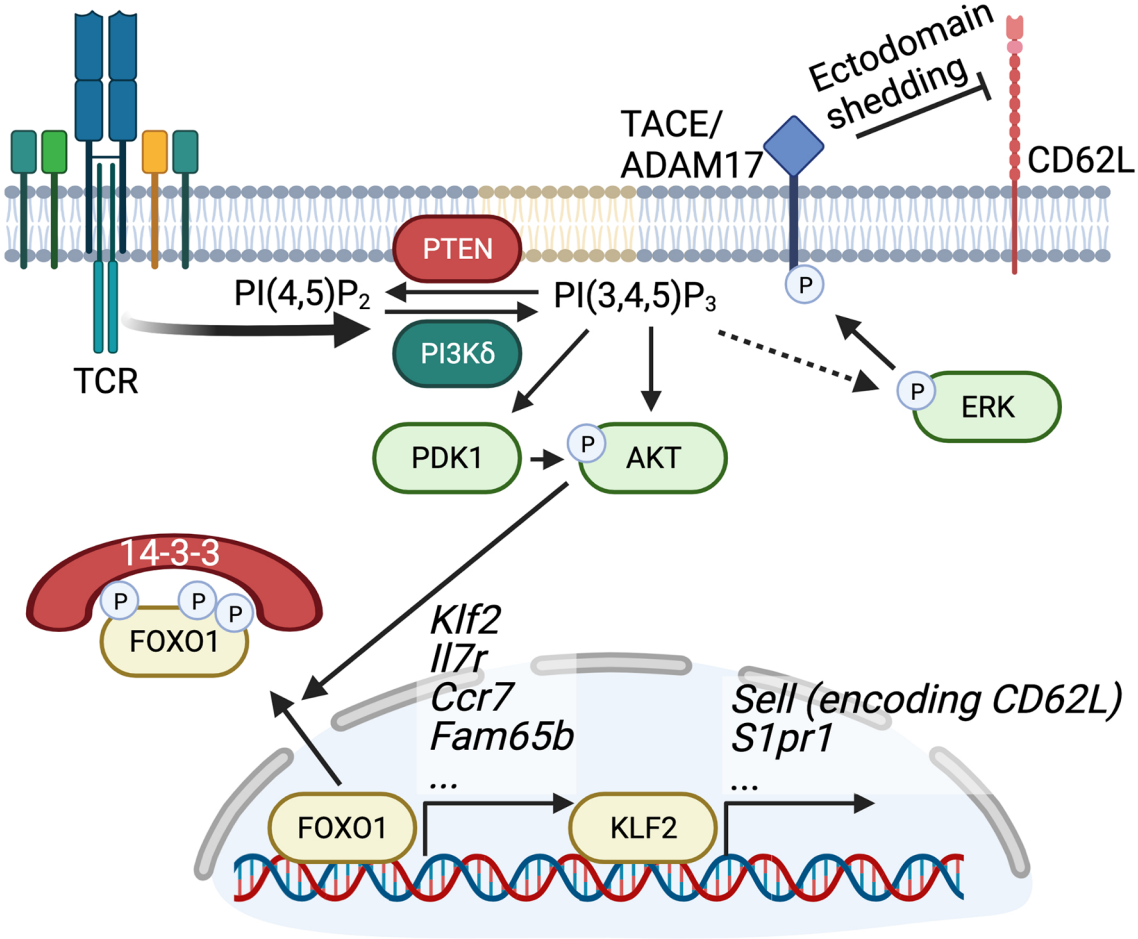

FIGURE 5 | PI3Kδ-mediated regulation of CD62L, CCR7, and S1PR1. PI3K-mediated PIP_3 production leads to recruitment of PDK1 and AKT, leading to AKT activation. AKT phosphorylates FOXO1, which allows for binding of the 14-3-3 leading to cytosolic sequestration of FOXO1. FOXO1 promotes transcription of *Klf2* (as well as *Il7r*, *Ccr7, Fam65b*). Decreased expression of the transcription factor KLF2 in turn results in decreased transcription of the CD62L encoding gene *Sell* and *S1pr1*. Figure made in BioRender.

and targeted for degradation (173). Transcriptional activity of FOXO1 is high in naïve T cells and results in robust expression of CD62L and CCR7 through control of KLF2 levels, a transcription factor that drives expression of these key homing molecules (174). In addition, FOXO1 activity is required to maintain IL-7Rα expression, a cytokine receptor key to maintenance of naïve T cell survival and homeostasis (175). Following T cell activation PI3Kδ-mediated signals result in the phosphorylation and inactivation of FOXO1, resulting in a suppression of important FOXO1 and KLF2 target genes involved in regulating migration such as CD62L, CCR7, Fam65b, and others (176–179) (**Figure 5**). As a result of this loss of homing molecule expression, activated T cells are diverted away from entering LNs and instead are biased towards migration into peripheral tissues where they can perform their effector function. In addition to CD62L and CCR7 downregulation, the KLF2 target S1PR1 is also downregulated downstream of PI3Kδ-mediated FOXO1 inhibition (178, 180). Downregulation of S1PR1 expression results in a loss of T cell egress capacity and, combined with negative regulation of CD62L and CCR7 expression, limits the recirculation of activated T cells (**Figure 5**). In fact, suppression of S1PR1 expression is particularly important for the establishment of tissue resident memory T cells (T_{RM}), which reside long term at barrier sites and are potent inducers of cell-mediated immunity (181). Therefore, PI3Kδ signals are instrumental in coordinating the acquisition of effector function with necessary changes in cell mobility that are required to execute functional immune responses (**Figure 5**).

Further regulation of CD62L expression on the surface of T cells is mediated through proteolytic cleavage of the ectodomain of CD62L by a process known as CD62L shedding. Following TCR activation, phosphorylation of TNF converting enzyme (TACE)/disintegrin and metalloprotease 17 (ADAM17) drives the trafficking of this protease to the cell surface, facilitating its cleavage of the ectodomain of CD62L (182–184). T cells expressing kinase dead $p110\delta^{D910A}$ show impaired shedding of CD62L from the cell surface, suggesting that PI3Kδ activity is critical in this process (185). Mechanistically, it has been shown that phosphorylation of TACE/ADAM17 by mitogen activated protein kinase (MAPK) ERK1/2 is required for the ability of this protease to cleave cell surface molecules like CD62L (186–188) and ERK phosphorylation is impaired in PI3Kδ-deficient T cells (**Figure 5**) (185, 189).

CCR7 Expression, CD62L-Shedding and LFA-1 Activation – Three Birds, One Stone?

It is intriguing that PI3K signalling regulates multiple processes involved in T cell migration. PI3K-mediated CD62L-shedding and reduced CD62L, S1P1, and CCR7 expression results in decreased LN entry and is an important step in T cell differentiation to effector subsets. Concurrently, as PI3K-signaling increases integrin affinity, PI3K signals can regulate migration and adhesion, including transendothelial migration into LNs. Consequently, inhibition of PI3K or disruption of PI3K signalling will affect all of these rheostats of migration, but not always in predictable ways. Hence, PTEN-deficient T cells with high PIP_3 levels are excluded from LNs after adoptive transfer (190). Nevertheless, APDS patients suffer from lymphadenopathy and this is revered upon treatment with a PI3Kδ inhibitor (36).

Studies of migration of PI3K-deficient T cells as well as use of inhibitors in mice provide some indication of how inhibition affects T cell distribution *in vivo*. p110γ-deficient T cells show reduced migration towards chemokines, whereas p110δ-deficient T cells respond to chemokines similarly to WT cells (191). Similarly, p110γ selective inhibitors affect responses to chemokines, whereas p110α/β/δ selective inhibitors do not affect responses to chemokines, except for at very high concentrations, likely as a result of off-target effects (192). Following LN entry, p110γ-deficient T cells seem to migrate interstitially similarly to WT T cells, and chemokine-induced interstitial migration seems independent of PI3K signalling (193). However, treatment with Wortmannin as well as disruption of regulatory p85 subunits of class IA PI3K showed that these cells migrated at lower velocities than WT cells, although T cell location within the LN did not seem altered (194). PI3Kδ under steady state does not contribute to T cell migration or chemokine-dependent migration per se, as $p110\delta^{D910A}$ T cells migrated like WT T cells in endothelial cell-coated transwell assays as well as following adoptive transfer (195). However, following antigenic challenge, p110δ was required for efficient migration to the site of inflammation and presence of antigen, consistent with a key role for PI3Kδ in regulating integrin affinity (195). Disruption of p110δ results in increased track velocities of OT-II $CD4^+$ T cells in LN slices with OVA-pulsed DCs, as a result of decreased interaction times with the peptide-presenting DCs in the slices (128). Similar results have been observed for p110γ-deficient T cells that are defective for antigen-dependent and chemokine-dependent migration of effector $CD4^+$ and $CD8^+$ T cells (196, 197). Interestingly, CD28 seems to also be important for homing of antigen-stimulated T cells to non-lymphoid tissues, whereas CD28 (Y173F) that is uncoupled from PI3Kδ was defective. This suggests that CD28-mediated activation of PI3K is involved in migration of activated T cells to non-lymphoid sites (198). Consequently, when inhibiting PI3Kδ, homeostatic migration of naïve T cells (T_N) seems unperturbed (as these have low PI3K activity in the first place), whereas activated T cells show decreased antigen-dependent migration into non-lymphoid tissues.

Central memory T cells (T_{CM}) that are $CD62L^+CCR7^+LFA\text{-}1^+$ are consequently supported by PI3Kδ inhibition, whereas effector T cells (T_{eff}) and effector memory T cells (T_{EM}) ($CD62L^-CCR7^-LFA\text{-}1^+$) are inhibited (**Figure 6A**). This is largely supported by the fact that APDS patients have reduced T_{CM} cells, and increased T_{eff} cells (32), whereas $p110\delta^{D910A}$ mice have normal memory T cells, but reduced T_{eff} cells (22) (**Figure 6B**). These PI3K-dependent alterations of T cell memory responses are possibly affected by altered expression of migratory receptors, however, differentiation of $p110\delta^{D910A}$ T cells to T_{eff} is largely defective, implicating PI3K more broadly in differentiation and migration.

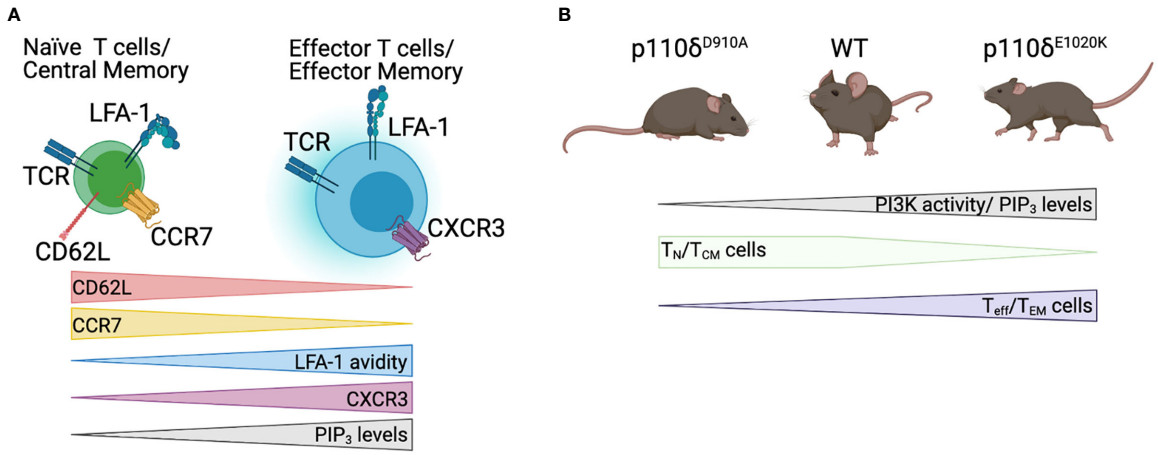

FIGURE 6 | Involvement of PI3K signalling in T cell migration. **(A)** Diagram of relative surface levels of CD62L, CCR7, CXCR3, and avidity of LFA-1 in naïve T cells (T_N)/Central memory T cells (T_{CM}) and effector T cells (T_{eff})/effector memory T cells (T_{EM}). **(B)** Spectrum of PI3K activity in PI3K mutant mouse models, and how this affects the levels of T_N/T_{CM} and T_{eff}/T_{EM} cells. Figure made in BioRender.

PI3K INHIBITORS – A THERAPEUTIC PERSPECTIVE

Because of the important role of PI3K signalling in antigen-dependent migration, PI3Kδ is a promising target for therapies, where broad blockade of T_{eff} and T_{EM} migration is favourable. There is a growing body of evidence that alloreactive memory cells are responsible for allograft rejection Reviewed in (199). Treatments have focused on blocking costimulatory pathways in T cells, but some patients are resistant to these treatments. LFA-1 and/or VLA-4 blocking antibodies (i.e. Efalizumab or Natalizumab) have been used in these patients with some success as these antibodies reduce the migration and activation of memory subsets. However, some anti-LFA-1 or anti-VLA-4-treated patients developed EBV-induced lymphoproliferative disease (200) or the fatal viral brain infection, progressive multifocal leukoencephalopathy (PML) (201), and it has been suggested that targeting TCR-induced inside-out signalling instead of broadly targeting both chemokine, and TCR-induced LFA-1 activation would restrict the blocking to antigen-induced migration of T cells, and therefore potentially limit the risk of PML or EBV-induced lymphoproliferation (202). As PI3Kδ inhibition reduces the antigen-dependent migration of T cells by limiting CD62L and CCR7 downregulation, as well as LFA-1 activity, it is likely that PI3Kδ inhibition would show efficacy in some types of allograft rejection. Indeed, PI3Kδ inhibition decreased chronic rejection of heart allografts in the absence of immunosuppressive treatment by interfering with antigen-dependent migration to the allograft (203). Other groups have also described data supporting the use of PI3Kδ inhibition in treatment of allograft rejection; both in mice with dual PI3K/mTOR inhibition (204) and with p110α/γ (205) or p110δ inhibition alone (206). However, it has been suggested that p110γ deletion is more effective than p110δ deletion, and p110δ deletion and inhibition even seemed to increase allograft rejection (207). Rag KO mice reconstituted with p110δ-deficient $CD25^-$ T cells (non-Tregs) prolonged allograft acceptance compared to WTs. This suggests that the negative effect of PI3Kδ inhibition is due to blockade of the immunosuppressive properties of Tregs (207). Further studies will have to evaluate and determine the contribution of CD62L, CCR7 and LFA-1 affinity in allograft rejection, and it will be of interest to systematically determine under what conditions p110δ or p110γ inhibition show efficacy.

Another treatment where T_{CM} cells are favourable to T_{eff} cells, is during adoptive T cell transfer. Studies from Restifo et al. have indicated that adoptively transferred T_{CM} cells are superior to transferred T_{eff} cells both in mice and primates (208–211). The reason why T_{CM} cells elicit a better anti-tumour response is thought to be a result of their circulation to LNs where they persist for longer than short-lived T_{eff} cells (212). Thus, one of the major hallmarks of adoptive cell transfer has been to find ways of differentiating and expanding T cells without terminally differentiating the cells to T_{eff} cells. Multiple ways have been described that support a favourable differentiation profile; Expanding T cells in presence of IL-15, which supports a more central memory-like phenotype, also promotes anti-tumour immunity (213). Similarly, stimulation of WNT signalling (214), inhibition of glycolytic metabolism (215), as well as tethered IL-15 (216), promoted favourable central memory-like phenotypes that augmented anti-tumour immunity. PI3K/AKT inhibition has been shown to favour generation of cells with increased anti-tumour efficacy. AKT inhibition post transfer was shown to promote expansion of favourable T_{CM} cells with improved *in vivo* efficacy (217, 218). Further, PI3Kδ inhibition during expansion of T_H17 cells followed by inhibition of β-catenin resulted in generation of T_H17 cells that persisted *in vivo* and elicited heightened anti-tumour immunity (219). Similarly,

PI3Kδ inhibition with Idelalisib *ex vivo* before adoptive transfer heightened the anti-tumour response to an even greater extent than AKT inhibition (220). This suggests that PI3K-mediated anti-tumour efficacy in adoptive transfers is partially independent of AKT. Further, the transcription factor TCF7 was increased in the PI3K-inhibited *ex vivo*-expanded T cells, whereas it was not increased to the same extent following AKT inhibition (220). This is surprising as FOXO, which is inhibited by AKT, regulates expression of TCF7. It is possible that additional mechanisms downstream of PI3Kδ are responsible for the increased anti-tumour efficacy seen with PI3Kδ inhibition, and further studies should evaluate the role of other mechanisms in this process, including decreased LFA-1 activation, or the role of other AKT-independent PI3K functions and effectors.

SUMMARY

In summary we have described how migration is regulated by PI3K signalling in T cells, with a focus on T cell integrin activation. As PI3K activity increases LFA-1 affinity, whilst decreasing CD62L surface levels and CCR7 expression, signalling *via* PI3K is critical in the process of T cell migration following antigen stimulation. We further described how this potentially could be targeted in situations where a naïve/central memory-like phenotype is preferred to effector T cell subsets, such as in allograft rejections and adoptive T cell transfer.

AUTHOR CONTRIBUTIONS

KHJ conceived the ideas for the review, outlined, and wrote the manuscript. DPG and JHT contributed with intellectual support, and wrote sections of the manuscript. PLS contributed with intellectual support and suggestions for the manuscript. KO conceived the ideas for the review, wrote sections of the manuscript, and provided intellectual support. All authors contributed to the article and approved the submitted version.

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