LEUKOCYTE TRAFFICKING IN HOMEOSTASIS AND DISEASE

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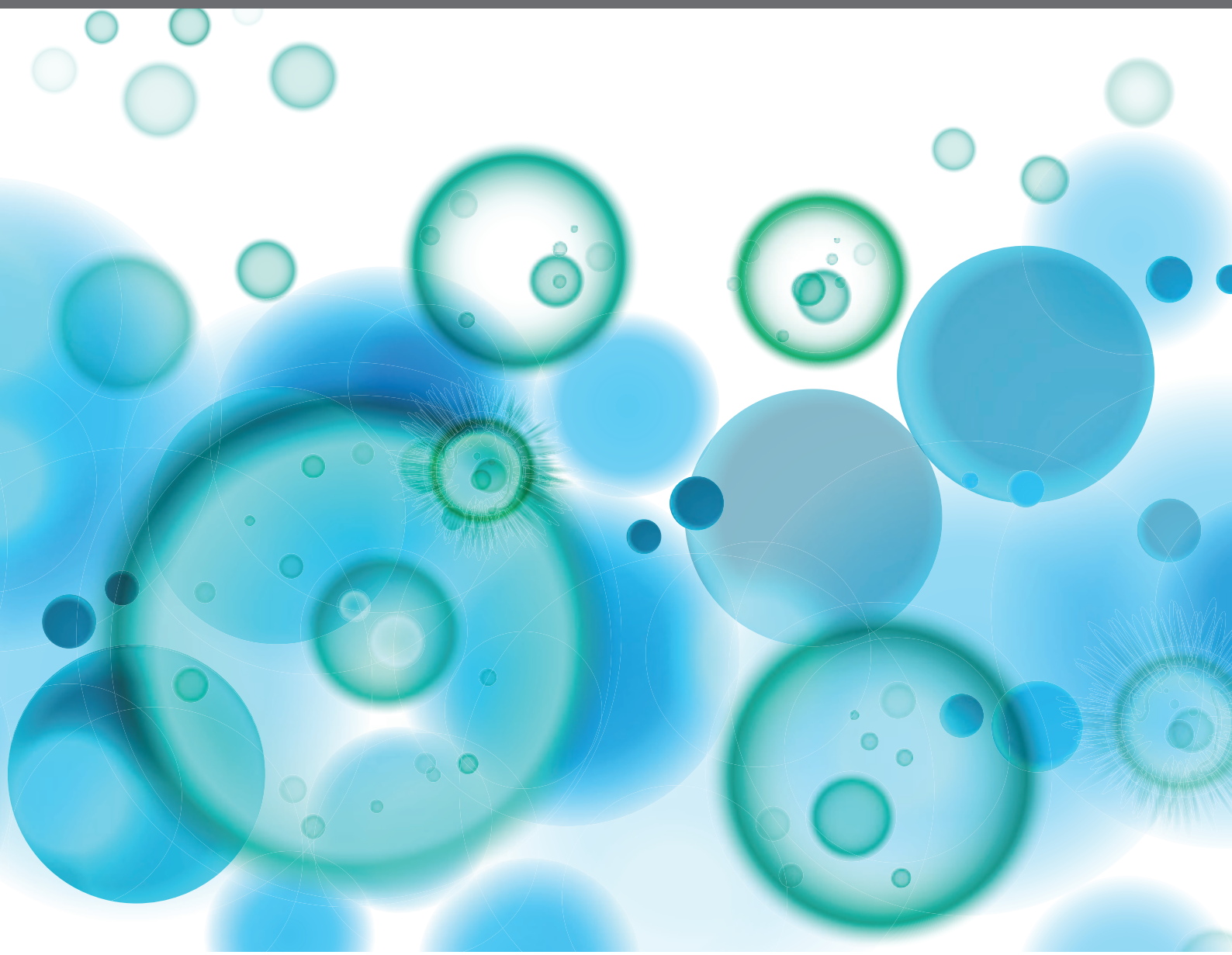

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LEUKOCYTE TRAFFICKING IN HOMEOSTASIS AND DISEASE

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EDITORIAL
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Editorial: Leukocyte Trafficking in Homeostasis and Disease

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Editorial on the Research Topic

Leukocyte Trafficking in Homeostasis and Disease

Leukocytes move avidly through the body. While this is classically associated with immune responses, leukocyte trafficking is just as prominent during steady-state conditions as they leave the bone marrow (BM), home back to tissues for elimination, or traffic through secondary lymphoid organs (1). However, immune cell trafficking becomes uncontrolled during inflammatory pathologies (2, 3), and in the homing of hematologic tumor cells to BM and lymph nodes. Diapedesis of immune cells and blood cancer cells across endothelium is facilitated by chemokines and adhesion molecules, which act in concert in tightly regulated directional motility (1–6).

The Research Topic on "Leukocyte Trafficking in Homeostasis and Disease" covers several reviews providing an up-to-date view of different molecular and cellular players that regulate key trafficking processes during cell differentiation, immune responses and lymphocyte recirculation, as well as in inflammatory pathologies and in hematological malignancies.

NEUTROPHIL TRAFFICKING

Integrins are key adhesion receptors controlling leukocyte trafficking. In their review, Fagerholm et al. describe the critical roles of β2 integrins in leukocyte trafficking and other leukocyte functions that are dependent on cell adhesion (7). The importance of β2 integrins for immune function is shown by rare genetic disorders (Leukocyte adhesion deficiencies, or LAD) that affect their expression (LAD-I) or function (LAD-III, caused by mutations in the integrin regulator, kindlin-3) (8, 9). However, β2 integrins are also associated with many immune suppressive functions. For example, they can inhibit tissue migration of dendritic cells, and also suppress cytokine responses in myeloid cells (10–12). Because of these various roles in immunity, β2 integrin dysfunction can contribute to the development of both immunodeficiency diseases and inflammatory diseases.

The classical leukocyte recruitment cascade consisting of leukocyte capture, rolling, arrest, firm adhesion, crawling and finally, transmigration through the endothelium (2), is widely accepted as the way leukocytes are recruited into tissues. Maas et al. describe the variations in the trafficking rules that neutrophils use to enter different tissues, focusing on lung, liver, kidney and aorta. These rules are distinct in different organs and tissues, and there appears to be significant redundancy in the system (chemokines, etc.), which may explain why it is so difficult to target leukocyte recruitment successfully in the clinic.

Focusing on the migratory dynamics of neutrophils, Morikis and Simon summarize an extensive body of literature suggesting that biomechanical signals at the original site of interactions between leukocytes and the activated endothelium critically regulate leukocyte adhesion and polarization. The extensive, but poorly characterized interplay between multiple types of receptors (selectins, glycoproteins, integrins, or calcium channels) in these regions is discussed to be critical at these

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early stages of the recruitment cascade. With the description of how protein modules in integrins and cytoskeleton reorganize, and the relevance of these events in controlling neutrophil migration, Morikis and Simon provide an exciting review of the intricate biomechanics of immunity.

Neutrophils must cross multiple barriers in the body to reach the areas where they will perform their immune tasks (13). An underappreciated aspect of this migration is how the cell adapts to the constraints imposed by each barrier, be it endothelial, matricial, or interstitial. Salvermoser et al. discuss how neutrophils adapt to the varying environments, and in particular focus on adaptations of nucleus, which is the stiffest cellular organelle. As thoroughly reviewed by the authors, nuclear architecture and deformability are key features that allow the swift and efficient migration of neutrophils through multiple environments.

Leukocytes not only travel into peripheral tissues, but can interestingly also regulate BM hematopoietic stem cells (HSC) (14). The review by Lucas describes the HSC niche, its components and its regulation by leukocytes and by leukocyte trafficking. HSCs give rise to leukocyte subtypes (including neutrophils), which feed back to the HSC niche, regulating both HSC number and function. This crosstalk may function as a biological rheostat during inflammation and in different disease states, and this feedback system allows the BM to monitor the periphery and to adjust leukocyte output according to peripheral needs, although many of the finer details still remain to be elucidated.

PLATELETS IN LEUKOCYTE RECRUITMENT AND RESOLUTION OF INFLAMMATION

Current research has expanded the appreciation of platelets beyond their contribution to primary hemostasis, indicating that they also actively participate in leukocyte recruitment, especially neutrophils, and in the regulation of the host defense in response to exogenous injuries (15). Platelets physically interact with different leukocyte subsets during inflammatory processes (16), which hold extensive implications for the leukocyte recruitment into peripheral tissues and for the regulation of leukocyte cell autonomous functions, including the formation and liberation of neutrophil extracellular traps. In addition, platelets have also been implicated in the resolution of inflammation (17). The review by Rossaint et al. focuses on the role of platelets in leukocyte recruitment during the initiation of the host defense, and also discusses their participation in the resolution process after acute inflammation.

MONOCYTE AND MACROPHAGE TRAFFICKING

Teh et al. describe recent advances in the field of monocyte trafficking. Monocytes are highly plastic cells which can perform effector functions in their own right, or traffic into tissues and differentiate into various monocyte-derived cell types, both during homeostasis and in different diseases (18). Major advances in understanding the role of monocytes and monocyte-derived cells were possible in recent years due to development of imaging techniques, but the authors point out that these cells are still challenging to investigate due to their plasticity.

In an original research paper included in this Topic, Cui et al. studied the role of the αLβ2 and αDβ2 integrins in macrophage migration in tissues. They show that 3D amoeboid macrophage migration is inhibited by high β2 integrin expression, whilst a moderate expression of the integrin promotes it.

CATECHOLAMINES, GLUCOCORTICOIDS AND SCAVENGER RECEPTORS

Ince et al. provide an exhaustive overview of the dynamics of multiple leukocyte subsets with particular emphasis on the molecular cues guiding their trafficking patterns during baseline or inflammatory conditions. The first of these cues are catecholamines, which are neurotransmitters produced by the adrenal gland, sympathetic nerves and even leukocytes themselves. Recent studies established important roles for catecholamines in regulating the expression of adhesion molecules and chemoattractants by endothelial cells, but also through direct actions on leukocytes (19, 20). A second class of cues is the glucocorticoids, a type of steroid hormones produced by the adrenal gland. These hormones influence many aspects of leukocyte behavior by generally reducing their adhesive capacity (21, 22). This can induce, for example, potent demargination of certain leukocyte types by attenuating interactions with vascular cells (23). Because the presence of both signals display potent circadian patterns, the review also discusses how these signals contribute to diurnal rhythms in leukocyte trafficking.

The review by Patten and Shetty describes roles of scavenger receptors expressed on endothelial cells (24), which regulate the leukocyte trafficking. However, the roles of these receptors in leukocyte migration are less well-understood than those of other traditional adhesion receptors.

HEMATOLOGIC TUMOR CELL TRAFFICKING

The trafficking of hematologic tumor cells is facilitated by adhesion molecules and chemokines, a process that contributes to progression of hematologic malignancies (4, 6). A common feature of multiple myeloma, chronic lymphocytic leukemia and acute lymphoblastic leukemia is the homing and lodging of blood cancer cells in the BM, which favors their growth and survival. The α4β1 integrin and the chemokine receptor CXCR4 are key molecules for cell trafficking into and out of the BM in these hematologic neoplasias (25, 26). Redondo-Muñoz et al. review the molecular players that regulate the trafficking of neoplastic cells during development and progression of these hematologic malignancies.

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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ORIGINAL RESEARCH
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Distinct Migratory Properties of M1, M2, and Resident Macrophages Are Regulated by $\alpha_D\beta_2$ and $\alpha_M\beta_2$ Integrin-Mediated Adhesion

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Chronic inflammation is essential mechanism during the development of cardiovascular and metabolic diseases. The outcome of diseases depends on the balance between the migration/accumulation of pro-inflammatory (M1) and anti-inflammatory (M2) macrophages in damaged tissue. The mechanism of macrophage migration and subsequent accumulation is still not fully understood. Currently, the amoeboid adhesion-independent motility is considered essential for leukocyte migration in the three-dimensional environment. We challenge this hypothesis by studying the contribution of leukocyte adhesive receptors, integrins $\alpha_M\beta_2$, and $\alpha_D\beta_2$, to three-dimensional migration of M1-polarized, M2-polarized, and resident macrophages. Both integrins have a moderate expression on M2 macrophages, while $\alpha_D\beta_2$ is upregulated on M1 and $\alpha_M\beta_2$ demonstrates high expression on resident macrophages. The level of integrin expression determines its contribution to macrophage migration. Namely, intermediate expression supports macrophage migration, while a high integrin density inhibits it. Using *in vitro* three-dimensional migration and *in vivo* tracking of adoptively-transferred fluorescently-labeled macrophages during the resolution of inflammation, we found that strong adhesion of M1-activated macrophages translates to weak 3D migration, while moderate adhesion of M2-activated macrophages generates dynamic motility. Reduced migration of M1 macrophages depends on the high expression of $\alpha_D\beta_2$, since α_D-deficiency decreased M1 macrophage adhesion and improved migration in fibrin matrix and peritoneal tissue. Similarly, the high expression of $\alpha_M\beta_2$ on resident macrophages prevents their amoeboid migration, which is markedly increased in α_M-deficient macrophages. In contrast, α_D- and α_M-knockouts decrease the migration of M2 macrophages, demonstrating that moderate integrin expression supports cell motility. The results were confirmed in a diet-induced diabetes model. α_D deficiency prevents the retention of inflammatory macrophages in adipose tissue and improves metabolic parameters, while α_M deficiency does not affect macrophage accumulation. Summarizing, β_2 integrin-mediated adhesion may inhibit amoeboid and mesenchymal macrophage migration or support mesenchymal migration in tissue, and, therefore, represents an important target to control inflammation.

Keywords: integrin $\alpha_D\beta_2$(CD11d/CD18), integrin $\alpha_M\beta_2$(CD11b/CD18), macrophages (M1/M2), migration, inflammation, adhesive receptors

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INTRODUCTION

Monocyte/macrophage migration to, and accumulation within the site of inflammation are critical steps in the development of the inflammatory response. While acute inflammation is usually generated as a defensive mechanism, the development of chronic inflammation is an essential step in the initiation or progression of many devastating diseases including atherosclerosis, diabetes, obesity, arthritis and others (1–4). Macrophage accumulation at the damaged tissue is a hallmark of inflammation (5, 6). However, the particular subset of accumulated macrophages is critical for the further development or resolution of chronic inflammation. Classically activated (M1) macrophages produce a harsh pro-inflammatory response, while alternatively activated (M2) macrophages may have anti-inflammatory functions (7, 8). The balance between the accumulation of pro-inflammatory and anti-inflammatory macrophages regulates the fate of inflammation. So far, the mechanism of macrophage accumulation is not fully understood.

Macrophage accumulation at the site of inflammation depends upon monocyte recruitment, macrophage retention and emigration. Monocyte recruitment includes activation, diapedesis through the endothelial monolayer (2D migration) (9, 10), and migration through the extracellular matrix to the site of inflammation (3D migration). While the role of leukocyte adhesive receptors in 2D migration is well-established (9, 11), their contribution to macrophage migration through 3D extracellular matrix (ECM) is still unclear. Macrophages utilize two types of motility in a 3D environment—amoeboid and mesenchymal. Amoeboid migration is adhesion-independent movement that is based on flowing and squeezing. This migratory mode was shown to be dominant for neutrophils, dendritic cells and lymphocytes (12). Mesenchymal migration involves the classical adhesion-mediated mechanism that includes cell protrusion and adhesion of the leading edge, followed by detachment of the trailing edge and retraction of the contractile cell rear (13). It has been shown that cell-substratum adhesiveness regulates the fate of mesenchymal cell migration. Namely, an intermediate level of adhesiveness generates the optimal conditions for cell migration (14). Low adhesiveness does not support cell motility, while a very high level of adhesiveness thwarts cell locomotion because it inhibits cell detachment from the substrate (15, 16). The density of adhesive receptors on the cell surface is one of the most critical parameters of cell-substratum adhesiveness. Therefore, a high density of cell adhesion receptors that generate a high adhesiveness may lead to the retention of cells (15, 17).

Integrins are the most important cell adhesive receptors that are involved in monocyte/macrophage migration. Of particular note is the subfamily of β_2 integrins that are exclusively expressed on leukocytes and consist of four members: $\alpha_L\beta_2$ (CD11a/CD18), $\alpha_M\beta_2$ (CD11b/CD18), $\alpha_X\beta_2$ (CD11c/CD18), and $\alpha_D\beta_2$ (CD11d/CD18) (18). Integrins $\alpha_M\beta_2$ and $\alpha_D\beta_2$ are the most interesting members with regard to cell migration, since $\alpha_L\beta_2$ has no ligands in ECM (19) and $\alpha_X\beta_2$ demonstrated a very low expression on macrophages (20). In contrast, α_M and α_D have marked macrophage expression and share many ECM ligands (21, 22).

Different subsets of macrophages have a diverse expression of integrins (23) and, most importantly, possess different migratory characteristics (24). We hypothesize that integrin expression regulates the distinct migratory properties of M1-polarized, M2-polarized, and resident macrophages. We realize that *in vitro* activated M1 and M2 macrophages do not fully represent the varieties of pro-inflammatory and anti-inflammatory macrophages *in vivo*; however, these cells are appropriate models that can help us to understand the migratory mechanisms of different macrophage subsets during inflammatory diseases.

In our previous project, we found that the pro-atherogenic role of integrin $\alpha_D\beta_2$ depends upon the upregulation of α_D on pro-inflammatory M1 macrophages *in vitro* and on macrophages in atherosclerotic lesions, which apparently mediates macrophage retention (23). In agreement with this, α_D-deficiency reduced the development of atherosclerosis and released the migration of M1 macrophages *in vitro* (23).

In this paper we further develop this project by analysing the role of β_2 integrins on different subsets of macrophages and attempt to depict the mechanisms that stimulate cell migration/retention based on the analysis of integrin expression, cell adhesion, secretion of proteases, and mode of cell migration. We found a strong correlation between macrophage migration and expression of $\alpha_M\beta_2$ and $\alpha_D\beta_2$. A moderate expression of $\alpha_M\beta_2$ and $\alpha_D\beta_2$ on M2 macrophages supports cell movement, while the upregulation of $\alpha_D\beta_2$ on M1 macrophages and α_M on resident macrophages prevents mesenchymal and/or amoeboid migration. These results were verified by using α_M- and α_D-deficient macrophages in 3D *in vitro* migration and by using an *in vivo* model for the resolution of peritoneal inflammation and diet-induced diabetes.

Therefore, the regulation of β_2 integrin expression may help to shift the pro-/anti- inflammatory balance at the site of inflammation and reduced the pathophysiological outcome.

MATERIALS AND METHODS

Reagents and Antibodies

Reagents were purchased from Sigma-Aldrich (St. Louis, MO, United States) and Thermo Fisher Scientific (Waltham, MA, United States). Rock inhibitor (Y27632) and aprotinin were from Sigma-Aldrich. Recombinant human and mouse IFNγ, IL-4, MCP-1, and FMLP were purchased from Invitrogen Corporation (Carlsbad, CA, United States). Anti-human α_D mAb (clone 240I) was generously provided by Eli Lilly Corporation (Indianapolis, IN, United States). Polyclonal antibody against the α_D I-domain was described previously (10). The antibody recognizes both human and mouse α_D I-domains and has no cross-reactivity

Abbreviations: ECM, Extracellular matrix; EDTA, Ethylenediaminetetraacetic acid; FACS, Fluorescence-activated cell sorting; FMLP, N-Formylmethionine-leucyl-phenylalanine; IFNγ, interferon-γ; IL-4, interleukin 4; MCP-1, Monocyte chemoattractant protein-1; ROCK, Rho-associated protein kinase; TG, thioglycollate; WT, wide type; 2D, 2 dimensional; 3D, 3 dimensional.

with recombinant human and mouse α_M, $\alpha_{X,}$ and α_L I-domains. The antibody was isolated from rabbit serum by affinity chromatography using α_DI-domain-Sepharose. Mouse PE-cy7 and APC- conjugated anti-α_M mAb (clone M1/70) and F4/80 mAbs were from eBioscience (San Diego, CA, United States). The mAb 44a directed against the human α_M integrin subunit was purified from the conditioned media of the hybridoma cell line obtained from American Type Culture Collection (ATCC, Manassas, VA, United States) using protein A agarose (GE Healthcare, Piscataway, NJ, United States).

Animals

Wild type (C57BL/6J, stock # 000664) and integrin α_D-deficient (B6.129S7-*Itgad*^{tm1Bll}/J, stock # 005258 and integrin α_M-deficient (B6.129S4-*Itgam*^{tm1Myd}/J, stock # 003991) mice were bought from Jackson Laboratory (Bar Harbor, ME). α_D-deficient and α_M-deficient mice have been backcrossed to C57BL/6 for at least ten generations. All procedures were performed according to animal protocols approved by East Tennessee State University IACUC.

Flow Cytometry Analysis

Flow cytometry analysis was performed to assess the expression of α_D and α_M on mouse peritoneal macrophages. Cells were harvested and pre-incubated with 4% normal goat serum for 30 min at 4°C, then 2×10^6 cells were incubated with specific antibody for 30 min at 4°C. Non-conjugated antibodies required additional incubation with Alexa 488 or PE-cy7-donkey anti-mouse IgG (at a 1:1,000 dilution) for 30 min at 4°C. Finally, the cells were washed and analyzed using a Fortessa X-20 (Becton Dickinson).

Generation of Classically Activated (M1) and Alternatively Activated (M2) Mouse Macrophages

Peritoneal macrophages from 8 to 12 week old mice (WT and $\alpha_D^{-/-}$, $n = 3$ mice per group) were harvested by lavage of the peritoneal cavity with 5 ml of sterile PBS 3 days after intraperitoneal (IP) injection of 4% thioglycollate (TG; 0.5 ml). The cells were washed twice with PBS and resuspended in complete RPMI media. The cell suspension was transferred into 100 mm petri dishes and incubated for 2 h at 37°C in humidified air containing 5% CO_2 atmosphere. Non-adherent cells were washed out with RPMI media, and the adherent macrophages were replenished with RPMI media. The macrophages were differentiated to M1 and M2 phenotypes by treatment with recombinant mouse interferon-γ (IFN-γ) (100 U/ml, Thermo Fisher) and interleukin 4 (IL-4) (2 nM, Thermo Fisher), respectively, for 4 days. Medium with IFN-γ and IL-4 were changed every 2 days or as required. The M1 phenotype macrophages from WT and $\alpha_D^{-/-}$ were labeled with red fluorescent marker PKH26 and green fluorescent marker PKH67, respectively, according to the manufacturer's instructions (Sigma-Aldrich). The fluorescently-labeled cells were dissociated from the plates using 5 mM EDTA in PBS and used for the experiments thereafter.

Cell Adhesion Assay

The adhesion assay was performed as described previously (22) with modifications. Briefly, 96-well plates (Immulon 2HB, Cambridge, MA, United States) were coated with different concentrations of fibrinogen or Matrigel for 3 h at 37°C. The wells were post-coated with 0.5% polyvinyl alcohol for 1 h at 37°C. Mouse peritoneal macrophages or HEK 293 cells transfected with $\alpha_M\beta_{2,}$ or $\alpha_D\beta_2$ integrins were labeled with 10 μM Calcein AM (Molecular Probes, Eugene, OR) for 30 min at 37°C and washed with DMEM and resuspended in the same medium at a concentration of 1×10^6 cells/mL. Aliquots (50 μL) of the labeled cells were added to each well. For inhibition experiments, cells were mixed with antibodies and incubated for 15 min at 22°C before they were added to the coated wells. After 30 min of incubation at 37°C in a 5% CO_2 humidified atmosphere, the non-adherent cells were removed by washing with HBSS. The fluorescence was measured in a Synergy H1 fluorescence plate reader (BioTek, Winooski, VT, United States), and the number of adherent cells was determined from a labeled control.

Migration of Macrophages in 3D Fibrin Gel and Matrigel

The migration assay was performed as described previously (25). WT and $\alpha_D^{-/-}$ or WT and $\alpha_M^{-/-}$ peritoneal macrophages activated to M1 or M2 phenotype as described above were labeled with PKH26 red fluorescent dye and PKH67 green fluorescent dye, respectively. Cell migration assay was performed for 48 h at 37°C in 5% CO_2 in a sterile condition. An equal number of WT and $\alpha_D^{-/-}$ macrophages was evaluated by cytospin of mixed cells before the experiment and at the starting point before migration. Labeled WT (1.5×10^5) and $\alpha_D^{-/-}$ (1.5×10^5) activated macrophages were plated on the membranes of transwell inserts with a pore size of 8 μm and 6.5 mm in diameter (Costar, Corning, NY) precoated with fibrinogen (Fg). Fibrin gel (100 μl/sample) was made by 0.75 mg/ml Fg containing 1% FBS and 1% P/S and activated by 0.5 U/ml thrombin. Matrigel (50%) was diluted by RPMI-1640 supplemented with 1% FBS and 1% P/S. 30 nM of MCP-1 (or 100 nM FMLP) were added on the top of the gel to initiate the migration. Migrating cells were detected by Leica Confocal microscope (Leica-TCS SP8) and the results were analyzed and reconstructed using IMARIS 8.0 software.

Adoptive Transfer in the Model of Resolution of Peritoneal Inflammation

Adoptive transfer was performed as described previously (23). Briefly, fluorescently-labeled WT (red PKH26 dye) and $\alpha_D^{-/-}$ or $\alpha_M^{-/-}$ (green PKH67 dye) M1- or M2-activated macrophages were mixed in a 1:1 ratio and further injected intraperitoneally into wildtype mice at 4 days after thioglycollate (TG)-induced inflammation. 3 days later, peritoneal macrophages were harvested with 5 ml PBS supplemented with 5 mM EDTA. The percentages of red and green fluorescent macrophages in the peritoneal exudate were assessed by fluorescence microscopy, multi-color flow cytometer (Fortessa X-20) and imaging flow cytometry (ImageStream Mark II, Amnis).

The PKH26 and PKH67 dyes were switched in one experiment to verify the effect of dye on cell migration. We did not detect any difference between two dyes. The quantification of the data was analyzed by using Image Analysis Software (EVOS, Thermo Fisher).

Adoptive Transfer in the Model of Diet-Induced Diabetes

The approach is based on previously published method (26) with some modifications. Monocytes were isolated from the bone marrow progenitors of WT and α_D-deficient mice using magnetic bead separation kit (Miltenyi Biotec, Gaithersburg, MD, United States). Monocytes were labeled with red, PKH26 (WT) or green, PKH67 ($\alpha_D^{-/-}$) fluorescent dyes. Red (1.5×10^6) and green (1.5×10^6) cells were mixed together and injected in tail vein of wild type C57BL6 mice fed high fat diet (45% kcal/fat) for 8 weeks. After 3 days adipose tissue was isolated, digested as described previously (26) and analyzed using FACS (Fortessa X-20, BD, United States) and imaging flow cytometry (ImageStream Mark II, Amnis).

Glucose Tolerance and Insulin Sensitivity Tests

Wild type and $\alpha_D^{-/-}$ mice fed a high fat diet for 16 weeks were fasted overnight in a new cage containing water but no food, (∼16 h). The following morning mice were weighed, and an initial blood glucose level was measured using a glucometer and blood from the tail vein. Glucose (2 grams/kg body weight of 20% D-glucose) was administered IP and at 15, 30, 60, and 120 min post injection blood glucose was again measured.

For insulin sensitivity test, mice fed a high fat diet were fasted for 5 h, starting at 7 a.m. (lights on). After fasting, mice were weighed, and the initial level of blood glucose measured as described above. Insulin (0.75 mU/g) was injected I.P. and the level of blood glucose was evaluated at 15, 30, 45, and 60 min.

Quantitative RT-PCR

Cellular mRNA was extracted from macrophages using the Qiagen Oligotex mRNA Midi Kit. mRNA was reverse transcribed with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA, United States) and real-time quantitative PCR was performed using SYBR Green Supermix (Bio-Rad) on an MyIQ2 two color real-time PCR detection system (Bio-Rad), with the thermal cycler conditions suggested by the manufacturer. The sequences of integrin primers are shown below: α_D forward, 5′-GGAACCGAATCAAGGTCAAGTA-3′, and reverse, 5′-ATCCATTGAGAGAGCTGAGCTG-3′. α_M forward, 5′-TCCGGTAGCATCAACAACAT-3′ and reverse, 5′-GGTGAAGTGAATCCGGAACT-3′. α_4 forward, 5′-AAGGAAGCCAGCGTTCATATT-3′, and reverse, 5′-TCATCATTGCTTTTGCTGTTG-3′. α_5 forward, 5′-CAAGGTGACAGGACTCAGCA-3′, and reverse, 5′-GGTCTCTGGATCCAACTCCA-3′. α_X forward, 5′-CTGGATAGCCTTTCTTCTGCTG-3′, and reverse, 5′-GCACACTGTGTCCGAACTCA-3′. GAPDH or 5S rRNA were used as an internal control (Ambion/Life Technologies, Grand Island, NY, United States).

Statistical Analysis

Statistical analyses were performed using Student's *t*-test or Student's paired *t*-tests where indicated in the text using SigmaPlot 13. A value of $p < 0.05$ was considered significant.

RESULTS

Strong Adhesion of Classically-Activated (M1) Macrophages Is Converted in Weak Migration in Contrast to Well-migrated, but Low-Adherent Alternatively-Activated (M2) Macrophages

To evaluate the adhesive and migratory properties of M1 and M2 macrophages, we stimulated thioglycollate-induced peritoneal macrophages with IFNγ (M1-activated) or IL-4 (M2-activated) and evaluated the adhesion of these cells to fibrinogen and their migration in 3D fibrin matrix. The adhesion assay revealed a much stronger attachment of M1 macrophages (28.68 ± 5.33%) when compared to M2 macrophages (9.12 ± 2.79%) (**Figure 1A**). Moreover, M1 and M2 adherent cells possess different morphologies. While M1 macrophages have a rounded, flat, pancake-like shape after adhesion assay, M2 macrophages were elongated, and less spread out (**Figure 1B**). The development of M1 and M2 phenotypes were verified by upregulation of iNOS and ArgI, respectively (**Figure 2A**).

We tested how different adhesive properties affect macrophage cell migration (**Figures 1C–F**). Fluorescently labeled M1 (red, PKH26) and M2 (green, PKH67) macrophages were mixed in an equal number (**Supplementary Figure 1A**) and placed on a 3D fibrin gel where cell migration was stimulated via a MCP-1 gradient (**Figures 1C,E**). After 48 h, we detected a robust migration of M2 macrophages, which markedly exceeded the locomotion of M1 macrophages (**Figures 1D–F**). It has been shown previously that M1 and M2 macrophages demonstrate a similar chemotaxis to MCP-1 in 2D transwell assay (no ligand coated on membrane) (27). These data proved that the different migration of M1 and M2 macrophages in our 3D chemotaxis/haptokinesis assay does not regulated by different expression of CCR2 (chemotaxis), but by distinct adhesion-mediated migration (haptokinesis). To additionally verify it, the migration was repeated using a gradient of N-Formylmethionine-leucyl-phenylalanine (FMLP) and revealed similar results (**Supplementary Figure 1B**), therefore the adhesive receptors are potential cause of different migratory properties of M1 and M2.

THE LEVELS OF INTEGRIN EXPRESSION DETERMINE THE EFFECTS ON MACROPHAGE MIGRATION

Recently, we demonstrated that integrin α_D is upregulated on M1-polarized macrophages but does not change on M2-polarized macrophages (23). We evaluated the potential changes in the expression of other fibrin-binding macrophage adhesive receptors during M1 and M2 polarization (**Figure 2A**). The

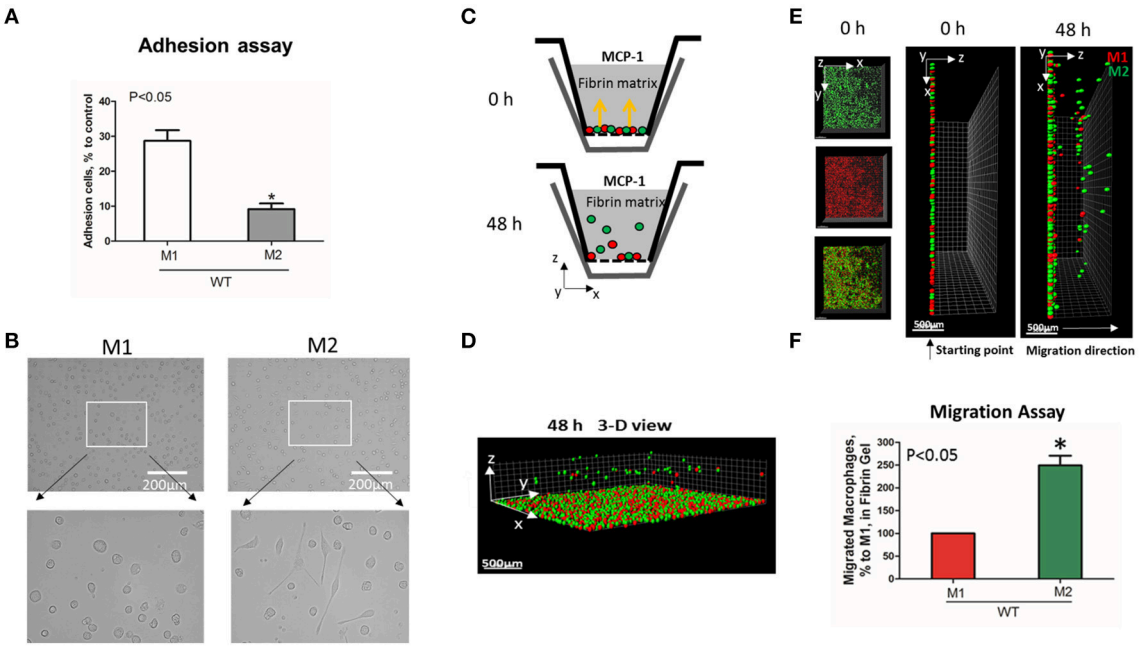

FIGURE 1 | M1-activated macrophages demonstrate much stronger adhesive properties but weaker migration in comparison to M2-activated macrophages. **(A)** Adhesion assay of WT M1 and M2-activated macrophages to Fg. 96-well plate was coated with 4 μg/ml Fg for 3 h at 37°C. Fluorescently labeled M1 and M2 macrophages were added to the wells and cell adhesion was determined after 30 min in a fluorescence plate reader. Data are presented as mean ± SEM. $^*P < 0.05$. **(B)** Morphologies of M1 (Left panel) and M2 (right panel) activated macrophages, scale bar=200 μm. **(C–F)** 3-D migration assay in Fibrin matrix using M1 and M2 activated macrophages labeled with PKH26 (Red) and PKH67 (Green) fluorescent dyes, respectively. C. Sketch diagram of the migrating cells in Boyden transwell chamber. Before migration (upper panel) and after 48 h migration (lower panel). **(D)** 3-D view of the migrating cells in Fibrin matrix after 48 h. **(E)** Labeled Cells were mixed in equal amounts and verified by scanning samples with confocal microscope before the initiation of migration (**E**. left and middle panels). Migration of macrophages was stimulated by 30 nM MCP-1 added to the top of the gel. After 48 h, migrating cells were detected by a Leica Confocal microscope (**E**. right panel). **(F)** The results were analyzed by IMARIS 8.0 software and statistical analyses were performed using Student's paired *t*-tests ($n = 4$ per group). Scale bar= 500 μm. Data are presented as mean ± SEM. $^*P < 0.05$.

RT-PCR results demonstrated that α_D is the only adhesive receptor that upregulates during M1 macrophage activation to compare with M2 subset (**Figure 2B**). We also detected the increased expression of integrin α_X on M2 macrophages; however, the total expression of α_X on macrophages is very low (20), which quashes its potential effect on macrophage migration. Therefore, the upregulation of integrin α_D is the most significant modification that may affect the migratory properties of M1 and M2 macrophages.

Based on these data, further analysis was focused on integrin α_D and related integrin α_M, that possess similar ligand binding properties, but distinct surface expressions. The contributions of integrin α_D and α_M to M1 and M2 migration were evaluated using α_D- and α_M-deficient macrophages. α_D deficiency reduced the adhesion of M1 macrophages to fibrinogen (**Figure 3A**), but significantly increased cell migration (**Figures 3C,** left panel; **3E**). In contrast, integrin α_M deficiency has very limited effect on adhesion, due to its moderate expression on M1 macrophages (23) (**Figure 2B**), and did not demonstrate a significant effect on cell locomotion (**Figures 3C,E**). Both integrins, α_D and α_M, have moderate expression on M2 macrophages (23) (**Figure 2B**). The adhesion of M2 macrophages depends on both integrins, which is demonstrated in the presence of antibodies and integrin-deficient cells (**Figure 3B**). In parallel assays, the reduced migration of α_M- and α_D-deficient macrophages verified that both integrins help to support the mesenchymal migration of M2 macrophages (**Figures 3D,F**).

The deficiency of α_D or α_M may also modify the expression of other fibrin-binding integrins that can affect cell migration. To test this possibility, we evaluated the expression of α_4, α_5, α_X, and α_M on $\alpha_D^{-/-}$, as well as α_D on $\alpha_M^{-/-}$ macrophages activated to M1 and M2 phenotypes using RT-PCR. We did not detect any marked changes, except for the reduced expression of α_5 and α_X on α_D-deficient M1 macrophages (**Supplementary Figure 2**). Clearly, these changes cannot significantly modify migration.

α_D-MEDIATED ADHESION IS CRITICAL FOR THE RETENTION OF M1 MACROPHAGES

Inflamed extracellular matrix contains different β_2 ligands, including fibronectin, vitronectin, thrombospondin, fibrinogen and others. Moreover, we recently showed that oxidative stress during inflammation may form ECM protein modifications with carboxyethylpyrole, which is also a ligand for β_2 integrins (25). To verify the role of α_D-mediated adhesion on cell migration, we performed macrophage migration in Matrigel, the model of

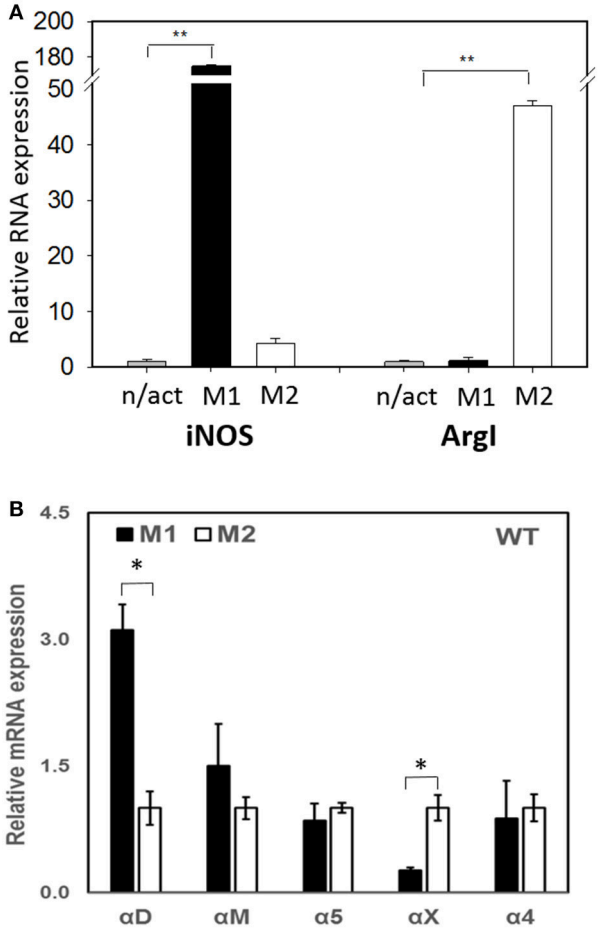

FIGURE 2 | (A) The expression of M1 (iNOS) and M2 (Arg I) markers on IFN-γ (M1) and IL-4 (M2) stimulated macrophages using Real Time-PCR. Statistical analyses were performed using paired Student *t*-tests ($n = 3$ per group). Data are presented as mean ± SEM. $^{**}P < 0.01$, compared to non-activated (n/act). **(B)** The expression of fibrin-binding integrins during M1 and M2 polarization. Open bars—non-activated; black bars M1-polarized, gray bars M2-polarized macrophages. Statistical analyses were performed using Student's paired *t*-tests (non-activated to activated) ($n = 3$ per group). Data are presented as mean ± SEM. $^{*}P < 0.05$.

basement membrane matrix, which consists of laminin, collagen IV and proteoglycans. Notably, these proteins are not ligands for integrin $\alpha_D\beta_2$ or $\alpha_M\beta_2$. To confirm this, we tested the adhesion of $\alpha_D\beta_2$- and $\alpha_M\beta_2$-transfected HEK293 cells to a plate coated with Matrigel (**Figure 4A**). Both cell lines demonstrated strong adhesion to Matrigel, but this adhesion was independent of α_D and α_M, since anti-α_D and anti-α_M antibodies did not inhibit this binding. In contrast, the adhesion of $\alpha_M\beta_2$ and $\alpha_D\beta_2$-transfected cells to fibrinogen was significantly inhibited by these antibodies (21, 28) (**Figure 4B**). Apparently, the adhesion to Matrigel is mediated by integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$, which are receptors for laminin and collagen, and are expressed endogenously on HEK293 cells (29–31). To verify this hypothesis, we evaluated the adhesion of MOCK-transfected HEK293 cells to Matrigel and fibrinogen. These cells did not support the adhesion to fibrinogen, but demonstrated the same level of adhesion to Matrigel as $\alpha_D\beta_2$ and $\alpha_M\beta_2$ transfected cells (**Figures 4A,B**). Therefore, cells do not use $\alpha_D\beta_2$ for the adhesion to Matrigel. Accordingly, we detected a similar level of wild type and α_D-deficient M1 macrophage migration through Matrigel, which is distinct to our data in α_D-dependent fibrin matrix. Therefore, this result is in agreement with our hypothesis regarding the critical role of α_D-mediated adhesion for macrophage retention during 3D migration (**Figure 4C**).

However, one of the mechanisms that affects mesenchymal migration is the secretion of MMPs that degrade Matrigel. To test the potential effect of α_M or α_D deficiency on MMPs secretion, M1 and M2 macrophages were incubated in 48-well plates for 24 h and the media was tested using gelatin zymography as we described previously (32) (**Figure 4D**). First, we found a much stronger secretion of MMPs (specifically MMP-9) in M2 macrophages in comparison to M1 macrophages. Second, we did not detect any significant effect of α_D- or α_M-knockout on MMPs secretion, particularly in regard to M1-polarized macrophages.

Interestingly, the robust secretion of collagen-specific MMP-9 by M2 macrophages can be responsible for the strong migration of these cells in Matrigel. The migration of M1 and M2 macrophages was performed in separate gels to avoid the effect of M2-released MMP-9 on the migration of M1 macrophages (**Figure 5**). In contrast, similar secretion of MMPs in WT and α_D-deficient M1 macrophages allowed us to compare these two cell types in one sample. Therefore, the similar migration of WT and α_D macrophages in Matrigel was not regulated by a different level of MMPs secretion, but by the lack of α_D-mediated adhesion.

A HIGH EXPRESSION OF α_M ON RESIDENT MACROPHAGES REDUCES THEIR AMOEBOID MIGRATION

To test the effect of high expression of other integrins on cell locomotion, we evaluated α_M-dependent migration of resident macrophages. α_M has a very high expression on peritoneal resident macrophages (**Figure 6A**). A comparable analysis of 3D migration in fibrin matrix between WT and α_M-deficient resident peritoneal macrophages revealed a strong improvement in the migration of the $\alpha_M^{-/-}$ subset (**Figures 6B,C** right panel). Notably, α_D-deficiency, which has a very low expression on resident macrophages (**Figure 6A**), did not affect macrophage migration (**Figures 6B,C** left panel). These results demonstrated that α_M at high density on the cell surface can also prevent migration. It has been shown that resident macrophages apply the amoeboid migratory mode (24). Accordingly, the migration of WT and $\alpha_M^{-/-}$ in the presence of ROCK inhibitor, the inhibitor of amoeboid migration (33), resulted in a dramatic reduction in both the number of migrated cells and migratory distance (**Figures 6B,C** right panel). Therefore, macrophage adhesion-independent amoeboid migration can be reduced by integrin-mediated strong adhesion.

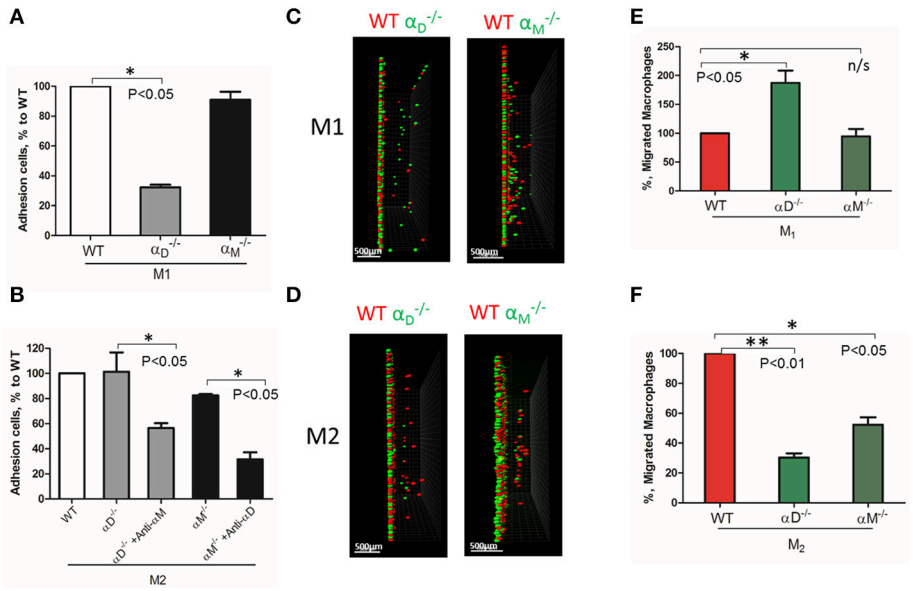

FIGURE 3 | The level of integrin expression determines the effect on macrophage migration. **(A,B)** Adhesion assay to fibrinogen of WT, $\alpha_D^{-/-}$ and $\alpha_M^{-/-}$ macrophages activated to M1 **(A)** and M2 **(B)** phenotypes. Some samples in **(B)** were pre-incubated with anti-α_M and anti-α_D blocking antibodies before the adhesion assay. Data are presented as mean ± SEM. *$P < 0.05$. **(C,D)** Migration assay of α_D- and α_M-deficiency M1 **(C)** and M2 **(D)** macrophages in 3D fibrin matrix. After 48 h, migrating cells were detected by a Leica Confocal microscope and the results were analyzed by IMARIS 8.0 software, scale bar= 500 μm. **(E,F)** Statistical analyses were performed using Student's paired *t*-test ($n = 4$ per group). Data are presented as mean ± SEM. *$P < 0.05$, **$P < 0.01$.

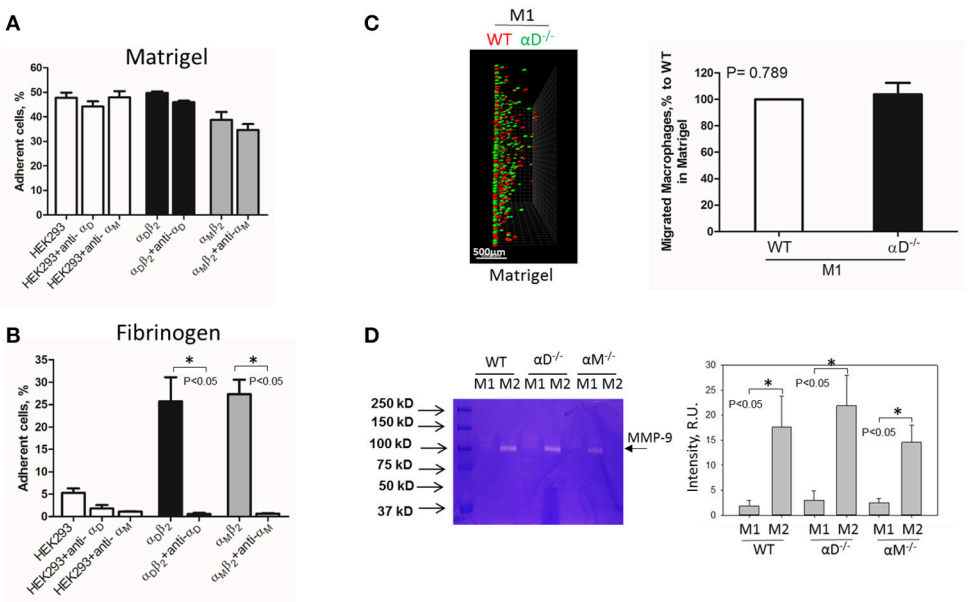

FIGURE 4 | Matrigel does not support integrin α_D-mediated adhesion and retention of M1 macrophages. **(A,B)** Adhesion of $\alpha_D\beta_2$- and $\alpha_M\beta_2$-transfected and mock-transfected HEK293 cells to Matrigel **(A)** and fibrinogen **(B)**. The adhesion was performed as described above. Data are presented as mean ± SEM. *$P < 0.05$. **(C)** 3-D migration assay of WT and α_D-deficient M1 macrophages in Matrigel. Migration was stimulated by 30 nM MCP-1 added to the top of the gel. After 48 h, migrating cells were detected by a Leica Confocal microscope (Leica-TCS SP8) **(C,** left panel**)**. Scale bar= 500 μm. The results were analyzed by IMARIS 8.0 software. **(C,** right panel**)**. **(D)** Evaluation of MMPs in culture media after macrophage adhesion. WT, $\alpha_D^{-/-}$ and $\alpha_M^{-/-}$ M1- and M2-activated macrophages were plated on fibrinogen. Media was collected after overnight incubation and analyzed by gelatin-zymography (**D,** right panel). The intensity of gelatin degradation was evaluated by Fuji software (**D,** left panel). Statistical analyses were performed using Student's paired *t*-tests ($n = 4$ per group). Data are presented as mean ± SEM. *$P < 0.05$.

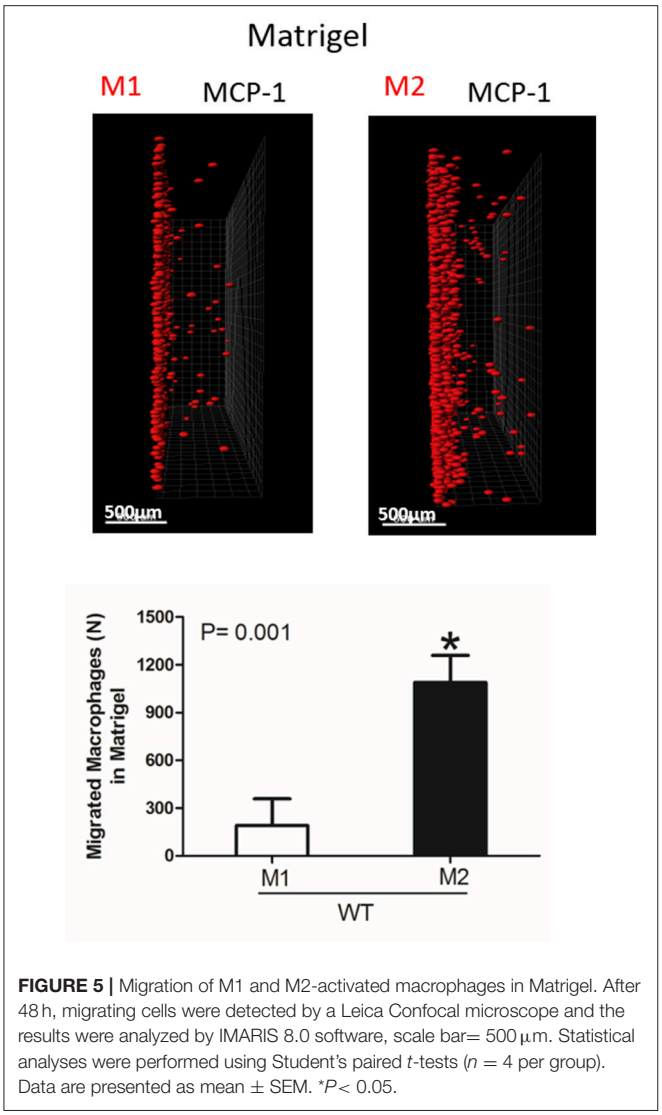

FIGURE 5 | Migration of M1 and M2-activated macrophages in Matrigel. After 48 h, migrating cells were detected by a Leica Confocal microscope and the results were analyzed by IMARIS 8.0 software, scale bar= 500 µm. Statistical analyses were performed using Student's paired *t*-tests ($n = 4$ per group). Data are presented as mean ± SEM. *$P < 0.05$.

IN VIVO MIGRATION OF M1, M2, AND RESIDENT MACROPHAGES CONFIRMED THE RESULTS OF THE 3D MIGRATION ASSAYS

To verify our *in vitro* results, we performed *in vivo* migration using the model of resolution of peritoneal inflammation as we have done previously (23). After the development of thioglycollate-induced peritoneal inflammation, macrophages migrate to, and accumulate within, the peritoneal cavity. The resolution of inflammation is started after 96 h and is characterized by the intensive emigration of macrophages from the peritoneal cavity to the lymphatics (34). We injected adoptively transferred M1 and M2 macrophages to assess their migratory properties in the *in vivo* environment (**Figure 7A**). *In vitro*-activated M1 and M2 macrophages were labeled with PKH26 and PKH67 fluorescent dyes, respectively. The recipient mice were first injected with thioglycollate and then, 96 h later, with an equal number of fluorescently labeled M1 and M2 macrophages. After an additional 72 h, the cells from the peritoneal cavity were collected and the number of M1 and M2 adoptively transferred macrophages was evaluated. The cytospin of harvested samples demonstrated the preferential accumulation of M1 macrophages (red fluorescence) in the peritoneal cavity (**Figure 7B** and **Supplementary Figure 3**), which corresponds to our *in vitro* migration assays (**Figures 1D–F**). Our FACS data confirmed these results, since mostly M1 macrophages reside in the peritoneal cavity, while M2 macrophages emigrate during resolution (5.02 ± 0.31% vs. 2.57 ± 0.41%) (**Figure 7C**). The Amnis imaging flow cytometry verified the size and morphology of fluorescently labeled macrophages in the peritoneal cavity (**Figure 7D**).

According to our *in vitro* results and previous data (23) we demonstrated that α_D-deficiency on an M1 background stimulated the emigration of macrophages from the peritoneal cavity, while α_M-knockout had no effect (**Figure 7E**). In contrast, we detected an increased accumulation of α_M-deficient M2 macrophages in the cavity, which demonstrates the supportive role of α_M in the migration of M2 macrophages and remained consistent with our *in vitro* results. Surprisingly, we did not detect the same effect for $\alpha_D^{-/-}$ macrophages. The difference between the migrations of WT and $\alpha_D^{-/-}$ M2 macrophages was not significant (**Figure 7E**, lower panel).

WT and $\alpha_M^{-/-}$ resident macrophages were isolated and tested using the same resolution of inflammation assay. After 72 h, we detected predominantly wild type cells in the peritoneal cavity, while α_M-deficient macrophages emigrated (**Figure 8A**). This result was verified by flow cytometry. The number of red-fluorescent WT cells isolated from the peritoneal cavity significantly exceeded the number of green-fluorescent $\alpha_M^{-/-}$ cells (Q4 vs. Q1), (**Figure 8B**). Based on this result, we suggest that α_M serves for the supporting resident macrophage accumulation in the tissue, and α_M-deficiency increases the efflux of resident macrophages.

To confirm this conclusion, we evaluated the number of macrophages in the non-inflamed peritoneal cavity of wild type and $\alpha_M^{-/-}$ mice. Isolated peritoneal cells were stained with F4/80 antibodies and analyzed by flow cytometry to detect the percentage of macrophages. We found that α_M-deficiency resulted in a twofold reduction in the number of resident macrophages in the cavity (**Figure 8C**). In contrast, α_D-deficiency on resident peritoneal macrophages did not affect macrophage number. These data are in agreement with our *in vitro* and *in vivo* migration assays.

α_D DEFICIENCY REDUCES MACROPHAGE ACCUMULATION IN ADIPOSE TISSUE AND IMPROVES METABOLIC PARAMETERS

To further confirm the contribution of $\alpha_D\beta_2$ to macrophage retention in the site of chronic inflammation, we used the

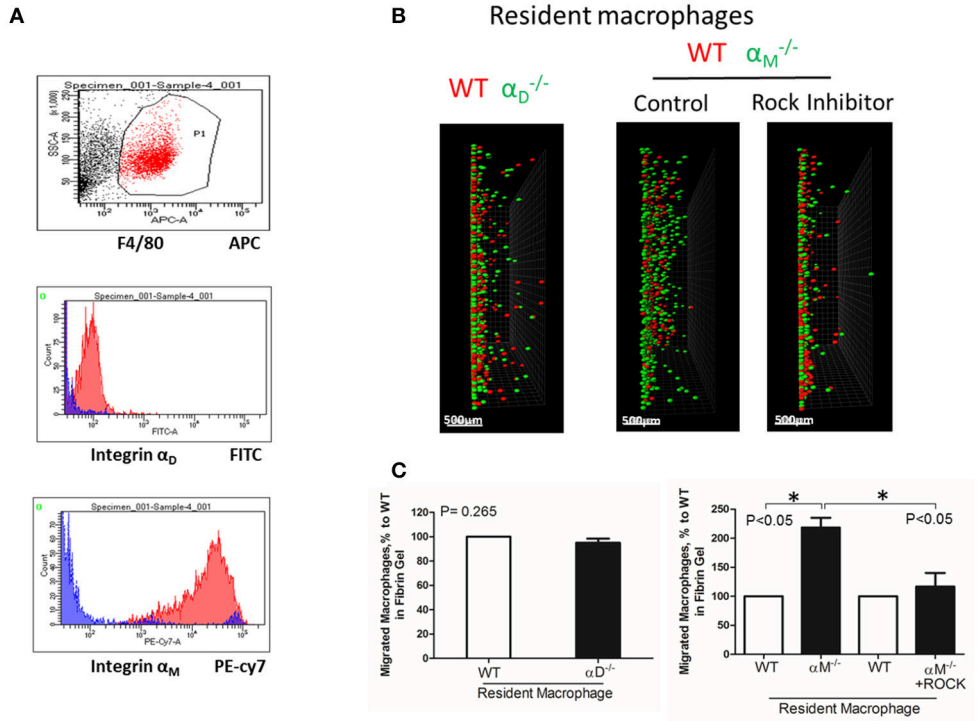

FIGURE 6 | A high expression of α_M on resident macrophages reduces their amoeboid migration. **(A)** The expression of integrin α_D and α_M on resident macrophages was detected with anti-α_D and anti-α_M antibodies, respectively, and tested by flow cytometry analysis. **(B)** Migration of peritoneal resident macrophages in 3-D fibrin matrix. Migrating resident macrophages from WT and $\alpha_D^{-/-}$ mice are shown in the left panel. The middle and right panels represent the migrating resident macrophages from WT and $\alpha_M^{-/-}$ mice with or without Rock inhibitor (Y27632). Migrating cells were detected by a Leica Confocal microscope (Leica-TCS SP8). Scale bar= 500 μm. **(C)** The results were analyzed by IMARIS 8.0 software. Statistical analyses were performed using Student's paired *t*-tests ($n = 4$ per group). Data are presented as mean ± SEM. $^*P < 0.05$.

model of diet-induced diabetes. The accumulation of pro-inflammatory (M1-like macrophages) in the inflamed adipose tissue is a hallmark of the inflammatory component of diabetes (26). It has been shown that α_D is upregulated in the adipose tissue during diet-induced obesity (35), which concurs with the upregulation of α_D on M1-activated macrophages *in vitro* and in atherosclerotic lesions (23). We also detected a strong expression of $\alpha_D\beta_2$ on adipose tissue macrophages of C57BL6 mice after 8 weeks of a high fat diet (45 kcal% fat) (**Supplementary Figures 4A,B**). To assess the role of $\alpha_D\beta_2$ and $\alpha_M\beta_2$ in macrophage migration during chronic inflammation, monocytes isolated from WT and $\alpha_D^{-/-}$ (or $\alpha_M^{-/-}$) mice were labeled with red (PKH26) or green (PKH67) dyes, respectively, mixed in equal number and injected intravenously into mice on a high fat diet (**Supplementary Figure 4C**). The accumulation of adoptively transferred WT and integrin-deficient macrophages in the adipose tissue of these mice was evaluated after 3 days. The isolated adipose tissue was digested and analyzed by multi-color FACS. We detected a 3-fold decrease in the number of α_D-deficient macrophages (in comparison to WT) in the visceral adipose tissue (**Figures 9A,B**). The result was verified by Imaging flow cytometry that confirmed the presence of labeled cells in the digested adipose tissue (**Figure 9C**). More importantly, it also demonstrates the maturation of labeled macrophages, since migrated cells expressed macrophage receptor F4/80 (**Figure 9C**, Lower panels), while injected monocytes lack this expression (**Figure 9C**, Upper panel). Interestingly, the deficiency of integrin α_M, which did not significantly upregulate on M1 macrophages (23) (**Figure 2B**) had no effect on macrophage accumulation in adipose tissue (**Figure 9A**, Lower panel). Our previous data demonstrate that α_D deficiency does not affect monocyte recruitment from circulation during inflammation (23). Therefore, these results are in agreement with our *in vitro* and *in vivo* experiments and with recently published data that α_M deficiency does not affect the accumulation of macrophages during diet-induced obesity (36, 37).

The assessment of metabolic parameters of α_D-knockout and WT mice after 16 weeks on a high fat diet confirm the physiological significance of our results by showing that a reduced number of macrophages in the adipose tissue of $\alpha_D^{-/-}$ improved glucose tolerance and insulin sensitivity (**Figure 9D**). On the other hand, the recently published data did not reveal a change in glucose tolerance test of α_M-deficient mice in comparison to WT control after 20 weeks of high-fat diet, but detected decreased insulin sensitivity in skeletal muscle and liver (37).

Taken together, these results provide the link between integrin expression and potential pathophysiological functions.

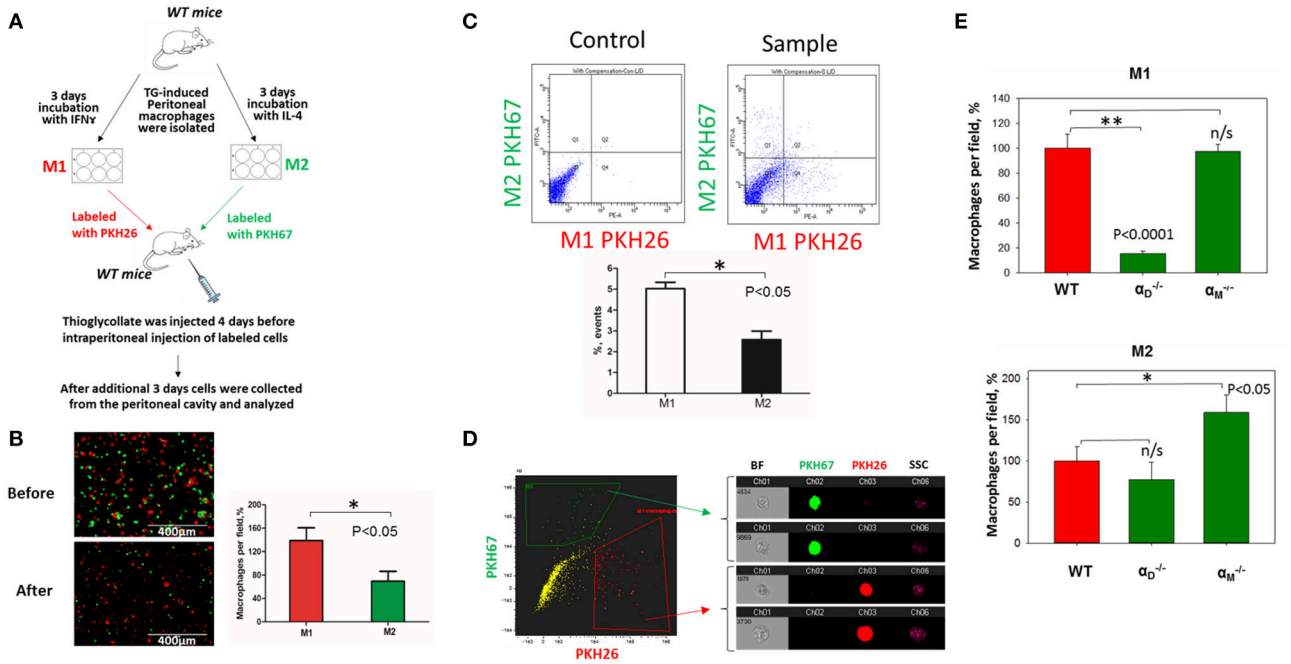

FIGURE 7 | *In vivo* migration of M1 and M2 macrophages confirmed the results of the 3D migration assays. **(A)** The model of *in vivo* resolution of peritoneal inflammation. Peritoneal macrophages were isolated from WT mice at 3 days after injection of thioglycollate (TG) and placed on petri dish for 3 days incubation with 100 U/ml IFNγ or 2nM IL-4 to generate M1 and M2 activated macrophages, respectively. Collected M1 and M2 macrophages were labeled with PKH26 or PKH67 fluorescent dyes. Labeled M1 and M2 macrophages were mixed in a 1:1 ratio and further injected intraperitoneally into WT mice with 4 days predisposed TG-induced peritoneal inflammation. **(B)** The equal ratio of red and green macrophages before the injection was verified by sample cytospin preparation (**B,** upper panel). 3 days later, peritoneal macrophages were harvested, and the percentages of red and green fluorescent macrophages were assessed by cytospin (**B**, lower panel) and flow cytometry **(C,D)**. The quantification of the data was analyzed by using Image Analysis Software (EVOS, Thermo Fisher) at least 4 fields of view per sample ($n = 4$) (**B**, right panel). **(C)** Flow cytometry. Live isolated cells were selected with live/dead kit and analyzed using 488 and 567 channels (Fortessa X-20). The results were analyzed with Diva software and statistical analysis was performed using Student's *t*-test. Data are presented as mean ± SEM. $^*P < 0.05$. **(D)** Imaging flow cytometer. The population of single, alive cells was analyzed on red and green channels and individual cells were evaluated in green and red positive areas (ImageStream Mark II, Amnis). Channel 1- Brightfield (BF). Channel 2- 488 wavelength (PKH67). Channel 3—566 wavelength (PKH26), channel 6—side scattering (SSC). **(E)** M1- and M2-activated macrophages in the peritoneal cavity during the resolution of peritoneal inflammation. The quantification of the data was analyzed by using Image Analysis Software (EVOS, Thermo Fisher) 4–6 fields of view per sample ($n = 4$). Data are presented as mean ± SEM. $^*P < 0.05$.

Apparently, the same integrin can support or inhibit 3D migration in tissue depending on the macrophage subset and the level of integrin expression on the cell surface.

DISCUSSION

The accumulation of macrophages at the site of inflammation is a complex physiological process that is critical for the development and resolution of inflammation. Macrophage apoptosis, proliferation and chemokine stimulation are important components of this mechanism, but the adhesive receptors that regulate the macrophage accumulation via cell migration and cell retention are the critical factors that generate the final outcome.

During the last decade, the role of adhesive receptors, particularly integrins, in the three-dimensional migration of immune cells in tissue has been questioned due to a new mechanism, the amoeboid mode of migration, being suggested (12, 38). However, recent data demonstrate that some immune cells, particularly macrophages, utilize adhesion-mediated mesenchymal migration in 3D matrices (13, 39). It has been shown that the migratory mode of macrophages depends on the environment and density of matrix (33). Previously, based on 2D models, it was suggested that cell migration is regulated by cell-substratum adhesiveness, which depends on substrate concentration, adhesive receptor density and affinity (15). This theory postulates that an intermediate level of adhesiveness (or intermediate expression of the adhesive receptors) is optimal for cell migration, while very low adhesiveness does not support cell locomotion and very high adhesiveness inhibits migration due to the prevention of the detachment of adhered cells. However, this theory was not evaluated during 3D migration in the tissue, which has more complex regulatory mechanisms and much stronger physiological implications. In this project, we tested integrins $\alpha_M\beta_2$ and $\alpha_D\beta_2$ as physiologically relevant models for studying the role of adhesive receptors during the migration of different subsets of macrophages. We discussed resident peritoneal macrophages and two subsets of monocyte-derived activated macrophages—classically activated (called M1), which can be generated by IFNγ/LPS or TNFα stimulation; and alternatively activated, which are produced by stimulation with IL-4 and/or IL-13 (called M2a) (7). For simplicity, we

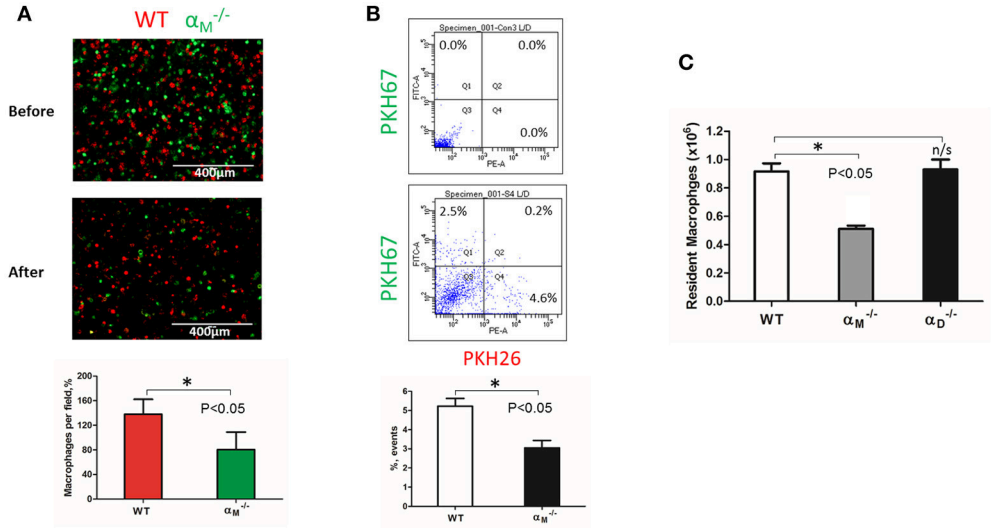

FIGURE 8 | α_M deficiency improve efflux of resident macrophages. **(A)** Fluorescently-labeled resident peritoneal macrophages isolated from WT and $\alpha_M^{-/-}$ mice were mixed in equal numbers and confirmed by cytospin (**A**, upper panel). Labeled cells were injected intraperitoneally into WT mice 4 days after TG-induced inflammation. After 3 days, the harvested peritoneal cells were cytospun (**A**, middle panel). The quantification of the data was analyzed using *t*-test at least 4 fields of view per sample ($n = 4$) by Image Analysis Software (EVOS, Thermo Fisher) (**A**, lower panel). Data are presented as mean ± SEM. $^*P < 0.05$. **(B)** The harvested macrophages were also assessed by flow cytometry and the percentages of red (Q4) and green (Q1) fluorescent cells were assessed. Data are presented as mean ± SEM. $^*P < 0.05$. **(C)** The amount of resident WT, $\alpha_M^{-/-}$ and $\alpha_D^{-/-}$ macrophages was evaluated by assessing the number and percentage of macrophages in non-inflamed peritoneal cavity of mice. Isolated peritoneal cells were counted and the number of WT, $\alpha_M^{-/-}$ and $\alpha_D^{-/-}$ resident macrophages were calculated based on the percentage of F4/80 positive population in flow cytometry analysis. Data are presented as mean ± SEM. $^*P < 0.05$.

are calling the latter group M2. We realize that M1 and M2 activated macrophages are simplified models; and macrophages in the atherosclerotic lesion and adipose tissue may represent "mixed phenotypes." However, these two subsets characterize the most variable difference in macrophage functional properties, and therefore, are an appropriate model for analyzing β_2 integrin expression and functions in different macrophage subsets.

Our experimental approach is based on several observations. First, α_D and α_M share similar ligands (21, 22); second, these two integrins form a complex with the same β_2 subunit, thus leading to similar integrin-mediated outside-in signaling during the interaction with the ligand; and third, the expressions of α_D and α_M are distinct on M1-polarized, M2-polarized and resident macrophages. We demonstrated that α_D is upregulated on M1 macrophages, while the expression of α_M is moderate (**Figure 2**) and (23). In contrast to these observations, the resident macrophages express a low level of α_D, but have a high density of α_M (**Figure 6**). At the same time, the expressions of both α_D and α_M integrins on M2 macrophages are intermediate (**Figure 2**).

Using these three subsets of macrophages, we found that 1) M2 macrophages possess much stronger migratory ability within 3D matrix in comparison with M1. 2) Integrins $\alpha_D\beta_2$ and $\alpha_M\beta_2$ are important receptors that regulate cell migration. 3) Similar to the 2D migration, integrins can support mesenchymal 3D cell migration at the intermediate density and prevent mesenchymal and amoeboid cell migration at high levels of expression. 4) Even the adhesion-independent amoeboid mode can be negatively-regulated by a high expression of β_2 integrins.

In this project, we show that strong adhesion via integrins is critical for cell retention that defines the different migratory properties of M1 and M2 macrophages. (**Figures 3**, **6**). The analysis of α_M, α_X, α_D, α_5, and α_4, integrins demonstrates that the upregulation of α_D on M1 macrophages is a major change in integrin expression during M1 activation. Therefore, $\alpha_D\beta_2$-mediated adhesion is crucial for the prevention of M1 macrophage migration. In a parallel line of evidence, we found that the lack of α_D-dependent substrate (exemplified in Matrigel) eliminates the effect of α_D on cell migration in this matrix (**Figure 4**). Importantly, α_D-deficiency does not significantly change the expression of other macrophage integrins and the levels of MMP expression, which rules out the possibility for an indirect effect of α_D knockout on M1 macrophage migration.

Taken together, these results propose that the accumulation of M1 macrophages at the site of inflammation is mediated by strong adhesion which promotes cell retention and the progression of chronic inflammation. In agreement with that, α_D-deficiency prevents the accumulation of adoptively transferred fluorescently-labeled macrophage accumulation in adipose tissue during diabetes. The reduced number of macrophages is associated with reduced inflammation and improved glucose tolerance and insulin sensitivity in α_D-knockout mice. These data correspond to our previous

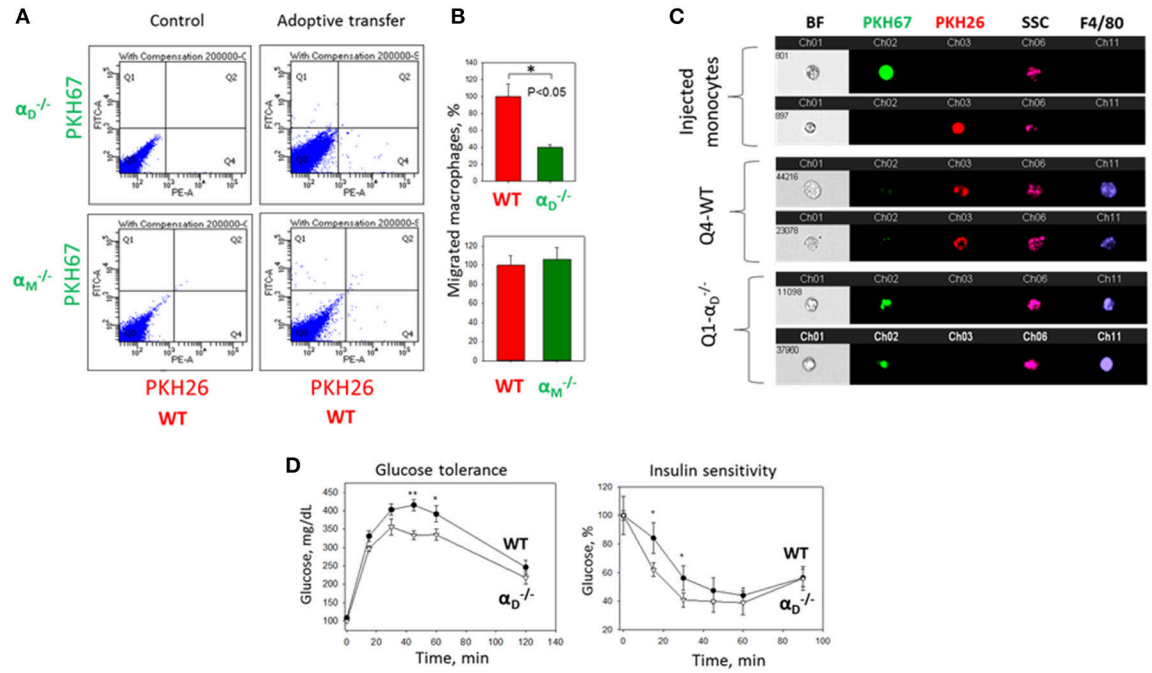

FIGURE 9 | α_D deficiency reduces accumulation of monocyte-derived macrophages in adipose tissue and improves metabolic parameters during diet-induced diabetes. **(A)** WT and $\alpha_D^{-/-}$ (or $\alpha_M^{-/-}$) monocytes were isolated from bone marrow, labeled with red (WT) or green ($\alpha_D^{-/-}$) fluorescent dyes, respectively, mixed in an equal amount and injected into the tail vein of WT mice fed for 8 weeks with high fat diet (45% kcal/fat). After 3 days visceral adipose tissue was isolated, digested and analyzed using flow cytometry. **(B)** Statistical analyses were performed using Student's paired *t*-tests ($n = 4$ per group). Data are presented as mean ± SEM. $^*P < 0.05$. **(C)** Imaging flow cytometry. Upper panel represents the injected monocytes, isolated from WT and $\alpha_D^{-/-}$ (or $\alpha_M^{-/-}$) mice, labeled with red and green fluorescent dyes, respectively. Middle (Q4) and lower(Q1) panels represent the labeled cells in digested adipose tissue. Channel 11- F4/80 represents macrophage staining. **(D)** WT mice (black circles) and α_D-knockout mice (white triangles) were fed with high fat diet for 16 weeks and glucose intolerance (left panel) and insulin resistance (right panel) were evaluated. $N = 6$ for $\alpha_D^{-/-}$ and $n = 9$ for WT per group. A statistical analysis was performed using Student's *t*-test. Data are presented as mean ± SEM. $^*P < 0.05$; $^{**}P<0.01$, compared to $\alpha_D^{-/-}$ group.

results, that α_D-deficiency reduced macrophage accumulation in atherosclerotic lesions and the development of atherosclerosis (23). Therefore, the upregulation of α_D on pro-inflammatory macrophages during diabetes (35) or atherosclerosis (23) demonstrates a similar outcome, which is manifested in the macrophage retention at the site of inflammation and disease development. Interestingly, α_M deficiency has pro-atherogenic effect on female and no effect on male mice (40). In agreement with this result, it has been recently shown that α_M deficiency elevates glucose level and decreased insulin sensitivity after 16 weeks on a high fat diet. Taken together, these data confirm the opposite role of $\alpha_D\beta_2$ and $\alpha_M\beta_2$ on pro-inflammatory M1 macrophages.

In contrast, the stronger migratory properties of M2 macrophages indicate that these cells more easily leave the tissue toward the lymphatics. The increased phagocytic properties of M2 macrophages, coupled with their high migratory abilities, confirm the major function of anti-inflammatory macrophages—phagocytosis followed by efflux from the tissue. α_D and α_M support the motility of M2 macrophages, and therefore promote the emigration of M2 macrophages from the inflamed tissue. Interestingly, the role of α_M in macrophage efflux during resolution was proposed previously (41).

The published data demonstrates that M2 macrophages may apply both locomotion modes, amoeboid and mesenchymal, which is supported by our observations regarding the α_M and partially α_D-mediated mesenchymal migration of M2 macrophages (**Figure 3**). In contrast, resident macrophages use preferentially amoeboid motility. Using ROCK inhibitor, we confirmed the preferential amoeboid migration of resident macrophages, but also demonstrated that amoeboid migration can be increased after the knockout of α_M integrin, which has a high density on these cells (**Figure 6**). Therefore, these data propose an anchoring role for integrin $\alpha_M\beta_2$ for resident macrophages in tissue. This mechanism may be important for the normal homeostasis and mobilization the initial immune defense, which is mediated by resident macrophages. We showed that α_M-deficiency reduced macrophage numbers in the non-inflamed peritoneal cavity (**Figure 6**). Therefore, the different immune pathologies associated with α_M-deficiency can be at least partially related to the impaired resident macrophage number. Most importantly, since integrins can block (or reduce) amoeboid migration, it suggests the potential role of integrins in the regulation (particularly, inhibition) of 3D migration of other immune cells that use only amoeboid movement (for example neutrophils or dendritic cells).

In summary, our study demonstrates the important contribution of $\alpha_D\beta_2$ and $\alpha_M\beta_2$ to the locomotion of distinct macrophage subsets and proposes a β_2-integrin dependent mechanism of macrophage retention in the tissue and efflux during the resolution of inflammation.

AUTHOR CONTRIBUTIONS

KC designed and performed the experiments, analyzed the data and edited the manuscript. CA performed the experiments and analyzed the data. NP analyzed the data and edited the manuscript. VY designed the research, performed the experiments, analyzed the data, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02650/full#supplementary-material

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MINI REVIEW
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Nuclear Deformation During Neutrophil Migration at Sites of Inflammation

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Cell migration is indispensable for various biological processes including angiogenesis, wound healing, and immunity. In general, there are two different migration modes described, the mesenchymal migration mode and the amoeboid migration mode. Neutrophils rapidly migrate toward the sites of injury, infection, and inflammation using the amoeboid migration mode which is characterized by cell polarization and a high migration velocity. During site-directed trafficking of neutrophils from the blood stream into the inflamed tissue, neutrophils must first withstand shear stress while migrating on the 2-dimensional endothelial surface. Subsequently, they have to cross different physical barriers during the extravasation process including the squeezing through the compact endothelial monolayer that comprises the blood vessel, the underlining basement membrane and then the 3-dimensional meshwork of extracellular matrix (ECM) proteins in the tissue. Therefore, neutrophils have to rapidly switch between distinct migration modes such as intraluminal crawling, transmigration, and interstitial migration to pass these different confinements and mechanical barriers. The nucleus is the largest and stiffest organelle in every cell and is therefore the key cellular element involved in cellular migration through variable confinements. This review highlights the importance of nuclear deformation during neutrophil crossing of such confinements, with a focus on transendothelial migration and interstitial migration. We discuss the key molecular components involved in the nuclear shape changes that underlie neutrophil motility and squeezing through cellular and ECM barriers. Understanding the precise molecular mechanisms that orchestrate these distinct neutrophil migration modes introduces an opportunity to develop new therapeutic concepts for controlling pathological neutrophil-driven inflammation.

Keywords: inflammation, neutrophil, migration, nuclear deformation, myosin1f

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INTRODUCTION

Neutrophils are important players in innate immunity as they represent the first immune cells arriving at site of tissue injury or infection. Besides their ability to control local infections, neutrophils are critically involved in tissue remodeling including wound healing, angiogenesis, and tumor metastasis (1–6). During acute inflammation, neutrophils are recruited from the blood stream into the inflamed tissue following a well-defined multi-step recruitment cascade.

This cascade is initiated by neutrophil capturing via specific adhesion receptors on inflamed blood vessels, followed by fast and slow rolling, arrest, adhesion strengthening, intraluminal crawling, and protrusion through endothelial junctions in search for exit cues (7). These intravascular events are then followed by neutrophil squeezing through junctions ending in successful transendothelial migration (TEM), followed by abluminal crawling of the neutrophil in between the endothelial layer and its associated pericyte sheet, and interstitial migration to the final destination at the site of inflammation (8). An indispensable prerequisite for efficient neutrophil recruitment is their ability to migrate in different microenvironments. Following 2-dimensional (2D) crawling on the inflamed endothelium in search of potential exit sites, neutrophils protrude and transmigrate through the endothelial monolayer establishing sub-endothelial crawling which involves simultaneous engagement of the endothelial layer and the subjacent basement membrane (BM). Soon thereafter the neutrophil begins to crawl on and navigate in between individual pericytes on its way to the interstitial space where they migrate in a 3D collagen-rich environment toward the site of inflammation (**Figure 1**) (9–11). During these processes, neutrophil migration is characterized by rapid shape changes underlying polarization into a lamellipodium and a uropod (12, 13). In this review, we describe the diverse environmental conditions which dictate the different migration modes neutrophils employ with a stress on the molecular mechanisms of nuclear deformation events critical for neutrophil squeezing through different cellular (i.e., endothelial and pericytic), and extracellular barriers at sites of inflammation.

ENVIRONMENTAL CHALLENGES FOR NEUTROPHIL MIGRATION TO SITES OF INFLAMMATION

An important characteristic of neutrophils is their high flexibility to adapt their mode of migration rapidly to the environmental conditions. Intraluminal crawling occurs either under low hemodynamic shear stress conditions in postcapillary venules during acute inflammation or under high shear stress conditions in inflamed arteries e.g., during the development of a chronic disease such as atherosclerosis (14). How neutrophils resist shear stress has been reviewed in detail elsewhere (15). Briefly, the process of intraluminal crawling involves specific β_2 integrin-mediated shear resistant adhesive interactions of neutrophils with endothelial cells (ECs) (**Figure 1A**). The key integrin ligands on inflamed ECs that enable efficient intravascular neutrophil crawling are ICAMs (16). During this mode of 2D migration β_2 integrins anchor neutrophils to the adhesive substratum enabling force transmission from the actin cytoskeleton to the environment (17–20). Additional molecules that facilitate intravascular crawling are the cytokine midkine and the serine protease Cathepsin G (CatG) (21, 22). Weckbach et al. demonstrated that the genetic absence of midkine abrogates neutrophil adhesion and extravasation in TNF α-stimulated mouse cremaster muscle venules arguing for a pro-adhesive role of midkine probably by binding to the neutrophil LDL-receptor-related protein-1 (LRP-1) (6, 21, 23). In contrast, CatG displayed by ECs has been found to be exclusively important for neutrophil adhesion to arteries under high flow conditions (22). Integrin-dependent neutrophil adhesion and crawling require the binding of chemokines presented by inflamed blood vessels with respective G-protein-coupled receptors (GPCR) on neutrophils eliciting intracellular signaling that triggers integrin adhesiveness, as well as shape changes and polarization (24). Upon GPCR engagement, primarily CXCR2 (25), the G-protein dissociates into distinct Gαi and Gβγ subunits, which regulate the activity of different molecules such as ion channels, adenylyl cyclase and phosphatidylinositol 3-kinase (PI3K) (26, 27). Activation of the PI3K leads to the recruitment of small guanosine triphosphatases (GTPases) of the Rho family including Rac, Cdc42, and RhoA. Neutrophil GPCRs can also activate these different Rho GTPases via PI3K-independent pathways (28). The appropriate subcellular and spatiotemporal regulation of these signaling molecules mediate cell polarization into an F-actin-rich lamellipodium and a myosin-rich trailing edge—a prerequisite of the amoeboid migration mode (29–31). The non-muscle myosin class II (NMII) protein complex is fundamentally important to maintain cell polarization by linking and translocating F-actin filaments (32, 33). Recently, Zehrer et al. demonstrated the important role of Myh9, the heavy chain of NMIIa in neutrophils for their proper 2D migration (crawling), TEM, and 3D migration. Myh9 was found to be critical for the retraction of the uropod and the consolidation of the leading edge ensuring proper neutrophil polarization and migration (34). Notably, integrin-mediated neutrophil crawling can occur with, against and perpendicular to the direction of blood flow ensuring optimal scanning capacity of the endothelial surface for appropriate extravasation sites (35). Neutrophil crawling to and protrusion through endothelial junctions are regulated by specific cytoskeletal adaptors such as the Rho-GTPase specific guanine exchange factor (GEF) Vav1, the mammalian actin binding protein 1 (mAbp1), the hematopoietic progenitor kinase 1 (HPK1), and GEF-H1 (36–39). It has been shown that CXCL2-stimulated neutrophils use Vav1 for their shear stress-induced perpendicular crawling as the genetic absence of Vav1 results in migration exclusively in the direction of blood flow. Under artificial shear free conditions, the migration behavior of neutrophils is intact in the genetic absence of Vav1 pointing toward the specialized role of Vav1 for Rho activities orchestrating integrin-mediated neutrophil crawling under shear flow (36). The same is true for mAbp1 and its interacting protein HPK1 indicating that these two proteins are additionally required for neutrophil crawling under shear flow (37, 38). Recently, Fine et al. demonstrated that spreading and mechanotactic migration are impaired in the genetic absence of GEF-H1, a specific RhoA GEF (29, 39). These data indicate that neutrophils possess tightly regulated molecular mechanisms that allow their integrin-mediated migration on inflamed vessels under shear stress conditions, a critical checkpoint in their extravasation into inflamed tissues. Once reaching potential exit sites, primarily paracellular endothelial junctions, neutrophils traverse the endothelial monolayer via

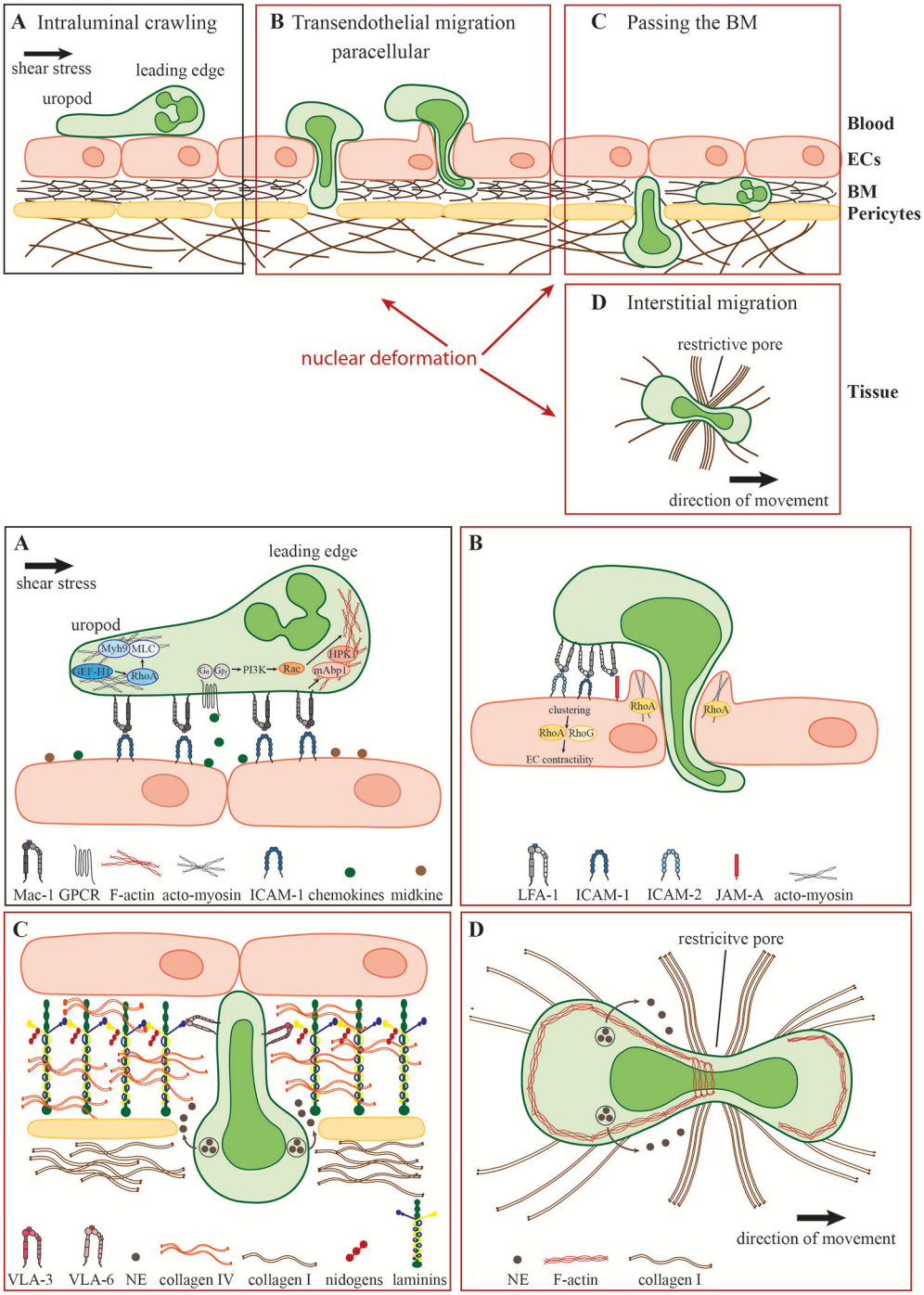


FIGURE 1 | Neutrophil migration in different environmental conditions. During the acute inflammatory response, **(A)** recently arrested neutrophils migrate along the inflamed endothelium (intraluminal crawling) toward potential exit sites. Intraluminal crawling is mainly mediated by the interaction of Mac-1 on neutrophils with ICAM-1 on inflamed ECs. Binding of chemokines and other chemoattractants to their respective GPCRs results in the dissociation of the Gα and Gβγ subunits with subsequent intracellular signaling inducing cell polarization with an F-actin-rich leading edge and an acto-myosin-rich uropod. **(B)** Neutrophils protrude and transmigrate through the endothelial monolayer via the paracellular route. Here, LFA-1- and Mac-1-engagement of endothelial ligands including ICAM-1, ICAM-2, and JAM-A activates endothelial Rho-GTPases e.g., RhoA, RhoG, and NMII leading to EC contractility and plasma leakage restriction. Neutrophils must use their own Rho GTPases to squeeze their nuclei through paracellular endothelial junctions triggering gap formation. **(C)** Following transmigration, neutrophils pass the subjacent basement membrane (BM) and often also crawl on adjacent pericytes embedded in the BM. The main components of the BM are laminins, collagen type IV and nidogens. Neutrophils penetrate this meshwork through LER which can be enlarged by the secretion of elastase (NE) and by nuclear squeezing, both potentially coordinated by the neutrophil integrins VLA-3 and VLA-6 and their interactions with BM collagens and laminins. **(D)** In the inflamed tissue, neutrophils migrate within a 3D collagen I-rich environment toward the site of inflammation (interstitial migration). Here, along with NE secretion, neutrophils deform and push forward their nuclei to pass through restrictive barriers in the meshwork of collagen fibers, most probably by dynamic interactions of their actin cytoskeleton with the nuclear lamina.

sequential steps of protrusion through these junction, formation of large pseudopodia in the subendothelial compartments and squeezing of their multi-lobular nuclei through adjacent ECs (**Figure 1B**). The ECs lining the blood vessel are connected by elaborated endothelial junctions composed of variable tight junctions, adherens junctions and gap junctions (40). In general, neutrophils take almost exclusively the paracellular route for their TEM (41). Neutrophil adhesion triggers also EC signaling events believed to facilitate the disassembly of the endothelial junctions enabling neutrophils to send their protrusions through the endothelial monolayer in search for exit signals, primarily chemokines highly enriched within the endothelial BM (42). Engagements of neutrophil integrins with different endothelial ligands like ICAM-1, ICAM-2, and JAM-A initiates the formation of "docking structures" (43) or "transmigratory cups" (44) consisting of pseudopod-like, F-actin-rich endothelial membrane extensions surrounding the leukocyte (45). Subsequent activation of endothelial Rho-GTPases like RhoA, and RhoG, and NMII triggers EC contractility temporally linked to gap formation (46–48). However, recent works have proposed an alternative mechanism whereby endothelial RhoA and acto-myosin contractility are not required for gap formation by transmigrating neutrophils. Instead, gap formation is dictated by neutrophil protrusion and nucleus squeezing through the paracellular endothelial junctions and at rare instances also through transcellular pores, which generate large displacements of the highly elastic endothelial stress fibers and collapse of thin actin filaments interlaced in between these actin bundles (49, 50). One of these works suggested that RhoA activation in endothelial cells is essential for restricting plasma leakage through the gaps generated by squeezing neutrophils (49). Collectively these studies suggested that neutrophils rather than endothelial cells control their TEM dynamics and that nuclear squeezing determines both the gap size generated by transmigrating leukocytes and the speed of TEM (50).

After successful TEM, neutrophils have to pass the perivascular BM predominantly consisting of laminins (isoform 411 and 511), collagen type IV, heparan sulfate proteoglycans, and nidogens (51–56) as well as embedded pericytes adjacent to the blood vessels (**Figure 1C**) (57, 58). The venular BM exhibits low-expression regions (LER) of laminins and collagen IV which are enriched between pericytes and are favored exit sites for neutrophils to overcome the BM (59, 60). However, the exact mechanism how neutrophils penetrate the BM is still highly debated. Neutrophils contain specific proteases including matrix metalloproteases and the serine protease neutrophil elastase (NE) and use these proteases to degrade the BM and squeeze through LERs (61, 62). Indeed, elastase-deficient neutrophils can normally cross inflamed endothelium but fail to penetrate the BM (62). In addition, the binding of the neutrophil integrins VLA-3 ($\alpha 3\beta 1$) and VLA-6 ($\alpha 6\beta 1$) to the BM is thought to facilitate neutrophil remodeling of the BM enlargement of LER and interstitial migration at sites of inflammation (59, 63, 64). Interestingly, VLA-3, VLA-6, and NE are located in intracellular vesicles which need to be translocated to the cell surface for efficient neutrophil transmigration through the BM, implicating these integrins as potential scavengers of elastase that restricts its proteolytic activity to BM regions enriched with VLA-3 and VLA-6 binding collagens and laminins (65, 66). Recently, Kurz et al. showed that the mammalian sterile 20-like kinase 1 (Mst1) is critically involved in this unique mobilization of VLA-3, VLA-6, and NE to the neutrophil surface (67). Accordingly, Mst1-deficient neutrophils that successfully extravasate through the venular wall get stuck between the endothelial monolayer and the BM and fail to pass the BM. Neutrophil crossing of the endothelial BM is also tightly associated with neutrophil crawling along venular pericytes (59, 68). This type of 2D migration is also ICAM-1-dependent, and during the onset of inflammation pericytes upregulate this ligand for neutrophil LFA-1 and Mac-1 (68). Whether these neutrophil integrins also rely on stimulatory chemokines co-elevated on inflamed pericytes for crawling, a migration mode that takes place in the absence of shear forces is unknown. It is likely, however, that the GTPase machineries discussed above as critical for β_2 integrin-mediated neutrophil crawling on inflamed endothelial cells under shear flow- are not identical to those involved in neutrophil crawling on inflamed pericytes.

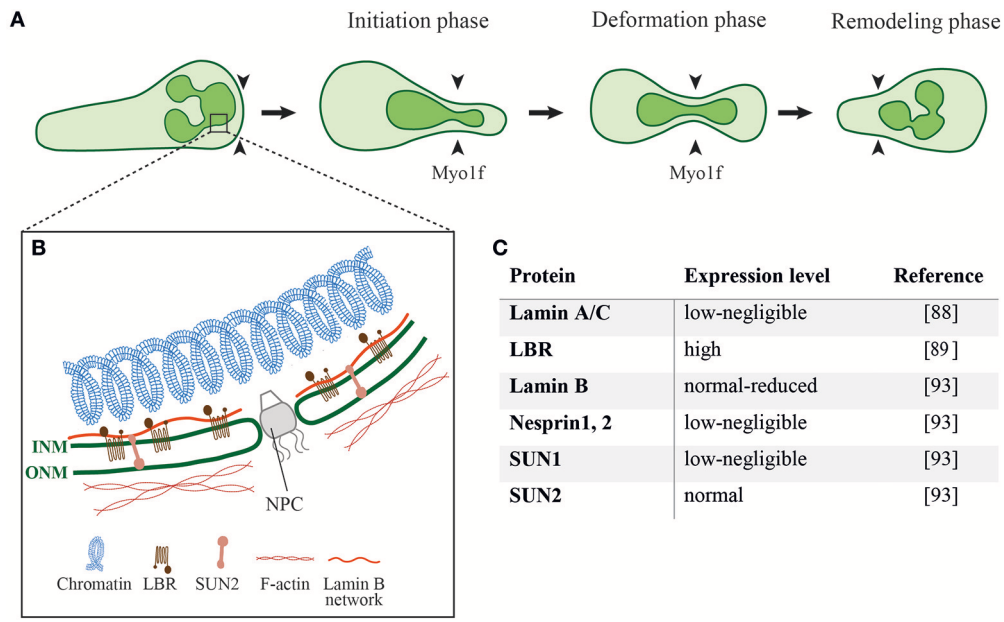
In the inflamed tissue, neutrophils migrate in 3D collagen-rich environments toward their final destinations at the site of inflammation (**Figure 1D**). Of note, the microenvironment in which the neutrophils migrate differs both mechanically and biochemically between different organs (69). However, the extracellular matrix as the non-cellular component of all tissues consists predominantly of type I collagen, elastin, proteoglycans, and non-collageneous glycoproteins (70). Here, type I collagen assembles into mechanically stable fibrils providing physical stability of the connective tissue (71). *In vivo* this fibrillary collagen meshwork exhibits interfibrillar spaces ranging from 2 to 30 μm as shown for mouse cremaster tissue (71, 72). Neutrophils migrate within this confined tissue in a low-adhesive and largely β_2 integrin-independent manner. Furthermore, integrin-deficient as well as talin-deficient neutrophils show intact migration in 3D environments compared to control cells, ruling out contributions from either β_1 and β_3 integrins to this mode of neutrophil motility (17, 73). These data indicate that the traction forces needed for successful 3D migration are transmitted to the environment without integrin-dependent anchoring of the cell to the surface, the prevalent mechanism for neutrophil migration in 2D environments (17, 74). However, the exact mechanism how neutrophils translate their intracellular actomyosin-driven forces to the traction forces critical for their locomotion inside various collagenous 3D environments is still not entirely understood.

In order to study the underlying mechanism experimentally, 3D collagen gels are widely employed. These gels mimic different meshwork architectures with different pore sizes, dependent on the collagen concentration. A collagen concentration of 1.5 mg/mL yields a low-density meshwork with pore cross sections of 10–12 μm^2 and a high-density collagen matrix with a collagen concentration of 3.0 mg/mL exhibits pore cross sections ranging between 2 and 3 μm^2 (17, 72). As the exact structure of collagen gels cannot be experimentally controlled, various microchannels were recently developed to closely mimic parameters including pore sizes and micro-geometry to improve the analysis of interstitial migration (75, 76). During migration

in such confined 3D environments, neutrophils need to pass physical restrictions much smaller than their nucleus similar to the situation in the tissue or 3D collagen gels. Nevertheless, while microchannels are rigid, dense 3D collagen polymers are not only more elastic but can be also locally degraded by neutrophil proteases. Thus, neutrophil passage through microchannels and collagen barriers involve similar but not identical requirements of nucleus deformation.

MOLECULAR MECHANISMS OF NUCLEAR DEFORMATION

During cell migration through different mechanical constrictions the dynamic interaction of the nucleus with the actin cytoskeleton is required to ensure proper positioning of the nucleus and nuclear deformation to successfully squeeze the cell through these constrictions (77). Indeed, nucleus deformation is the rate-limiting step for cells to pass through different constrictions smaller than the nucleus (78–81). The neutrophil nucleus is composed of 2–6 nuclear lobes with a diameter of 2 μm connected by a segment with a size of ∼ 0.5 μm (82, 83). Nuclear deformation follows three different phases while the cell squeezes through physical barriers, namely the initiation phase, the deformation phase and the remodeling phase (**Figure 2A**) (78, 84). When the cell reaches the constriction, the nucleus is the first organelle pushing against the constriction (50, 84). During the deformation phase the nucleus elongates into an hour-glass shaped nuclear morphology while squeezing through the constriction. After passing the constriction, the rear of the nucleus pushes forward to refold into its original spherical morphology. The nucleus is mechanically stabilized by a thin, elastic shell encoded by three genes, LMNA, encoding lamins A/C, and LMNB1 and LMNB2, encoding lamin B1 and lamin B2, respectively (85). The rigidity of the nuclei is determined by the relative levels of their A and B lamins (86, 87). The nuclear lamina of neutrophils is much softer than the lamina of most tissue resident cells due to their negligible content of lamin A/C (**Figures 2B,C**) (88). The neutrophil nucleus is further adjusted for rapid squeezing through small confinements by its unique multi-lobular shape. Expression of a lamin B receptor (LBR) on the inner nuclear membrane is critical for this multi-lobular shape (**Figures 2B,C**) (89). Interestingly, interference with the multi-lobular shape of the nucleus by LBR knockdown keeping lamin A content low bears minimal effects on nuclear squeezing via rigid pores (90). Notably, bone marrow neutrophil precursors regulate both their nuclear shape and lamin A/C content during maturation. The nuclei of immature neutrophils are stiff and circular as they express higher levels of lamin A/C and lack LBR. Upon full maturation neutrophils adapt their nuclear shape and rigidity to optimize their squeezing through bone marrow sinusoids (89). Similarly, naïve T cells temporally upregulate their lamin A/C expression during TCR activation and remain stationary until they downregulate lamin A/C expression and regain nuclear deformability as they become migratory (91). Thus, nuclear



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Protein	Expression level	Reference
Lamin A/C	low-negligible	[88]
LBR	high	[89]
Lamin B	normal-reduced	[93]
Nesprin1, 2	low-negligible	[93]
SUN1	low-negligible	[93]
SUN2	normal	[93]

FIGURE 2 | Different phases of nuclear deformation and structural components of the neutrophil nucleus. **(A)** While neutrophils squeeze through restrictive barriers (indicated by black arrowheads) smaller than their nucleus, the individual nuclear lobes undergo different phases of deformation. During the initiation phase neutrophils use one of its preexistent lobes to penetrate the barrier. This is followed by pushing, deformation, and elongation of the lobe and its neighbor lobes. Myo1f is critically required for nuclear pushing and deformation during this squeezing process. **(B)** Schematics of the structural proteins that regulate the shape and mechanical properties of a neutrophil nucleus as well as its crosstalk with the neutrophil cytoskeleton. ONM, outer nuclear membrane; INM, inner nuclear membrane; NPC, nuclear pore complex; LBR, Lamin B receptor; **(C)** Expression levels of different nuclear proteins in neutrophils.

shape and deformability are adapted to the squeezing needs of particular cells.

In contrast to epithelial and mesenchymal cells, which keep their stiff nuclei at their rear, motile leukocytes readily translocate their nuclei to their pseudopodia and do so irrespectively of barrier rigidity (50). In a recent study on granulocyte-like differentiated HL-60 cells, we found that this property of the neutrophil nucleus is conserved and is independent of the barrier rigidity the neutrophil is squeezed through. Nevertheless, when the stiffness of the nucleus was elevated by overexpression of lamin A, and when the neutrophil was embedded in a dense collagen matrix the nucleus could no longer translocate to the neutrophil pseudopodia (92). Thus, the exceptional ability of neutrophils to squeeze through mechanically rigid barriers such as collagen-rich interstitial spaces, or the BMs of blood vessels and epithelial barriers likely depends on both low lamin A content, nuclear lamina deformability, and high LBR expression (90). These requirements are dispensable, however, for neutrophil squeezing through the much softer endothelial junctions and consequently for TEM, because of the higher elasticity of the endothelial cytoskeleton than the elasticity of individual collagen fibers within collagen-rich interstitial spaces and the BMs of blood vessels and epithelial barriers.

The nuclear translocation to the neutrophil's pseudopodia, a shared feature among all motile leukocytes which appears to facilitate their squeezing may be regulated by specific interactions of the nuclear cytoskeleton (nucleoskeleton) and the perinuclear actin filaments. The neutrophil nucleoskeleton is deficient of several linker of the nucleoskeleton and cytoskeleton (LINC) complex proteins, including nesprin1, 2, and SUN1 (**Figures 2B,C**) (93) which are implicated in force transmission in adherent cells (94). Mature neutrophils are possibly devoid of these nuclear-cytoskeletal interactions as part of their highly motile nature and preference of chemotactic cues over integrin-dependent adhesions specialized to transduce forces to the nucleus (95). The nuclei of neutrophils can be also pushed to the leading edge by actomyosin machineries that orchestrate nuclear positioning and squeezing and bridge the neutrophil's uropod with the microtubule-organizing center (MTOC) at the back of the squeezed nucleus (96).

In addition to the unique shape and deformability of the neutrophil nucleus, neutrophil migration to sites of inflammation critically depends on the unconventional class I myosin Myosin 1f (Myo1f), found to facilitate nuclear deformation (84). This recent work suggests that the deformation of the nucleus is almost completely absent in Myo1f-deficient neutrophils compared to control cells resulting in diminished *in vitro* neutrophil migration within 3D collagen gels and impaired *in vivo* trafficking toward sites of lesions. Whereas, class II myosins are involved in the generation of contractility forces, class I myosins exist as monomers and link membranes to the actin cytoskeleton (97) potentially implicating these myosins in nuclear deformation critical for neutrophil squeezing. How precisely this unique myosin communicates with the nucleus and its closely associated microtubules remains an open question for future investigations.

CONCLUSION

Neutrophil migration to sites of inflammation is indispensable for innate immunity as neutrophils are the predominant immune cells combating pathogens. Efficient neutrophil migration critically relies on the exceptionally dynamic deformation of the nucleus of neutrophils. Accordingly, neutrophils obtained from mice lacking LBR expression show hyposegmentation of the nucleus associated with a decreased nuclear deformability and impaired neutrophil responses (98). The same is true for patients suffering from Pelger-Huet anomaly (PHA), a mutation in the human LBR leading to hyposegmentation of the neutrophil nucleus (99). Thus, impaired nuclear deformability can hamper neutrophil migration and function in inflammation. The improvement of our current knowledge of the molecular mechanisms underlying nuclear deformation events critical for neutrophil crossing through distinct mechanical barriers may therefore help to identify novel therapeutic targets for the treatment of neutrophil-driven acute and chronic inflammatory pathologies as well as for the manipulation of neutrophil crosstalks with tumor cells.

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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REVIEW
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Role of Platelets in Leukocyte Recruitment and Resolution of Inflammation

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Platelets are most often recognized for their crucial role in the control of acute hemorrhage. However, current research has greatly expanded the appreciation of platelets beyond their contribution to primary hemostasis, indicating that platelets also actively participate in leukocyte recruitment and the regulation of the host defense in response to exogenous pathogens and sterile injury. Early recruitment of leukocytes, especially neutrophils, is the evolutionary stronghold of the innate immune response to successfully control exogenous infections. Platelets have been shown to physically interact with different leukocyte subsets during inflammatory processes. This interaction holds far-reaching implications for the leukocyte recruitment into peripheral tissues as well as the regulation of leukocyte cell autonomous functions, including the formation and liberation of neutrophil extracellular traps. These functions critically depend on the interaction of platelets with leukocytes. The host immune response and leukocyte recruitment must be tightly regulated to avoid excessive tissue and organ damage and to avoid chronification of inflammation. Thus, platelet-leukocyte interactions and the resulting leukocyte activation and recruitment also underlies tight regulation by several inherited feedback mechanisms to limit the extend of vascular inflammation and to protect the host from collateral damage caused by overshooting immune system activation. After the acute inflammatory phase has been overcome the host defense response must eventually be terminated to allow for resolution from inflammation and restoration of tissue and organ function. Besides their essential role for leukocyte recruitment and the initiation and propagation of vascular inflammation, platelets have lately also been implicated in the resolution process. Here, their contribution to phagocyte clearance, T cell recruitment and macrophage reprogramming is also of outmost importance. This review will focus on the role of platelets in leukocyte recruitment during the initiation of the host defense and we will also discuss the participation of platelets in the resolution process after acute inflammation.

Keywords: platelets, leukocytes, neutrophils, inflammation, resolution

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INTRODUCTION

The adequate regulated recruitment of leukocytes is an indispensable element of the innate immune response (1–3). Neutrophils are the predominant leukocyte subset that is recruited to inflamed tissue by the initial innate immune system response during the onset of inflammation. Their primary function lies in combating and removal of invading pathogens. If defective, reduced neutrophil recruitment and activation can be the cause of severe immune deficiency syndromes (4). Platelets are traditionally well recognized for their important role in primary hemostasis, yet research over the past decade has created a broader understanding of platelets as an essential element of the innate immune system (5, 6). Platelets serve as a major contributor of several pro-inflammatory chemokines and possess a whole inventory of surface receptors and adhesion molecules that enable platelets to bind to leukocytes as well as circulating pathogens, e.g., bacteria (7). Platelets circulate in the blood in a resting, quiescent state under physiological conditions. When platelets are activated, e.g., in the situation of acute vascular inflammation, they may physically directly interact with circulating leukocytes in the blood stream (8–13). The consequences of this interaction are manifold and include leukocyte activation and may enable leukocytes to fulfill their multiple cell-intrinsic functions and immunological task. Furthermore, the interaction of platelets in particular with neutrophils is a prerequisite for neutrophil extravasation and recruitment into inflamed organs in multiple inflammatory scenarios (14). Activated neutrophils can produce and release neutrophil extracellular traps (NETs) (15). NETs are capable of physically entrapping and killing circulating pathogens, e.g., bacteria. The interaction of platelets and neutrophils has been demonstrated to be a prerequisite for NET formation and release under different inflammatory conditions (9, 11, 12, 15, 16).

Beyond their role as auxiliary cells interacting with leukocytes and supporting them in fulfilling their immunological fate, platelets are also capable of direct interaction with circulating pathogens (7). The liver plays a central role in this process. Here, platelets patrol the microvasculature and perform multiple "touch-and-go" maneuvers with sinusoidal Kupffer cells. This interaction is mainly mediated by bond formation between GPIb on platelets and vWF expressed on hepatic Kupffer cells as an element of the innate immune surveillance system of the liver (17). Kupffer cells in the liver act like tissue-resident macrophages and may catch pathogens in the circulation that may have reached the bloodstream, e.g., from the intestines. This process changes platelet behavior in the liver and the interaction of platelets with Kupffer cells becomes permanent with subsequent activation of platelets. Therefore, the activated platelets may initiate the recruitment of circulation neutrophils to eliminate the entrapped pathogens. In this situation, platelets also serve as sentinel cells together with Kupffer cells to guide the innate immune responses elicited by leukocytes. Currently this phenomenon is best described in the liver (18). If platelets may also patrol other cell types apart from Kupffer cells in organs apart from the liver still has to be investigated.

The focus of this review is the role of platelets in leukocyte recruitment during inflammatory processes and during resolution from inflammation. We will emphasize the molecular mechanism regulating the complex formation between platelets and leukocytes and will highlight the functional consequences associated with these processes under different inflammatory conditions.

Platelet Physiology

Platelets do not possess a cellular nucleus and are essentially produced by fragmentation of megakaryocytes in the bone marrow, from where they are released into the circulation in large amounts. They are traditionally well known for their essential functions in primary hemostasis. Subendothelial structures of the extracellular matrix, e.g., collagen fibers and von-Willebrand factor, are usually inaccessible for circulating platelets. If the vessel wall injury leads to exposure of these molecules, platelet adhesion is triggered. The establishment of bonds between adhesion receptors on the cell surface of platelets and their ligands in the exposed extracellular matrix leads to signaling events in platelets and the cells become activated. Consequently, further adhesion molecules on platelets, e.g., integrins, become activated and platelets may release the contents of their intracellular granules including highly active pro-coagulatory mediators (e.g., ADP, thrombin and prostaglandins). The adhesion and activation of single platelets quickly recruits and activates further platelets to the site of vascular injury and leads to the formation of a leak-sealing thrombus. In addition to platelets, leukocytes are recruited and red blood cells are incorporated into the thrombus (19, 20). Apart from stopping blood loss from the injured vessel during traumatic tissue injury, a second objective is to limit and control the possible entry of exogenous pathogens, e.g., bacteria, via the wound surface into the circulation and thus into the organism. This process could potentially lead to local and/or systemically disseminated infections. From this perspective, the formation of an occlusive platelet thrombus resembles not only a barrier preventing blood loss to the outside, but may also serve as a shield to reduce local blood flow in the injured vessel and prevent the dissemination of pathogens from the outside into the organism, which may explain why platelets have evolutionary evolved to a cell type that also executes immunological functions.

A common cell line known as "hematocytes" were abundant in certain invertebrates and very early vertebrates, and are still conserved e.g., in horshoe crabs, a member of the anthropod family which originated about 450 million years ago. Hematocytes combined immunological as well as hemostatic functions primarily found in leukocytes and platelets of today's mammals (7, 21, 22). During evolution and the appearance of mammals, several more specialized hematopoietic cell lines originated from hematocytes, including lymphocytes, monocytes, neutrophils, and eventually also platelets. These cell lines are characterized by the fact that they are actually able to execute less cell-autonomous functions, but with higher specialization. As a matter of fact, the relationship between the hemostatic and the immune system remained very tight during the development of higher organism with a high degree of

interconnectivity. The term "immunothrombosis" has lately been proposed to describe the pathophysiological events modulated by immune cells in cooperation with the coagulation system to facilitate the recognition, containment and destruction of exogenous pathogens during vascular inflammation (23).

Platelets possess a wide inventory of cellular adhesion molecules. These molecules have individual functions and enable platelets to act in different hemostatic and inflammatory situations. Furthermore, they contain intracellular granules (α-granules, dense granules and lysosome granules) packed with various pro-coagulant and immune-modulatory mediators that may be released in response to exposure to different activating stimuli (24). Platelets circulate in the blood stream in very high numbers and it does not come as a surprise that the immune system utilizes platelets to serve as cellular sentinels which are needed for broad surveillance of the circulation and detection of pathogens and possible threats.

Platelet Cellular Activation, Adhesion Molecules and Surface Receptors

Initial platelet activation is the key element in platelet function. Platelets can be activated either by binding of soluble platelet agonists, e.g., ADP or thrombin, or by exposure to subendothelial extracellular matrix components, e.g., collagen (25, 26). Ligand binding to platelets leads to the activation of intracellular signaling pathways which cause platelet shape change and cytoskeletal rearrangement, release of platelet granule content and the activation of cell surface adhesion molecules. Platelet granules are a source of many pro-inflammatory and pro-coagulant mediators. These also include chemoattractive cytokines and chemokines and allow platelets to actively fulfill their role in in primary hemostasis and also in inflammatory processes. Interestingly, experimental evidence suggests that the response of platelets to different activating stimuli is actually not uniform. This indicates the existence of a stimulus-dependent platelet response, e.g., degranulation (27, 28). Further *in vitro* studies revealed more insights of stimulus-dependent release characteristics of platelet granule content. It could be shown that although the composition of released platelet granule-derived mediators following agonist exposition appeared to be mixed in a stochastic manner, the temporal kinetics of platelet granule release clearly followed different stimulus-characteristic patterns (29). These findings are supported by imaging studies using immunofluorescence staining to visualize pair-wise packing of different molecules stored in α-granules. Here, the packaging pattern of platelet granule content also followed a stochastic distribution (30). However, alternative mechanism other that individual platelet granule packing and release might contribute to a stimulus-dependent platelet response, e.g., incomplete granule fusion upon content release and the interaction of individual mediators in a complete signaling network, and this questions remains the topic of current investigations.

Apart from activation by soluble mediators of cellular interaction by direct binding of ligands for platelet cell surface adhesion molecules, platelets are capable of direct interactions with bacteria. For example, platelets may bind and take up Listeria monocytogenes, a facultative intracellular bacterium. In turn, platelets selectively bind to DCs ($CD8\alpha^+$ dendritic cells) for pathogen delivery and presentation initiating an adaptive immune response (31). In another example, platelets have been shown to be necessary for viral clearance by cytotoxic T cells in lymphocytic choriomeningitis virus (LCMV) infections (32, 33). These findings also underline the fact that platelets do not only play a crucial immunological role by interaction with the innate immune system but are also capable of directly affecting the adaptive immune response.

To acknowledge the exact role of platelets in initiating and modulating the immune response to inflammatory stimuli, the function of the main platelet surface adhesion molecules and platelet surface receptors are of great importance. Integrins are a family of surface adhesion molecules which are abundantly expressed an many cell types where they mostly mediate direct cell-matrix and cell-cell interactions (34). Platelets express several integrins, which are the most important class of cell adhesion molecules on platelets. Integrins are formed as a heterodimer consisting of an α- and a β-chain. Integrins reside in an inactive state not capable for ligand binding under resting conditions (low affinity conformation). If activated, conformational change of both the α- and β-subunit occurs and access to the ligand binding site is granted (high affinity conformation) (35). Individual integrins may also possess the ability to change into an intermediate conformation with limited ligand binding affinity. Platelets express mostly integrins of the β_1- and β_3-subfamily, including $\alpha_{IIb}\beta_3$ (GPIIb/IIIa), $\alpha_2\beta_1$ (VLA-2, GPIa/IIa), $\alpha_5\beta_1$ (VLA-5), and $\alpha_6\beta_1$ (VLA-6) (36, 37). Platelet integrins fulfill divergent functions in the interplay of platelets with the subendothelial, extracellular matrix, leukocytes and endothelial cells (36, 37). A second important feature of integrins is their ability to transduce activating signals into the cell in a process called outside-in signaling (38). Thus, following ligand binding to integrins an intracellular signaling cascaded may be triggered inside platelets leading to further cell activation or degranulation.

Furthermore, platelets express additional glycoprotein complexes, including the glycoprotein (GP) Ib-V-IX complex. This molecule cluster serves as the most important binding partner on platelets for von Willebrand factor (vWF) (39). This complex generally mediates the first contact of platelets with structures of the subendothelial, extracellular matrix which is exposed following blood vessel injury. Another important glycoprotein, GPVI, can bind to collagen. Noticeably, platelet activation also leads to an increased surface expression and activation of glycoproteins. The glycoprotein GPIIbIIIa, which is a synonym for the integrin $\alpha_{IIb}\beta_3$ is a binding partner for fibronectin, retronectin and vWF (36, 40). GPIIbIIIa is the most abundantly expressed platelet surface adhesion molecule and in its activated conformation binds various ligands, e.g., fibrinogen, vitronectin, fibronectin, vWF or thrombospondin (40). Platelet adhesion molecules are involved in many immunological tasks elicited by platelets. *In vitro* studies under static conditions demonstrated that β_3-integrins on platelets are necessary to mediate firm platelet adhesion to the cell surface of inflamed endothelial cells (41, 42) and both the used of blocking antibodies

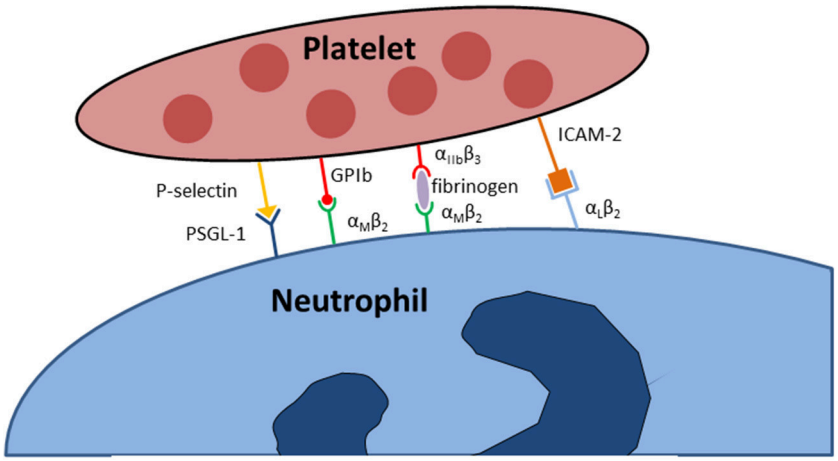

FIGURE 1 | Adhesion molecules implicated in the physical interaction between neutrophils and platelets.

directed against the integrin $\alpha_{IIb}\beta_3$ on platelets and the genomic knockout of this adhesion molecule caused less platelet adhesion to inflamed endothelial cells *in vivo* (43). Besides this very important role in mediating the contact to endothelial cells the integrin $\alpha_{IIb}\beta_3$ is also crucially involved in the initiation and regulation of direct physical interactions of platelets with leukocytes under inflammatory conditions. Here, the integrin $\alpha_{IIb}\beta_3$ serves as a binding partner the integrin $\alpha_L\beta_2$ (Mac-1) on neutrophils via a bridge of soluble fibrinogen (**Figure 1**) (40). Beyond physical bond formation, the binding of platelet $\alpha_{IIb}\beta_3$ to neutrophil Mac-1 also initiates outside-in signaling into neutrophils and is necessary for NET formation and leukocyte recruitment (12, 16).

Selectins are adhesion molecular that are abundant on numerous cells types, including endothelial cells, leukocytes, and platelets (44). P-selectin is stored in platelet α-granules in resting platelets. When activated, platelets incorporated P-selectin into the plasma membrane where it becomes available for interaction with its binding partners, e.g., P-selectin glycoprotein ligand-1 (PSGL-1) on neutrophils and monocytes (**Figure 1**) (7, 45–49). Experimental evidence suggests that the binding of PSGL-1 to P-selectin is necessary to initiate the first interaction between platelets and neutrophils (50, 51). Further adhesion molecules expressed on platelets include several cellular adhesion molecules, including the junctional adhesion molecules (JAM-A, JAM-C), intercellular adhesion molecule (ICAM)-2, and PECAM-1 (platelet endothelial cell adhesion molecule-1) (37). In particular, ICAM-2 on platelets is capable of binding to Mac-1 on neutrophils, but the physiological relevance of these adhesion molecules for the immunological functions of platelets and neutrophils are not fully understood.

Besides adhesion molecules platelets also express different receptors on their cell surface, e.g., complement receptors, pattern recognition receptors (PRRs) of the Toll-like receptor family (TLR1-9) and receptors for detecting immunoglobulins (FcR) (7, 52, 53). These receptors provide platelets with the ability to sense and respond to endogenous pro-coagulant and/or pro-inflammatory mediators, exogenous pathogens and incorporate these signals into cell activation (52). TLRs are a family of evolutionary highly conserved pattern recognition receptors to sense common motifs of exogenous pathogens, termed "pathogen associated molecular patterns" (PAMPs). The detection of PAMPs by TLRs leads to the initiation of an adequate immunological response (54). Functional TLR4 is expressed on platelets (52) and its main function is to recognize lipopolysaccharide (LPS) (55). However, TLR4 binding of LPS does not directly lead to platelet activation and aggregation (52, 56), but merely causes significant platelet priming in the lung and in the liver of mice during LPS-induced vascular inflammation (55). The reasons for this phenomenon are not fully understood to date, but it has been proposed that LPS-induced platelet priming induced the increased production of the pro-inflammatory cytokine TNF-α in the context of bacteremia, and the LPS-binding to TLR4 leads to increased phagocytosis by mononuclear cells (57). Furthermore, it is known that TLR4 activation during systemic inflammation causes the production and release of neutrophil extracellular traps (NETs) helping to catch circulating pathogens from the bloodstream (9). The exact direct or indirect molecular interactions between neutrophils and platelets following platelet TLR4 activation remain poorly defined.

Platelets express various prostaglandin receptors. Prostaglandins are synthesized from membrane-derived phospholipids and are involved in the modulation and regulation of a wide range of physiological processes, e.g., in the cardiovascular, central nervous and immune system. Platelets possess receptors to sense thromboxane, prostacyclin (PGI_2), PGD_2, and PGE_2. Thromboxane A_2/prostaglandin H_2 (TxA_2/PGH_2) receptor activation causes the activation of phospholipase A_2. This in terms leads to the amplification of platelet activation by autocrine mechanisms. The major inhibitory prostaglandin receptor on platelets are prostacyclin (PGI_2) receptors. Prostacyclin is synthesized and released by resting, non-inflamed endothelial cells. PGI_2 receptors on

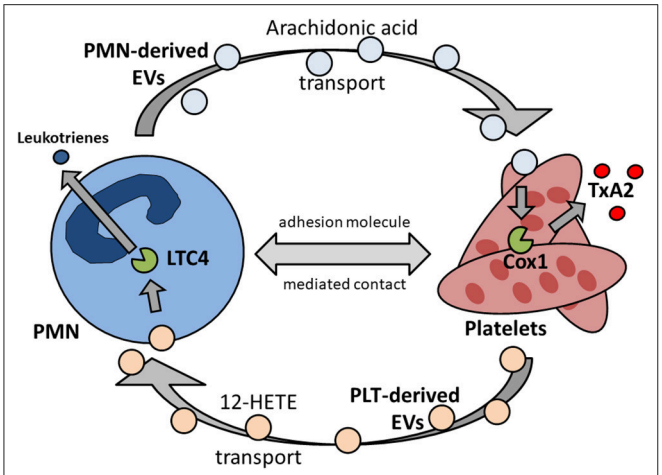

FIGURE 2 | Reciprocal transcellular exchange of substrate and metabolites by extracellular vesicles governs synthesis and release of pro-inflammatory mediators. PMN, polymorphonuclear granulocyte, neutrophil; EV, extracellular vesicles; Cox1, cyclooxygenase 1; TxA_2, thromboxane A_2; PLT, platelet; 12-HETE, 12-hydroxyeicosatetenoic acid; LTC4, leukotriene 4 synthase.

platelets sense prostacyclin and suppress platelet activation (58). TxA_2 has been shown to be an major regulator of inflammatory processes *in vivo* and is involved in endothelial cells activation and amplification of inflammation (8, 13, 59). Platelets may synthesize TxA_2 by cyclooxygenases, but they are lacking sufficient substrates on their own (60). The main substrate needed for prostaglandin synthesis is arachidonic acid, which is produced by phospholipase A_2 by enzymatic hydrolysis of plasma membrane phospholipids. TxA_2 production in platelets is significantly increased in the presence of neutrophils. Here, arachidonic acid is shuttled from neutrophils by transcellular metabolism into platelets (13, 61). This process substantially increases platelet TxA_2 production. Of note, the binding of P-selectin on platelet to PSGL-1 on neutrophils plays a role in this process, possibly by providing and maintaining the physical proximity between the two cell types (13, 62) (**Figure 2**).

Platelet-Derived Soluble Mediators

Platelet granules are a storage for various mediators. These include pro-coagulant factors, e.g., ADP and mediators without a direct hemostatic function, e.g., PDGF (platelet-derived growth factor). PDGF plays a role in the regulation of wound healing during and after local inflammation (63). Beyond pro-coagulant factors, platelet granules are also a packed with different pro- and anti-inflammatory mediators, e.g.. transforming growth factor-β (TGF-β) (64). TGF-β, together with IL-10, is one of the most important negative regulatory chemokines of inflammatory processes. Platelets are a major source of TGF-β in the organism, and ITP (idiopathic immune thrombocytopenia) in humans is also characterized by reduced plasma TGF-β levels, which may rise again if low platelet counts during ITP recover to normal values (65, 66). Platelets are also a major source of the chemokines CXCL4 (platelet factor 4), CCL5 (RANTES), and CXCL7 (Neutrophil-activating peptide-2, NAP-2) which may be liberated by activated platelets and are actively involved in neutrophil recruitment and activation (67). Platelet CXCL4 and CCL5 have been shown to be crucial involved in the recruitment and activation of neutrophils during the pathogenesis of acute lung injury (12, 68). Here the two chemokines are deposited on the luminal cell surface of endothelial cells to form a heterodimer and are needed to induce endothelial arrest of intravascular neutrophils (68–71). Furthermore, platelet CXCL4/CCL5 heterodimer binding to neutrophils has been shown to be necessary for neutrophil NET formation and release during the pathogenesis of acute lung injury (12). CXCL7 (NAP7) is a potent CXCR2 agonist and is produced from its precursor molecules CTAP (connective tissue-activating peptide)-III and PBP (platelet basic protein). CXCL7 has also been implicated in the complex formation of neutrophils and platelets under inflammatory conditions *in vitro* and *in vivo* (72) and promotes chemotaxis of neutrophils (73). CXCL7 and CXCL4 possess unique structural properties that modulate neutrophil recruitment and processes including chemokine heterodimer formation, glycosaminoglycan (GAG) interactions, and gradient formation. CXCL7 is known to form several biologically active heterodimers with other chemokines, e.g., CXCL7-CXCL1, CXCL7-CXCL4, and CXCL7-CXCL8 heterodimers (74). Interestingly, the binding properties of these heterodimers to GAGs on endothelial cells are substantially different compared to monomeric CXCL7 and this also modulated the receptor binding of the chemokines (74, 75). It was described that the binding of GAGs to monomeric CXCL7 might dynamically modulate the chemokines receptor binding properties and that the GAG-bound monomeric CXCL7 shows less receptor binding affinity than GAG-bound CXCL7 heterodimers (75). CXCL7 liberation by platelet degranulation, interaction with GAGs on the endothelial surface and the resulting gradient formation between free and GAG-bound forms of CXCL7 complexes are also events that contribute to the directed neutrophil recruitment to the site of vascular inflammation (76). CXCL7 also forms tetramers, but to this date nothing is known about the pathophysiological role of these complexes. Interestingly, a negative feedback loop exits to limit the pro-inflammatory action of CXCL7 by CTAP-II (the precursor molecule of CXCL7) inducing the downregulation of CXCR2 on neutrophils. Likewise, also PBP may dampen CXCL7-induced neutrophil activation, degranulation and chemotaxis (77). These studies provide evidence that the release of platelet-derived chemokines may itself lead to the desensitization of chemokine receptors on neutrophils. This may represent an important negative-feedback regulation limiting neutrophil activation.

Platelets are not only a source of chemokines, but the cells themselves also possess chemokine receptors and respond to chemokine stimulation (78). The most prominent chemokine receptors expressed on the cell surface of platelets include CXCR4, CX3CR1, CCR1, CCR3, and CCR4 (79, 80). Several chemokines are known as binding ligands of these receptors and induce platelet activation, including SDF-1 (CXCL12) released by inflamed endothelial cells, TARC (CCL17) and MCD (CCL22)

which may be produced by mononuclear cells (81). Besides platelet shape change and activation of cell adhesion molecules, another major consequence of platelet activation by chemokines is the platelet degranulation during which platelet P-selectin stored in platelet granules is integrated into the platelet plasma membrane. The binding of P-selectin of activated platelets to PSGL-1 on neutrophils is an essential step during the formation of physical platelet-neutrophil interactions during inflammatory processes. The formation of platelet-neutrophil complexes is of great importance for neutrophil recruitment and neutrophil function during the pathogenesis of numerous inflammatory diseases (see paragraph below). Current research has demonstrated that platelets are capable of active migration, and activation of CXCR4 on platelets by CXCL12 is critically involved in this process (82–84), but the pathophysiological relevance of this finding during inflammatory diseases has yet to be investigated.

Adenosine Diphosphate Receptors

Adenosine diphosphate (ADP) is a platelet agonist and kept in dense platelet granules in the quiescent state. Upon platelet activation, ADP is set free following platelet degranulation. Extracellular ADP may bind to P_2Y_1 an P_2Y_{12} receptors. Both receptors are GTP-coupled, platelet-activating receptors. Furthermore, APD may also act by binding to the receptor P_2X_1, which acts as an ion channel for free calcium ions upon ligand binding and subsequently leads to platelet cytoskeletal rearrangement and induction of platelet shape change. ADP alone is a rather weak platelet agonist. However, it significantly increases the platelet-activating response induced by additional platelet agonists, e.g. thrombin, and leads to the synthesis and liberation of TxA_2 from activated platelets, which in turn resembles a strong paracrine platelet agonist. Furthermore, ADP binding to platelets induces the platelet integrin activation (e.g., GPIIbIIIa, integrin $\alpha_{IIb}\beta_3$) and platelet aggregation (85). Platelet activation by ADP plays an important pathophysiological role during several inflammatory diseases, including sepsis (86–89).

Platelet-Leukocyte Interactions

Direct physical interactions between platelets and leukocytes are regulated by several distinct molecular interactions but are also enforced by non-biological physical propensities of the vascular system. This is in parts explained by the rheological properties of blood as a mixture of fluids and corpuscular components (90, 91). Here, erythrocytes and larger cells (e.g., leukocytes) stay relatively centered in the middle of the blood flow, whereas platelets are more enriched in the peripheral vicinity of the blood flow closer to the endothelial cell surface lining the inner lumen of the blood vessel. The enrichment of platelets near the vessel wall make encounters with leukocytes in this area more likely. This increases the chance of transient platelet-leukocyte interactions in this area, which might become permanent in case of vascular inflammation with activation of platelets, leukocytes and endothelial cells (91). But also under physiological condition in the absence of inflammation, a small number of transient platelet-neutrophil interactions has been described close to the vascular endothelium (92, 93). The first and probably most important physical interaction between platelets and leukocytes, in particular neutrophils, is established by bond formation between P-selectin on activated platelets and PSGL-1 constitutively expressed on neutrophils (**Figure 1**). This causes the phenomenon of secondary capturing of free-flowing neutrophils by initial binding of PSGL-1 on these cells to P-selectin expressed by adherent, activated platelets on the vascular endothelial cell surface (94). However, ligand binding to neutrophil PSGL-1 does not only mediate cell-cell interactions, but also induces intracellular signaling. Following PSGL-1 engagement, a cascade of signaling events in neutrophils, including BTK (bruton's tyrosine kinase), Src and MAP kinases, leads to the activation of integrins expressed on neutrophils, e.g., $\alpha_L\beta_2$ (LFA-1) and $\alpha_M\beta_2$ (Mac-1) (50, 95–100). LFA-1 is a binding partner of ICAM-2 (intercellular adhesion molecule 2) on platelets, although the exact pathophysiological contribution of this interaction under different inflammatory conditions remains unclear (101–103). Activated Mac-1 on neutrophils is of particular importance for the interaction of neutrophils with platelets, as it is a direct binding ligand for the platelet surface adhesion molecule GPIbα and also indirectly binds to activated platelet GPIIbIIIa through a "bridge" of fibrinogen (**Figure 1**) (104, 105). The role of GPIIbIIIa binding to Mac-1 in the regulation of neutrophil recruitment and activation has been shown in different inflammatory diseases, e.g., pulmonary inflammation, whereas GPIbα binding to Mac-1 is known to regulate platelet adherence *in vitro* and is involved in leukocyte recruitment following femoral artery injury in the murine system (105–107). While research over the past decades has revealed several mechanisms by which the platelet and neutrophil may directly and indirectly interact, we are just beginning to understand the specific role and contribution of this phenomenon in different inflammatory diseases. While some diseases models appear to be critically dependent on this interaction, others may not, and even within the same organ system differences may exist in between different inflammatory stimuli (3, 8, 12).

Beyond mediating physical cell-cell interactions, the binding of platelets to neutrophils also modulates and induces cellular immunological functions in neutrophils. A major cellular function of neutrophils is the production and release of ROS (reactive oxygen species). Due to their nature as free radicals, ROS are extremely cell-toxic, and they aid in the destruction of pathogens, e.g., invading bacteria at sites of infection. The complex formation and interaction of platelets and neutrophils induces subsequent integrin-mediated outside-in signaling into neutrophils, which in addition to chemokine stimulation triggers ROS production and release by activated neutrophils. It has been shown that platelet binding to neutrophils increases neutrophil ROS generation efficiency (108, 109). The molecular interaction of P-selectin on platelets and neutrophil PSGL-1 is also of great importance for this process *in vitro* and *in vivo* (110, 111). Likewise, pharmacological blockade of ADP binding to its cellular platelet receptor P_2Y_{12} also impaired ROS production in neutrophils (112). As platelet activation by ADP also induces P-selectin mobilization and membrane integration, an implication of the P-selectin/PSGL-1 binding system could be involved in

the underlying molecular mechanism. However, exact evidence for this hypothesis is lacking as ADP stimulation of platelets also induces the activation of additional platelet surface adhesion molecules. The second important immunological function by which neutrophils eliminate pathogens is phagocytosis and this process is also affected by platelet-neutrophil interactions. Here, indirect interaction pathways mediated by soluble inflammatory mediators, e.g., prostaglandins and purine nucleotides, play a more important role than direct ligand-receptor interactions (113–116). However, direct cellular interactions also seem to be involved, at least under distinct inflammatory conditions. This was shown by results from study utilizing a periodontitis model where efficient phagocytosis by neutrophils relied on the complex formation of neutrophils and platelets (117), indicating a possible tissue- and stimulus-specificity of platelet-dependency.

The third cell-autonomous immunological feature by which neutrophils may directly engage and kill bacteria is the formation and released of neutrophil extracellular traps (NETs) generated by "NETosis" (15). NETs are essentially decondensed nuclear chromatin, which is decorate with granular proteins from neutrophils and spun into the extracellular space. Although the generation of NETs leaves the neutrophils without a nucleus, the cells are still alive and are capable or cellular functions, e.g. intravascular crawling and transmigration (118). Physically, NETs may act like real-life fishing nets and entangle pathogens circulating in the blood stream. The relevance of this effect has been shown in different models of inflammatory diseases involving the blood-borne distribution of pathogens in the organism (11). Interestingly, NETs are also implicated in the pathogenesis of inflammatory disorders not involving infectious pathogens or stimuli. Here, NET formation has been shown to be a prerequisite for efficient neutrophil recruitment from the vasculature to the site of inflammation and platelet-neutrophil complex formation has been demonstrated to be critically involved in this process (12, 16). The pattern recognition receptor TLR4 is expressed on platelets and may be activated by binding of bacterial products, e.g., LPS. TLR4 activation on platelets leads to neutrophil NET formation and liberation, but the exact mechanism remains unclear (9). Furthermore, the direct physical interaction of platelets and neutrophils by binding of activated GPIIbIIIa to Mac-1 on neutrophils (via a bridge of fibrinogen) also induces NET formation by neutrophils together with simultaneous activation of GPCRs (G-protein coupled receptors) on neutrophils by CXCL4/CCL5 heterodimers released by activated platelets to facilitate neutrophil recruitment during sterile pulmonary inflammation (12). An example for indirect platelet-neutrophil interactions inducing NET release is hBD-1 (human β-defensin 1). Platelets secrete hBD-1 in response to contact with toxin from S. aureus, and hBD-1 has been shown to cause NET release by neutrophils (119).

Platelet Microparticles

Although platelets are small fragments originating from larger cells (megakaryocytes) themselves, they are still capable to generate microparticles with dimensions in the sub-micrometer range (120). Current research has demonstrated that microparticles are associated with multiple physiological and pathophysiological functions (121). Also not restricted to platelets as originating cells, the majority of microparticles in the blood are actually coming from platelets (122). The fact that microparticles may carry certain proteins that are normally not expressed or only expressed in much smaller quantities in their originating cells indicates that microparticles are produced and packed with dedicated proteins in an active process and not just by random cell sequestration (123). Yet the exact regulatory processes guiding these pathways in platelets still have to be investigated. Microparticles may also well interact with and bind to leukocytes, since they inherit the adhesion receptors, e.g., P-selectin and the platelet integrin $\alpha_{IIb}\beta_3$, from the platelets cells surface (124–126). However, it is unknown if the platelet integrin $\alpha_{IIb}\beta_3$ is activated on the microparticle surface and contributes to adhesion. Microparticles originating from platelets are also capable of binding to other cell types than leukocytes, e.g., endothelial cells. In fact, excessive microparticle binding to the surface of endothelial cells may lead to endothelial cell activation (127).

As a specialized class of microparticles, extracellular vesicles (EVs) are actively released by cells, e.g., neutrophils, following active cell-internal production, packaging and release. EV transport in between neutrophils and platelets has gained attention as it could be demonstrated that intermediate metabolites necessary for sufficient prostaglandin synthesis and release by platelets are shuffled from neutrophils into platelets via specific EV release and uptake (13, 61). Likewise, the transcellular transport vice versa from platelets to neutrophils also plays an important pathophysiological role. Here, neutrophils receive 12-hydroxyeicosatetenoic acid (12-HETE) from platelets to synthesize leukotrienes (128). Interestingly, this interaction also modulates LTC4 synthase activity further downstream in neutrophils, as does the transport *vice versa* from neutrophils to platelets regulate cyclooxygenase 1 activity in platelets (62, 129). It seems fair to argue that transcellular metabolite exchange between neutrophils and platelets via EVs is a two-way interaction (**Figure 2**) (130).

The Role of Platelets in the Pathogenesis of Acute Inflammatory Diseases

Acute lung injury is a respiratory disorder characterized by pulmonary leukocyte recruitment and edema formation leading to impaired gas exchange with severe consequences for patients, depending on its severity (131, 132). It may occur in response to different stimuli, e.g., pulmonary bacterial infections, sepsis, and aspiration of gastric content (133). The pathogenesis of pulmonary inflammation and acute lung injury has been demonstrated to rely on platelet-neutrophil complex formation in various disease models. They include transfusion-related acute lung injury (TRALI) (10, 16, 134), LPS-induced lung injury (68, 135), acid-induced lung injury (8), and ventilator-induced lung injury (VILI) (12). Experimental evidence has demonstrated that platelet-neutrophil complexes can be detected in a circulating manner in the blood as well as directly attached to the vessel walls in the lung microcirculation as early as 30 min after exposure to the inflammatory stimulus (8). Here,

complex formation involving platelet P-selectin is critically involved and pharmacological blockade of this molecule or cellular depletion of platelets showed a protective effect in reducing immune cell recruitment and limiting the vascular permeability increase. Platelet-neutrophil complex formation also regulated the production of TxA_2 and this caused endothelial cell activation and expression of the endothelial cell adhesion molecule ICAM-1 (8). The importance of the direct cellular interaction between platelets and neutrophils in this process was also underlined by a later study demonstrating that platelet-neutrophil complex formation is necessary for the transcellular transport of metabolites from neutrophils into platelets to booster TxA_2 production during the host immune response following induction of bacterial pneumonia (13). Pulmonary inflammation may also be induced by non-inflammatory stimuli, e.g., barotrauma during ventilator-induced lung injury (VILI). The interaction of platelets and neutrophils has also been shown to be required for neutrophil recruitment into the lung during VILI by intravascular formation and release of NETs (12). Moreover, Grommes et al. could show that platelet-neutrophil complex formation is also involved in a murine model of LPS-induced lung injury (68).

Massive transfusion of blood products, e.g., during severe hemorrhage following trauma, may cause transfusion-related acute lung injury (TRALI) and is a feared complication in transfusion medicine (134). It has become evident, that the deterioration of gas exchange during TRALI is not a result of an intravascular fluid overload, but essentially involved immunological pathways leading to inflammatory activation of the pulmonary endothelium, immune cell recruitment and increased vascular permeability. Lately, the platelets and platelet-neutrophil interaction shave been shown to be critically involved in the development of TRALI (109). Here, platelets may liberate CD154 in response to TLR-ligand binding (136). Subsequent CD154 binding to CD40 on neutrophils may cause activation of these cells and lead to neutrophil recruitment into the lung (137).

Whether platelet depletion or the attenuation of a platelet-elicited immunological response is associated with improvement of deterioration of the outcome critically relies on the nature of the underlying inflammatory stimulus in a particular model. Whereas the attenuation of the innate host immune response may be beneficial in disease models using aseptic inflammatory stimuli (e.g., LPS inhalation, intratracheal acid instillation or TRALI), the same intervention may substantially worsen the outcome in an infectious model, e.g., following the intratracheal instillation of viable bacteria to induce pulmonary inflammation (13, 138). In addition, it remains unclear if and how platelets may reach other compartments in the lung than the intravascular space, e.g., the lung interstitium or the alveoli. Evidence from first studies suggest that platelets may also be present in the lung as far as in the alveoli (139). There is first evidence that platelets may also be released by megakaryocytes situated outside of the bone marrow, e.g., within the pulmonary microcirculation and it was claimed that this extra-medullar platelet synthesis contributes to a large amount of circulating platelets (140). Yet, the specific contribution to this putative new platelet reservoir in the lungs as well as the functional role of platelets in the different compartments of the lung and their specific contribution to the disease progression here still has to be investigated in more detail.

Platelets are traditionally thought to possess only little motile capabilities, mainly related to rolling, adhesion and aggregation. However, several recent reports have substantially challenged this dogma with the discovery of platelet migration. First reports indicated that human platelets adapt to the application of high shear forces by cellular polarization and flow-directed migration and show migratory behavior toward a SDF-1 (stromal cell-derived factor 1) gradient *in vitro* (82, 83). First *in vivo* studies demonstrated that platelet migrate into the extravascular compartment of the lung during allergen-induced airway inflammation (141). Lately, Gärtner et al. showed that platelet migration occurs under inflammatory conditions in mice *in vivo* and is crucial for bacterial host defense and bundling of bacteria for improved phagocytosis (84). For this process, GPIIb/IIIa, as well as ADP and thromboxane A_2 are needed, and it has previously been shown that platelet-TxA_2 contributes to the neutrophil recruitment into the lung (13). Interestingly, it was noted that platelets adherent to leukocytes migrate faster than independent platelets, and only a certain percentage of all platelets does migrate (84). However, the relevance of platelet migration in the lung remains unclear to this date.

The liver represents another organ in which platelets and platelet-neutrophil complexes are prominently involved in host defense. The liver is uniquely characterized by the fact that the hepatic microcirculation is placed second in line beyond the intestinal microcirculation, connected by the portal vein. Thus, the liver is also exposed as the first organ that might be passed by invading exogenous bacteria from the intestinal tract (142). The liver is equipped with a unique and specialized immune surveillance system that resides in the livers sinusoidal space where Kupffer cells sense distinct bacterial structures and components. Circulating platelets are in constant temporary contact with Kupffer cells, performing "touch-and-go" maneuvers. Once Kupffer cells become activated following pathogen contact, platelets permanently attach to Kupffer cells by GPIIb-mediated adhesion and attract neutrophils to the liver sinusoids to aid in pathogen clearance (17).

Overwhelming systemic inflammation may occur due to uncontrolled local inflammation and can be potentially life-threatening for host. Interestingly, systemic inflammation and sepsis are often accompanied by transient low platelet counts in the blood, which may rise again after the initial phase of systemic inflammation is overcome (143). One factor contributing to decreased blood platelets counts may be the occurrence of DIC (disseminated intravascular coagulation) consuming platelets. However, emerging evidence also hints to a possible consumption of platelets caused by immunological processes in the circulation during systemic inflammatory disorders (144). This is also supported by results from clinical studies indicating that low circulating platelet counts are often associated with increased circulating microparticles and that circulating platelets from patients with sepsis show increased P-selectin surface expression levels indicating platelet activation (126).

Platelets in the Resolution of Inflammation

Timely resolution of inflammation is important to impede uncontrolled host tissue destruction and organ dysfunction leading to chronic inflammation and fibrosis (145). It is essential that neutrophils are rapidly and efficiently removed from the inflammatory site upon clearance of the invading microorganisms thus avoiding excessive tissue damage (146). Neutrophil apoptosis and consequent engulfment by macrophages is the major route by which the host clears neutrophils. Efficient phagocytosis of apoptotic neutrophils by macrophages not only prevents their secondary necrosis but also turns pro-inflammatory macrophages into cells with an anti-inflammatory, reparative signature (147). Dysfunction in the neutrophil apoptosis machinery is considered critical for the pathogenesis of many chronic human inflammatory diseases, e.g., pulmonary fibrosis after ARDS (148). While various pro-resolving mediators and pathways that govern resolution from inflammation in the lung have been described, the role of platelets in this process remains vaguely investigated. Interestingly, a current report also indicated that delayed neutrophil apoptosis and clearance are also associated with delayed recovery from ischemia/reperfusion-induced acute kidney injury and accelerated renal fibrosis (149).

In the lunge, several studies have provided evidence that platelets do not only act during vascular inflammation within the intravascular compartment, but eventually also appear in the lung alveoli. Platelets have been found to extravasate and accumulate beneath the airways in a model of allergic inflammation (141). Further evidence supported the observation that platelets, eventually coupled to leukocytes, can be detected in the bronchoalveolar lavage fluid after induction of pulmonary inflammation (139). Supporting this finding, platelets could also be found in the BAL of mice after intratracheal instillation of LPS (150). Platelets were long thought to be passive corpuscular blood components that reach their site of action by chance, enforced by their sheer numbers (151). Contradicting this dogma it could recently be demonstrated that platelets are capable of active migration (84). This observation may contribute to the concept that the distribution of platelets is not only restricted to the intravascular compartment, but that platelets also translocate toward the alveolar space, i.e., into the organ tissue. However, previous studies in other organs provided hints that platelets may not only be important for the propagation of vascular inflammation. It could be shown that platelet activating factor (PAF) plays a role in mediating the uptake of urate crystals during the resolution of gouty inflammation (152). Platelets are also a major source for anti-inflammatory mediators of the lipoxin family, e.g., specialized pro-resolving mediators (SPMs) such as resolvins and maresins (153). These lipoxins are produced and released already during the inflammatory onset phase of acute inflammation and their concentrations sharply rise during the convergence toward the resolution phase (154). Interestingly, these lipoxins also promote phagocytic clearance of apoptotic immune cells, e.g., neutrophils, during resolution (155).

Neutrophils and macrophages have traditionally been regarded as dominant cell types during the resolution of inflammation. Regulatory T cells (T_{regs}) represent a T cell subpopulation with predominantly immune regulatory functions and are mainly acting immunosuppressive. T_{regs} are a source of the anti-inflammatory cytokines interleukin 10 (IL-10) and transforming growth factor ß (TGF-ß). Yet, it is unknown how exactly platelets, macrophages and T_{regs} participate in the resolution of pulmonary inflammation. Platelets have been previously described to interact with regulatory T cells under inflammatory conditions. In this context, it could be shown that platelets are needed to control the anti-inflammatory actions of $CD4^+$ regulatory T cells following burn injury trauma in mice (156). In another organ, platelets have also been shown to interact with $CD4^+$ T cells in the liver following ischemic injury and during atherosclerosis (157, 158). Interestingly, platelets are also thought to be capable of inducing $CD4^+$ T cell differentiation by both the release of distinct chemokines and by direct cell-cell contact with T cells. As a consequence, IL-10 production and release by T cells was enhanced (159).

CONCLUSION

While platelets are traditionally perceived as essential elements of primary hemostasis, the contemporary perception of their pathophysiological role should also clearly include their prominent contribution to inflammatory processes. Current and past research has shed light on their participation in the generation of an adequate immune response. Here, both the direct and indirect interactions with leukocytes, in particular neutrophils, are of outmost importance. Future research will further characterize the detailed, spatio-temporal role of platelets in the pathogenesis of distinct tissue- and stimulus-specific inflammatory situations. Furthermore, platelets may also be involved in the resolution of acute inflammation, a field of research of growing importance. A more detailed understanding of the underlying molecular mechanisms will be the key to the development of targeted therapeutic approaches and interventions to improve the treatment of patients suffering from inflammatory diseases.

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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REVIEW
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Organ-Specific Mechanisms of Transendothelial Neutrophil Migration in the Lung, Liver, Kidney, and Aorta

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Immune responses are dependent on the recruitment of leukocytes to the site of inflammation. The classical leukocyte recruitment cascade, consisting of capture, rolling, arrest, adhesion, crawling, and transendothelial migration, is thoroughly studied but mostly in model systems, such as the cremasteric microcirculation. This cascade paradigm, which is widely accepted, might be applicable to many tissues, however recruitment mechanisms might substantially vary in different organs. Over the last decade, several studies shed light on organ-specific mechanisms of leukocyte recruitment. An improved awareness of this matter opens new therapeutic windows and allows targeting inflammation in a tissue-specific manner. The aim of this review is to summarize the current understanding of the leukocyte recruitment in general and how this varies in different organs. In particular we focus on neutrophils, as these are the first circulating leukocytes to reach the site of inflammation. Specifically, the recruitment mechanism in large arteries, as well as vessels in the lungs, liver, and kidney will be addressed.

Keywords: neutrophil, recruitment, lung, liver, kidney, aorta, inflammation, organ-specific

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INTRODUCTION

Inflammation is a tightly regulated process initiated by tissue injury, be that of sterile or pathogenic origin. To eliminate the pathogenic insult or to remove damaged tissue, a coordinated cascade of events is rapidly unleashed aimed at restoring tissue homoeostasis (1). The innate immune system is the first line of host defense and mediates the inflammatory process. The immune system is activated by damage-associated molecular patterns (DAMPs) discharged from injured tissue or pathogen-associated molecular patterns (PAMPs) released by invading microorganisms (2). DAMPs and PAMPs stimulate sentinel cells including mast cells, macrophages, and dendritic cells resulting in the activation of a cascade of events. One of the first events is the recruitment of leukocytes, predominantly neutrophils, to the inflamed site. Acute inflammatory responses are terminated actively, a process known as resolution of inflammation. During resolution, tissue homeostasis is resorted and progression toward an uncontrolled chronic inflammatory state prevented (1, 3). The active resolution process is coordinated by the interplay of multiple events, including inhibition of neutrophil recruitment, promotion of neutrophil apoptosis, macrophage-mediated apoptotic neutrophil clearance, as well as egress of infiltrated leukocytes from the

inflamed tissue (1, 4). A failure in cell clearance and egress results in accumulation of inflammatory cells and might potentially result in excessive tissue damage and ultimately in chronic inflammation (1, 5), such as chronic obstructive pulmonary disease, renal fibrosis, chronic kidney disease, non-alcoholic fatty liver disease, and cardiovascular diseases.

There has been a substantial public and scientific awareness in the use of therapeutic agents against chronic inflammatory diseases. As an example, randomized clinical trials have shown the beneficial effect of statins, anti-platelet, or anti-hypertensive compounds for treatment and prevention of cardiovascular events (6). However, the residual burden of cardiovascular diseases remains immense. Therefore, during the last 20 years research focused on the development of anti-inflammatory strategies to treat atherosclerosis. However, anti-inflammatory therapies that were reported successful also present considerable limitations (7). In the case of atherosclerosis, the patients are often elderly people who frequently cope with additional inflammatory comorbidities. In such situation, compromising host defenses might jeopardize the patient.

Interestingly, the neutrophil recruitment mechanism deviates in different organs. It has been shown that some surface molecules, which are involved in the recruitment, are tissue-specific and the lung, liver and kidney show an atypical recruitment cascade (8). Furthermore, differences are observed between arterial and venular endothelial sites (9–12), suggesting the involvement of different mediators of neutrophil recruitment. In addition, recruitment mechanisms in the same organ can vary with different inflammatory stimuli (8). Thus, this review will highlight the available evidence for tissue-specific neutrophil recruitment in vessels of the cremaster muscle (the model system to study neutrophil adhesion), the lung, the liver, the kidney, and the aorta. Furthermore, we will discuss the influence of endothelial heterogeneity, shear stress, and oxygen tension and the role of sentinel cells, pericytes and platelets.

THE LEUKOCYTE RECRUITMENT CASCADE: A PARADIGM ESTABLISHED IN MODEL SYSTEMS

Research over the last decades has established a uniform paradigm of leukocyte recruitment into inflamed tissues. The classical paradigm of leukocyte recruitment and the molecules herein involved have been established by a combination of *in vitro* flow chamber models and *in vivo* intravital microscopy. The latter allows direct visualization of the microvasculature of translucent tissues, including the cremaster muscle. The optical properties and the relative ease mode of preparation for microscopy have made the murine cremaster muscle the backbone for leukocyte recruitment studies worldwide (13). However, the cremaster muscle is a rather unique organ and is only fully developed in males. The microvasculature of this muscle is comprised of arterioles, capillaries and venules. The arterioles have a diameter of 10–100 μm and divide into narrow capillaries. The exchange of nutrients and gases takes place in these capillaries, which thereafter drain into post-capillary venules to return perfusion to the venous circulation (13). This microvasculature arrangement is common in almost all tissues, such as intestine, skeletal muscle and skin. In organs of this nature, interactions of circulating neutrophils with the endothelial surface almost exclusively take place in the post-capillary venules. These interactions are predominantly due to locally-restricted expression of adhesion molecules (14). Although intravital microscopy studies performed in the murine cremaster muscle have been indispensable for the development of the widely accepted rolling-adhesion-transmigration paradigm, findings made in this tissue cannot be plainly transferred to other organs.

Classical Leukocyte Recruitment Cascade

The classical cascade of leukocyte recruitment is defined by the following steps: capture, rolling, arrest, adhesion, crawling, and transendothelial migration. The primary step in leukocyte recruitment is to establish adhesive interactions between neutrophils, and endothelial cells (EC) of inflamed tissue. Neutrophils circulate passively in the bloodstream and are swept to the center of the blood vessels by the laminar blood flow (15). In inflamed post-capillary venules, the rate of the blood flow is greatly disturbed as a result of local changes in hemodynamic. The reduced flow increases the chance of neutrophils to get in contact with the ECs lining of the vessel and to be primed and become more responsive (15). Neutrophils circulating in the blood are in a resting state, in which processes such as transcription, protein, and lipid synthesis, protein activation do not occur. Their activation is therefore crucial in the inflammatory response, and this process consists of multiple steps. Neutrophils become partially activated—a state also known as primed—when they migrate toward inflammatory foci. Priming agents, such as cytokines, PAMPs, DAMPs, and growth factors, as well as interaction with activated EC, awaken the neutrophil from its latency (16–18). Interestingly, the neutrophil response to individual chemoattractants varies and depends on the concentrations and the time of exposure (19–21). Furthermore, stimulation of the neutrophil by a chemoattractant often results in endocytosis of the corresponding receptor, thereby leading to a desensitization of the neutrophil to repeated stimulation with the same molecule (22, 23). Priming leads to the activation of a variety of neutrophil responses, including adhesion, transcription, cytoskeletal reorganization, expression of receptors and other molecules, metabolic activity, phagocytosis, and the rate of constitutive apoptosis, hereby amplifying the inflammatory response (24–27). Neutrophils are likely exposed to a grade of concentrations of priming agents as they progress through the multistep process of recruitment, allowing the cell to acquire functions in an ordered fashion (25). Full activation seems to be a two-step process, since maximal neutrophil activation may only occur in cells that have been primed (28). Upon a secondary stimulus, such as inflammatory factors, the neutrophil becomes fully active, resulting in ROS generation, granule release, acquisition of phagocytic capabilities, and neutrophil extracellular traps (NET) formation (19, 25, 29).

Activation of ECs is a decisive step in the inflammatory process and can occur in a rapid (within minutes) or slow (within

hours) manner. The rapid activation is independent of new gene expression whereas slow EC activation is not (30). Activation, rapid or slow, is mainly induced by histamine or inflammatory cytokines, respectively (30), that originate from mast cells and tissue macrophages—immune sentinel cells. These processes are further discussed below.

Activation of ECs involves upregulation of P- and E-selectin. P-selectin can be rapidly translocated from Weibel-Palade bodies (endothelium) or α granules (platelets) to the cell membrane (31). P-selectin is translocated in response to mediators, such as thrombin, histamine, or activated complement. Contrary, in most organs, ECs must be stimulated to express E-selectin (31). Yet on the surfaces of venular hematopoietic tissues, such as spleen, bone marrow, and cutaneous immunosurveillance (i.e., skin), E-selectin is constitutively expressed (32–34). This constitutive expression of E-selectin seems to be important for homing of hematopoietic stem cells (35).

Neutrophils express L-selectin and other ligands, such as P-selectin glycoprotein ligand 1 (PSGL-1), CD44, and E-selectin ligand-1, which bind in high on-and-off-rate to P- and E-selectins on the ECs (36, 37). This allows the rapid moving neutrophils to be initially captured from the bloodstream and to bind tentatively to the endothelium. Due to this binding they can move along the endothelium, a process called rolling (37). The rolling step is often reversible, unless followed by endothelial presentation of chemokines and/or chemoattractants, which activate neutrophil integrins. Integrins present in neutrophils are: lymphocyte function-associated antigen-1 (LFA-1) or CD11a/CD18 (present in all effector leukocytes) and macrophage-1 antigen (Mac-1) or CD11b/CD18 (present in neutrophils and monocytes) (38). G protein–coupled receptors on rolling neutrophils bind chemokines presented on the apical endothelium, leading to "inside-out" signals that induce conformational changes of β2-integrins (39), mediating slow rolling (low concentration) and arrest (high concentration). Chemokines synergize with selectins to activate β2-integrins when chemokine availability is limited (40). Engagement of endothelial P- or E-selectin with neutrophilic PSGL-1 triggers signals that separate LFA-1 α and β cytoplasmic tails (41), which induces integrin extension from the bent to an extended intermediate-affinity conformation (42). Talin-1 is recruited upon parallel Rap1a- and PIP5Kγ90-dependent pathways activated by selectins and chemokines (40). The head domain of talin-1 facilitates the cytoplasmic tail separation (43) and conformational change by binding to membrane-distal and membrane-proximal sites on the tail of the β subunit (43–45). A rapid reversible interaction of LFA-1 with intercellular adhesion molecule-1 (ICAM-1) on ECs results in slow rolling (46, 47). Binding of endothelium-presented chemoattractants to their corresponding receptors on neutrophils triggers signals that convert integrin LFA-1 to an extended conformation, which mediates neutrophil arrest on ICAM-1 (46, 48). Kindlin 3 (also known as fermitin family homolog 3) is a FERM domain-containing protein, which also binds to the tail of the β subunit. Activation of both talin 1 and kindlin 3 induces LFA-1 to adopt a high-affinity conformation, by opening the headpiece of LFA-1, which promotes neutrophil arrest on the endothelium (49).

Once the neutrophils are stably arrested on the endothelial surface they flatten, to reduce their surface exposure to the blood flow, shear force, and collisions with circulating blood cells. Shear-resistant arrest requires signaling through clustered E-selectin/L-selectin bonds that result in lymphocyte-specific protein tyrosine kinase phosphorylation (Lck) and the rapid activation of β2-integrin to a high-affinity state capable of shear-resistant bond formation with ICAM-1 (50). Neutrophils then crawl on the apical surface of the blood vessel until a suitable extravasation site is signaled. This crawling is guided by gradients in adhesion receptors, chemokines, and EC stiffness. The apical neutrophil crawling is particularly mediated by Mac-1 (51). Chemoattractants induce re-localization of intracellular stored Mac-1 to the cell surface (52). For neutrophils, ICAM-2 is an important endothelial ligand for Mac-1-mediated crawling. And although blocking ICAM-2 function *in vivo* does not reduce the number of crawling cells, it results in an increase in the number of neutrophils with a disrupted stop–and-go crawling profile (53). **Figure 1** summarizes the classical recruitment cascade here described.

Chemoattractants are key players in the neutrophil recruitment cascade. These molecules contribute to neutrophil activation; they are required for firm arrest and they also guide the neutrophil to the site of inflammation. Neutrophils respond to chemoattractants in a hierarchical manner. They prefer "end-target" chemoattractant factors such as bacterial products and complement components (e.g., N-formyl-methionine-leucine-phenylalanine (fMLP), C3a and C5a, respectively) over "intermediate" attractants such as chemotactic stimuli [e.g., chemokines (C-X-C motif) ligand 1 (CXCL1), CXCL2, and leukotriene B4 (LTB4)] (54). Chemotaxis is controlled by the activation of the PI(3)K and p38 mitogen-activated protein kinase (MAPK) pathways. Intermediate chemoattractants activate PI(3)K, while end-target chemoattractants activates both pathways. The activity of the pathways is pivotal for the prioritization between opposing signals from end-target and intermediate chemoattractants (54–57). More recently, *in vitro* studies showed fMLP acting as the most potent chemoattractant followed by interleukin-8 (IL-8) (human), CXCL2, and LTB4 (58). Interestingly, fMLP inhibits C5a-, IL-8- and LTB4-induced neutrophil chemotaxis and LPS promotes this inhibitory effect of fMLP via p38 activation. Although C5a was also recognized as an end-target chemoattractant (59), fMLP was found to be more attractive for neutrophils. As depicted above different inflammatory stimuli influence the activation of the signaling pathways.

Generally, neutrophils transmigrate via endothelial junctions (paracellular route, ∼90%) rather than directly through the EC (transcellular diapedesis, ∼10%) (60). It is therefore no surprise that neutrophils stop for a prolonged time at EC junctions (53). Interestingly, blocking Mac-1 increases the number of neutrophils that stop crawling impulsively and favors transcellular over paracellular migration (51). Two key structures involved in paracellular migration are the adherens junctions and the tight junctions. The adherens junctions contain the vascular endothelial (VE)-cadherin and the tight junctions consist of junctional adhesion molecules A-C (JAM-A,

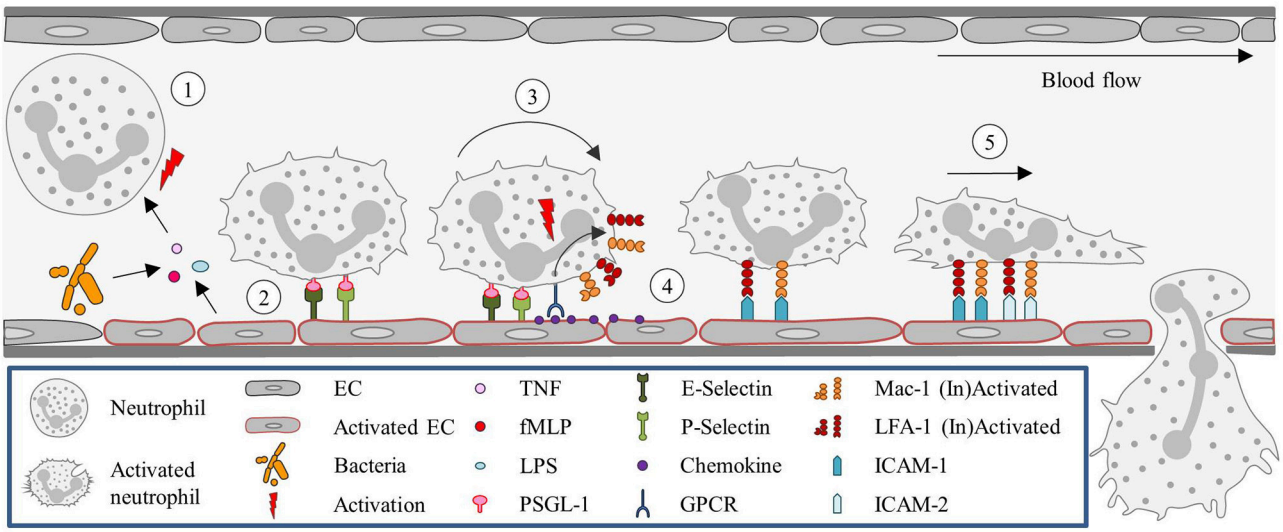

FIGURE 1 | Neutrophil recruitment in the post-capillary venules of the cremaster muscle. (1) Neutrophils are primed upon exposure to inflammatory agents, such as cytokines (e.g., TNF) and DAMPs (fMLP) or PAMPs (LPS), in the context of sterile or non-sterile inflammation, respectively, or interaction with activated ECs. (2) Neutrophil capture is mediated by P- and E- selectin, (3) followed by rolling, which is also largely regulated via selectin signaling. (4) Subsequently, neutrophils firmly adhere to ECs. This step is dependent on integrin (LFA-1 and Mac-1) activation, which is mediated by GPCRs interacting with chemokines presented on the endothelium. (5) Neutrophils then crawl along the endothelium, via ICAM-1 and ICAM-2 interactions with Mac-1 and LFA-1, until they reach their site of TEM. EC, Endothelial cell; fMLP, N-formyl peptides; GPCR, G protein-coupled receptor; ICAM, Intracellular adhesion molecule; LFA-1, Lymphocyte function-associated antigen-1; LPS, Lipopolysaccharide; Mac-1, Macrophage-1 antigen; PSGL-1, P-selectin glycoprotein ligand-1; TEM, Transendothelial migration; TNF, Tumor-necrosis factor.

JAM-B, JAM-C), EC-selective adhesion molecule, and claudins. Paracellular migration is accompanied by the disruption of the EC adherens and tight junctions to form a gap, through which cells migrate. Opening of the adherens junction involves dissociation of vascular endothelial protein tyrosine phosphate (VE-PTP) and VE-cadherin. Dissociation is induced by binding of neutrophils as well as lymphocytes to ECs (61). ICAM-1 engagement with neutrophilic LFA-1 leads to the activation of proline-rich tyrosine kinases (Pyk2) and Src kinases (62, 63). These kinases induce phosphorylate of VE-cadherin at its cytoplasmic tail. Two key tyrosine residues, Tyr731 and Tyr658, present on this tail have been implicated in this process. Phosphorylation of VE-cadherin, due to internalization and often degradation of VE-cadherin (64), promotes junction opening resulting in an increased vascular permeability and transendothelial migration (TEM) (65). Several permeability-inducing mediators, such as vascular endothelial growth factor (VEGF), histamine and tumor-necrosis factor (TNF), have also been found to induce tyrosine phosphorylation of VE-cadherin (66–68). Alternatively, stimuli of endothelial origin can act on junctional proteins, leading to localized, and transient junctional disassembly. This is accompanied by the reorganization of an adhesive platform and the recycling of adhesive proteins, including platelet endothelial cell adhesion molecule (PECAM-1, also known as CD31), via the lateral border recycling compartment (LBRC). LBRC vesicles are mobilized to the junctional plasma membrane of ECs upon diapedesis of leukocytes, resulting in increased membrane surface area at such sites. Homotypic PECAM-1 interactions and CD99 initiate LBRC vesicle mobilization (69, 70).

The majority of the leukocytes that undergo paracellular TEM go through the EC junctions in a luminal to abluminal direction. However, a smaller proportion of transmigrating neutrophils exhibited reverse TEM. During reverse TEM leukocytes migrate through EC junctions in opposite direction, disengage from the junction, and crawl across the luminal surface of the endothelium away from the junction. Although reported for other leukocytes, neutrophil reverse TEM is a contentious subject. However, studies in zebrafish (71, 72) and cultured human ECs (73) showed evidence of reverse neutrophil TEM. More recently, it has been shown that under certain conditions neutrophils do not go into apoptosis after having performed their key repair functions. The neutrophils can transmigrate back into the vascular system and relocate to the lung, where they seem to be reprogrammed or deactivated, and eventually migrate back to the bone marrow. The neutrophil transmigration is potentially assisted by chemokinesis and also might be mediated by proteases (74). Furthermore, neutrophil reverse transmigration has been observed to be enhanced upon loss of JAM-C expression or function (60). In venules of the cremaster muscle, LTB4 can trigger neutrophils to release elastase, which causes degradation of JAM-C, a response that seems to drive reverse transmigration (75). Other factors that are suggested to mediate neutrophil reverse migration include chemokines, hypoxia inducible factor, and reactive oxygen species (ROS) (76–78).

For the final stage of TEM, transient receptor potential cation channel, subfamily C, member 6 (TRPC6), a calcium $(Ca)^{2+}$ channel, is recruited to the endothelial surface, resulting in increased levels of intracellular Ca^{2+} (79, 80). Increased intracellular Ca^{2+} triggers actomyosin contractility by myosin light chain kinase and contributes to active opening of junctions via Ras homolog gene family, member A (RhoA), a RhoGTPase (81). RhoA activity is the highest during the final stage of extravasation, and mediates endothelial filamentous actin remodeling to form ring structures around transmigrating neutrophils, preventing vascular leakage during neutrophil diapedesis and promoting pore closure and transmigration (82).

Several factors have been shown to favor transcellular migration, including the stiffness of ECs or the density of integrin ligands at the apical endothelial surface (83, 84). Surprisingly, the adhesive molecules and mechanisms that guide transcellular migration are very similar to those controlling junctional migration. An exception is VE-cadherin, which is only inactivated in paracellular TEM. Whereas, paracellular migration is always preceded by ICAM-dependent lateral neutrophil crawling onto the endothelial surface, scanning for an extravasation site (51, 53), ICAM-1 is also involved in transcellular TEM. Next to ICAM-1 surface density and distribution, EC shape contributed to transcellular migration (84). Mac-1-deficiency in mice showed delayed paracellular migration and favored transcellular migration (51). Other important structures for this type of migration are transmigratory cups, rich in ICAM-1, and docking structures (85). Furthermore, LBRC are recruited to sites of neutrophils-EC contact, carrying PECAM-1, CD99, and JAM-A (86). Transcellular migration was found to be dependent on PECAM and CD99, since antibodies blocking these two molecules resulted in arrest of this type of migration (86). Hence, although EC junctions remain intact, junctional molecules are required for TEM.

Once the neutrophil has passed across the endothelial barrier, it needs to cross the subendothelial basal lamina as well as the surrounding interstitial tissue to reach the site of inflammation. This process is generally more time consuming than the TEM (60). Neutrophils move between the abluminal surface of the ECs and the basal lamina searching for areas that are deposited with a low density of collagen IV, and laminin. Indeed this is the path of least resistance and it also minimizes the amount of proteolysis necessary to reach the site of injury. Generally, these areas contain a gap in pericyte coverage allowing the neutrophils to easily exit the interstitium (87). Upon inflammation, pericytes are stimulated to produce and release macrophage migration-inhibitory factor in the interstitium, assisting neutrophils in their migration. In particular, a murine model of sterile inflammation showed that DAMPs, and PAMPs stimulated $NG2^{+}$ pericytes to produce macrophage migration-inhibitory factor (88). As a consequence neutrophils interacted extensively with these cells and migration was facilitated by the interaction between ICAM-1 (expressed by pericytes) and leukocytic LFA-1 and Mac-1 (89).

The general concept of the classical leukocyte recruitment cascade is not ubiquitous. The expression of molecules facilitating different stages of cell recruitment seems, to a large extent, dependent on the leukocyte subtype and the nature of the inflammation, such as inflammatory stimuli, the organ of interest and the genetic background of the animal models, reviewed by Ley et al. (46), Muller et al. (90), Nourshargh et al. (91), Voisin et al. (92), Vestweber et al. (93). In addition, EC phenotype, morphology, and junctional composition can vary between different vascular beds. These differences can impact on the dynamics and profile of vascular permeability and the interaction between neutrophils and ECs (13). Furthermore, the classical leukocyte recruitment paradigm is mainly established in the microcirculation of the cremaster muscle, which is only present in men, hence gender aspects are not taken into account.

THE ROLE OF TISSUE-RESIDENT CELLS AND PHYSICAL PROPERTIES ON NEUTROPHIL RECRUITMENT

In addition to what was *supra* described a variety of tissue-resident cells such as mast cells, macrophages and pericytes as well as platelets and physical properties including endothelial heterogeneity, shear stress and oxygen tension influences neutrophil recruitment. These determinants will be addressed in more detail below.

Endothelial Heterogeneity

The EC lining shows remarkable heterogeneity. This heterogeneity can be observed on different levels, such as morphology, function, gene, and antigen expression. Endothelial phenotype can differ among organs and is dependent on health and disease conditions (94). EC heterogeneity can also be observed within one organ (95–97), such as the kidney, where three different vascular beds serve different functions in the filtration of the blood. Phenotypic EC heterogeneity is further supported by proteomic studies [reviewed by Ruoslahti and Rajotte (98), Simonson and Schnitzer (99)]. Interestingly, this EC property can be exploited for therapeutic applications, by means of targeted delivery (100, 101).

The vessels are lined by a monolayer of ECs. The structural lining of ECs varies among vessel types. The endothelial lining in arteries and veins is continuous, uninterrupted, with each EC interacting with the next by tight junctions. Arterial ECs are generally thicker compared to ECs in veins, with the exception of those in high endothelial venules. Arterial ECs also are long and narrow or ellipsoidal, a reflection of their alignment in the direction of undisturbed flow, while venous ECs are short and wide. In capillaries the endothelium can be classified into three groups: continuous, fenestrated, or discontinuous. Organs involved in filtration and secretion have a fenestrated endothelium. These organs include endocrine and exocrine glands, gastric and intestinal mucosa, choroid plexus, glomeruli, and a subpopulation of renal tubules. Discontinuous and fenestrated endothelium share several similarities. However, the fenestrae in discontinuous endothelium have a larger diameter (200 nm compared to 70 nm) and lack a diaphragm (102). In addition, the basement membrane underlying discontinuous ECs is less dense. This type of endothelium can be observed in

sinusoidal vascular beds, as for instance in the liver, and facilitates cell migration and sensing.

ECs also show a significant heterogeneity in function, including basal and inducible permeability and leukocyte recruitment. Differences in permeability are observed between capillaries and post-capillary vessels. In capillaries water, small solutes and lipid-soluble materials can freely cross the endothelium, albeit the rates may differ among vascular bed. Whereas, post-capillary venules are generally impermeable: permeability is either damage-associated or requires active transportation. Larger molecules pass the barrier via transcytosis, which is regulated by specific transporters such as vesiculo–vacuolar organelles and caveolae. This difference in permeability is supported by the higher abundance of vesiculo-vacuolar organelle in post-capillary venules and the relative paucity of tight junctions. Likely this relative paucity of tight junctions supports leukocyte recruitment, underscoring the role of endothelial heterogeneity in this process. Also glycosylation of adhesion molecule might vary among vascular beds and hereby be a critical element in the understanding of the role of endothelial heterogeneity in leukocyte recruitment. As an example during inflammatory stress, N-glycosylation of adhesion molecules may be under distinct, and up to date, unknown modes of regulation, affecting the inflammatory response in a vascular bed- and disease-specific manner (103). The spatial and temporal differences in morphology and function of ECs are the result of microenvironmental as well as epigenetic influences, which mediate EC gene, messenger RNA (mRNA) and protein expression (94). The microenvironment is mediating non-heritable changes in EC phenotype. These changes have their origin in receptor-mediated posttranslational modification of protein and transcription factor–dependent induction of gene expression. Epigenetics mediate heritable changes in EC phenotype, via DNA methylation, histone methylation, and/or histone acetylation. In turn, these changes negatively or positively influence gene expression. Although epigenetic modifications are triggered by extracellular signals and are dynamically regulated, they might persist after removal of these external cues, and are transmitted during mitosis (104).

Genes can be characterized as constitutively expressed or inducible, grouped as endothelial-specific or unspecific, and their expression regarded throughout the endothelium or only in specific EC subsets (105). Remarkably there are few endothelial-specific genes constitutively expressed across the vascular tree, two of these genes are VE-cadherin and Robo4. There is a bigger variety of endothelial-specific genes whose expression, constitutive and/or inducible, is limited to an EC subset.

RNA sequencing of organ-specific vascular beds revealed a distinct expression pattern of gene clusters, both in human and mice. Regarding human samples, Marcu et al. isolated human ECs three months after gestation from four different organs, and observed an expression pattern supporting organ-specific development. Additionally, distinct barrier properties, angiogenic potential and metabolic rate among organs seems to support organ-specific functions (106). In adult mice, where ECs were labeled *in vivo* and thereafter isolated, Nolan et al. identified distinct gene clusters of transcription factors, angiocrine factors, adhesion molecules, metabolic profiles, and surface receptors expressed on the microvascular ECs of nine organs at steady state or during regeneration (107). Although the two reports analyze tissues at different stages of differentiation and assess in general distinct genes and functions, both studies support endothelial heterogeneity, at genetic level, and a function hereof associated to. However, unfortunately none of the articles relates their findings to leukocyte recruitment. It would be interesting to study their organ-specific gene profile in relation to potential organ-specific adhesion protein expression.

The majority of the studies focus on the influence of EC origin and differentiation on heterogeneity. The relation between endothelial heterogeneity and leukocyte recruitment is especially studied in cancer tissues. As a future perspective, protein expression of adhesion molecules on the endothelial lining of different organs in homeostatic and inflammatory conditions could be compared, to establish a better understanding of neutrophil recruitment into the tissues in health and disease and have to possibility to generate tissue-specific therapeutic strategies.

Mast Cells and Perivascular Macrophages: Sentinels Initiating Neutrophil Recruitment

Mast cells are tissue-resident immune sentinels that reside in most peripheral tissues. They typically reside in perivascular locations and have been implicated in sensing of sterile damage and microbial invasion. Damage is sensed by pattern recognition receptors, such as TLR or IL-1 receptor-like 1, respectively (108, 109). Mast cells are granule rich cells that store a multitude of vasoactive (e.g., histamine, prostaglandins, leukotrienes, and thromboxanes) and inflammatory mediators (e.g., cytokines, myeloid-attracting chemokines), which are critical for triggering the onset of acute as well as chronic inflammatory reactions (110, 111). Mast cell secretion is induced by a variety of stress signals, including tissue damage, microbial products and the binding of allergen-coated cross-linked immunoglobulin E to their Fc receptors (112). Upon inflammation, mast cells undergo immediate degranulation and slowly release newly synthesized vasoactive and angiogenic compounds, pro-inflammatory and nociceptive mediators (113). To illustrate, degranulation leads to histamine and sphingolipid-1-phosphate release, which through the histamine 1 and sphingolipid-1-phosphate receptor 3 results in the capacity to mobilize P-selectin from the Weibel-Palade Bodies to the luminal endothelial surface (114). Histamine also induces tyrosine phosphorylation of endothelial VE-cadherin, resulting in increased of vascular permeability (67).

Perivascular macrophages (PVM) are dendritic-shaped macrophages in close proximity to the blood vessel wall. Where present, PVM discontinuously cover post-capillary venules in close association with pericytes, where they reside outside the basement membrane. PVM themselves do not directly contact ECs and are not migratory, however, they influence the neutrophil recruitment by secreting neutrophil-attracting CXCL1, CXCL2 and chemokine (C-C motif) ligand 3 (CCL3) (115). Interestingly, in 80% of the cases, intraluminally crawling neutrophils extravagate in areas in close proximity to PVMs

(115). In the absence of PVMs, firm adherence and TEM are markedly reduced. Moreover, the discontinuous association pattern of PVMs with basement membrane is consistent with the patchy arrest of neutrophils to the post-capillary venule wall. These observations strongly support the existence of "hot spots" with increased chemokine deposition (115), although such hotspots can also occur due to other circumstances, including pericyte gaps (89), the presence of tricellular junctions (116), or regions of low basement-membrane protein expression (87). Nevertheless, as *supra* described, a number of observations underscores the enrolment of PVMs in neutrophil extravasation.

Pericytes: Assistants of Paracellular Migration

The venular wall is composed of two cellular components, ECs and pericytes, and a noncellular matrix protein structure called the vascular basement membrane. Pericytes are essential components of the vessel wall and occupy a strategic position, since they are wrapped around ECs, and are the interface between the circulating blood and the interstitial space. Pericytes are long cells (∼70 μm in length) (117), and a single pericyte can cover multiple ECs. Between 10 and 50% of the abluminal side of the blood vessel is covered by pericytes (91). Pericytes are responsible for communication of signals between multiple cells, for providing nutrients and regulating the transit of circulating immune cells into underlying tissues. Of relevance to neutrophil recruitment, these cells express toll-like and cytokine receptors and release chemokines and cytokines in response to stimulation (88, 89). In the microvascular bed, different populations of pericytes can be discriminated: neural/glial antigen 2 $(NG2)^-$α-smooth muscle actin $(SMA)^+$pericytes have been located along post-capillary venules and $NG2^+$α-SMA^+ pericytes are found along arterioles and capillaries (118). In the cremaster muscle, movement of neutrophils across the basement membrane is regulated by post-capillary $NG2^-$ (88, 89).

In the abluminal space, neutrophils crawl along pericytes to reach gaps between adjacent pericytes. These gaps colocalize with regions within the venular basement membrane, which contain lower levels of certain basement membrane constituents, such as laminin-8, laminin-10, and collagen type IV. These sites are known as low expression regions (LERs) and are the preferred regions for neutrophils to transmigrate (119, 120). After neutrophil transmigration, these gaps enlarge in size although not in number (119), a phenomenon not observed in monocyte transmigration (120). Interestingly, neutrophils follow other neutrophils and the following neutrophil exhibites markedly reduced meandering. There extremely coordinated chemotaxis and cluster formation is reminiscent of the swarming behavior of insects. Multiple neutrophils exit the venular wall through the same LER gap. Mechanisms that potentially facilitate migration of the follower-cells include the release of leukotriene B4 and other chemoattractants, from the leading neutrophil (45), and the remodeling the venular basement membrane in a protease-dependent manner (89, 121).

TEM of neutrophils occurs rather fast (∼4–6 min) (60), while crawling in the layer between the ECs and pericytes, the abluminal space, takes considerably more time (∼15–20 min) (122). Abluminal crawling appeared to be supported by pericyte-expressed ICAM-1 and integrins Mac-1 and LFA-1 (89). Furthermore, enhanced levels of ICAM-1 and the chemokine CXCL1 were observed on ECs and pericytes after TNF-stimulation as compared with non-stimulated tissues. These results indicate that, neutrophil crawling on pericytes is driven by pericyte-expressed ICAM-1 and chemokine release (89). Other pericyte-associated adhesion molecules might also contribute to crawling on the abluminal surface, since inhibition of ICAM-1 only partially reduced the neutrophil crawling (89).

Several studies have shown, *in vitro*, that pericytes are contractile cells and they have the ability to change shape after stimulation with vasoactive mediators, such as histamine (123, 124). These observations might provide an explanation for the increase in gaps between adjacent pericytes seen in the cremaster muscle upon TNF and IL-1β stimulation (89). The signaling pathway regulating pericyte shape change is still unclear, however, both TNF and IL-1β are known to activate small GTPases that play a key role in actin cytoskeleton rearrangement (125), providing a plausible explanation to the increased gap size.

In conclusion, pericytes were until relatively recent under-appreciated and their function down-played. However, the observations discussed above strongly support a role for these cells in assisting the arrival of neutrophils to the site of inflammation.

Shear Stress: When Less Is More

ECs are constantly exposed to vascular forces, such as shear stress, a frictional force exerted by blood flow. The flow patterns differ based on vessel type and geometry. These patterns range from uniform undisturbed laminar flow to disturbed oscillatory flow. ECs are able to sense and differentially respond to these flow patterns, that create a restricted and unique microenvironment (126).

Laminar flow is observed where geometry of the vessel is straight and uniform. Responses to laminar flow include EC alignment in the direction of flow, low EC proliferation, the formation of stress fibers, and upregulation of transcription factors—all contributing to anti-inflammatory gene expression (126). The transcription factors nuclear factor erythroid 2-like 2 (NRF2) and the flow-dependent transcription factor Krüppel-like factor 2 (KLF2) are activated via mitogen-activated protein (MAP) kinase/extracellular-signal-regulated (ERK) kinase and PI(3)K/Protein kinase B (PKB) signaling pathways and maintain endothelial phenotype (127, 128) and metabolic state (129). They inhibit nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and activator protein-1, contributing to a quiescent state of the ECs (130).

Disturbed flow primarily manifests in bifurcations or curves of the vessel. This type of flow is characterized by low and oscillatory flow patterns. Under disturbed blood flow, ECs sense different blood flow directions, cells do not align so tightly (131, 132), ECs are more proliferative (133) and produce more ROS compared to those cells in areas of laminar flow (126). This activation of ECs is accompanied by pro-inflammatory properties, including

the activation of transcription factor NF-κB (126). NF-κB is stimulated through the activation of a mechanosensory complex, consisting of VEGF receptor 2, PECAM-1 and VE-cadherin, extracellular matrix, and integrins (134). Under disturbed flow conditions, ROS production by the endothelium occurs via Rac-1-mediated p67phox NOX2 activation (135). Increased expression of NADPH oxidase 2 leads to an increased expression of VCAM-1 (136). Furthermore, ROS degrades NF-κB inhibitor, IκB kinase, and translocates activated NF-κB to the nucleus, hereby aiding to the increased transcription of cell adhesion molecules including ICAM-1 and VCAM-1 (137).

The glycocalyx, consisting of a mixture of glycoproteins, hyaluronin, and proteoglycans, also plays an important role in the mechanosensing process. Mechanical forces acting on ECs are primarily transmitted to the glycocalyx layer. The glycocalyx is thereby reducing the shear gradients that the cell surface experiences. However, disrupted flow impairs the glycocalyx layer properties contributing to the increased ability of neutrophils to adhere to ECs and inducing an unstable pattern of flow forces gradients acting on the endothelial surface (138, 139).

Once the neutrophils adhere to the endothelium, adhesion forces are generated, mainly by leukocytic ligands binding to ICAM-1 and VCAM-1 expressed on inflamed endothelium. This interaction is able to resist the convective hemodynamic forces imparted by flowing blood. Neutrophils show a rolling behavior, when forces are almost balanced. This balance is a main determinant of cell rolling velocity (140, 141).

Platelets: Small but Mighty Players in Neutrophil Recruitment

Interactions of platelets with neutrophils as well as with ECs are important mediators of the inflammatory response (142–144). Platelets express adhesion molecules and can therefore bind to the endothelium as well as neutrophils. The most abundant adhesion molecule expressed on platelets is the $\alpha_{IIb}\beta_3$ integrin (145, 146). This integrin can bind fibrinogen, which is able to bind the neutrophilic Mac-1, thereby facilitating the formation of neutrophil-platelet complexes or aggregates (147, 148). Such complex formation also takes place upon interaction of neutrophilic Mac-1 with glycoprotein Ib on platelets (149), complemented by the interaction of neutrophil LFA-1 with platelet ICAM-2 (150) or JAM-A (151). Aggregate formation can also be mediated by the interaction between platelets CD40 and neutrophil CD40L. This is a two-way interaction, which results in the activation of both cells (152). Heterotypic neutrophil-platelet interactions are also supported by selectins. In this case, upon platelet activation, P-selectin is incorporated into the plasma membrane, and is then available to bind PSGL-1 present on neutrophils (48). Since platelets can bind ECs as well as neutrophils, platelet-neutrophil aggregates can be recruited to activated endothelium (153).

Activated platelets can also directly simulate neutrophils by releasing a variety of growth factors, chemokines and cytokines into their microenvironment (154). These stimuli support apoptosis and NET formation as well as leukocyte recruitment (155–157). Platelets can further influence recruitment by altering the adhesive, chemotactic and proteolytic properties of ECs (158, 159).

Apart from their role in neutrophil recruitment, platelets can also be involved in maintaining the integrity of the vascular endothelium. In particular, they are able to influence vascular permeability and thus indirectly modulate neutrophil recruitment (160, 161).

Low Oxygen Tension: An Intrinsic Relation With Inflammation

Inflammation is a metabolically costly process and oxygen demands exceed its supply. Neutrophils are in particular relevant to the concept of "inflammatory hypoxia." Neutrophilic functions like release of ROS, granule proteins and NETs locally deplete molecular oxygen, consequently creating a hypoxic microenvironment sensed by neighboring cells (162).

The master regulator of oxygen homeostasis is hypoxia inducible factor-1 (HIF-1), a transcription factor turned on in response to hypoxia. HIF has emerged as a major player in neutrophil function and survival. Under normal conditions, HIF-1α is hydroxylated by oxygen-sensing prolyl hydroxylase domain enzymes (PHD1, −2, and −3) (163), followed by ubiquitination and proteasomal degradation. HIF-1α activity is also mediated by factor inhibiting HIF, since it is able to fine tune HIF activity by asparagine hydroxylation (164). However, during hypoxic conditions, PHDs and factor inhibiting HIF are inactive, allowing HIF-1α to stabilize and translocate to the nucleus, where it dimerizes with HIF-1β. Dimerization, results in the formation of a functional active transcriptional complex, which transcribes genes involved in angiogenesis, glycolysis, and cell migration (163). Regarding cell migration, HIF-1α acts as a transcriptional regulator of the β2-integrin beta subunit, hence, affecting the neutrophil process of migration (165). HIF also regulates neutrophil responses to proinflammatory stimuli (166, 167), mediates their phagocytic ability, regulates adaptation of neutrophils to hypoxia and influences neutrophil lifespan by delaying apoptosis (168). However, by delaying cell apoptosis HIF is also adjourning resolution of inflammation by propagating effete neutrophils (169). For this reason, in order to prevent chronic inflammation and limit tissue damage, there must be a balance between the fully competent neutrophils at the onset of the inflammation and the removal of damaged cells (170).

Altogether, these observations underscore an essential role of HIF-1 in the function, survival and recruitment of the neutrophil cell under inflammatory conditions.

NEUTROPHIL RECRUITMENT IN DIFFERENT ORGANS

Mechanisms described above can vary among organs. For example, the vasculature of the lung, liver, kidney, and the aorta are characterized by structural specializations, which are required for their functions. Therefore, it comes as no surprise that neutrophil recruitment might differ within these organs. Lungs, kidneys, the liver and the aorta play an important role in frailty in older adults. Developing interventions to prevent frailty

in older adults is a priority in aging societies as it increases the risk for disability, hospitalization and mortality (171, 172). A better understanding of distinct mechanisms of neutrophil recruitment in different organs would set a basis for tailored intervention in the future, without compromising host defenses. In the following sections we will describe organ-specific neutrophil recruitment and a summary of different molecules involved in the different stages of neutrophil recruitment in several organs can be found in **Table 1**.

How Neutrophils Travel on Air

The lung is characterized by a unique anatomical architecture, intrinsic to its vital function as oxygen provider. The vasculature is highly branched compared to peripheral circulation. The lung has a dual circulation: the bronchial vasculature, with high-pressure, low-volume, which delivers oxygen to the bronchial tree; and the pulmonary vasculature, with low-pressure, high-volume, which is involved in gas exchange (201). Both vascular beds are composed of a continuous layer of ECs. Most of the leukocyte migration takes place in pulmonary capillaries, as compared with their bronchial analogs. A possible explanation relies on the increased blood pressure in the bronchial circulation and/or the wider diameter of bronchial capillaries (202). In the bronchial circulation recruitment takes place in the post-capillary venules, whereas in the pulmonary circulation in the capillaries. Air-filled alveoli are separated from the extensive pulmonary microvasculature system by a thin interstitial tissue membrane, the alveolar space (202). Furthermore, they possess an unusually high number of caveolae, which are membrane structures that have important roles in cell signaling and transcellular transport (13).

The lung constantly samples the air we breathe. It oxygenates the blood by taking up oxygen and releasing carbon dioxide (201). The lungs are supporting the entire cardiac output, however, the blood flow velocity in the capillary network of the lung is relatively low. Interestingly, the diameter of the capillaries (ranging from 2 to 14 μm) is smaller than that of the neutrophilic cell (13.7 μm) (203). For this reason, these cells do not roll, as in post-capillary venules, instead they are forced to change their shape to progress in the capillaries and find a suitable transmigration site (204). This phenomenon might be supported by the low blood flow.

Unlike the majority of organs, the lungs possess a neutrophil reservoir, often termed "marginated pool," that are readily recruitable and in dynamic equilibrium with those in local circulation (205). This TLR4-Myd88-and abl tyrosine kinase-dependent niche can provide immediate CD11b-dependent neutrophil responses to Lipopolysaccharide (LPS) and Gram-negative bloodstream pathogens, clearing the inflammatory insult (206). The need for such reservoir might be closely related to the proximity and exposure of the lungs to pathogens, allergens, irritants and toxins, which make the lung vulnerable to inflammation (207).

The first-line of defense is provided by tissue resident alveolar macrophages, that phagocyte and eliminate pathogens without directly initiating leukocyte recruitment (208, 209). Macrophages, together with ECs and epithelial cells, secrete chemokines, cytokines and other inflammatory mediators, which promote local inflammation and neutrophil accumulation. Alveolar macrophages can also aid neutrophil transmigration. In a murine model of sepsis, alveolar macrophages increased neutrophil TEM by producing platelet-activating factor and hydrogen peroxide, which led to endothelial superoxide production and consequent oxidant EC stress (210). Neutrophils provide the second-line defense. Upon inflammation, neutrophils migrate out of the pulmonary capillaries and infiltrate the air spaces (209, 211).

Neutrophil recruitment to the pulmonary microvasculature does not follow the conventional paradigm (**Figure 2**). Mechanical trapping of neutrophils was proposed to contribute to neutrophil extravasation and naturally obviates the need for rolling on the endothelium (212). Nevertheless, the involvement of selectins and integrins in neutrophil recruitment seems to be dependent on the experimental model of lung inflammation (8). Neutrophil recruitment under *Streptococcus pneumoniae*-induced lung inflammation is independent of E- and P-selectin (174). On the other hand, neutrophil recruitment in the lung in LPS treated mice was dependent on E- and L-selectin. Additionally, PSGL-1 and platelets played a role in their recruitment (180). A different selectin dependent neutrophil recruitment pattern was observed in lung injury following systematic activation of the complement system (L- and P-selectin dependent) and an IgG immune complex model of lung injury (E-, L-, and P-selectin dependent) (175). Similar to selectins, the role of integrins on neutrophil recruitment in experimental lung inflammation varies and depends on the type of inflammatory stimuli. Neutrophil migration can occur in a β2-integrin dependent way when lung inflammation is induced by *Streptococcus pneumoniae*, hydrochloric acid, C5a complement fragments (176) or LPS (177). Integrin independent neutrophil recruitment takes place upon lung injury following administration of *Escherichia coli*, *Pseudomonas aeruginosa*, phorbol ester, IgG immune complexes or IL-1 (176).

Once the neutrophils are sequestered, both L-selectin and LFA-1 are critical to keep these cells within the capillary bed for more than 4–7 min (178, 179). Neutrophil adhesion in the lung seems to be influenced by connexin 43 (181) and the glycoprotein, gp130. Gp130 is a subunit of the IL-6 receptor family. Loss of endothelial gp130 in mice results in upregulation of CXCL1 at endothelial junctions of the microvascular cells. Neutrophils from these mice show impaired adhesion most likely by disrupting chemotactic gradients (213).

Neutrophil recruitment in the lungs is also assisted by monocytes. Blood monocytes often colocalize in vessels near sites of neutrophil extravasation and reports support a role for these cells in neutrophil recruitment. As an example, $CCR2^{+}$ circulating monocytes were shown to be essential for neutrophil recruitment (214). And in agreement with these observations, clodronate-liposome-mediated depletion of monocytes dramatically impaired neutrophil transendothelial migration (211).

Platelets are tightly associated with lung injury. They increase vascular permeability and neutrophil activation, NET formation and migration, due to platelet-derived CCL5-CXCL4

TABLE 1 | (Adhesion) molecules, cytokines and chemokines involved in different stages of the neutrophil recruitment in cremaster, lung, liver, kidney, and aorta.

	Tethering/rolling			Arrest/adhesion			Crawling			Transmigration		
Organ/Vessel	**EC**	**Neutrophil**	**References**	**EC**	**Neutrophil**	**References**	**EC**	**Neutrophil**	**References**	**EC**	**Neutrophil**	**References**
Cremaster recruitment	P-selectin	PSGL-1	(36)	ICAM-1	(Mac, −1), LFA-1	(51)	ICAM-1	Mac-1, (LFA-1), fibrinogen	(51)	VE-cadherin–VE-PTP*	-	(65)
	E-selectin	PSGL-1, ESL-1, CD44	(37)				ICAM-2	Mac-1, LFA-1	(53)	PECAM-1–CD99 *	-	(86)
										JAM- C	-	(75, 173)
Lung	P-selectin**	β2-integrin	(174–177)	L-selectin	LFA-1	(178, 179)						
	E-selectin**	β2-integrin, PSGL-1	(174–177, 180)	Cx43 (indirectly)	-	(181)						
	L-selectin**	?	(175, 180)									
Liver–Sinusoids	In sinusoids selectin-independent		(182, 183)	HA**	CD44	(184)	?	?	-	?	?	-
Sepsis and endotoxemia												
Liver–Sinusoids	In sinusoids selectin-independent		(182, 183)	ICAM-1***	Mac-1	(145, 185)	CXCL1 CXCL2 ***	-	(186)	-	FPR1	(186)
Sterile inflammation												
							Platelets ***	Mac-1	(145, 186)			
							-	FPR1	(186)			
Kidney	P-selectin	PSGL-1	(187)				Cytokines	-	(188)	VAP-1 (by pericytes)	-	(189)
Peritubular capillaries	E-selectin	β2-integrin	(187)									
	CD44	HA	(188, 190)									
Kidney	Does not occur		(191)	ICAM-1	Mac-1, β2-integrin	(191, 192)						
Glomeruli												
			P-selectin (via platelets)	PSGL-1	(191–193)							
Aorta	P-selectin	PSGL-1	(194–196)	ICAM-1, ICAM-2	β2-integrin	(194, 195, 197)				JAM-A	-	(198)
	E-selectin	?	(194)	CCR1***, CCR2, CCR5***, CXCR2	CCL5	(9)						
				CCR2	CCL2	(199)						
				CatG***	-	(10)						
				CRAMP	FPR	(200)						

*The classical cascade applies to neutrophils extravasating in post-capillary venules, whereas in other organs the extravasation can take place in different vessels. Additionally, some stages of the classical cascade are not present, not yet studied in detail or deviate from the classical cascade. Owing to the complexity of the extravasation process, the list is not exhaustive but represents the (adhesion) molecules, cytokines and chemokines addressed in this review "?" indicates unknown data. CatG, Cathepsin G; CCL, chemokine (C-C motif) ligand; CRAMP, Cathelicidin related antimicrobial polypeptide; Cx43, Connexin 43; CXCL, Chemokine (C-X-C motif) ligand; EC, Endothelial cell; ESL-1, E-selectin-ligand-1; FPR, Formyl peptide receptor; HA, Hyaluronic acid; ICAM, Intercellular adhesion molecule; JAM, Junctional adhesion molecule; LFA-1, Lymphocyte function-associated antigen 1; Mac-1, Macrophage-1 antigen; PECAM, Platelet endothelial cell adhesion molecule-1; PSGL-1, P-selectin glycoprotein ligand 1; Ref, Reference; VAP-1, Vascular adhesion protein-1; VE-cadherin, Vascular endothelial cadherin; VE-PTP, Vascular endothelial protein tyrosine phosphate. *ECs-ECs interaction **Stimulus dependent ***Artery specific.*

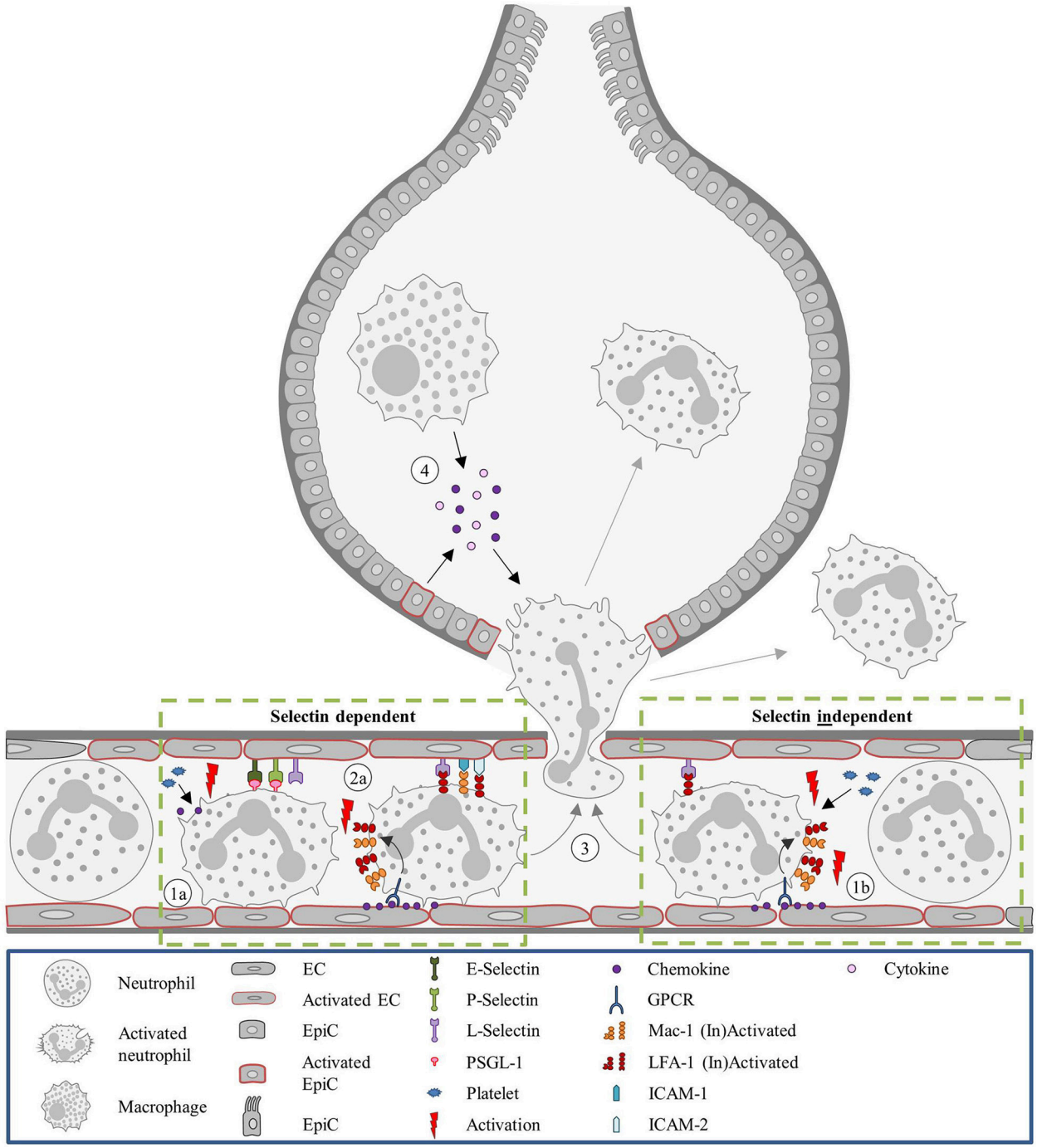

FIGURE 2 | Neutrophil recruitment in the lung. Unlike most organs, in the lung neutrophils are sequestered in the capillaries, instead of post-venules. (1a) In the capillaries, neutrophils are activated by platelets releasing chemokines and the recruitment is promoted by endothelial stress. Due to the diameter of the capillaries, neutrophils are subjected to mechanical entrapment and the involvement of selectins for the recruitment process is not always occurring. The involvement of selectins and integrins is dependent on the inflammatory stimulus. (2a) For LPS-treated mice neutrophil recruitment is selectin and integrin dependent. Integrin activation occurs as described in the classical recruitment cascade. (1b) However, in mice treated with *S. pneumoniae* neutrophil recruitment was shown to be selectin independent. And recruitment was described as integrin-independent in mice administered with *E. coli*. In any case, L-selectin and LFA-1 can keep neutrophils within the capillary for several minutes, supporting the cell transmigration. (3) Neutrophil recruitment proceeds with transmigration to the interstitium or to the alveolar space. (4) In the alveolar space, alveolar macrophages and EpiCs are essential for guiding the neutrophil by the secretion of inflammatory mediators (e.g., cytokines and chemokine's). E. coli, *Escherichia coli*; EC, Endothelial cell; EpiC, Epithelial cell; GPCR, G protein-coupled receptor; ICAM, Intracellular adhesion molecule; LFA-1, Lymphocyte function-associated antigen 1; LPS, Lipopolysaccharide; Mac-1, Macrophage-1 antigen; PSGL-1, P-selectin glycoprotein ligand-1; S pneumoniae, Streptococcus pneumoniae.

(RANTES-Platelet Factor 4) chemokine heteromers (215). Furthermore, TLR4^{+}platelets can detect TLR4 ligands in blood and induce platelet binding to adherent neutrophils, resulting in neutrophil activation and the formation of NETs (216).

How Neutrophils Navigate in the Liver

Similar to the lung, the liver also has as dual blood supply. The arterial system, via the hepatic artery, provides the liver with well-oxygenated blood and delivers approximately one-third of the blood supply to this organ. The portal system, via the portal vein, delivers blood from several abdominal locations to the liver. This blood represents two-thirds of the blood supply that is nutrient-rich, lipid droplet-rich and poorly oxygenated. Both the hepatic artery and portal vein drain into capillary-like hepatic sinusoids. Eventually, the blood flows into the terminal hepatic (post-sinusoidal) venules, continues through the hepatic vein and thereafter the inferior vena cava, that supplies the heart's right atrium (201).

Under homeostatic conditions granulocytic cells, such as neutrophils, are largely absent in the liver. However, the neutrophil population can be rapidly increased in response to a pathogenic (217) or sterile stimulus (218). Numerous infectious pathologies as well as sterile insults affect the liver by causing tissue injury (182). Interestingly, Wang et al. observed the beneficial effect of neutrophils on the healing of a sterile thermal hepatic injury. Neutrophils penetrate the injury site and dismantle injured vessels and create channels for vascular regrowth. Upon completion of their task, they neither die nor are phagocytized. Instead, many of these neutrophils undergo reverse transmigration and travel to the lung where they regain CXCR4, followed by re-entering the bone marrow where they undergo apoptosis (74).

The neutrophil recruitment in the liver differs per anatomical location. In the post-capillary venules neutrophils undergo selectin-dependent rolling. However, in the sinusoidal vascular bed these neutrophils adhere via a selectin-independent mechanism, which is rolling independent (182, 183). Interestingly, liver sinusoids support the majority of leukocyte trafficking, 70–80%, while the remaining traffic takes place in the post-capillary venules, in accordance with the classical recruitment cascade (182). Similar to the capillaries in the lungs, anatomical features of the liver, namely the diameter of the sinusoids, of 6.4–15.1 μm, also influence the recruitment (219). Originally, it was thought that migration was mediated by physical trapping of the neutrophil in the narrow channels, however recently other recruitment mechanisms were identified. Sinusoid endothelium expresses a different portfolio of adhesion molecules, with little E- and P-selectins present (182) as well as low expression of VCAM-1. Instead, ICAM-1 and vascular adhesion protein (VAP)-1 are found to be highly expressed in a constitutively manner (220, 221).

The sinusoidal vasculature, composed of liver sinusoidal ECs (LSEC), has a unique morphology. The LSECs are discontinuous and fenestrated, lacking tight junctions and basal lamina (222). Openings in the endothelial layer, fenestrations (100 nm) (223), allow plasma to flow freely into the sub-endothelial Space of Disse, where it comes in direct contact with hepatocytes. The fenestrae size is dynamically regulated in response to drugs, toxins, vascular tone, disease and aging (224).

The inflammatory process is initiated by the release of DAMPs from damaged and necrotic cells. Kupffer cells (KCs, tissue resident macrophages) are the first cells to detect these damage signals, and respond with the production of cytokines, chemokines and ROS, resulting in the homing, activation, and adhesion of neutrophils (225). Activated KCs can also promote recruitment by altering the shear forces within the microvasculature (226). Depending on the inflammatory stimulus, neutrophils undergo different recruitment pathways.

Under sterile inflammation, DAMPs, such as extracellular ATP, released from damaged or necrotic cells, bind to TLR9 on neutrophils, and promote neutrophil recruitment and activation. This initiates a positive feedback loop, where neutrophils sense and react to DAMPs by activating the TLR9/NF-κB pathway, further sustaining neutrophil recruitment (227, 228). Extracellular ATP also signals to KCs, stimulating these cells via P2X purinoceptor 7 to produce caspase-1 and IL-1β. The presence of IL-1β induces the up-regulation of ICAM-1 on LSECs (186). Neutrophils can adhere via an endothelial ICAM-1 leukocytic Mac-1-dependent adhesion mechanism (145). TLR2 plays an important role in ICAM-1/Mac-1-dependent neutrophil recruitment. TLR2 and myeloid-related protein 14 (S100A9) are key regulators of CXCL2 release by KCs (185). An initial chemotactic gradient of CXCL2 stimulates, via CXCR2, the influx of neutrophils into the liver. CXCL2 is expressed as an intravascular gradient that leads toward the injured area. Expression starts at approximately 650 μm distance from the injury and gradually increases till 150 μm. However, the CXCL2 gradient on the luminal surface of the sinusoids abruptly ends at approximately 100–150 μm proximal to the border of necrotic tissue. Neutrophils continue to migrate into the area of necrosis independently of CXCR2 (186). Platelets then take over from the chemokines-dependent neutrophil crawling. Immobilized platelets physically "pave the way" for neutrophils to enter the liver and aid repair. The platelets adhere to the injured LSECs by GPIIbIIIa and pave the last 200 μm of the sinusoids toward the necrotic area by completely encapsulating the injury site (145). Neutrophils crawl on the immobilized platelets through Mac-1, independently of LFA-1 (186). Additionally, migration of neutrophils through the last 200 μm requires formylated peptide receptor 1 (FPR1) to be expressed on neutrophils, to follow a ECs mitochondria-derived formyl-peptide gradient, which promotes precise neutrophil migration into the necrotic zones (186). **Figure 3A** summarizes the neutrophil recruitment under sterile inflammation.

During gram-negative-induced sepsis, or endotoxemia, high levels of bacterial LPS are circulating and stimulate KCs. Stimulation of KCs results in the production of large amounts of IL-10, inducing down-regulation of neutrophilic Mac-1 (229). However, in LPS-treated mice, neutrophils are still recruited and arrest in the sinusoids, where they act as filters for systemic infections (230, 231). Initially it was hypothesized that the neutrophils' migration was merely mechanically instigated, due to physical entrapment (232). Nevertheless, a systematic examination of several candidate molecules revealed that CD44

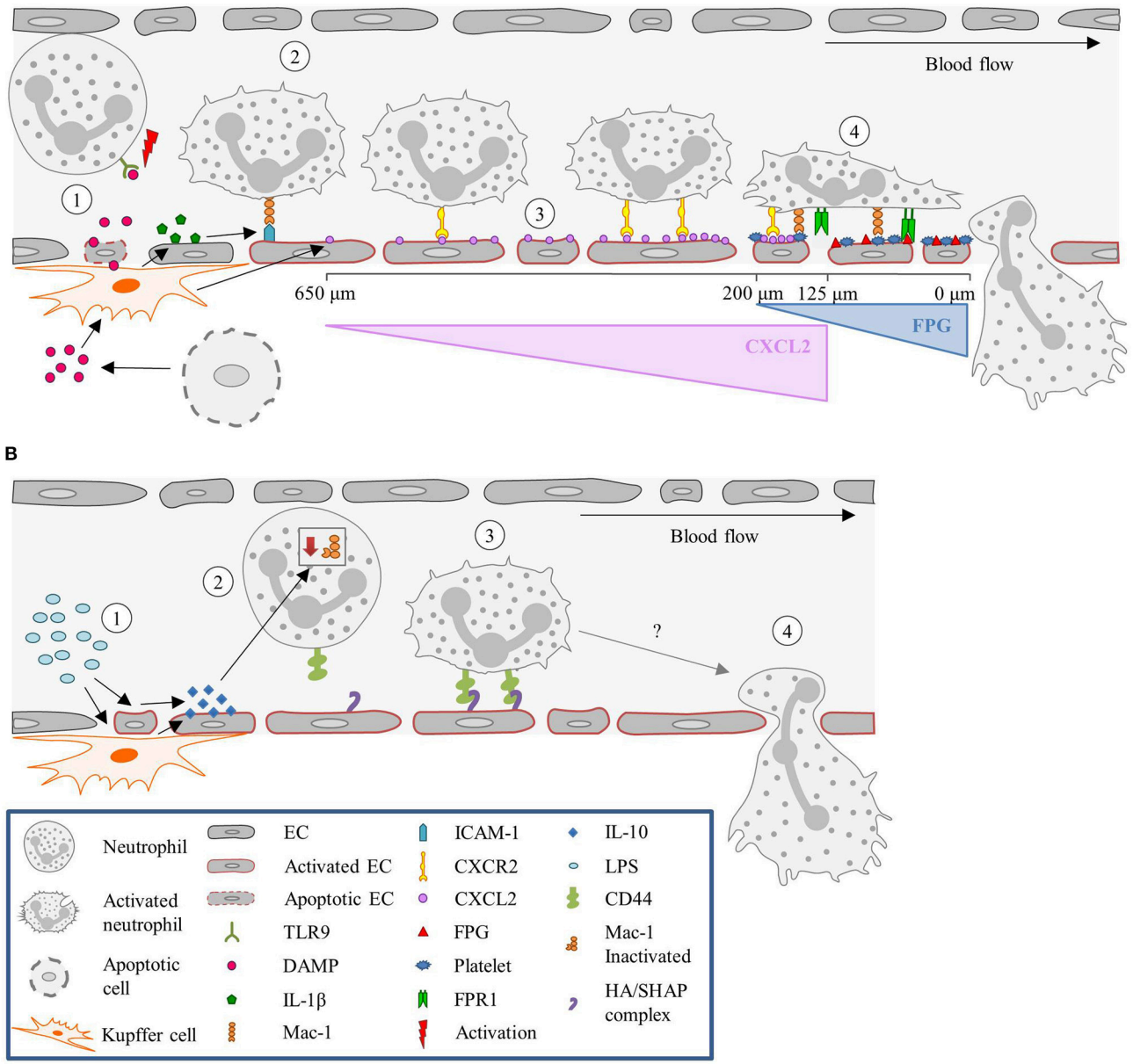

FIGURE 3 | Neutrophil recruitment in the liver. **(A)** Sterile inflammatory stimuli. (1) During sterile inflammation, DAMPs are released from apoptotic ECs or cells in the tissue. DAMPs can directly activate neutrophils via interaction with TLR9 or stimulate KCs to produce inflammatory mediators such as IL-1β. (2) In turn, IL-1β upregulates ICAM-1 expression on the sinusoids, resulting in the adhesion of neutrophils mediated by ICAM-1-Mac-1 interaction. (3) KCs also release CXCL2, and create a gradient that increases toward the site of injury, guiding the neutrophils. This gradient starts ~650 µm and ends at 100–150 µm away from the injury site. (4) From here on, neutrophils are guided by platelets and a formyl-peptide gradient (FPG) (released by the endothelium), in a Mac-1 and FPR1-dependen manner, respectively. **(B)** Pathological inflammatory stimulus. (1) During endotoxemia or Gram-negative sepsis, high levels of LPS stimulate KCs and ECs to produce large amounts of the anti-inflammatory cytokine IL-10. (2) The exposure of neutrophils to high levels of IL-10 results in down regulation of Mac-1 surface expression, yielding CD44 as the dominant adhesion molecule for recruitment. (3) CD44 then interacts with the HA/SHAP complex on the endothelium mediating the adhesion process, (4) eventually leading to neutrophil extravasation. CXCL2, Chemokine (C-X-C motif) ligand 2; CXCR2, Chemokine (C-X-C motif) receptor 2; DAMP, Damage-associated molecular pattern molecules; EC, Endothelial cell; FPG, Formyl-peptide gradient; FPR1, Formyl peptide receptor 1; HA, Hyaluronic acid; ICAM-1, Intercellular adhesion molecule-1; IL, Interleukin; KC, Kupffer cell; LPS, Lipopolysaccharide; Mac-1, Macrophage-1 antigen; SHAP, Serum-derived hyaluronan-associated protein; TLR9, Toll-like receptor 9.

deficient mice lack neutrophil accumulation in the sinusoids following LPS challenge (233). Therefore, neutrophil recruitment seems CD44 dependent. LSECs are enriched with extracellular matrix glycosaminoglycan hyaluronan, which is a ligand for CD44, a cell surface glycoprotein found on most leukocytes, including neutrophils (184, 233). LPS activates LSECs to undergo transesterification of HA, resulting in the production of serum-derived hyaluronan-associated protein (SHAP). SHAP binds to the sinusoidal endothelium, forming a HA/SHAP complex. The complex facilitates CD44-dependent neutrophil adhesion in the sinusoids (184). Interestingly, hyaluronidase pre-treatment in the liver sinusoids attenuated LPS-induced neutrophil arrest, an effect that was not observed in the post-capillary venules (233). Therefore, these studies support a role for CD44 in sinusoid-specific neutrophil recruitment. Intravital immunofluorescence imaging demonstrated that stimulation of endothelial TLR4 alone was sufficient to induce the deposition of SHAP within sinusoids, which was required for CD44/hyaluronan-dependent neutrophil adhesion (184). This validated that LPS stimulation is TLR4-dependent. **Figure 3B** summarizes the neutrophil recruitment under gram-negative-induced sepsis.

Neutrophils themselves appear to recruit platelets to sites of infection. And in turn, platelets modulate the recruitment, activation and adhesion of neutrophils (152, 230, 234). The interaction of platelets with neutrophils seems to occur via interactions with LFA-1 (231). The bacterial and viral trapping, normally executed by KCs, is greatly increased as neutrophils and platelets are recruited and induce NET formation (235).

To summarize, neutrophil trafficking mechanism in the liver is stimuli dependent and the recruitment differs from the classic paradigm in two fundamental ways: (1) the majority of infiltrating neutrophils adhere within the capillary-like sinusoids rather than the post-capillary venules; (2) a selectin-mediated rolling step is not apparent and the adhesion of neutrophils within sinusoids is mainly described as selectin-independent.

Neutrophils in the Human Filter Unit

The kidney receives 15–20% of the cardiac output (201) and has three distinct capillary networks, a feature unparalleled by any other organ. With this complex capillary networks, the kidney functions as a filter, for liquids and small particles (including nutrients), cleaning the body from toxins as well as needless components, and keeping the water and nutrients (236). Blood enters the first capillary network, located in the cortex, via the renal artery that then branches into the interlobar artery. In turn, the interlobar artery is followed by the arcuate and interlobular arteries which later drain into afferent arterioles. From the afferent arterioles the blood arrives to the capillaries located in the glomeruli (201, 236). These capillaries participate in the production of plasma ultrafiltrate, which enters the nephrons. The blood leaves the glomeruli via efferent arterioles and enters the second and third renal capillary network. The second network, the peritubular capillaries, surrounds the nephrons, and is often described as part of the renal cortex. This second network further assists in the filtration process, by reabsorbing solutes and water from the proximal tubular lumen and returning them to general circulation (237). Peritubular capillaries are also in close proximity to the tubules and serve as a supply for oxygen and nutrients. The third network is reached via the descending vasa recta, which gives rise to the small capillary network that supplies oxygen and nutrients to the inner medulla and maintains the medullary concentration gradient. The blood from the peritubular capillaries and vasa recta ascending from the third network eventually drains into venules and thereafter veins, which parallel the arterial system (8, 236).

The glomerular capillaries are lined by specialized highly fenestrated ECs. The fenestrae have a diameter of ∼60 nm (238) and seem to facilitate filtration of small solutes and water. The ECs on the luminal side are covered by glycocalyx, glomerular basement membrane, and podocytes, all further supporting the EC barrier function (239–241). Podocytes are specifically expressed in kidneys and are mainly found covering the glomeruli. Apart from preserving the glomerular ECs barrier function, these cells regulate the tight spatial control of fenestrae, both via the production of VEGF-A (242, 243).

In the kidney, inflammation is induced by activation of immune cells as well as of intrinsic renal cells (such as podocytes, mesangial or epithelial cells). This process can result in the production and consequent release of profibrotic cytokines and growth factors that drive fibrosis, which when uncontrolled leads to end-stage renal disease (244). Neutrophil recruitment occurs in all capillary networks: in the cortex [in the capillaries of the glomeruli (192) as well as in peritubular capillaries (245)], and in the medulla [in the dense capillaries network that arises from the descending vasa recta (246, 247)]. To dissect the process of neutrophil recruitment direct visualization of the neutrophil interaction is required. However, the kidney is a very dense organ and its anatomy and features are a challenge for such studies. Even superficial glomeruli are found as deep as at 100 μm below the surface (13). Likely due to this reason, early studies reported that leukocyte adhesion in glomerular capillaries shared much in common with adhesion in "conventional" post-capillary venules (248–251). However, later on, and with the introduction of the murine model of hydronephrosis, it has been observed that neutrophil recruitment is not dependent on rolling (191). By ligating one of the ureter, in this animal model, the kidney becomes easier to image. These studies then showed that in unstimulated glomeruli, and unlike in other organs, neutrophils, as well as monocytes, patrol the capillaries. Particular to the kidney, while patrolling, these cells have short adhesion periods (also termed "dwell time"). Upon encounter with an acute inflammatory stimulus, these patrolling neutrophils are activated and respond by increasing their "dwelling time" on the endothelium. Under acute inflammatory conditions, activated neutrophils can remain attached to the endothelium for long periods of time, up to 20 min (192). These increased adhesion time was shown to be Mac-1 dependent (192). The activated neutrophils initiate ROS production, which in turn increases Mac-1 expression and hence the cell adhesion times. Consequently, Devi et al. postulated that rather than affecting the number of recruited cells, acute inflammation increases the duration of neutrophil retention in the capillaries. To what extent this increased

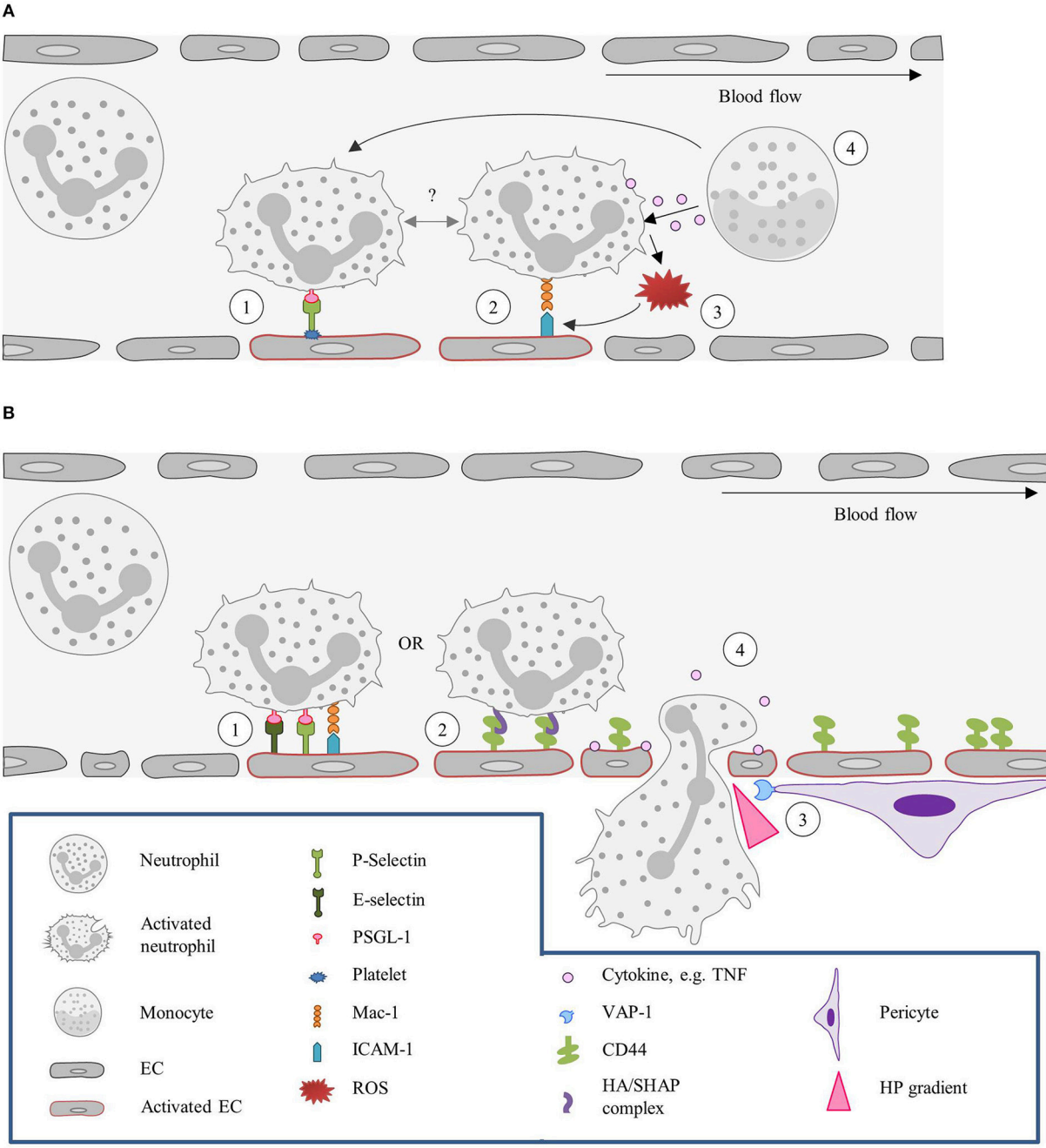

FIGURE 4 | Kidney: the neutrophil actions in different capillary beds. **(A)** Tethering and adhesion/retention of neutrophils in the glomeruli. (1) In the glomeruli P-selectin is required for neutrophils recruitment. As neutrophils do not express this molecule, P-selectin has to be provided by other sources, such as platelets. Platelets adhere to the endothelium, in a GPVI and $\alpha_{IIb}\beta_3$/fibrinogen/ICAM-1-dependent fashion, and neutrophils are thereafter recruited by interaction of leukocytic PSGL-1 with P-selectin. (2) Upon acute inflammation, neutrophils have been found to be retained in the vasculature for increased periods of time (also referred to as "dwell time"), via Mac-1-β2-integrins interaction. Whether this "dwell time" is preceded or followed by P-selectin-dependent tethering remains to be described. (3) Neutrophils retained in the endothelium by Mac-1-β2-integrins interaction release ROS upon activation, which in turn increases Mac-1 expression and consequently expands the cell adhesion times. (4) Neutrophil "dwell time," recruitment and ROS production can also be fostered by patrolling monocytes due to release of TNF or direct interaction with the neutrophil. **(B)** Neutrophil recruitment in the peritubular capillaries. (1) In the peritubular capillaries, neutrophil recruitment is initiated by ICAM-1, P- and E- selectin interactions. (2) Neutrophils can, however, also be recruited in a CD44-HA dependent manner. Under homeostatic conditions, CD44 is poorly expressed by ECs, but upon injury its expression strongly increases. (3) Neutrophil transmigration is assisted by pericytes, which express VAP-1 that generates a local hydrogen peroxide gradient, guiding the neutrophil to the TEM site. (4) In addition, migrating neutrophils release cytokines that further guide other neutrophils and induce vascular permeability facilitating the extravasation. EC, Endothelial cell; GPVI, Glycoprotein VI; HA, Hyaluronic acid; HP, Hydrogen peroxide; ICAM-1, Intercellular adhesion molecule 1; Mac-1, Macrophage-1 antigen; PSGL-1, P-selectin glycoprotein ligand 1; ROS, Reactive oxygen species; SHAP, Serum-derived hyaluronan-associated protein; TEM, Transendothelial migration; VAP-1, Vascular adhesion protein.

retention time influences the inflammatory response remains to be addressed.

Neutrophil recruitment in the glomeruli occurs via immediate arrest and requires P-selectin and ICAM-1 and leukocytic PSGL-1 and β2-integrins (191). Notably, glomeruli ECs do not express P-selectin, but platelets act as a source of P-selectin on the inflamed glomerulus endothelium, once again underscoring the relevance of the cooperative mechanism between platelets and neutrophils in the recruitment of these leukocytes (191, 193). Platelet recruitment was shown to be dependent on the combined actions of Glycoprotein VI and the $\alpha_{IIb}\beta_3$/fibrinogen/ICAM-1 pathway (193). Monocytes can also stimulate neutrophil dwell time in glomerular capillaries, as well as recruitment and ROS generation, in particular by TNF production. This observation suggests that monocyte-neutrophil interactions within the glomerular microvasculature might lead to increased neutrophil recruitment (252). **Figure 4A** summarizes the neutrophil recruitment in the glomeruli.

In the peritubular capillary, also aligned by fenestrated endothelium, neutrophil recruitment depends on E-selectin, P-selectin, and ICAM-1 (187). More general, and in the context of a model of renal ischemia reperfusion, endothelial CD44 was shown to be relevant for neutrophil recruitment (190). Under physiological conditions ECs barely express CD44. However, after renal injury, expression of CD44 on these cells sharply increases (190, 253). Endothelial CD44 then binds to hyaluronic acid on neutrophils and assists their recruitment. Transmigration of neutrophils from the vascular to the interstitial compartment is, as anticipated, directly associated with increased vascular permeability and assisted by cytokine release. Cytokine release can mediate changes across the vascular endothelial layer, hence promoting neutrophil adhesion as well as transmigration (188). Interestingly, intracellular levels of the cytokines interferon-γ, IL-6, and IL-10 are lower in interstitial neutrophils than in vascular neutrophils, suggesting that transmigration, *per se*, leads to cytokine release (188). In corticomedullary junctions, neutrophil infiltration is also aided by pericytes, namely by the expression of VAP-1. VAP-1 generates a local gradient of hydrogen peroxide that guides the neutrophils to the extravasation site (189). **Figure 4B** summarizes the neutrophil recruitment in the peritubular capillaries.

Knowledge concerning neutrophil recruitment in the dense capillaries network, which arises from the descending vasa recta, is limited, and published reports are controversial. As an example, Awad et al. reported observations made in the outer medulla as processes occurring in the peritubular capillaries (188). However, others suggest that the peritubular capillaries are located in the cortex instead of the medulla (236, 254). This associated to the anatomy of this organ contributes to the difficulty in clarifying neutrophil recruitment in the kidney.

The Neutrophil in the Main Stream

The vessel wall of the arteries is covered with a continuous non-fenestrated endothelial layer and displays a well-developed tight junctions system (104)—of great importance to its function, as a fluid conductor, and to manage the exposure to a broad range of shear stress forces throughout the entire body. Dysfunction of the endothelial lining of the arteries is the initiator of the chronic inflammation named atherosclerosis, the main underlying cause of cardiovascular disorders (255). The atherosclerotic disease is characterized by an intricate pathophysiology but one of its main features is the continuous leukocyte recruitment to the damaged endothelium. Despite respiratory and pulsatile movements hampering *in vivo* visualization (9, 256), intravital microscopy studies, focused on the carotid arteries, have been major contributors to the better understanding of this arterial disease, and leukocyte recruitment in particular. However, most studies investigating the inflammatory process in larger vessels mainly focused on the role of monocytes and macrophages—cells with a well-accepted role in atherosclerosis (257). Neutrophils, despite being the first circulating leukocytes to infiltrate the inflammatory site, were only recently shown to be an important mediator in atherosclerosis (9, 258).

Several animal studies demonstrated that regions at high risk for atherosclerotic plaque development are exposed to disturbed flow, low or oscillatory shear stress (131, 132, 137, 259). These regions are primarily in bifurcations or curves (131), where low shear stress induces activation of ECs. Thereafter, several processes take place: reduced production of nitric oxide (NO), increased EC apoptosis and phonotypical changes, and subendothelial accumulation of low-density lipoproteins (LDL) followed by LDL oxidation (255). Notably, the presence of oxidized LDL can activate neutrophils, leading to ROS production and further aggravated endothelial dysfunction (260, 261). Indirectly, low shear stress also contributes to the neutrophil recruitment, via NF-κB and TNF pathway, which in turn upregulates the expression of cytokines, such as CCL2 (262).

As already mentioned, the classical leukocyte recruitment cascade has been defined in the microcirculation, however, to a large extent, this paradigm holds true in the larger arteries (46, 263). As in the microcirculation, Sager et al. also observed the involvement of P-selectin, E-selectin, VCAM-1, ICAM-1, and ICAM-2 in monocyte and neutrophil recruitment. They showed a reduction in recruitment after delivery of small interfering RNAs, which disturbed the translation of all five molecules (194). Neutrophils firmly adhere to the endothelium via the interaction of leukocytic CC chemokine receptors 1 (CCR1), CCR5 and with CCL5, which is seeded on the arterial endothelium by platelets (9). Interestingly, the involvement of CCR1 and CCR5 in the CCL5-mediated firm adhesion is only observed in arteries and not in veins (9). Another interesting fact is that myeloid cells adhere to atherosclerotic lesions in a circadian manner. Neutrophils and monocytes were observed to deposit CCL2 rhythmically on the arterial endothelium, resulting in their recruitment in a CCR2-CCL2-dependent fashion (199).

Neutrophil activation results in rapid release of secretory vesicles, containing granule proteins such as myeloperoxidase, azurocidin, proteinase-3, and cathelicidins. The cathelicidin related antimicrobial polypeptide CRAMP, has been shown to promote neutrophil adhesion in large arteries in a FPR-dependent fashion (200). More recently another granular protein, cathepsin G (CatG), has been identified as a guiding cue favoring myeloid cell adhesion, including neutrophils, specifically under conditions of high shear stress and in large

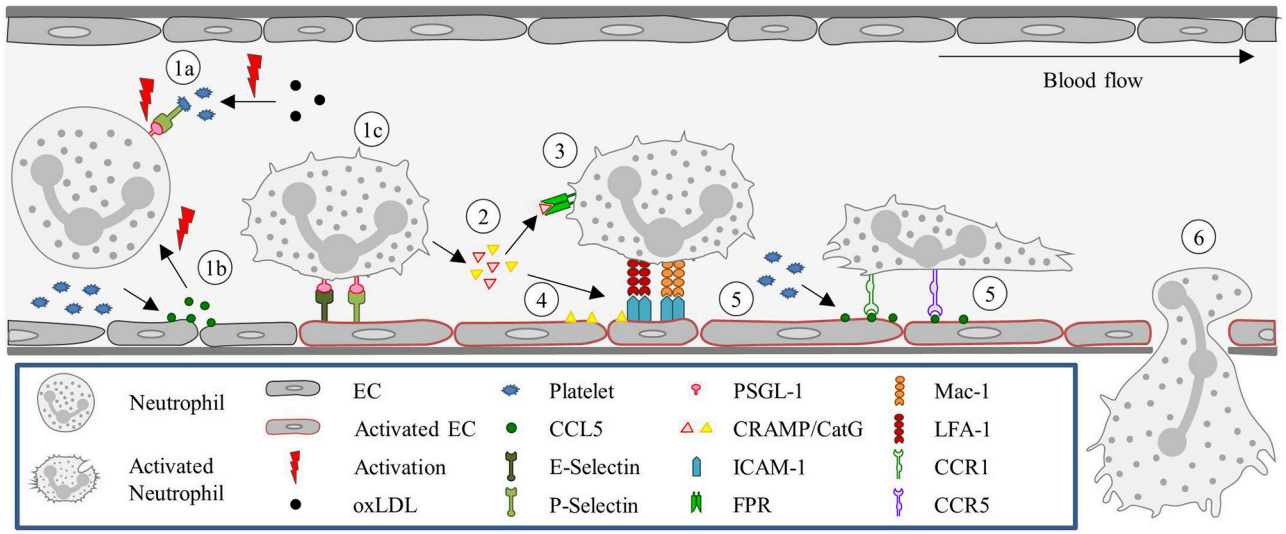

FIGURE 5 | Neutrophil recruitment in the aorta. (1a) Neutrophil recruitment can be directed by platelets activated by oxidized LDL. The activated platelet adheres to the neutrophil, forming a platelet-neutrophil-aggregate. This aggregate formation is mediated by P-selectin. (1b) Neutrophils can also be activated by CCL5 released by activated platelets. (1c) Alternatively, upon damaged endothelium circulating neutrophils tether with ECs in a selectin-dependent manner, followed by their activation. (2) Activated neutrophils can release granular proteins, such as CRAMP and CatG, which can further support neutrophil recruitment. (3) CRAMP supports the recruitment via FPR (4) while CatG, seeded on the endothelium, facilitates firm adhesion of the neutrophil by engaging integrin clustering. (5) Platelets can also seed CCL5 on the endothelium, which can interact with CCR1 and CCR5 present on neutrophils, leading to the firm adhesion of neutrophils to the endothelium, and (6) eventually resulting in neutrophil extravasation. CatG, Cathepsin G; CCL, Chemokine (C-C motif) ligand; CCR, Chemokine (C-C motif) receptor; CRAMP, Cathelicidin related antimicrobial polypeptide; FPR, Formyl peptide receptor; ICAM-1, Intercellular adhesion molecule-1; oxLDL, Oxidized LDL.

arteries, as opposed to veins (10). The release of CatG from neutrophils was shown to be triggered by CCL5 of platelet origin. In turn, platelets were stimulated to release CCL5 under high shear stress conditions, which are absent in veins, results in the specificity of CatG to assist neutrophil recruitment in large arteries. Platelet-neutrophil interplay during neutrophil recruitment is well reported in the literature (264). Another example is the neutrophil recruitment directed by platelets activated by oxidized LDL. The activated platelet adheres to the neutrophil, forming a platelet-neutrophil-aggregate. This aggregate formation is mediated by P-selectin (265). **Figure 5** summarizes the neutrophil recruitment in the aorta.

Similar to CatG, but important for cell transmigration, also JAM-A was suggested to direct monocyte and neutrophil recruitment in the artery, specifically at sites of disturbed blood-flow (198). However, the same molecule, JAM-A, was also reported to mediate neutrophil transmigration in mice cremasteric venules. In this case, the function of JAM-A was studied in the context of a sterile inflammatory stimulus, IL- 1β, or upon ischemia/reperfusion injury (173).

Notably, neutrophils are positioned in distinct areas of the atherosclerotic plaques (266). The distribution pattern of neutrophils in the atherosclerotic plaque suggests recruitment routes via the arterial endothelium as well as via *neo*vessels in advanced lesions. Intravital microscopy in mice showed that, in early stages of atherosclerosis, neutrophils are recruited in a transarterial-fashion (9, 256). Whereas, in humans in later stages, it was suggested that formation of *neo*angiogenesis and adventitial vessel takes place, leading to a new and preferred neutrophil entry route (267).

FUTURE PERSPECTIVES

Neutrophil recruitment is a hallmark in all acute and chronic inflammatory disorders and hence appears as a process that is worth targeting to alleviate symptoms and disease progression. Interference with leukocyte accumulation in inflammatory conditions has previously focused on targeting of cell adhesion molecules, integrins, and chemokines. However, clinical studies have been largely unsuccessful and thus far the only approved interventions are the blockade of very late antigen-4 (VLA-4) and lymphocyte Peyer's patch adhesion molecule 1 (LPAM-1) with the monoclonal antibodies natalizumab or vedolizumab for treatment of multiple sclerosis and inflammatory bowel disease (ulcerative colitis and Crohn disease), respectively. Possible reasons for failures of clinical studies are manifold. The redundancy of adhesion molecules is well documented, and so is the apparent indiscrimination between a number of chemokines and their shared receptors. These facts increase the likelihood for rendering interference with just one molecule insufficient, as well as prominent off-target effects due to cross-reactivity with receptors of similar structure. In addition, stimulus-dependent effects have to be taken into consideration as well as the importance of the targeted molecule in host defense. And finally, of relevance

when taking therapeutic strategies into the clinic, is to never avert the discrepancy between animal models and human diseases.

Thus, a refined understanding of how neutrophils enter different tissues may set the basis for tailored intervention in the future.

AUTHOR CONTRIBUTIONS

SM wrote the manuscript. OS and JV made critical corrections.

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REVIEW
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Neutrophil Mechanosignaling Promotes Integrin Engagement With Endothelial Cells and Motility Within Inflamed Vessels

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Neutrophils are the most motile of mammalian cells, a feature that enables them to protect the host against the rapid spread of pathogens from tissue into the circulatory system. A critical process is the recruitment of neutrophils to inflamed endothelium within post-capillary venules. This occurs through cooperation between at least four families of adhesion molecules and G-protein coupled signaling receptors. These adhesion molecules convert the drag force induced by blood flow acting on the cell surface into bond tension that resists detachment. A common feature of selectin-glycoprotein tethering and integrin-ICAM bond formation is the mechanics by which force acting on these specific receptor-ligand pairs influences their longevity, strength, and topographic organization on the plasma membrane. Another distinctly mechanical aspect of neutrophil guidance is the capacity of adhesive bonds to convert external mechanical force into internal biochemical signals through the transmission of force from the outside-in at focal sites of adhesive traction on inflamed endothelium. Within this region of the plasma membrane, we denote the inflammatory synapse, Ca^{2+} release, and intracellular signaling provide directional cues that guide actin assembly and myosin driven motive force. This review provides an overview of how bond formation and outside-in signaling controls neutrophil recruitment and migration relative to the hydrodynamic shear force of blood flow.

Keywords: neutrophil recruitment, mechanosignaling, selectin, integrin, outside-in signaling

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LEUKOCYTE RECRUITMENT CASCADE AT SITES OF INFLAMMATION

Leukocyte recruitment is an evolutionarily conserved process in which the target of natural selection is a fast and efficient immune system that transports neutrophils in numbers appropriate for host defense. A multi-step cascade of adhesive events, which includes ligation and signaling through selectins, integrins, and chemokine receptors, guide neutrophil recruitment to inflamed endothelium (**Figure 1A**). Adhesive engagement between the neutrophil and the endothelium is initiated by selectins that recognize sialylated and fucosylated carbohydrate ligands expressed on adjacent plasma membranes. E-selectin (CD62E) and P-selectin (CD62P) receptors, upregulated on inflamed endothelium, and L-selectin (CD62L), constitutively expressed on the leukocyte, are strategically positioned on the plasma membrane to form bonds that initiate cell tethering and

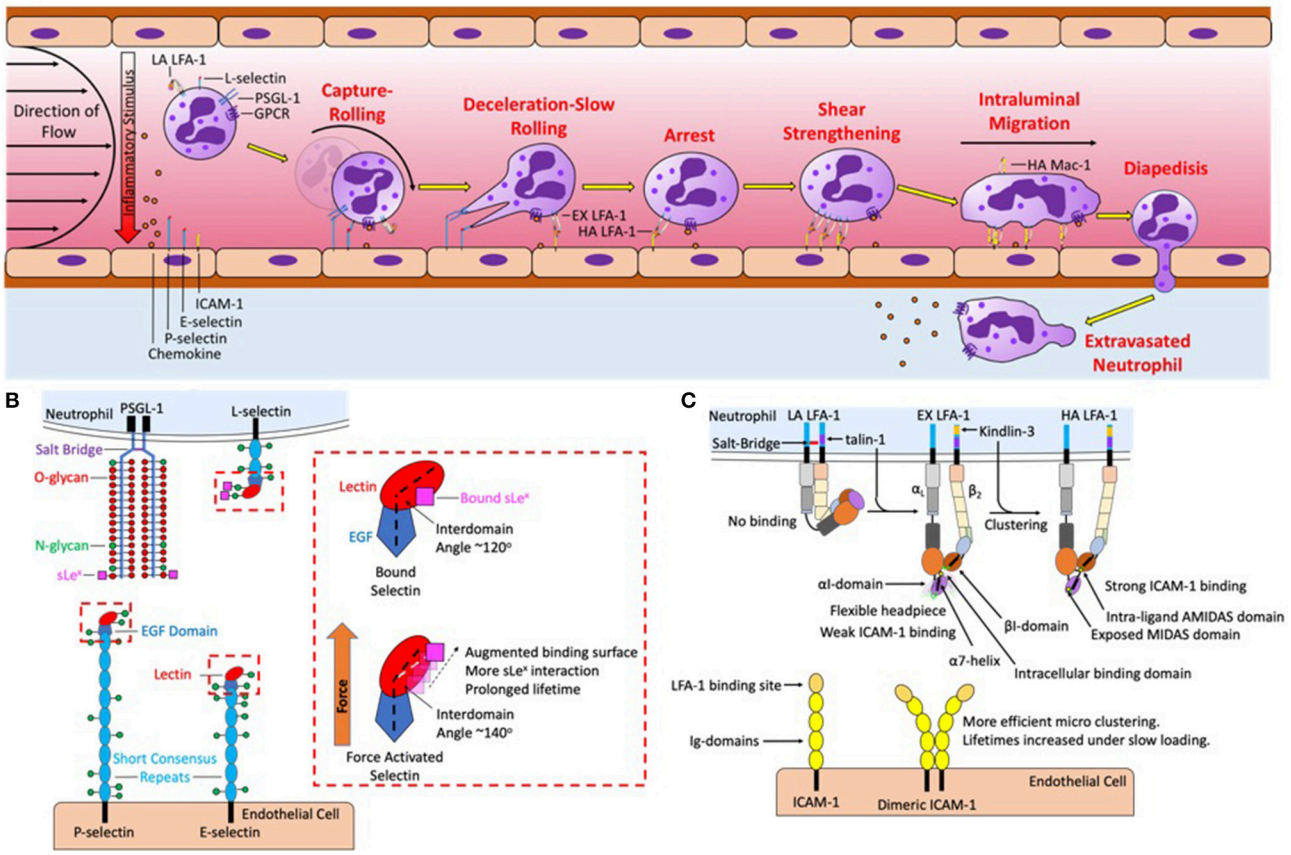

FIGURE 1 | Neutrophil recruitment under shear flow is coordinated by selectins, chemokines, and integrin ligand binding and signaling. **(A)** The sequential steps of neutrophil recruitment under shear flow is initiated by hydrodynamic forces that bring L-selectin and PSGL-1 on the neutrophil surface in contact with P-selectin and E-selectin on the endothelial plasma membrane, mediating capture, and rolling. To resist shear flow selectins and PSGL-1 form transient yet strong bonds that form at the leading-edge to initiate cell rolling and break at the trailing edge under tensile force. Chemokine captured via glycosaminoglycans on the endothelial surface and force acting on L-selectin then activates LFA-1 into an extended conformation to initiate deceleration and slow rolling. A second signal is initiated by L-selectin and CXCR that upshifts integrin into a high affinity state thereby initiating arrest. Initially integrin LFA-1 is spread randomly throughout the surface of the neutrophil, force acting on high affinity LFA-1 initiates their redistribution that reinforces shear resistant cell arrest. Clusters of LFA-1 transduce calcium signaling and neutrophil shape change, leading to high affinity Mac-1 mediated intraluminal crawling along a gradient of chemokine on the endothelial surface, directing the neutrophil to the desired site of migration. Endothelial diapedesis promotes neutrophil access the site of injury or tissue infarct. **(B)** Mechanics of PSGL-1 and L-selectin bond formation play a key role in force transduced signaling. PSGL-1 is primarily decorated with O-glycans while L-selectin is primarily decorated with N-glycans, sLe^x deposited on these glycans binds to the lectin domain of P-selectin and E-selectin. Under tensile bond force catch-bonds are formed by a change in intracellular angle of selectin EGF-Lectin domain in all three selectins. Selectin catch-bonds are necessary to mechanosignal activation of LFA-1 under shear conditions in the absence of chemokine. The red box details one potential mechanism in which the selectin interdomain angle between the lectin and EGF domain shifts in response to force allowing for prolonged bond lifetimes. **(C)** LFA-1 is expressed predominantly on membrane microvilli in a low affinity conformation, stabilized by a salt-bridge that clasps together the intracellular α_L and β_2 tails. Signaling through selectin and chemokine initiates talin-1 recruitment to the proximal NPxF motif on the β_2-integrin that breaks the salt-bridge and extends the integrin, revealing the ICAM-1 binding site in the headpiece. However, the headpiece remains flexible and the MIDAS binding domain remains obscured, supporting weak ICAM-1 bonds. Kindlin-3 binding to the distal NPxF motif promotes transition to a high affinity state and stabilizes microclustering of talin-1 and ICAM-1 bound LFA-1 monomers. Tensile bond force acting on high affinity LFA-1 is transduced intracellularly through a shift in the α_7 helix of the αI domain. This in turn induces an allosteric shift that exposes the MIDAS domain allowing for recognition of ICAM-1 and stable bond formation. The binding between ICAM-1 and high affinity LFA-1 at low force regimes is amplified by tandem bond formation with dimeric ICAM-1.

rolling under the hydrodynamic shear exerted by flowing blood (**Figure 1B**). Fluid drag forces are resisted by selectin bond tension, which rapidly induces receptor redistribution and formation of focal clusters within the site of adhesive contact between adjacent plasma membranes. For instance, E-selectin and P-selectin binding to sialyl-Lewis^x (sLe^x) on PSGL-1, or E-selectin recognition of sLe^x on L-selectin (only on human neutrophils), promotes neutrophil tethering and rolling that induces subsequent selectin interactions such as with glycolipids. Cell rolling allows interrogation of the vascular surface, and at optimum site density between selectins and their ligands a second event occurs that involves intracellular signaling, a process necessary to activate β_2-integrins. In the absence of high affinity activation of β_2-integrins, shear resistant adhesion

or cell arrest is not observed; a requisite step to initiate neutrophil spreading, polarization, and transendothelial migration (1–4). The importance of β_2-integrin expression and activation in innate immune function is evident in patients suffering from leukocyte adhesion deficiency 1 (LAD-I), where CD18 expression on the cell surface is lost or reduced, resulting in chronic infections, impaired wound healing, and a defect in neutrophil recruitment (5–7).

The first β_2-integrin discovered, LFA-1 (also known as CD11a/CD18 or $\alpha_L\beta_2$), is a key integrin involved in early signaling. LFA-1 converts drag forces of flowing blood into bond tension that transduces intracellular chemical signals. Once bound, high affinity LFA-1 not only acts as an adhesive anchor but also functions as a mechanosensitive receptor capable of transducing external force into internal chemical signals (8–11). The conversion of mechanical force to chemical signals at the site of contact (e.g., inflammatory synapse) can be considered a mechanism of tactile sensing through selectin and integrin bond force transduction that determines through molecular recognition where and when neutrophil emigration occurs (**Figure 1C**). A cooperative mechanism underlies early activation of LFA-1 that is initiated by selectin mediated capture and rolling, which facilitates G-protein coupled receptor (GPCR) binding of chemokines presented on the glycocalyx of inflamed endothelium. Rolling on E-selectin initiates the rapid extension of LFA-1 that effects deceleration of neutrophils through interaction with its endothelial ligand intracellular adhesion molecule 1 (ICAM-1) (9, 12–14). Chemokine binding of CXCR1 and CXCR2 is sufficient to initiate so called inside-out activation of β_2-integrins that corresponds with a shift of LFA-1 to a high affinity conformation and promotion of tight bond formation with ICAM-1. Superposition of selectin ligand outside-in signaling during rolling via E-selectin effectively amplifies GPCR inside-out signaling, such that very low levels of chemokine engagement become stimulatory at concentrations that independently do not elicit measurable calcium flux or β_2-integrin activation (7, 14–16). For example, stimulating neutrophils rolling on E-selectin under shear at a concentration of 0.05 nM IL-8, corresponding to ligation of ∼10–100 CXCR receptors per cell, activates a similar level of Ca^{2+} release and up-shift of β_2-integrin receptors to high affinity as does stimulation of cell suspensions with 5 nM IL-8 under static or very low shear conditions (9, 15). Thus, combined selectin ligand outside-in signaling via E-selectin recognition of sLe^x on L-selectin and LFA-1/ICAM-1 bonds effectively amplify signaling via CXCR1/2 by ∼100-fold and induces inside-out activation of β_2-integrin. Ligation of CXCR1 and CXCR2 also activates Mac-1 (also known as CD11b/CD18 or $\alpha_M\beta_2$) on the plasma membrane (17, 18). While LFA-1 binds the ICAM family of proteins and regulates adhesive events within seconds of cell capture and rolling, Mac-1 recognizes a wide variety of ligands including complement iC3b, fibrinogen, and fibronectin, which facilitates intravascular crawling during paracellular and transcellular migration across inflamed endothelium (19, 20). Force acting on high affinity LFA-1 induces intracellular protein assembly that provides a physical linkage between calcium release-activated channels and calcium stores associated with the endoplasmic reticulum (ER), (21–24). We propose that local regulation of intracellular calcium serves as a secondary messenger downstream of GPCR signaling to regulate neutrophil shape change during the transition from rolling to arrest. While GPCR triggers PLC-β activation of IP3 to elicit calcium release from ER stores on the order of 500 nM of Ca^{2+}, to achieve the maximum burst in cytosolic Ca^{2+} flux (∼1.0 μM) requires integrin activation, ligation to ICAM-1, and force transduced outside-in signaling (16, 25). In this manner, intracellular Ca^{2+} release functions as a gatekeeper in regulating the conversion of a passive neutrophil in circulation to one that is firmly arrested and poised to transmigrate at sites of vascular inflammation expressing appropriate levels of chemokine agonist, E-selectin, and ICAM-1.

MECHANOSIGNALING VIA SELECTINS PROMOTES LFA-1 ACTIVATION

Selectin and integrin receptors are by nature's design mechanically tuned to function as de facto tactile sensors that convert the drag forces of flowing blood to tensile bond force that transduces biochemical signals at sites of focal adhesion. Mechanosignaling superposes with chemokine signaling to provide for precise spatiotemporal regulation of neutrophil recruitment at vascular sites proximal to tissue insult and injury. Inactive LFA-1 exists in a compact bent conformation with close association of the α and β extracellular domains that maintain a low binding affinity for ICAM-1 (**Figure 1C**). Rolling on E-selectin and P-selectin in the absence of chemokine induces extension of LFA-1 into an intermediate affinity conformation that supports slow neutrophil rolling at velocities of ∼5 μm/s. In the extended conformation the extracellular domain of LFA-1 swings outward, but remains in a relatively closed state, such that bulkier ligands are sterically hindered from accessing the metal ion-dependent adhesion site (MIDAS) domain associated with high affinity binding (**Figure 1C**) (12, 26–28). This physiological mechanism for neutrophil deceleration is observed in a parallel plate flow chamber whereby the dynamics of LFA-1 extension is reported by increased binding of antibody extension reporter, KIM127 (29, 30). Inside-out stimulation elicits the release of a salt bridge between the α and β chains in the intracellular component of the integrin that triggers opening of the extracellular domain in a switchblade-like motion thereby exposing the MIDAS ligand binding domain (**Figure 1C**) (28). Increased binding affinity corresponds to a decrease in the k_{off}, the rate constant for dissociation of the complex, which coincides with formation of LFA-1 bond clusters with ICAM-1 that supports shear resistant cell arrest under flow conditions (12, 31, 32). Blocking high affinity LFA-1 using a small molecule allosteric agonist that prevents the MIDAS domain from opening elicits suppression of cell arrest, while slow rolling via the extended conformation is maintained (12, 33). The increase in association rate and maintenance of slow rolling under conditions whereby the high affinity state is blocked implicates

LFA-1 extension as the braking mechanism that neutrophils utilize to initiate the transition to firm arrest. Data supports the contention that intermediate-affinity of LFA-1 can participate in neutrophil capture and rolling; however, further up-regulation to high affinity by other activation mechanisms, including selectins, chemokines, and inflammatory lipids, is critical for the efficient transition to arrest and prompting of transendothelial migration. In particular, selectin ligand outside-in signaling functions to activate β_2-integrins, elicit Ca^{2+} flux, and promote F-actin formation all of which promote cell polarization and a migratory phenotype (4, 13). In particular, L-selectin and P-selectin glycoprotein ligand 1 (PSGL1) function as mechanosensitive receptors that trigger LFA-1 extension and transition to integrin mediated activation processes.

Neutrophil rolling in humans is primarily mediated by L-selectin and PSGL-1 on the neutrophil surface and P-selectin and E-selectin on inflamed endothelium (34, 35). The minimum recognition unit of selectins is the tetrasaccharide sialyl Lewis^x (sLe^x), a sialic acid α2-3 linked to galactose anchored by a β1-4 linked N-acetylglucosamine bearing a α1-3 linked fucose (36). sLe^x is expressed on glycosphingolipids (GSL), O-glycans of PSGL-1, and N-glycans of L-selectin (**Figure 1B**) (35, 37). In human's enzyme fucosyltransferases 4, 7, and 9, as well as sialyltransferase ST3-Gal-IV, are required to assemble sLe^x on N- and O-linked glycans, and GSL (38–40). Recent studies employed CRISPR-Cas9 gene editing to truncate each of the commonly expressing glycan types, reveal that O-glycans are responsible for leukocyte capture and initiation of rolling while N-glycans and GSL stabilize slow cell rolling and the transition to arrest (41, 42). Extracellular PSGL-1 is decorated in serine and threonine residues that are glycosylated to primarily bear fucosylated O-glycans capped with sLe^x that are capable of binding the calcium ion present within the lectin domain of all three selectins (**Figure 1B**) (43). Human L-selectin is decorated with N-glycans capped with sLe^x, which enable recognition by E-selectin (44, 45). This data implicates PSGL-1 as a ligand associated with selectin mediated capture and slow rolling, while L-selectin functions as a mechanosignaling ligand of E-selectin on inflamed endothelium. However, a number of studies using transgenic mice deficient in selectins or their ligands indicate that rolling via PSGL-1 is sufficient to mechanosignal integrin activation. One key difference is that fucosyltransferase 9 plays a key role in human, but not mouse neutrophil/E-selectin interactions (39, 40). This indicates a major difference in E-selectin ligands and their potential to signal, specifically that L-selectin in mice is not an E-selectin binding partner. Interestingly, while L-selectin is not an E-selectin binding partner in mouse, both E-selectin ligand-1 and CD44 play critical roles during murine neutrophil rolling and transition to arrest (46). Studies in mice genetic knockouts indicate that ESL-1 cooperates with PSGL-1 to maintain myeloid homeostasis and initiate neutrophil recruitment, but it is PSGL-1 that does the heavy lifting when it comes to integrin activation and slow rolling. In fact, ESL-1 is the predominant E-selectin ligand used by immature hematopoietic progenitors to home to the bone marrow. As myeloid maturation occurs a functional shift in selectin ligands from ESL-1 to PSGL-1 reduces the importance of ESL-1 in selectin signaling (47). Despite L-selectin not being a functional E-selectin ligand, inhibition of L-selectin binding in mice inhibits rolling, which was largely attributed to a loss of neutrophil-neutrophil mediated secondary capture that is L-selectin/PSGL-1 dependent (48). Further highlighting the function of L-selectin in selectin ligand outside-in signaling in mouse neutrophils, Stadtmann et al. reported that PSGL-1 ligation under shear flow precipitates membrane co-localization of PSGL-1 and L-selectin, which in turn elicits outside-in signaling of LFA-1 activation (49). One important component of L-selectin signaling is the multiple intracellular binding sites on the cytosolic domain of L-selectin for binding to tyrosine kinases and other downstream activators such as PLCγ2 (**Figure 2A**). The colocalization between PSGL-1 and L-selectin in mouse is induced by CD44 on the cell body engaging with E-selectin, which promotes clustering of PSGL-1 and L-selectin on the neutrophil surface in a p38 dependent manner. This was shown to promote secondary leukocyte tethering and formation of the L-selectin/PSGL-1 signaling complex (46). Murine neutrophils with down regulated L-selectin expression do not form this complex and therefore cannot signal for extension of integrin in the absence of chemokine (49). Thus, even in the absence of direct recognition of L-selectin by E-selectin on mouse neutrophils, L-selectin represents a potent selectin ligand outside-in signaling receptor.

Selectin engagement and mechanosignaling in neutrophils is only partially defined, and while other E-selectin ligands may play a role in capture, tethering, and signaling, we will focus on mechanosignaling through L-selectin engagement. E-selectin preferentially recognizes sLe^x on L-selectin and PSGL-1 in humans and signals active transport of adhesion molecules, leading to rapid cell arrest in shear flow (45). Binding of E-selectin promotes colocalization of L-selectin and PSGL-1 into membrane clusters on microvilli, an event that temporally correlates with MAPK phosphorylation and focal clustering of high-affinity CD18 (50). Genetic deletion of L-selectin in mice results in loss of phosphorylation of Akt, Syk, and phospholipase C (PLC) γ2, indicating these signaling molecules are downstream of the PSGL-1/L-selectin signaling complex (**Figure 2A**) (49). PSGL-1 engagement during rolling in inflamed mouse vasculature involves receptor clustering within lipid rafts that recruits membrane FcRγ and cytosolic DAP-12 (**Figure 2A**). Subsequent phosphorylation by Fgr then recruits Syk (51). The signaling pathway downstream of Syk includes SLP-76 and Bruton tyrosine kinase (Btk), which bifurcates the activation of PLCγ2 and phosphoinositide-3 kinase (PI3K) gamma-dependent pathways (51–53). Ligation of L-selectin and PSGL-1 enhances activation of PI3K, which acts on the effector Vav1 and results in F-actin assembly and downstream L-selectin reorganization and clustering, facilitating a feedback loop that amplifies signaling via L-selectin (54). It is known that PI3K activation results in cytoskeletal changes following leukocyte rolling, however its direct role in mechanosignaling via L-selectin outside of inducing clustering is unknown. A second pathway involving PLCγ2 activates downstream CalDAG-GEFI and p38 MAPK resulting in Rap1a activation (55). Rap1 then facilitates integrin extension and activation by recruiting Talin-1, and potentially Kindlin-3, which in turn allosterically reorients the integrin cytoplasmic

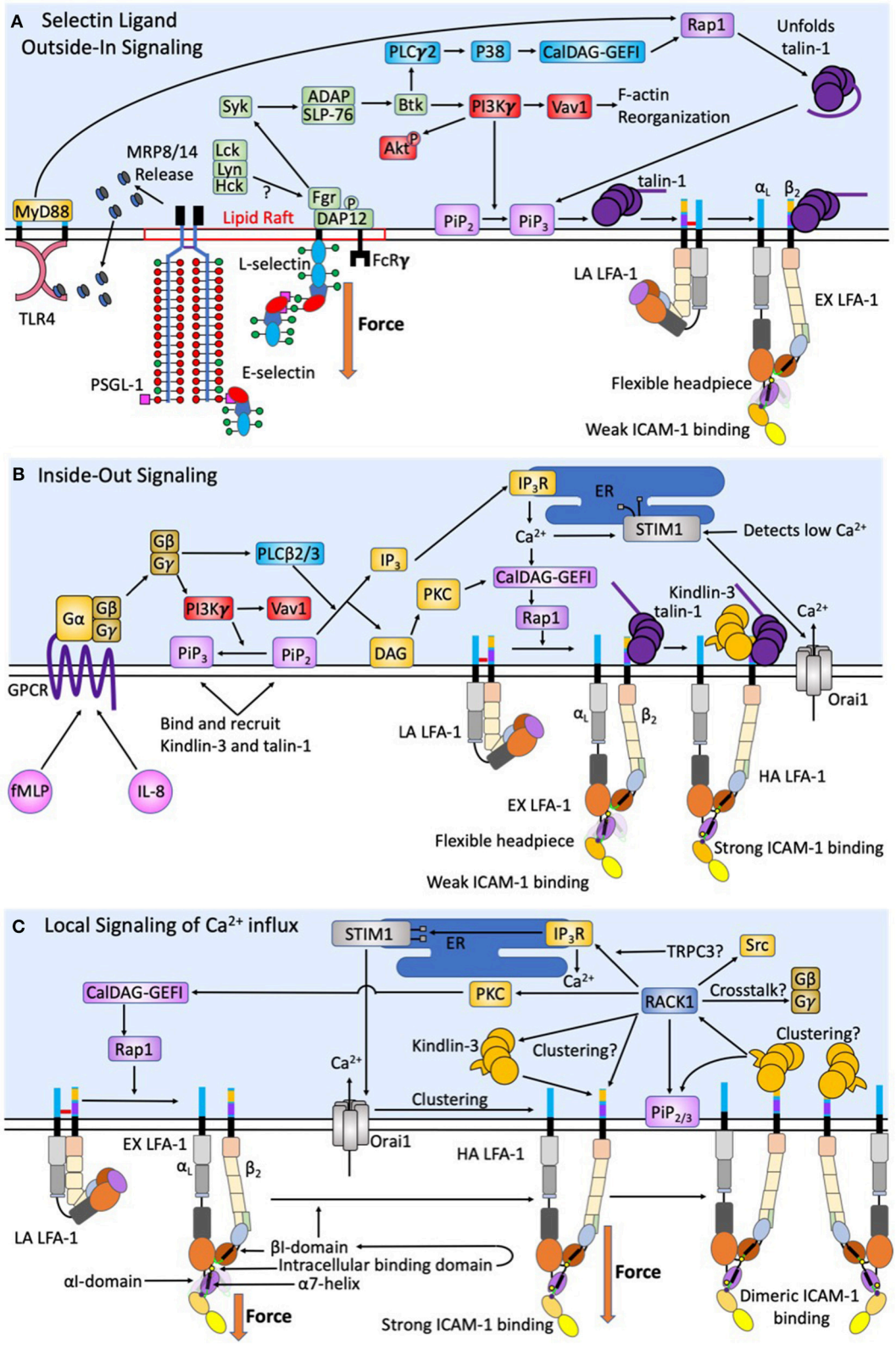

FIGURE 2 | Intracellular signaling events act synergistically to promote human neutrophil arrest and shape change. During initial capture and rolling on inflamed endothelium a low baseline level of intracellular calcium is maintained. **(A)** Force acting on L-selectin and PSGL-1 induces clustering and recruitment of FcRγ and

(Continued)

FIGURE 2 | DAP-12 into lipid rafts. Phosphorylation by Fgr results in Syk activation. Other Src family kinases have also been shown to enhance selectin signaling, however only Fgr binds L-selectin cytodomain. Syk activation of SLP-76 and ADAP results in Btk activation, where signaling becomes PI3Kγ dependent. This catalyzes Vav1 activation and downstream F-actin reorganization that plays a key role in L-selectin clustering. The PLCγ2 activation of p38 and CalDAG-GEFI activate Rap1 and the unfolding of autoinhibited talin-1, which promotes recruitment to $PiP_{3/2}$ and engagement and extension of LFA-1. Force acting on L-selectin catch-bonds transduces the signaling of high affinity LFA-1. Whereas, engagement of PSGL-1 and L-selectin primes MRP8/14 release. Its binding to TLR4 elicits the extension of LFA-1 and supports deceleration and cell rolling. **(B)** Selectin signaling is synergistic with chemokine signaling via GPCR to induce complete activation of integrin. CXCR1/2 ligation by fMLP and IL-8 elicit the dissociation of Gα from Gβγ subunits of G proteins resulting in PI3Kγ activation and PLCβ2/3, a convergence point between CXCR ligation and selectin signaling pathways. F-actin reorganization induced by PI3Kγ also results in transition of PiP_2 to PiP_3, which has a higher binding efficiency for Kindlin-3 and talin-1. DAG then activates PKC. Additionally, PLCβ2/3 splits PiP_2 into IP_3 and DAG. IP_3 then binds IP_3R on the ER to activate calciosome release. The gradient of intracellular calcium and activation of PKC catalyze activation of CalDAG-GEFI and Rap1 mediated integrin activation. This is a second convergence point between CXCR ligation and selectin signaling to activate LFA-1 through talin-1 recruitment. Calcium influx via Orai1 CRAC channels at focal sites of adhesion elicits the release of ER Ca^{2+} stores, which precipitates STIM1 association with the ER. This synergy between CRAC and the ER at the inflammatory synapse represents a positive feedback loop to enhance local calcium entry and the activation of additional LFA-1. **(C)** Tensile force acting on LFA-1/ICAM-1 provides for mechanotransduction of local calcium entry through Orai1 CRAC. Force acting on high affinity LFA-1 transduces from outside-in Kindlin-3 engagement. A conformational shift in the high affinity LFA-1 β_2-tail exposes the Kindlin-3 binding domain. RACK-1 and Kindlin-3 both localize to the plasma membrane through its engagement with $PiP_{2/3}$ to promote clustering of high affinity LFA-1. RACK1 may be a physical link between Kindlin-3, clusters of high affinity LFA-1, and TRPC3/IP_3R/STIM1/Orai1; thereby completing a circuit to transduce force mediated calcium entry. RACK1 can activate PKC and CalDAG-GEFI and Rap1 providing a means of crosstalk between integrin outside-in and GPCR inside-out signaling.

domain (56). Talin-1 is an adaptor molecule that binds to the β_2-integrin cytodomain to initiate extension and the high-affinity state, as well as providing a cytoskeletal anchor for vinculin (12, 56, 57). Integrin activation and clustering via talin-1 facilitates its binding to dimerized ICAM-1, this effectively prolongs the lifetime of LFA-1 bonds by ∼10-fold and potentially enhances the association of adaptor molecule recruitment (27). Kindlin-3 is a similar adaptor protein to talin-1 that plays a central role in LFA-1 clustering and is critical for regulation of integrin off-rates (12, 58, 59).

It is well-established that mechanosignaling through L-selectin induces extension of β_2-integrin, while the mechanism for activation of high-affinity β_2-integrin independent of chemokine mediated inside-out signaling remains a point of contention. A recent discovery highlights a central role for MRP8/14 (also known as S100A8/S100A9 or calprotectin) as a signaling pathway that elicits extension of β_2-integrin but not necessarily activation of the high affinity state (60). MRP8/14 is secreted by neutrophils during rolling on E-selectin where it then binds toll-like receptor 4 (TLR4) and signals in an autocrine manner activation of Rap1 and LFA-1 extension (13, 60). Ligation of PSGL-1 was sufficient to elicit release of MRP8/14 and induce extension of β_2-integrin in a TLR4 dependent manner, whereas clustered L-selectin delivers a distinct signal that activates high affinity β_2-integrin (13). Thus, bond tension and clustering of L-selectin cooperates with MRP8/14/TLR4 in priming LFA-1 extension, but a distinct signal is necessary for LFA-1 to achieve a high affinity state. Shear-resistant arrest is mediated in human neutrophils through clustered E-selectin/L-selectin bonds via signaling of high-affinity β_2-integrin. The signaling pathway underlying MRP8/14 and TLR4 activation of Rap1 may involve PLCγ2, but this remains ill defined. Additionally, TLR4 has been shown to drive neutrophil aging which brings up a key point in neutrophil signaling, specifically dependence of senility in altering neutrophil receptor expression (61). Aged neutrophils show an enhanced expression of LFA-1, Mac-1, and CD44 and a decreased expression of L-selectin, however whether these receptor expression changes can be induced by MRP8/14 ligation of TLR4 remains unknown (62–64). Enhanced integrin expression translates to a more efficient shear-resistant recruitment of neutrophils under LPS challenge and implicates aged neutrophils as the first line of defense (65).

SELECTIN-SLE^X BOND MECHANICS

Hydrodynamic drag force acting on flowing neutrophils induces catch bond formation between E-selectin and L-selectin. The mechanics of selectin-sLe^x bonds and how they transduce outside-in signals via Src and Lck kinases are active areas of investigation (53, 66). A logical question is how catch-bond behavior of selectins dictates their capacity to engage in selectin ligand outside-in signaling. Neutrophil selectin binding kinetics have been studied using a variety of techniques including, atomic force microscopy, bioforce probes, surface plasmon resonance, and parallel plate flow channels (3, 67). Selectin bonds exhibit a triphasic adhesive response that is denoted slip-catch-slip bonds (68, 69). Slip bonds exhibit a shortened lifetime as the tensile force acting on them is increased. Catch bonds on the other hand increase lifetime as applied tensile force is increased. Neutrophils bound via PSGL-1 by P-selectin at low wall shear stress (<0.3 dynes/cm^2) capture sporadically, dissociate frequently, and roll with high velocity (70). In contrast, higher shear stress (between 0.3 and 1 dynes/cm^2) promotes more efficient capture and steady rolling at constant velocity, indicative of selectin catch bond behavior (69, 71–73). As wall shear stresses exceeds 1 dynes/cm^2, slip bond behavior is again observed. An experiment that begins to explain the mechanism of catch-bond behavior was application of Rivipansel to antagonize L-selectin/E-selectin bonds, along with selectin ligand outside-in mechanosignaling of high affinity LFA-1 (13). Rivipansel is a rationally designed pan-selectin inhibitor that mimics the sLe^x tetrasacharide structure and an extended sulfate domain recognized by all three selectins lectin domain and in clinical trials for treatment of vaso-occlusive crisis in Sickle Cell disease (13, 74, 75). We recently reported that treatment with Rivipansel, blocks catch-bond formation between E-selectin and L-selectin and effectively inhibits selectin ligand outside-in signaling of integrin activation

and neutrophil rolling to arrest (13). A structural explanation that begins to shed light on selectin catch-bond behavior was derived from co-crystallization between E-selectin and sLe^x that was imaged by small-angle X-ray scattering and modeled with molecular dynamics simulation. This analysis predicted that E-selectin bound to sLe^x under force caused an opening in the angle between the lectin and EGF domains from ∼120 to ∼141° (**Figure 1B**) (76). This rotation facilitates more efficient engagement of sLe^x expressing ligands by E-selectin resulting in an increase in bond strength and resistance to shear flow, indicative of a catch bond. Molecular dynamics simulations of the lectin-EGF domain angle between P-selectin bound with sLe^x is predicted to be ∼114.6°, similar to the E-selectin/sLe^x angle. When tensile force is applied to this complex the interdomain angle opens to an extended conformation of ∼140° allowing it to align in the direction of force application resulting in an enhanced off rate (43, 77). This catch-bond strengthening phenomenon has not been reported for L-selectin bound to sLe^x, but it is highly likely given the homology in lectin structure between the three selectins. A second mechanism that accounts for catch-bond behavior is when force induced dissociation of ligand results in rebinding by the lectin headpiece as the angle between the lectin-EGF domain increases (**Figure 1B**) (77). Bond lifetime is prolonged through multiple dissociations required for complete ligand detachment. Another model, known as the allosteric model, involves reorientation of the crystal structure of P-selectin when ligand binding elicits a shift in residues 83–89 in the lectin domain, thereby augmenting the binding surface and increasing the affinity between the lectin domain and sLe^x (78). Swapping the alanine 28 in the lectin domain with a bulky histidine to effectively open the 83–89 loop, resulted in decreased dissociation constant and slower rolling velocity on a substrate of PSGL-1 (78). It is noteworthy that the slower rolling was not observed at low shear stress regimes, corroborating the slip to catch transition induced at the higher shear stress. Taken together, modeling, and experimentation have shown that tensile force of sufficient magnitude and applied at defined rate can elicit an allosteric shift in selectins that in turn influence the strength and lifetime during bond formation with sLe^x presenting ligands. LFA-1 bonds also convert from a low or intermediate affinity state to a high affinity state during rolling at low shear stresses within the catch-bond regime between 0.5 and 2 dyne/cm^2. This was first demonstrated by neutrophil capture on a substrate co-expressing E-selectin-IgG and high affinity β_2-integrin reporter mAb24 (13). However, a different study reported that the majority of neutrophils sheared in microfluidic channels at 6 dynes/cm^2, outside the catch-bond regime, did not activate and arrest on a substrate presenting mAb24, but bound efficiently on a substrate presenting the extension reporting antibody KIM127 (30). Taken together the data indicate that, while catch-bond formation via E-selectin/L-selectin engagement provides a distinct signal to activate high affinity LFA-1 and neutrophil arrest. This elucidates the importance of hydrodynamics in selectin-sLe^x catch-bond mechanics to provide a force sensitive mechanism for signaling optimum recruitment at appropriate shear stress.

SIGNALING BETWEEN SELECTINS AND GPCR ACTIVATION CONVERGES TO ACTIVATE LFA-1

So far, we have only focused on integrin activation via selectins, it is important to note that GPCR inside-out signaling is capable of transitioning LFA-1 from a low to high affinity state. This begs the question; how does selectin ligand outside-in signaling cooperate with inside-out signaling generated by CXCR1 and CXCR2 engagement by chemokine? While the role of GPCR signaling is well-reviewed elsewhere (79, 80), here we focus on how signaling facilitates LFA-1 mediated neutrophil arrest and a migratory phenotype (**Figure 2B**). Chemokine stimulation of GPCR in neutrophils results in stimulation of G proteins, separating Gα from the Gβγ subunits triggering PLC-β and PI3Kγ activation. PLC-β cleaves phosphatidylinositol 4,5 biphosphate (PIP_2) into diacylglycerol (DAG) and inositol-1,4,5 triphosphate (IP_3) (18, 81). IP_3 then binds IP_3 receptor (IP_3R) localized on the membrane of the endoplasmic reticulum (ER) triggering cytosolic release of calcium (82, 83). The release of calcium within the ER is sensed by stromal interaction molecule 1 (STIM1), which functions to localize the ER to the primary calcium release activated calcium (CRAC) channel, Orai1 on the neutrophil membrane (**Figure 2B**) (83). Orai1 forms a hexamer that regulates influx of extracellular calcium (22, 84). PI3Kγ signals downstream of GPCR signaling by catalyzing PiP_2 conversion to PiP_3 and its association with the membrane near the integrin cytoplasmic domain. PiP_3 functions to recruit Kindlin-3, Skap2, and other PH domain interacting molecules that are necessary for integrin transition from extension to high affinity more efficiently than PiP_2 (14, 85, 86). PI3Kγ represents a convergence point between selectin ligand outside-in signaling and inside-out GPCR signaling pathways, and its activity cooperatively regulates activation of integrin. Each signaling pathway elicits a calcium influx, selectins through TRPC channels and chemokine through Orai1, and both pathways converge upon Rap1 dependent activation of integrin. It is widely known that high concentrations of chemokine can promote activation of LFA-1 and the onset of neutrophil deceleration and arrest. It appears that nature has designed a cooperative system by which very low levels of chemokine signaling can superpose with selectin catch-bond dependent signaling to amplify the response and likelihood of recruitment of surveilling neutrophils, perhaps most relevant in skin where E-selectin is expressed at low levels (14, 15). While there is lots of evidence supporting L-selectin and CXCR1/2 cooperativity in neutrophils, there has been no synergy observed between L-selectin and CCR7 signaling for enhancing LFA-1 activation in lymphocytes.

LFA-1 IS REQUISITE FOR NEUTROPHIL ARREST

LFA-1 and Mac-1 are both involved in the transition from arrest to a migratory phenotype. However, it appears that the sequence of adhesive events is important for the precise synchronization of transendothelial migration. LFA-1 bonds function to initiate

neutrophil arrest, while Mac-1 bonds provide migratory traction. Bond number and strength dictate the adhesive lifetime and translates to the amount of force that is transmitted across the membrane. Forces transmitted via LFA-1 and Mac-1 bonds in neutrophils are in part a function of their respective ligands, ICAM-1 and ICAM-2 are the main ligands on inflamed endothelium bound by activated LFA-1, while Mac-1 primarily binds to RAGE and JAM-C (87). It is noteworthy that LFA-1 can also bind to JAM-A and JAM-C, while Mac-1 recognizes the Ig-domain 3 of ICAM-1, albeit with lesser bond strength (19, 80, 88). Direct measurements of adhesion efficiency and rupture force for Mac-1 and LFA-1 bonds locked into a high or low affinity state were performed using atomic force microscopy (AFM) targeting the slip bond regime (87). An AFM tip was functionalized with LFA-1 or Mac-1 locked into specific states via allosteric antibodies or activated via manganese. Bond formation was induced by bringing this tip into contact with a surface of counter ligands ICAM-1, ICAM-2, RAGE, JAM-A, or JAM-C and then retracted at various rates. Deflection of the cantilever, as measured by a deflection of a laser beam reflected off the back of the cantilever, brought to light distinct features of bond rupture force and lifetime. The differences between mean rupture force of high affinity and low affinity LFA-1 was most pronounced when bound to ICAM-1 (56.1 ± 4.1 pN), ICAM-2 (37.7 ± 2.0 pN), JAM-A (37.4 ± 4.3 pN), and JAM-C (34.0 ± 5.9 pN) (87). The difference between high affinity and low affinity Mac-1 was most pronounced when bound to JAM-C (32.0 ± 2.8 pN) and RAGE (25.2 ± 4.2 pN) (87). When activated to high affinity, LFA-1/ICAM-1 and Mac-1/JAM-C bonds show the greatest strength. This correlates well with the observed function in the adhesion cascade of each integrin subunit, specifically LFA-1 mediates shear resistant cell arrest, while Mac-1 functions primarily in cell migration. It is important to note that the differential spatial localization of LFA-1 (on microvilli) and Mac-1 (on microvilli and cell body) on the neutrophil surface may result in each bond experiencing a distinct force regime during arrest in shear flow. This in turn can influence catch-bond behavior and result in different functional roles for each subunit during ligand binding.

Given that the magnitude of shear stress dictates the efficiency and lifetime of adhesion (89), it is critical to review how molecular mechanics are regulated and activated compared to quiescent neutrophils. Evans et al., was the first to measure off-rates and bond lifetimes between LFA-1 and ICAM-1 at the single integrin scale utilizing a bioforce probe (90). Activated LFA-1 possesses persistent mechanical strength exceeding 20 pN per bonds with lifetimes on the order of ∼1 s when tensile force is applied at rates of ∼10 pN/s. When force was ramped between ∼10 and 1,000 pN/s it was observed that unbinding increased exponentially, indicating that LFA-1 bond lifetime is highly sensitive to force application. When locked in a high affinity state in Mn^{2+} enriched buffer, LFA-1 lifetime decreased to ∼3 ms at a bond strength of ∼64 pN (90). When these curves were extrapolated to zero force, bond lifetime increased to ∼2 min corresponding to an off rate of ∼0.05/s. As force ramps were increased to very high levels exceeding 7,000 pN/s, the force sensitivity of the off rates between LFA-1 and ICAM-1 disappeared, suggesting that large forces induce a change in molecular configuration of the complex (90). Locking recombinant LFA-1 at high affinity with Mn^{2+}, or for native LFA-1 on neutrophils with allosteric activating antibody 327C or stimulation with IL-8, and testing bond formation with recombinant dimeric ICAM-1 revealed nearly identical changes in off-rates as force was ramped (91). Taken together, these studies indicate that a conformational switch elicited by either inside-out or selectin ligand mediated outside-in signaling results in LFA-1 heterodimers binding in tandem with domains 1–2 of parallel ICAM-1 molecules to establish long-lived bond formation that supports neutrophil firm arrest (**Figure 2C**).

Using the bioforce probe technique to ligate single LFA-1 molecules and measure bond kinetics it was subsequently reported that LFA-1/ICAM-1 bonds experience catch-slip bond behavior (92). Three states and corresponding distinct off-rates were identified at defined force profiles: At zero force, allosteric activation of LFA-1 to a high-affinity state elicited the most efficient binding. However, when force was ramped to ∼10–15 pN on LFA-1/ICAM-1 bonds, lifetime increased to a maximum, revealing catch-bond behavior dependent upon the high affinity state (92). Beyond a threshold level of force, bond lifetimes monotonically decreased, indicative of slip bond behavior. Moreover, when LFA-1 was locked into a low affinity or extended conformation catch bonds were not detected, rather bond lifetime decreased monotonically. A structural model was proposed whereby pulling on extended LFA-1 anchored to the cytoskeleton elicits a shift in the α7 helix, thereby exposing the MIDAS domain and a shift to high affinity LFA-1 (**Figure 1C**). A structural model by which force stabilizes the high affinity conformation was proposed that involves movement of the α7 helix in the α_L-I domain linking to an intracellular loop that shifts orientation of the adjacent AMIDAS domain in the β_2-I domain (93). This was experimentally supported utilizing the antagonist XVA143 that binds internally to a site between the α_7 helix and the AMIDAS domain on the β-I domain. This effectively blocked catch-bond behavior, reduced ligand binding affinity under force, and decreased bond lifetimes. This reveals the importance of the α_7 helix and its binding to the intra-ligand AMIDAS domain in forming strong long-lasting catch-bonds (92). However, when force is applied the intra-ligand interaction is enhanced, indicating that force is necessary to precipitate the complete maturation of high affinity LFA-1. This external shift in geometry of β_2-integrin suggests that uptake of tensile force can reinforce bond strength and lifetime via a shift in the angle of the transmembrane domain. As discussed below, we propose that a shift in transmembrane domain angle with force is responsible for initiating outside-in signaling by transducing a deformation in the Kindlin-3 binding domain within the β_2-integrin cytoplasmic tail. This in turn may catalyze the association of PiPs to the integrin to enhance adaptor molecule recruitment to the site of high affinity LFA-1.

INTEGRIN LFA-1 CYTOSKELETAL ADAPTOR PROTEINS FUNCTION IN MECHANOSIGNALING

Kindlins are a family of proteins that are highly conserved and function as cytoplasmic adaptor proteins that bridge the

cytoskeleton to integrins via their FERM domains. A rare mutation in Kindlin-3 is the culprit in leukocyte adhesion deficiency type-III, a disease characterized by defects in leukocyte and platelet β_1-, β_2-, and β_3-integrin functions (6, 94–97). For LFA-1 to transition from low to intermediate and high affinity, engagement of the cytoplasmic domains by talin-1 and Kindlin-3 are necessary. These adaptor proteins are 4.1/ezrin/radixin/moesin (FERM) domain proteins with four subdomains (F0–3), whose F3 domains are capable of binding one of two specific NPxF/Y domains present on β-integrin cytoplasmic tails (98). Talin-1 also has an extended rod domain that binds actin, indicating a key role in F-actin association and local cytoskeletal rearrangement (56). NMR spectroscopy revealed that the talin-1 rod domain interacts with the F3 subdomain, masking its binding domain. This autoinhibition is disrupted by PiP engagement allowing the talin-1 F3 domain to bind the proximal NPxF/Y on β-integrin tails and break the salt bridge holding the α-β chains together, thereby initiating the transition to an extended and a high affinity state (99, 100). Kindlin-3 is endowed with a plexstrin homology (PH) domain embedded into its F2 subdomain. Kindlin-3 F3 domain binds to the membrane distal NPxF/Y motif on the β-integrin tail (56). Constitutive Kindlin-3 is not autoinhibited nor does it bind to low affinity LFA-1, indicating that its β-integrin binding domain is not exposed until integrin extension occurs. Transgenic mice in which talin-1 is genetically deleted lacks the capacity to both extend or activate high affinity integrin, while Kindlin-3 knockouts retain the capacity for LFA-1 extension, but not activation to high affinity (58). Remarkable was the finding that a 95% knockdown of Kindlin-3 in a mouse model, retained basal levels of integrin function in platelets (101). However, extended bleeding and impaired healing was observed when these mice were exposed to injury and infection. This indicates that a threshold level of Kindlin-3 and talin-1 are necessary to maintain normal function of LFA-1 (101). In fact, Kindlin-3 and talin-1 abundance is sufficient to occupy only ∼50% of integrin cytodomain in granulocytes. By comparison, platelets contain twice as much adaptor proteins, this highlights a key difference in LFA-1 activation kinetics compared with GPIIbIIIa that also requires Kindlin3 for function (101). A stoichiometric balance exists between Kindlin-3/talin-1 and integrin in neutrophils, such that diffusion may be a limiting factor in the rate of LFA-1 activation.

Loss of Kindlin-3 function in patients suffering from LAD-III is characterized by suppression of LFA-1 functions, but not VLA-4 under shear conditions in both neutrophils and primary T cells (94). Tensile force, talin-1 and Kindlin-3 are necessary conditions to observe activation of high affinity LFA-1. One potential mode of LFA-1 activation is via talin-1 recruitment to the β-subunit tail thereby catalyzing the extended conformation and recognition of ICAM-1. As fluid drag transmits tensile force to the intermediate affinity bond, molecular deformation exposes the MIDAS and precipitates a transition to the high affinity state. Given that LFA-1 extension is observed to promote the engagement of Kindlin-3 at sites of focal adhesion, it is possible that force transmission on LFA-1 itself catalyzes increased binding of Kindlin-3 (16). However, the precise mechanism by which these adaptor proteins recruit to LFA-1 is ill defined, as is whether they simultaneously reside on a single LFA-1 cytodomain. It has been suggested that Kindlin-2 and talin-1 are capable of simultaneously binding a single β_2-integrin tail, and due to the homology between Kindlin-2 and talin-1 F3 domain it is highly likely that Kindlin-3 can also bind to the integrin tail simultaneously with talin-1 (102). Adaptor protein binding occurs following phosphorylation of the tyrosine in their respective binding sites via Src family kinases. Binding is modulated by another key TTT phosphorylation site between the two binding regimes (58). Kindlins, filamin, 14-3-3 and other proteins can bind this domain and can affect the order in which binding to the other NPxF motifs occurs (103, 104). Kinases provide spatiotemporal regulation of integrin activation, but more research is required to elucidate its precise role in mechanosignaling. Given that talin-1 binding is retained in Kindlin-3 knockouts, a prevailing theory is that these adaptor proteins may serve as co-activators by removing potential competitive binding proteins such as 14-3-3 protein (103). An additional mechanism is via talin-1 induced extension to expose the binding site of Kindlin-3 on the integrin tail allowing it to then function as a mechanosensitive clutch. Experimental data indicates that talin-1 and Kindlin-3 play independent roles during signaling of neutrophil arrest and migration. Utilizing neutrophil-like HL-60 cells to knockout talin-1 or Kindlin-3, activated LFA-1 bonds under tensile force catalyzed calcium influx through the CRAC channel Orai1 only in the presence of Kindlin-3. The presence of talin-1 and absence of Kindlin-3 was insufficient to link LFA-1 to Orai1 and induce calcium influx (16). These data provide insight on mechanotransduction through LFA-1 under shear force conditions, which involves assembly of a complex via Kindlin-3-β_2-integrin cytodomain and Orai1 to complete a circuit whereby force induces calcium flux.

Kindlin-3 association with LFA-1 is necessary for the rapid clustering of LFA-1, but it is unlikely to function as a scaffold protein in this process since it has only one binding site for the β_2-integrin tail, unless Kindlin-3 is capable of complexing other Kindlin-3. Another adaptor protein that can enhance LFA-1 clustering is receptor of activated protein C kinase 1 (RACK1) (**Figure 2C**). RACK1 is a seven bladed propeller protein that can bind multiple Kindlin-3 with its domains 5 to 7. Kindlin-3 binds RACK1 through its PH domain and in cells with the PH domain deleted, LFA-1 clustering is inhibited (59). However, Kindlin-3 PH domains play a key role in binding numerous proteins such as SKAP2 or PiP_2. Thus, knockout of the PH domain may suppress Kindlin-3 migration to the LFA-1 tail domains, independent of RACK1 (80, 86). Despite this, it is noteworthy that immunoprecipitation of a ternary complex between β_2-integrin tail, RACK1, and Kindlin-3 is intact even when the Kindlin-3 F3 domain is genetically deleted (59). Given that RACK1 itself does not activate adaptor proteins, it may function as a chaperone for other adaptor proteins to bind the integrin cytodomain. RACK1 has also been shown to bind focal adhesion kinases (FAK) and Src via propeller domain 2 to promote IGF-1R receptor association with integrin, and in a similar way may induce LFA-1 clustering by promoting Kindlin-3 association under tensile bond force (105). RACK1 structure shares a similar homology to Gβ subunit and has been

shown to form a heterodimer with it (106). While it is clear that RACK1 plays a role in membrane clustering of LFA-1, whether that is due to aggregation of Kindlin-3 bound to LFA-1 or by promoting the assembly of additional adaptors requires further study. Kindin-3 induced LFA-1 clustering correlates with enhanced calcium signaling, yet the complete signaling circuit has yet to be elucidated (16).

CYTOSKELETAL ACTIVATION AND MOTILE FUNCTION REGULATED BY LOCAL Ca^{2+} INFLUX

Hydrodynamic force acting on LFA-1 and Mac-1 regulates calcium entry, kinase activation, and cytoskeletal protein recruitment all of which are necessary to achieve a migratory state (15, 107–109). We propose that LFA-1 functions not only as a breaking mechanism to achieve neutrophil arrest, but also in the mechanotransduction signals delivered through focal sites of adhesive traction that oppose shear force gradients present on the endothelial surface (**Figure 2C**). Cooperativity between selectin engagement and chemokine binding of GPCRs activate the transition of LFA-1 from low to high affinity resulting in deceleration of the cell that occurs on the order of seconds (12). Neutrophil deceleration and arrest trigger a concomitant rise in intracellular calcium detected within seconds and which precipitates cell shape change and polarization within minutes (15). Coordination in signaling rolling to arrest and to a migratory state is interrupted by inhibiting CRAC channels with pharmacological inhibitors, or genetic deletions that alter calcium flux (22, 110). The precise number of LFA-1 receptors associated with signaling calcium flux is unknown, however, once sufficient numbers of LFA-1 transition to high affinity bonds (on the order of ∼100 receptors) within ∼2–3 submicron focal microclusters, local calcium entry via Orai1 is initiated promoting the coalescence of LFA-1 into micron sized macroclusters (16). This feedback loop between enhanced LFA-1 clustering and Orai1 mediated calcium entry results in a large local transient burst of intracellular calcium that is required to promote organization of high-affinity integrin within focal adhesions. This is in contrast to GPCR that are distributed around the neutrophil within microvilli, and upon ligand binding provide an inside-out signal that is more globally dispersed within the cell volume. This implicates integrin mediated calcium signaling as a central regulator of neutrophil migratory function beyond firm arrest (**Figure 2C**). Remarkably, RACK1 has been shown to regulate IP_3R function in a manner dependent on TRPC3 that in turn promotes calcium release (111). Once calcium has been released through IP_3R activation via RACK1, IP_3R associates with activated STIM1 and subsequently binds Orai1 (111). Further, TRPC3 deletion in HELA and Hek cells, abrogates the association between Orai1 and IP_3R (111). While this has yet to be shown in primary human neutrophils, these data highlight the potential for high affinity LFA-1 bonds under force to catalyze association of a complex composed of Kindlin-3/RACK1/TRPC3/IP_3R/STIM1/Orai1 that effectively directs calcium influx and release of ER stores within focal adhesions in a manner that orients cytoskeletal force generation and neutrophil polarization (**Figure 2C**).

A lack of calcium release or entry via CRAC impairs various physiological events in immune cells, implicating calcium as a pivotal secondary messenger (23, 24, 81, 82, 107–109, 112–114). The role of calcium in T cell regulation can provide insight into calcium signaling in neutrophils. Through the use of genetically-encoded calcium indicators it has been shown that T cell interaction with antigen presenting cells *in vivo* results in low levels of local calcium release (115). Local calcium enhances T-cell mechanosignaling within the immune synapse by promoting T cell receptor clustering and the binding of anionic phospholipids within the plasma membrane, similar to how local calcium bursts in neutrophils regulates activation and integrin build-up within the inflammatory synapse at sites of focal adhesions. Furthermore, calcium entry via Orai1 is responsible for T cell homing to lymph nodes and is necessary for high-affinity integrin LFA-1 activation (116). The magnitude of calcium bursts builds over time and function to recruit more LFA-1, which in turn activates additional Orai1 in a feedback loop to promote adhesion and signaling. Once LFA-1 is engaged between the T cell and antigen presenting cell, external calcium concentration rises above cytosolic, lending credence to the theory that co-localization between membrane receptors and CRAC provides a spatially localized signal that is scaled by the surface area of the cluster which dictates its contribution to cell activation. Neutrophils appear to engage in a similar mechanical process in which LFA-1 bond traction provides spatiotemporal cues, but this occurs within seconds as opposed to hours for T cells and serves to synchronize the multistep process leading to transmigration.

LFA-1 bond formation provides a spatial queue, while calcium provides a temporal queue to signal cell shape change and polarization. Localized calcium flux provides a signal to initiate local cytoskeletal reorganization and subsequent cellular motility (**Figure 2C**). Contractile and protrusion forces created by filamentous actin (F-actin) during cytoskeletal reorganization enables the formation of pseudopods that lead migration and contractile rings that organizes formation of the uropod at the rear that generates traction force (117–119). We propose that local generation of calcium gradients generated by CRAC channels concentrated within sites of focal adhesion provides a signal to catalyze cytoskeletal actin formation and interaction with myosin to drive immune cell motility (119). In T-cells sustained calcium is necessary for continued actin polymerization and microcluster formation within the immunological synapse between the T-cell and antigen presenting cell (120). In neutrophils, deficiency of Wiskott-Aldrich syndrome protein (WASp) results in defects in β_2-integrin clustering, signaling of calcium flux, and cell motility (117, 121). This implicates F-actin mediated cytoskeletal reorganization in integrin clustering and highlights the importance of calcium signaling in this process. Enhanced calcium signaling promotes additional F-actin polymerization and cell spreading through binding to gelsolin a 6-domain actin binding protein that uses calcium to regulate actin filament assembly (122, 123). Once calcium is bound, gelsolin undergoes

a conformational change that exposes its actin binding site, thereby promoting cytoskeletal F-actin assembly (124–126). The asymmetry of front/back actin polymerization may be a consequence of the spatial pattern of integrin mediated calcium entry. F-actin also plays an important role in internalization of CRAC channels, providing a putative mechanism for down regulating extracellular calcium entry as neutrophils prepare to transmigrate at appropriate sites of inflammation (21). This illustrates a key feedback mechanism in which calcium entry and cytoskeletal reorganization provides feedback to organize a migratory phenotype in immune cells.

CONCLUSIONS AND PERSPECTIVES

Neutrophils function as the sentinels of the innate immune system by patrolling miles of vasculature in the microcirculation. To accomplish this critical function, they have evolved adhesive mechanisms that facilitate efficient recruitment at the precise location of tissue insult through the conversion of tensile bond force into biochemical signals. This review provides a scheme by which neutrophil tethering and rolling via selectins leads to integrin activation and shear resistant arrest, a set of mechanosignaling based events necessary for subsequent generation of neutrophil protrusions and diapedesis. The latter process is thought to require a chemotactic gradient that guides neutrophils to the site of tissue insult. In a previous *Frontiers of Immunity* review, we detailed how cytosolic release of Ca^{2+} converges with influx through CRAC to dynamically modulate the number and location of β_2-integrin bonds, which function to synchronize the transition from rolling to arrest and neutrophil shape polarization necessary for diapedesis (9). Recent studies have lent quantitative insight into the physical mechanisms by which L-selectin and integrin catch-bonds convert shear stress into chemical signals within distinct regions of plasma membrane enriched in kinases, phosphoinositides, and cytosolic adaptors (13, 39, 42, 49, 76). Although the specific mechanism of outside-in mechanosignaling is lacking, experimental evidence and structural models indicate that LFA-1 cytosolic domains directly complex with Kindlin-3 and Orai1 and this is regulated by the magnitude of tensile force. We propose that bond tension at durable sites of focal adhesive contact cause reorientation of the integrin headpiece with ICAM-1 and strengthening of the bond. This concomitantly elicits deformation of the LFA-1 cytodomain, thereby exposing the binding site for the PH domain of Kindlin-3 (16, 87, 96). In this review, we put forth the premise that the conversion of LFA-1 to a high affinity state capable of stable bond formation with ICAM-1 is a gatekeeper of this mechanically sensitive linkage that governs transmembrane Ca^{2+} influx. This in turn, facilitates recognition and binding by Kindlin-3 and talin-1 that leads to engagement with RACK1 and FAK and activation of STIM1/Orai1 channels within the focal region of contact on an arrested neutrophil. This contact-mediated circuit is triggered by tensile force conducted via LFA-1 bonds, promotes the calcium feedback loop to recruit additional high-affinity LFA-1 into macroclusters that serve as a nexus for Rho-GTPase activation and F-actin polymerization at contractile regions through which lamellipodia form (127). At sufficient levels of intracellular calcium, F-actin polymerization links to talin-1 tails that reinforce the binding of vinculin. Shape change and cell migration is then mediated by Mac-1 redistribution and bond formation at the uropod where myosins assist in contractile force generation and actin movement (128). In this manner, high affinity integrin bonds effectively function as tactile sensors of the magnitude and direction of hydrodynamic drag forces. Thus, neutrophils dynamically redistribute focal adhesions in a pattern that directs intracellular calcium flux that orients the major axis of neutrophil polarization and generation of motile force to direct innate immune cells at appropriate sites experiencing inflammation.

AUTHOR CONTRIBUTIONS

VM wrote the initial draft of the manuscript with the aid of SS. VM and SS both edited the manuscript to its current form. VM designed the figures and SS edited the figures.

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REVIEW
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More Than Just a Removal Service: Scavenger Receptors in Leukocyte Trafficking

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Scavenger receptors are a highly diverse superfamily of proteins which are grouped by their inherent ability to bind and internalize a wide array of structurally diverse ligands which can be either endogenous or exogenous in nature. Consequently, scavenger receptors are known to play important roles in host homeostasis, with common endogenous ligands including apoptotic cells, and modified low density lipoproteins (LDLs); additionally, scavenger receptors are key regulators of inflammatory diseases, such as atherosclerosis. Also, as a consequence of their affinity for a wide range of microbial products, their role in innate immunity is also being increasingly studied. However, in this review, a secondary function of a number of endothelial-expressed scavenger receptors is discussed. There is increasing evidence that some endothelial-expressed scavenger receptors are able to directly bind leukocyte-expressed ligands and subsequently act as adhesion molecules in the trafficking of leukocytes in lymphatic and vascular tissues. Here, we cover the current literature on this alternative role for endothelial-expressed scavenger receptors and also speculate on their therapeutic potential.

Keywords: leukocyte adhesion cascade, SR-AI, LOX-1, mannose receptor, SCARF1, SR-PSOX, stabilin-1, stabilin-2

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INTRODUCTION

The first scavenger receptor was described in the late 1970s by Brown and Goldstein and was defined by its ability to bind and subsequently internalize low density lipoproteins (LDLs) (1, 2). However, the term "scavenger receptor" was not coined until a couple of years later in the early 1980s by Fogelman et al. who were studying the functionality of Brown and Goldstein's LDL receptor in monocytes and macrophages (3). Scavenger receptors are now a large superfamily of proteins which are highly diverse in structure and are sub-divided into a number of classes (class A-J), with each class sharing structural features; however, there is little or no sequence homology between the classes and the superfamily grouping is purely a consequence of shared functional properties (4). Functionally, scavenger receptors have an important role in both homeostatic and disease states, as they detect and remove, or scavenge, unsolicited self-antigens, which predominantly manifest as damage-associated molecular patterns (DAMPs), such as phosphatidylserine on apoptotic cells (5–7) and products of oxidative stress (e.g., oxidized (ox)LDLs) (8, 9), from general circulation. The removal of apoptotic host cells by scavenger receptors is particularly pertinent in the context of autoimmune diseases, such as systemic lupus erythematosus (SLE), which has been shown to spontaneously develop in some lines of scavenger

receptor-deficient mice (7, 10), thus highlighting their role in homeostasis. Also, other clinical manifestations, for example severe renal glomerular fibrosis and premature mortality, have been shown to spontaneously develop in some multiple scavenger receptor-deficient mice as a result of impaired clearance of harmful factors, such as growth differentiation factor (GDF)-15, from the systemic blood supply (11). These severe phenotypes are somewhat surprising given that several scavenger receptors are able to bind a number of common ligands; therefore, one would assume there would be a certain amount of redundancy in their function and, in the absence of one scavenger receptor, the others would be up-regulated in a compensatory manner to maintain homeostasis. Nevertheless, this is clearly not the case for several members of the scavenger superfamily.

In a number of murine models of inflammatory diseases, the lack of certain scavenger receptors has been shown to be highly detrimental, thus implicating these receptors in the limitation of disease pathology. For example, in a murine model of Alzheimer's disease, reduction or deletion of scavenger receptor class B type I (SR-BI) resulted in increased severity of disease due to impaired clearance of amyloid-β by infiltrating macrophages (12). More recently, we have shown that a lack of the class H scavenger receptor, stabilin-1, in murine models of liver injury promotes fibrogenesis, due to impaired clearance of malondialdehyde (MDA) modified oxLDLs (MDA-LDLs) (13). Conversely, some scavenger receptors have been shown to actively contribute to disease pathology, with several implicated in the establishment, and progression of atherosclerosis due their role in the uptake and storage of LDLs in macrophages (14–17). Furthermore, scavenger receptors also play an important role in the host innate immune system (18–21), as the majority of scavenger receptors are differentially expressed in a number of professional innate immune cells, such as monocytes, macrophages and dendritic cells (22, 23), and are able to recognize a huge array of microbial antigens (24, 25). However, the paradigm is now being established that scavenger receptors require the presence of other pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), in order to elicit an immunological response (26–30).

In addition to their intrinsic scavenging capacity, a number of endothelial-expressed scavenger receptors also exhibit a secondary function in host immunity as they are able to directly interact with leukocytes and mediate their passage across a range of endothelia. This secondary function has led to the study of some scavenger receptors in lymphocyte migration in lymph nodes and in the extravasation of leukocytes during inflammation. In this review, we initially discuss the processes of leukocyte trafficking, subsequently explore the current knowledge of scavenger receptor involvement in these processes and speculate on future research and potential for this relatively understudied function of scavenger receptors.

Lymphocyte Trafficking in Lymph Nodes

The antigen-driven adaptive immune system requires regulated trafficking of T cells in order to orchestrate lymphocyte development, immune surveillance, rapid immunological responses, and memory (31). Consequently, lymphocytes are continually recirculating between the vascular and lymphatic systems and organ tissues. T cells which have not previously encountered antigens, termed naïve T cells, are programmed to undergo migratory cycles into and out of secondary lymphoid organs (SLOs), such as peripheral lymph nodes, tonsils, and Peyers patches, in search of cognate antigens (31). T cells enter lymph nodes (LNs) through afferent lymphatic vessels or high endothelial venules (HEVs) (32) and subsequently interact with antigen presenting cells, primarily dendritic cells (DCs), which present antigens encountered in inflamed tissues on their surface via major histocompatibility complex (MHC) proteins (33). Once T cells encounter cognate MHC/antigen, in concert with the relevant co-stimulatory or co-inhibitory molecules, they become activated, and undergo differentiation into antigen-specific effector or memory cells (33). The trafficking of T cells to and from lymph nodes is known to involve intimate interactions with lymphatic endothelial cells (LECs); however, the endothelial-expressed molecules involved in these processes are not well characterized (31). Nevertheless, the involvement of scavenger receptors has been suggested and is discussed throughout this review.

The Leukocyte Adhesion Cascade

During injury or infection, leukocytes in the blood are required to migrate from general circulation, across the vascular endothelium, and into the inflamed tissue, with the primary aim of eliminating the inflammatory trigger and/or contributing to tissue repair (34). In general, the migration of leukocytes from the blood into inflamed tissues occurs in post-capillary venules, with the exception of the liver, spleen and lungs (34). Leukocyte migration is achieved via a multi-step process known as the leukocyte adhesion cascade (35) (**Figure 1**), in which the leukocytes initially tether and roll on the luminal surface of the blood vessel and undergo arrest, followed by firm adhesion and, finally, migrate through the endothelial barrier into the tissue (36). This sequence of events is mediated by a large number of chemoattractant cytokines (chemokines) (37) and adhesion molecules (**Figure 1**) which determine the subset of leukocyte to be recruited to the site of inflammation and subsequently regulate their numbers (34). Additionally, crossing the vascular wall is not only a highly selective and regulatory step in leukocyte migration, but also acts to prime the tissue-infiltrating leukocytes (38) in order to deliver an efficient and effective immunological response.

Endothelial Activation, Initial Capture, and Rolling

Endothelial activation is the initial step which results in the expression of adhesion molecules and chemokines on the luminal membrane of endothelial cells involved in the initial capture of leukocytes from shear flow. Endothelial activation can be triggered by a wide range of stimuli and is classified as "type I" or "type II," depending on the mediating signal molecule. Type I activation of endothelial cells is a protein-synthesis-independent process and is predominantly mediated via ligands of heterotrimeric G-protein-coupled receptors (GPCRs), such as histamine and thrombin (39). Type I activation results in the trafficking of pre-formed P-selectin to the cell membrane within minutes, thus allowing the rapid

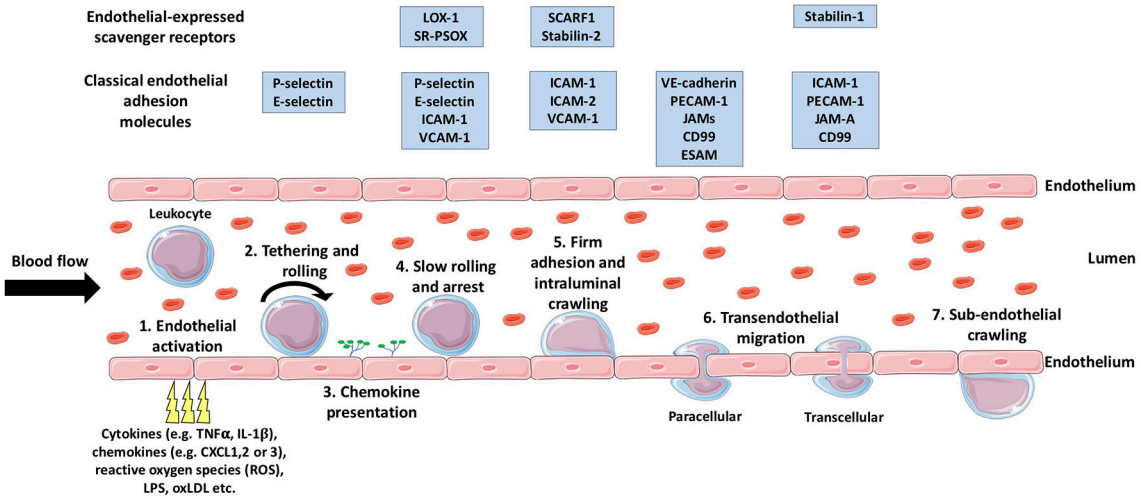

FIGURE 1 | The multistep leukocyte adhesion cascade. Leukocytes are recruited from the bloodstream to inflamed tissues via sequential multi-step process known as the leukocyte adhesion cascade. Firstly, endothelial activation is triggered by a range of endogenous or exogenous stimuli from the inflamed tissue (1), which triggers the selectin-dependent tethering and rolling of leukocytes along the luminal surface of the vessel (2). Subsequently, chemokines are presented on the luminal surface of the endothelium (3) which activate leukocyte-expressed integrins allowing stronger bond formation with their endothelial-expressed ligands. The formation of these stronger leukocyte-endothelium bonds results in leukocyte arrest (4), following which, intraluminal crawling occurs (5). Next, the leukocyte will undergo transendothelial migration (6) either via the paracellular or the transcellular pathway. Once the leukocyte has crossed the endothelial layer, it may undertake in sub-endothelial crawling (7), prior to entering the target tissue proper. TNFα, tumor necrosis factor-α; IL-1β, interleukin-1β; LPS, lipopolysaccharide; oxLDL, oxidized low density lipoprotein; LOX-1, Lectin-like oxidized low-density lipoprotein receptor-1; SR-PSOX, scavenger receptor that binds phosphatidylserine and oxidized lipids; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1 ; SCARF1, scavenger receptor class F; member 1; VE-cadherin, vascular endothelial cadherin; PECAM-1, platelet endothelial cell adhesion molecule-1; JAMs, junctional adhesion molecules; ESAM, endothelial cell-specific adhesion molecule. (Stock images provided by Servier medical for use under the Creative Commons Attribution 3.0 Unported License).

recruitment of neutrophils to vascular endothelia (40–43). Type I activation is a highly transient event and, in order to limit the extent of neutrophil extravasation, the GPCRs involved are presumed to undergo desensitization (44, 45) to their stimuli after 10–20 min to prevent further endothelial stimulation (39). Type II activation of endothelial cells is a much slower process known to be triggered by a much wider range of stimuli, including inflammatory cytokines [e.g., tumor necrosis factor (TNF)α, interferon (IFN)γ, and interleukin (IL)-1β (46)], microbial antigens [e.g., lipopolysaccharide (LPS) (47, 48)], and oxLDLs (49, 50). Type II activation results in morphological changes, via the reorganization of actin filaments (51) and *de novo* expression of leukocyte adhesion molecules, such as E-selectin, intracellular adhesion molecule (ICAM)-1 and vascular cell-adhesion molecule (VCAM)-1 (52–55), and chemokines (56, 57) on the luminal surface of the endothelial cells. Unlike, type I activation which is stringently regulated via receptor desensitization, type II activation is much more long-lived and can chronically persist until the inflammatory stimulus is removed and the regulatory anti-inflammatory feedback mechanisms are able to effectively counteract the proinflammatory exacerbation, commonly via regulation of the nuclear factor (NF)-κB pathway (58, 59).

Following endothelial activation, the initial capture of leukocytes from shear flow is mediated by selectins, a family of three Type I transmembrane Ca^{2+}-dependent lectins which bind to glycoprotein ligands (60). The selectins are named according to the cell type in which they were originally described in (platelet (P)-selectin, leukocyte (L)-selectin and endothelial (E)-selectin) and consist of an extracellular N-terminal C-type lectin domain, an epidermal growth factor (EGF)-like domain, a series of short consensus repeats (SCRs), a transmembrane domain and a short C-terminal intracellular domain (61). As mentioned above, stores of pre-formed P-selectin are held within human endothelial cells (62) and are rapidly trafficked to the surface in the event of type I activation, but P-selectin is also differentially regulated in a range of chronic inflammatory diseases (63–66) and plays a major role in leukocyte recruitment during prolonged type II activation (67–70). L-selectin is expressed in the majority of circulating leukocytes and is one of the first leukocyte-expressed cell adhesion molecules to interact with the endothelial layer in the initial "tethering" event (71), whereas E-selectin is constitutively expressed in bone marrow endothelial cells (72), but is inducible in other endothelia (54). E-selectin is predominantly involved in the rolling and slow rolling steps of the adhesion cascade (73, 74). Rolling is the transient and reversible selectin-ligand interaction which involves the "catch-bond" phenomenon, where bonds are strengthened with increasing shear stress (75). Also, the rolling motion of leukocytes is able to generate new selectin-ligand bonds before old ones are broken via the "tether and sling" phenomenon utilized by neutrophils (76, 77) and differentiated T cell subsets (78). The rolling and slow rolling steps aim to initiate leukocyte-endothelial contact and, consequently, further activate the

leukocyte, thus promoting the successive steps in the adhesion cascade.

Leukocyte Arrest and Crawling

The arrest of leukocytes rolling along the surface of the endothelium is triggered by chemokines which are expressed upon endothelial activation and are immobilized on the luminal surface via highly negatively-charged polysaccharides, such as glycosaminoglycans (GAGs) (79, 80). As a consequence of chemokine-induced "inside-out" signaling, heterodimeric adhesion receptors expressed on the surface of leukocytes, known as integrins, undergo conformational changes and become "activated" (81, 82). Once activated, integrins are able to form high affinity bonds with their endothelial-expressed ligands and their clustering in focal adhesion contacts allows for stronger leukocyte-endothelial bonds (83), thus resulting in leukocyte arrest [reviewed in detail by Ley et al. (35)].

Once firmly adhered to the endothelial layer, innate immune cells, such as monocytes, have been shown to patrol the vessel wall surface (84), scavenging microparticles, and supporting the recruitment of other cells, such as neutrophils (85). This intraluminal "crawling" behavior has also been observing in neutrophils and is thought to mediate their transmigration across the endothelial layer, as they search for sites of exit from the blood vessel (86–88). Additionally, a novel phenomenon in hepatic sinusoidal endothelial cells (HSEC) was recently described in which peripheral blood lymphocytes were shown to migrate horizontally from one endothelial cell to another (89). This intracellular crawling appeared to be HSEC-specific as it did not occur in more conventional vascular endothelial cells (HUVEC; human umbilical vein endothelial cells). It was subsequently speculated that this process could represent a liver-specific method of immune surveillance (89); however, studies of this phenomenon were all undertaken *in vitro* and it is yet to be confirmed *in vivo*. Interestingly, once leukocytes have traversed the endothelial barrier, they have also been shown to undergo sub-endothelial crawling (90–92) prior to their migration into the tissue proper.

Transendothelial Migration

The final step in the leukocyte adhesion cascade is the crossing of the endothelial barrier, which is known as transendothelial migration (93). Transendothelial migration of leukocytes is a highly regulated process as maintenance of barrier integrity is paramount and endothelial cells undergo significant cytoskeletal remodeling to facilitate the passage of leukocytes, whilst also preventing vascular leakage (94). There are two possible routes for leukocytes to transmigrate the endothelial barrier, the paracellular pathway, or the transcellular pathway [reviewed extensively by Ley et al. (35) and more recently by Vestweber (36)]. The paracellular route describes the passage of leukocytes between the cell-cell junctions of the endothelial layer and has inevitably been shown to be mediated via a number of key junctional proteins, such as platelet endothelial cell adhesion molecule (PECAM)-1 (also known as CD31) (95), CD99 (95, 96), and junctional adhesion molecules (JAMs) (97, 98). Also, vascular endothelial (VE)-cadherin has been shown to play an instrumental role in the inhibition of leukocyte extravasation and must be actively moved away from the site of leukocyte transmigration to allow the process to occur (99, 100). The vast majority (∼80–95 %) of cells undergo transendothelial migration via the paracellular route; however, the remainder transmigrate through the transcellular pathway which involves leukocytes passing directly through the cell body of endothelial cells This process is highly coordinated and requires extensive remodeling of the endothelial cell's actin cytoskeleton to form an appropriately sized pore to accommodate the passage of the leukocyte, and in particular its nucleus (101). Unsurprisingly, the transcellular migration of leukocytes is stringently regulated by the endothelial cell to minimize vascular leakage (101). The molecules involved in transcellular are less well-studied than those for paracellular migration; nevertheless, to date, ICAM-1 (53, 94, 102, 103) has been identified as the major contributor, but other molecules, such as PECAM-1, JAM-A, and CD99 (104, 105) have also been shown to play a role in this process.

With the technological advancements in microscopy, our knowledge of the processes involved in leukocyte transmigration are ever-increasing (94, 101, 106, 107); nevertheless the molecular mechanisms which determine whether leukocytes transmigrate through the paracellular or transcellular pathways still remain a mystery. The possibility of scavenger receptors playing a role in these processes is a tangible prospect and should be investigated in future studies.

SCAVENGER RECEPTORS IN LEUKOCYTE TRAFFICKING

Given that a number contain similar structural domains to those found in the selectin family [e.g., C-type lectin domains or epidermal growth factor (EGF)-like domains], it is perhaps unsurprising that several endothelial-expressed scavenger receptors are also able to directly bind to leukocytes. Consequently, several scavenger receptors have been shown to play a role in leukocyte trafficking through lymph nodes and/or in their extravasation through a range of endothelia. Discussed below are the scavenger receptors identified to date which play a role in these processes.

SR-AI

Scavenger receptor (SR)-AI, also known as macrophage scavenger receptor (MSR)-1 or CD204, was the first scavenger receptor to be cloned (108), and hence is the first member of the Class A family and arguably the most studied scavenger receptor (109). SR-AI is a Type II membrane protein, with its structure consisting of a short N-terminal cytoplasmic tail, a transmembrane domain, a spacer region, an α-helical coiled-coil domain, a collagen-like domain, and a C-terminal scavenger receptor cysteine-rich (SRCR) domain (110). As is characteristic of most scavenger receptors, SR-AI has been shown to bind a highly diverse range of endogenous products including: an array of modified LDLs (111, 112); apoptotic cells (113); heat shock proteins (Hsp) (114); collagen (115); β-amyloid (116); apolipoproteins (117), and advanced glycation end products

(AGE) (118). Additionally, SR-AI can also bind a range of exogenous ligands, such as bacterial lipopolysaccharide (LPS) (119), and lipoteichoic acid (LTA) (120), fungal β-glucan (121), and viral double stranded (ds)RNA (122–124). SR-AI is predominantly expressed in myeloid cells, such as monocytes and tissue-resident macrophages, but was also shown to be expressed in high endothelial cells of postcapillary venules (HEV) in peripheral lymph nodes a number of years ago (125). The adhesive ability of SR-AI has only recently been considered; however, this recent study has focused on lymphocyte binding to lymphatic endothelial cells (LEC) (126). In their investigation of SR-AI in LEC, Iftakhar-E-Khuda et al. utilized binding assays to primary murine lymphatic endothelial cells *in vitro* and antibody blockade on human and murine lymphatic tissue sections *ex vivo* to demonstrate its lymphocyte binding capacity in afferent lymphatics (126). However, they did not observe any differences in lymphocyte populations in the lymph nodes of wild type (WT) and SR-AI$^{-/-}$ mice, possibly suggesting a possible redundancy in SR-AI's lymphocyte binding activity *in vivo*, under homeostatic conditions. This discrepancy between the *in vitro* and *in vivo* data suggests that further investigation of SR-AI's adhesive properties is warranted and future studies could possibly explore lymph node trafficking of leukocytes in mice subjected to injury, such as LPS-induced toxemia. Additionally, given the SR-AI expression in HEVs and that inducible expression of SR-AI has been found in human arterial endothelial cells (127), it is not unreasonable for future investigations to explore SR-AI expression in a range of vascular endothelia from different tissues. If found in these vascular endothelial cells, basic static and flow-based adhesion assays, such as those utilized previously in our lab (128), could be employed to determine which step in the leukocyte adhesion cascade SR-AI potentially acts. Furthermore, a leukocyte-expressed ligand has yet to be explored and so future studies should also aim to identify the molecule(s) involved in SR-AI-mediated leukocyte binding to these endothelia.

LOX-1

Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) is another Type II membrane protein which comprises of a short N-terminal cytoplasmic domain, a single transmembrane region and an extracellular domain containing a coiled-coil "neck" region and a C-type lectin-like domain (129) and was the first member of the Class E family to be described. As its name suggests, LOX-1 was initially identified as a receptor for oxLDLs in endothelial cells (129), but has since been shown to bind a number of other modified LDLs, such as carbamylated LDLs (130) and glycoxidised LDLs (131). Subsequently, LOX-1 has also been found to bind a more diverse range of ligands, including phosphotidylserine on apoptotic cells (132, 133), Gram positive and Gram negative bacteria (134), and C-reactive protein (CRP) (135). Nevertheless, LOX-1 is a "non-essential" protein, as LOX-1$^{-/-}$ mice do not exhibit any phenotypic traits. Also, under physiological conditions, LOX-1 is expressed in relatively low levels in vascular endothelial cells, but is inducible upon endothelial activation by ligand binding (136, 137), inflammatory cytokines (138, 139) or shear stress (140). The leukocyte adhesive ability of LOX-1 was first described in 2002, when Hayashida et al. demonstrated that transfected Chinese hamster ovarian (CHO) cells over-expressing LOX-1 augmented the adhesion of primary peripheral blood mononuclear cells (PBMCs), and monocytic cell line, THP-1, when compared to control transfected cells (141). Interestingly, this effect appeared to be monocytic cell-specific, as they did not observe any effects on the Jurkat leukaemic T cell line (141). Additionally, they demonstrated that the enhanced adhesion of THP-1 cells to the LOX-1-CHO could be reversed by antibody or oxLDL blockade and recapitulated this blockade on bovine aortic endothelial cells (BAEC) *in vitro* (141). Finally, they demonstrated that THP-1 cells flowed over LOX-1-CHO cells at increasing shear stress exhibited increased numbers of cells rolling and at lower rolling velocities than those flowed over WT-CHO cells (141), thus suggesting that LOX-1 acts as an adhesion molecule in the early stages of the leukocyte adhesion cascade.

Following this initial study, Li et al. then demonstrated that antibody blockade of LOX-1 *in vivo*, in a rat myocardial ischaemia-reperfusion model, was able to significantly reduce the number of infiltrating leukocytes to the myocardial tissues, which also resulted in a significant decrease in the myocardial infarct (142). However, their data suggested that the diminished leukocyte infiltration was due to an indirect effect of LOX-1 blockade, as they showed a reduction in the expression of adhesion molecules, such as ICAM-1, VCAM-1, and P-selectin (142). Nevertheless, in a seminal study, Honjo et al. demonstrated in a rat model of endotoxemia and endotoxin-induced uveitis, that antibody blockade of LOX-1 expression induced in retinal endothelial cells significantly reduced the number of rolling infiltrating leukocytes, which predominantly consisted of neutrophils, and also increased the velocity of rolling (143). This data is suggestive of a direct interaction with leukocytes *in vivo* and adds to *in vitro* studies which show that LOX-1 functions as adhesion molecule in the early stages of the leukocyte adhesion cascade. Also, more recently, Ding et al. demonstrated that LOX-1$^{-/-}$ mice fed a high cholesterol diet exhibit a lower level of macrophage accumulation in their aortas compared to WT mice (144); nevertheless, it is unclear whether this was due a lack of LOX-1-mediated recruitment by the aortic endothelial cells or a migratory defect in the LOX-1-deficient macrophages.

From the current data implicating it in the leukocyte adhesion cascade, it is clear that LOX-1 contributes to the rolling stage of the adhesion cascade in the recruitment of myeloid cells to a range of vascular endothelia. Nevertheless, despite a number of studies now demonstrating this both *in vitro* and *in vivo*, the leukocyte-expressed ligand(s) responsible for LOX-1 binding have not yet been identified. Additionally, initial studies have suggested that the adhesive properties of endothelial-expressed LOX-1 do not extend to cells of lymphoid lineage, this has only been tested utilizing a leukaemic T cell line and so further investigation with primary lymphocytes could in fact be warranted.

Mannose Receptor

The third member of the Class E scavenger receptor family to be described, the mannose receptor (MR) or CD206, is a Type I membrane glycoprotein which consists of a short intracellular

domain, a transmembrane domain, and an extracellular region comprising of a eight C-type lectin-like domains, a fibronectin type II domain and an N-terminal cysteine-rich domain (145). As its name suggests, MR was originally discovered to bind mannose and other carbohydrate groups in a range of glycoproteins (146); nevertheless, given that its extracellular region comprises of several functionally distinct domains, MR has since been shown to bind a wide range of other endogenous ligands, including collagen (147, 148), CD45 (149), tumoural mucins (150), and neutrophil-derived myeloperoxidases (151). Additionally, MR can bind a range of bacterial- (152, 153), viral- (154–157), fungal- (158–161), and parasite-derived (21) antigens. The mannose receptor is predominantly expressed by macrophages (162, 163), but has also been described in a range of endothelial cells, such as hepatic sinusoidal endothelial cells (HSEC) (89, 164), dermal endothelial cells (165) and lymphatic endothelial cells (LEC) (166–168). The leukocyte adhesive properties of MR were first described by the Jalkanen group based at University of Turku, Finland in 2001, when they suggested that MR plays a role in lymphocyte exiting from lymph nodes as their data confirmed the MR-mediated adhesion of lymphocytes to LECs (167). These studies also demonstrated that L-selectin, was the lymphocyte-expressed ligand required for MR-mediated static adhesion of lymphocytes to LECs *in vitro*, which the authors believed to most accurately mimic physiological conditions within lymph nodes *in vivo* (167). Further studies by the same group demonstrated the binding of B lymphoblastoid cell lines to LEC and high endothelial venules (HEVs) both on tissues sections *ex vivo* and on isolated cells *in vitro* (166), further strengthening the evidence for the adhesive functionality of MR. Subsequently, these *in vitro* findings were corroborated with *in vivo* experiments by Marttila-Ichihara et al. who demonstrated that the adhesion of both normal lymphocytes and tumor cells to afferent lymphatic vessels was significantly reduced in MR-deficient mice, compared to WT (168). More recently, the Jalkanen group also showed that L-selectin-negative leukocytes trafficking to the lymph nodes utilize CD44 to bind to MR expressed on LECs and subsequently migrate to draining lymph nodes (169). The authors also suggest that therapeutic targeting of MR on LEC could selectively reduce leukocyte migration from the periphery into the draining lymph nodes thus potentially acting to dampen inappropriate inflammatory reactions (169). Expression in vascular endothelial cells, such as HSEC, suggests that MR could also potentially facilitate leukocyte binding in the adhesion cascade and future studies could investigate this.

SCARF1

Scavenger receptor class F, member 1 (SCARF1 or SR-F1), also known as scavenger receptor expressed by endothelial cells (SREC)-I, was first identified in cDNA libraries from human umbilical vein endothelial cells (HUVEC) (170). SCARF1 is a type I membrane protein which comprises of several extracellular EGF-like domains, a transcellular domain and, unusually for a scavenger receptor, a long serine- and proline-rich cytoplasmic tail (171). SCARF1 has been shown to bind modified low density lipoproteins (LDLs), specifically acLDLs (172), and acts as an endocytic receptor for a wide range of damage-associated products (173), including heat-shock proteins (Hsps) (174–176) and apoptotic host cells via phosphotidylserine-bound C1q protein (7). SCARF1 has been shown to play a key role in the prevention of autoimmunity, as SCARF1-deficient mice spontaneously develop systemic lupus erythematosus (SLE) due to the severely impaired clearance of apoptotic cells in the spleen (7). In addition to binding and internalizing a diverse range of endogenous proteins, SCARF1 also binds a wide array of viral (29, 177, 178), fungal (179), and bacterial (28, 30, 180, 181) antigens and SCARF1 expression in alveolar macrophages has been shown to play an important role in immunological responses to fungal lung infection (179). SCARF1 is also expressed in murine splenic endothelial cells (179) and liver sinusoidal endothelial cells (178) and our lab has corroborated this and recently described for the first time the expression on SCARF1 in primary human hepatic sinusoidal endothelial cells (HSEC) (182). Subsequently, utilizing a combination of flow-based adhesion assays with immobilized recombinant proteins, HSEC, and siRNA silencing in HSEC, we were able to robustly demonstrate that SCARF1 plays a role in the selective recruitment of $CD4^+$ T cells to the sinusoidal endothelium under physiological shear stress (182). Additionally, we showed that SCARF1 facilitates this process via the formation of adhesive cups which were also rich in ICAM-1 and F-actin and proposed that SCARF1 acts in the firm adhesion step of the leukocyte adhesion cascade (182). However, we did not explore the possibility SCARF1's involvement in the transendothelial migration step and future investigations from our lab will explore this. SCARF1 is known to form moderate homophilic interactions (183); however, we ruled out the possibility of these interactions in this context, as $CD4^+$ T cells do not express SCARF1 (182). Therefore, the lymphocyte-expressed ligand of SCARF1 is yet to be identified and screening experiments could be employed to determine this in future investigations.

SR-PSOX

Scavenger receptor that binds phosphatidylserine and oxidized lipids (SR-PSOX) is the only member belonging to the class G family of scavenger receptors to date (184) and is structurally unique within the scavenger receptor superfamily. SR-PSOX is a type I transmembrane glycoprotein with its N-terminal extracellular domain, consisting of a CXC chemokine motif and a mucin-like stalk, linked to a transmembrane domain and a short C-terminal intracellular domain (185). SR-PSOX also exists in a soluble form which is shed or enzymatically cleaved from the cell surface via a disintegrin and metalloproteinase (ADAM)-10 and ADAM-17 (186–189). SR-PSOX was first identified in the human monocytic cell line THP-1 and was shown to bind and internalize oxLDL and phosphatidylserine (190). Subsequently, SR-PSOX has also been shown to bind eryptotic erythrocytes (191, 192) and bacterial antigens (193, 194) and has been found to be expressed in a wide range of cell types, including macrophages (195), DCs (196), smooth muscle cells (197), and endothelial cells (189, 198, 199). Early cloning studies on a chemokine known as CXCL16 (200, 201) found it to be structurally identical to SR-PSOX and, as CXCL16 is a highly specific ligand for the chemokine receptor CXCR6, it was soon discovered that SR-PSOX was able to support

the adhesion of a range of $CXCR6^+$ leukocytes (202–205). Subsequent to these findings, it was suggested that SR-PSOX acts in the "arrest" stage of the adhesion cascade by triggering the conformational activation of β_1 integrins on leukocytes (206).

Possibly the best studied role for SR-PSOX in the recruitment of leukocytes is in the context of hepatic inflammation (207), with its endothelial-expressed form known to interact with intrahepatic $CXCR6^+$ immune cells, such as effector T cells (206, 208), natural killer (NK) cells (209, 210) and NKT cells (199). It has recently been shown that genetic deficiency of SR-PSOX in mice inhibits the extent of inflammation in a model of acetaminophen (APAP)-induced acute liver injury (211). In addition, pharmacological intervention with neutralizing antibodies raised against SR-PSOX has shown inflammation-reducing efficacy in preclinical murine models of sepsis-mediated (212, 213) and carbon tetrachloride (CCl_4)-mediated (207) acute liver injury. Conversely, an elegant study by Ma et al. has recently shown that HSEC-expressed SR-PSOX plays a key role in the recruitment of anti-tumourigenic NKT cells to the liver in a number of murine models of primary and metastatic hepatic cancers (214). Thus, the therapeutic targeting of SR-PSOX to inhibit hepatic inflammation must be carefully considered with regards to context of the inflammatory injury being treated.

Stabilin-1

Stabilin-1 is a highly conserved type I transmembrane protein and was the first member of the Class H family of scavenger receptor to be described. It was originally described in 1991 as MS-1 antigen, when it was used as a histological marker for non-continuous splenic sinusoidal endothelial cells (215). Subsequently, three labs independently described the same molecule as FEEL-1, due to its structure containing fasciclin (FAS), epidermal growth factor (EGF)-like, laminin-type EGF-like, and link domains (171), stabilin-1 (216) and common lymphatic endothelial and vascular endothelial receptor (CLEVER)-1 (217); however, due to its official gene nomenclature, *STAB1*, stabilin-1 is increasingly utilized in the literature. An early indicator of stabilin-1's capacity as a scavenger receptor was its constitutive expression in the professional scavenging cells of the non-continuous sinusoidal endothelia in the spleen (215), lymph nodes (218, 219), and liver (220). Interestingly, stabilin-1 expression is also inducible in continuous endothelia, in response to angiogenic and proinflammatory stimuli (221). This inducible expression is thought to originate from the transient non-continuous state that vascular endothelial cells transition through during the rapid growth of blood vessels throughout the wound healing process, tumor vascularization, and chronic inflammatory skin conditions, such as psoriasis. As is a prerequisite of being classified as a "scavenger receptor," stabilin-1 has been shown to bind a wide variety of ligands, such as: modified LDLs (13, 222); phosphotidylserine expressed on apoptotic cells (223–225); secreted protein acidic and rich in cysteine (SPARC) (226); placental lactogen (227) and microparticles from both Gram positive and Gram negative bacteria (228).

Additionally, a number of early studies showed stabilin-1 to be a multi-functional scavenger receptor with the ability to directly interact with leukocytes and effectively act as a leukocyte adhesion molecule. However, the ability of stabilin-1 to perform this particular function has historically been considered a contentious issue (229, 230), which is possibly confounded by the fact that the leukocyte-expressed ligand(s) for stabilin-1 still remains unidentified. Nevertheless, there is a growing body of evidence for this adhesive function and its first description was by the Jalkanen group (217), when they demonstrated that antibody blockade of stabilin-1 on high endothelial venules (HEVs) and lymphatic vessels, in both *in vitro* static adhesion assays and under flow conditions *in vivo*, significantly diminished the number of adherent lymphocytes, granulocytes, and monocytes (217). Around this time, the same group presented further evidence, showing the stabilin-1-mediated adhesion of B lymphoblastoid cell lines to lymphatic endothelial cells and HEVs *in vitro* (166). Subsequently, this group then demonstrated that stabilin-1 plays a key role in the transmigration of leukocytes through vascular and lymphatic endothelial cells *in vitro* (218) and later confirmed *in vivo* that it mediates the transendothelial migration of T cells and B cells across HEVs to the draining lymph nodes (219). Furthermore, they also showed that antibody blockade of stabilin-1 effectively inhibited peritonitis in mice by decreasing granulocyte recruitment by ∼50%, whilst migration of monocytes and lymphocytes into the inflamed peritoneum was almost completely inhibited (219). More recently, the Jalkanen group have also shown that stabilin-1 plays a key role in the recruitment of immunosuppressive macrophages and T regulatory (T_{reg}) lymphocytes in *in vivo* models of tumor growth and metastasis, with reduced numbers of both cell types demonstrated in the absence and therapeutic blockade of stabilin-1 (231).

In addition to this, and consistent with the Jalkanen group's data, our lab has implicated stabilin-1 in the transendothelial migration of both T_{regs} and B-cells through hepatic sinusoidal endothelial cells (HSECs) *in vitro*, under conditions which mimic the physiological flow and proinflammatory microenvironment of the hepatic sinusoids during liver injury (89, 220, 232). Interestingly, in the context of hepatic microvasculature, monocyte recruitment does not appear to be supported by stabilin-1, with antibody blockade on HSEC *in vitro* exhibiting no effect on neither monocyte adhesion nor transmigration, under physiological flow (unpublished data). Also, the leukocyte adhesion function of HSEC-expressed stabilin-1 appeared to be redundant *in vivo*, in murine models of liver injury, as no significant differences in T_{reg} or B cell numbers were found between stabilin-$1^{-/-}$ mice and their wild type counterparts, in both carbon tetrachloride (CCl_4)- and methionine and choline-deficient (MCD) diet-induced liver injury models (13). Nevertheless, given that Karikoski et al. showed significantly decreased numbers of T_{regs} were present in their murine tumor models when stabilin-$1^{-/-}$ mice were compared to WT controls (231), it can be speculated that stabilin-1's role in the recruitment of T_{regs} across HSEC will be potentially important in the context of hepatocellular carcinoma (HCC). Karikoski et al. also showed that stabilin-$1^{-/-}$ mice presented with smaller primary and metastatic tumors than WT mice (231) and these findings were corroborated with preliminary data in human HCC tissues

ex vivo, which has shown that stabilin-1 expression is highly augmented in peritumoural endothelia and correlated with adverse histological features (233). This suggests that stabilin-1 potentially plays an adverse role in malignancy by potentiating the suppression of the host immune response to a neoplasm; consequently, a Phase I/II trial, TIETALC, (Tumor Immunity Enabling Technology Against Liver Cancer) is currently being designed at the University of Birmingham to test the efficacy of targeting stabilin-1 in HCC (234).

Stabilin-2

The second member of the Class H scavenger receptor family, stabilin-2, also known as FEEL2 or HARE (hyaluronan receptor for endocytosis), is very similar in structure to stabilin-1 with both exhibiting similar domain organization in their extracellular regions. Stabilin-2 was originally described as a clearance receptor for hyaluronan (216, 235, 236); however, it is now known to bind a wide range of structurally diverse ligands. For example, stabilin-2 has also been shown to bind to acLDLs (228), heparin (237), apoptotic (238, 239), and necrotic (240) cells and microparticles from both Gram positive and Gram negative bacteria (228). Like stabilin-1, stabilin-2 has also been shown to be expressed in HSEC (235, 241, 242) and can also mediate lymphocyte recruitment to the hepatic sinusoidal endothelium (241). Through a number of mutation experiments and antibody blockade studies *in vitro*, Jung et al. found that the fasciclin 1 (FAS1) domains of stabilin-2 were response for lymphocyte binding and identified $\alpha_M\beta_2$ integrin as the lymphocyte-expressed ligand (241). They also determined that stabilin-2 expression was not regulated in HSEC by proinflammatory stimuli previously shown to activate endothelia and subsequently suggested that stabilin-2 predominantly acts in the firm adhesion step of the leukocyte adhesion cascade as its forced down regulation via shRNA treatment did not affect lymphocyte rolling or transendothelial migration, but was still able to significantly reduce the number of adherent cells (241). Despite their identification of the lymphocyte-expressed ligand for stabilin-2, the study undertaken by Jung et al. remains the only exploration of stabilin-2's lymphocyte binding ability to date. Since monocytes (243) and neutrophils (244) also express $\alpha_M\beta_2$, it would be interesting to investigate whether or not stabilin-2 is also able to mediate the binding of these myeloid populations. Furthermore, the Jung study was restricted to stabilin-2-mediated lymphocyte binding in the context of HSEC (241); however, splice-variants have also been identified in non-continuous sinusoidal endothelia of other tissues, such as lymph nodes and the spleen (235, 245) and so future studies could also explore the potential role of stabilin-2 in leukocyte recruitment to these alternative tissues.

FUTURE WORK AND THERAPEUTIC POTENTIAL

Trafficking of leukocytes represents the fundamental basis of any type of immunological response and so targeting this process remains an attractive prospect in the suppression of a wide variety of inflammatory diseases. Whilst many of the key players in this process have been identified, we have summarized the gathering evidence that scavenger receptors can act as atypical adhesion receptors which contribute to leukocyte homing (**Figure 1**). In summarizing this literature, it is evident that further work is

TABLE 1 | Summary of endothelial-expressed scavenger receptor function, leukocyte/ligand binding, and translational stage of research.

Scavenger receptor	Endothelial cells (EC) studied	Role in leukocyte trafficking	Leukocyte binding	Leukocyte ligand(s)	Translational stage
LEUKOCYTE ADHESION CASCADE					
SR-PSOX	Hepatic sinusoidal (HSEC)	Arrest	$CD4^+$ and $CD8^+$ T cells, NK cells, NKT cells	CXCR6	*In vivo*, murine models of acute liver injury
LOX-1	Bovine aortic endothelial cells (BAEC); rat retinal ECs	Rolling	Neutrophils, monocytes/macrophages	Unknown	*In vivo*, rat model of endotoxemia
SCARF1	Hepatic sinusoidal (HSEC)	Firm adhesion	$CD4^+$ T cells	Unknown	*In vitro*, primary human cell models
Stabilin-1	Peritoneal vascular ECs; tumor vascular ECs; hepatic sinusoidal (HSEC)	Transendothelial migration	T_{reg}, B cells, granulocytes and monocytes	Unknown	Phase I/II clinical trials in HCC being designed
Stabilin-2	Hepatic sinusoidal (HSEC)	Firm adhesion	PBLs	$\alpha_M\beta_2$ integrin	*In vitro*, primary human cell models
LYMPH NODE TRAFFICKING					
SR-AI	Lymphatic (LEC)		PBLs	Unknown	*In vitro*, primary murine and human cell models *Ex vivo*, static adhesion assays
Stabilin-1	Lymphatic (LEC) and high endothelial venules		T cells, B cells	Unknown	*In vivo*, murine models
Mannose receptor	Lymphatic (LEC) and high endothelial venules		PBLs	L-selectin, CD44	*In vivo*, murine models

required to understand the exact mechanisms by which scavenger receptors contribute to leukocyte adhesion and migration.

Scavenger receptors can rapidly recycle from the cell membrane (246) and are also known to interact with other pattern recognition receptors (20); this therefore leads to the question of whether or not scavenger receptors contribute to leukocyte adhesion in a direct or indirect manner. In addition, given that scavenger receptors have important homeostatic functions in the remove of endogenous waste products from cell turnover, further experimental work is required to understand how the multifunctional properties of these receptors influence their *in vivo* contributions. It is currently unclear if there is a hierarchy in ligand recognition/affinity and how the leukocyte homing properties of scavenger receptors work alongside their homeostatic functionality. Whilst the experiments described in this review have confirmed a role for scavenger receptors in leukocyte homing, in several cases the identity of the ligand they bind on leukocytes have not been elucidated (**Table 1**), although imaging has demonstrated that some these receptors, such as stabilin-1 and SCARF1, directly interact with leukocytes on the endothelial surface (182, 220). The development of high resolution imaging will hopefully help answer some of these questions, focusing on the trafficking of scavenger receptors and their membrane dynamics during leukocyte recruitment as well as their interaction with other cell membrane molecules.

Despite the need for further experimental work in this area, the potential of scavenger receptors as therapeutic targets in inflammatory disease should be explored. Due to their enrichment in specialized vascular beds, such as lymphatics and other sinusoidal endothelial vasculature, and the fact that leukocyte recruitment differs here from conventional vascular beds, scavenger receptors may predominantly influence recruitment in an organ-specific manner. They present a promising avenue for the translational development of clinical therapies to target inappropriate inflammatory reactions, such as autoimmunity, as well as hepatic inflammation and recruitment in the bone marrow niche. With regards to the potential targeting of scavenger receptors in the leukocyte adhesion cascade, liver-specific targeting may present more viable therapeutic targets than endothelia of other organs, given the increased expression of scavenger receptors in HSEC (247). Additionally, the low shear stress environment results in a largely selectin-free leukocyte adhesion cascade, thus allowing for a greater contribution by atypical adhesion molecules to leukocyte recruitment.

However, targeting the leukocyte adhesion cascade to treat inflammatory diseases could potentially be associated with significant side effects related to impaired immune surveillance and increased risk of invasion by pathogenic organisms. Nevertheless, detailed analysis of leukocyte recruitment of some scavenger receptors have shown that, rather than a pan-leukocyte effect, some of them influence the trafficking of specific subsets of leukocytes (**Table 1**). This suggests that these receptors may indeed be therapeutically effective in shaping the immune microenvironment by altering the balance of immune populations at the site of inflammation, whilst also minimizing potential side effects. Stabilin-1, for example, predominantly mediates the recruitment of regulatory T cells across liver endothelium suggesting that blocking its action would be more appropriate in the setting of malignancy to boost tumor-specific immune responses, whilst other scavenger receptors, such as LOX-1, appear to be more pro-inflammatory. Several preclinical experimental approaches have utilized monoclonal antibodies to block the action of this family of receptors in leukocyte recruitment; therefore, the development of humanized therapeutic antibodies appears to be a reasonable approach to target these receptors in the clinic. However, a caveat when using monoclonal antibodies is the probability of off-target effects, considering the differential expression of many scavenger receptors in a range of professional immune cells. Therefore, a more LEC- or HSEC-specific approach, e.g., adenoviral vector (AVV) delivery of siRNA, would perhaps be the most germane approach, as this would feasibly negate any potential off-target effects. Finally, the emerging evidence that scavenger receptors interact with other receptors and their multifunctional properties suggest that, as well as monotherapies, scavenger receptors could also be combined with other therapies, for example TLR-directed treatments, to alter leukocyte trafficking and boost the effectiveness of other therapies which target other arms of the immune response.

CONCLUSIONS

There is an increasing amount of evidence describing the role of endothelial-expressed scavenger receptors in leukocyte trafficking. In this capacity, a number of scavenger receptors are able to directly interact with leukocytes and mediate their passage across a range of endothelia. This secondary function is relatively understudied and further work could lead to novel immunological therapies which could effectively treat inflammatory conditions and contribute to combinatorial approaches to manage these conditions.

DISCLAIMER

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AUTHOR CONTRIBUTIONS

DP conceived the review, wrote, and edited the manuscript. SS wrote the manuscript.

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Control of Leukocyte Trafficking by Stress-Associated Hormones

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Leukocyte migration is a crucial process in both homeostatic and inflammatory conditions. The spatiotemporal distribution of immune cells is balanced between processes of cellular mobilization into the bloodstream, their adhesion to vascular beds and trafficking into tissues. Systemic regulation of leukocyte mobility is achieved by different signals including neuronal and hormonal cues, of which the catecholamines and glucocorticoids have been most extensively studied. These hormones are often associated with a stress response, however they regulate immune cell trafficking also in steady state, with effects dependent upon cell type, location, time-of-day, concentration, and duration of signal. Systemic administration of catecholamines, such as the sympathetic neurotransmitters adrenaline and noradrenaline, increases neutrophil numbers in the bloodstream but has different effects on other leukocyte populations. In contrast, local, endogenous sympathetic tone has been shown to be crucial for dynamic daily changes in adhesion molecule expression in the bone marrow and skeletal muscle, acting as a key signal to the endothelium and stromal cells to regulate immune cell trafficking. Conversely, glucocorticoids are often reported as anti-inflammatory, although recent data shows a more complex role, particularly under steady-state conditions. Endogenous changes in circulating glucocorticoid concentration induce redistribution of cells and potentiate inflammatory responses, and in many paradigms glucocorticoid action is strongly influenced by time of day. In this review, we discuss the current knowledge of catecholamine and glucocorticoid regulation of leukocyte migration under homeostatic and stimulated conditions.

Keywords: catecholamine, glucocorticoid, adrenergic signaling, neutrophil, lymphocyte, circadian rhythm

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INTRODUCTION

Leukocytes migrate through the body by shuttling between the vascular system and tissues. Within the vasculature, immune cells freely circulate or are firmly attached to the vessel wall, effectively removing them from the circulation in what is known as the marginal pool. Adherent cells may be in the process of exiting the circulation to immigrate into organs [reviewed in (1)]. However, still vasculature-bound, marginated cells can also detach and be remobilized into the bloodstream—a process which is called demargination (**Figure 1**). Leukocytes adhere to the vasculature in a sequence of events known as the leukocyte adhesion cascade. This cascade is crucial for a functioning immune system, allowing immune cells to infiltrate tissues that are in need of pathogen

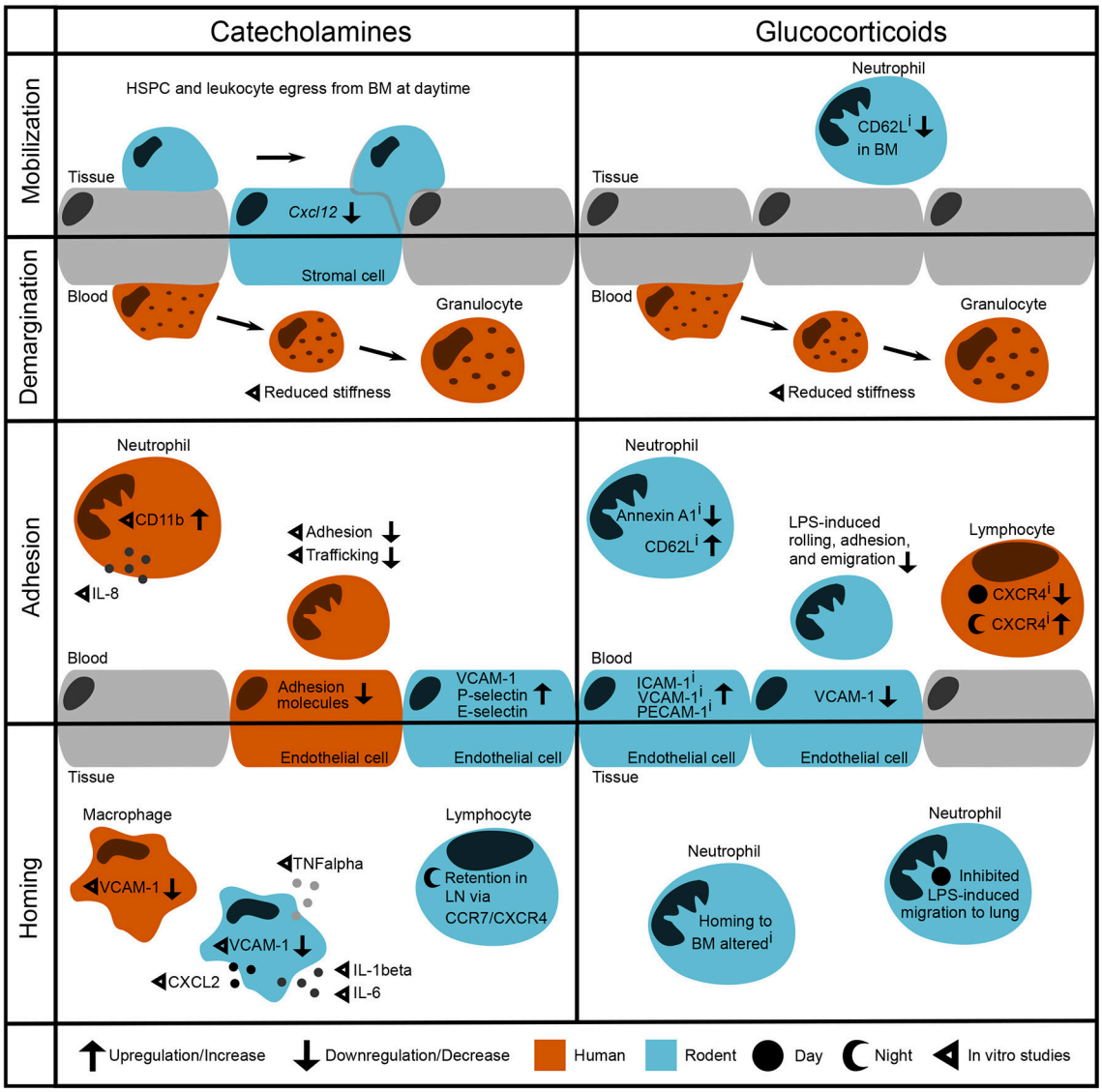

FIGURE 1 | Modulation of leukocyte trafficking by stress-associated hormones. Leukocyte migration can be broadly broken down into mobilization and homing (entering/leaving the vasculature, respectively) as well as adhesion and demargination (attachment to/detachment from the vessel wall, respectively). Catecholamines control hematopoietic stem and progenitor cell (HSPC) and leukocyte egress from the bone marrow during daytime under steady-state conditions by downregulation of the retention factor CXCL12 in stromal cells (2). *in vitro* studies showed that after incubation with catecholamines and glucocorticoids, human granulocytes detach more easily by reducing their stiffness (3). In the bloodstream, human neutrophils show increased levels of CD11b as well as IL-8 after stimulation with adrenaline (4). However, their adhesion and trafficking *in vitro* are reduced due to downregulation of endothelial adhesion molecules (5, 6). In contrast, mouse endothelial cells upregulate VCAM-1, P-selectin, and E-selectin after catecholamine stimulation (7). In both humans and rodent macrophages, VCAM-1 levels are regulated through β_2-adrenoceptor signaling (8). In addition, catecholamines induce cytokine release by murine macrophages (9). In mice, sympathetic stimulation leads to a retention of T cells in the lymph node via upregulation of CCR7 and CXCR4 (10, 11). Inhibition of glucocorticoid receptors downregulates Annexin A1 levels (12) and upregulates CD62L expression on circulating murine neutrophils whilst downregulating its expression in the bone marrow (13). Furthermore, murine neutrophils show increased LPS-induced adhesion when treated with a GR antagonist—although endothelial VCAM-1 is downregulated (14). Human naïve T cells show upregulated CXCR4 levels when treated with a GR antagonist during the night, whereas CXCR4 is downregulated when treated during the day (15). Similarly, GR agonism with dexamethasone inhibits LPS-induced neutrophil migration to the lung in the behavioral resting phase (16, 17). i denotes effects of inhibition.

clearance or regeneration. Leukocytes initially roll along the vessel wall with the help of cell adhesion molecules where they can be activated by chemokines on the vascular endothelium, leading to their arrest, and transmigration through the endothelial barrier to exit the bloodstream and enter underlying tissues [reviewed in (18) and illustrated in **Figure 1**]. These different stages of the adhesion cascade can be modulated by various factors, including circulating hormones such as catecholamines and glucocorticoids. It has been known for decades that the sympathetic nervous system, a key source

of catecholamines, regulates the maturation and function of leukocytes via adrenoceptors on their surface [see (19) for an in-depth overview, also on the expression profile of adrenoreceptors]. However, the regulation of leukocyte trafficking by catecholamines and glucocorticoids (typically classed as stress hormones) and their interplay in steady state and stress conditions is multifaceted and therefore incompletely understood. In this review we focus on the recent findings in this field, which we have summarized in **Table 1**.

CATECHOLAMINES

Catecholamines, such as adrenaline and noradrenaline, are an important class of systemic immune-modulators, released systemically by the adrenal gland and locally mainly by sympathetic nerves. These hormones have immune-enhancing or immune-suppressing effects, depending on the duration of the signal (acute vs. chronic), the microenvironment, and the timing of their release (27). In mice it was demonstrated that under steady-state conditions, the release of hematopoietic stem and progenitor cells requires local delivery of noradrenergic signals to the bone marrow by sympathetic nerves, where they are transmitted to stromal cells via β_3-adrenoceptors, leading to a downregulation of the key retention factor CXCL12 (2). A similar phenomenon may contribute to the release of leukocytes into the bloodstream in acute stress, as in rats administration of adrenaline and noradrenaline has been shown to increase circulating myeloid and lymphoid cell numbers within a few minutes. In this scenario, most subpopulations have left the blood after 2 h, except for neutrophils, whose numbers continue to increase (20). Differences in the effect on subpopulation specificity are evidenced by the fact that noradrenaline increases numbers of circulating neutrophils and B cells, whereas adrenaline increases the number of neutrophils and monocytes but decreases lymphocyte numbers in blood. (20) (**Table 1**). The underlying signaling pathways and receptors responsible for these distinct outcomes are, however, ambiguous. For example, it is currently not clear how much of the increase of blood leukocyte numbers is caused by a stress-induced mobilization from hematopoietic tissues into blood, or by demargination from the vessel wall.

Stress hormones can affect leukocyte migratory properties via diverse mechanisms. A recent publication provided the first evidence that catecholamines can induce the rearrangement of cellular cortical actin in human granulocytes, thereby decreasing cell stiffness and leading to leukocyte demargination (3). This could explain the very fast increase in circulating leukocyte numbers by these hormones without the need of mobilization from tissues, allowing the organism to respond quickly to acute signals. Additionally, catecholamines can alter cytokine levels and expression of adhesion molecules. Exposure to adrenaline *in vitro* increases interleukin-8 (IL-8) expression and CD11b (alpha-M-integrin) levels in human neutrophils (4). Under LPS-induced inflammatory conditions the production of IL-1, IL-8, and CCL2 is reduced, indicating that regulation of cytokines and chemokines by adrenaline is highly dependent on the inflammatory milieu (4). In contrast to this study, *in vitro* stimulation with the adrenergic agents adrenaline, noradrenaline, or the agonist isoproterenol reduced N-formyl-methionyl-leucyl-phenylalanine (fMLP)-induced human polymorphonuclear cell (PMN) migration, CD11b/CD18 (Mac-1) integrin expression, as well as production of reactive oxygen species, without affecting IL-8 levels (21). Furthermore, adrenaline and dopamine, a structurally-related catecholaminergic neurotransmitter, facilitated the down-modulation of adhesion molecule expression in human umbilical cord vein endothelial cells (HUVECs), reducing neutrophil adhesion (6). Thus, experiments using catecholamines or their agonists have thus far provided different outcomes, which is most likely dependent on the dosage used and the microenvironmental context. What is clear, however, is that they exert effects on both the immune cell and the endothelial aspects of the adhesion cascade, by modulating expression of adhesion molecules, cytokine levels and leukocyte stiffness.

In addition to their direct influence on the leukocyte adhesion cascade, catecholamines also modulate functions of macrophages, a resident leukocyte subset. As major producers of cytokines, these phagocytic cells are likely largely responsible for the effects of catecholamines on cytokine levels. Adrenaline and noradrenaline can directly activate NF-κB in isolated peritoneal mouse macrophages, resulting in the release of pro-inflammatory cytokines including TNFα, CXCL2, IL-1β, and IL-6 (9). In murine skin wounds, tissue-resident macrophages produce IL-6 in response to chronic β_2-adrenergic receptor activation, which in turn leads to a persistent trafficking of neutrophils to the site of injury (22). This is one potential mechanism by which long-term stress may be associated with a delayed wound healing. However, phagocytes themselves can also produce catecholamines and in a rat model of acute lung injury, elevated levels of macrophage-derived catecholamines were associated with increased expression of pulmonary intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) via α_2-adrenoceptors. Work using knockout models of adrenoceptors could show that in mice and humans the expression of VCAM-1 in macrophages is sensitive to stimulation of β_2-adrenoceptors, which plays an important role in the cardiac infiltration of leukocytes to facilitate an early inflammatory repair response to an acute myocardial injury (8). Taken together, these findings demonstrate that catecholamines act on resident macrophages but can also be released by these cells, providing an additional, indirect mechanism in regulating the behavior of migratory cells.

β_2-adrenoceptors are the most common adrenergic receptor type expressed on leukocytes [reviewed in (28)]. However, mRNA for other adrenoceptor subtypes is also present in human immune cells (21). Pharmacological agonists for the α_2-adrenoceptor reduced trafficking of IL-8 activated human neutrophils by inhibition of CD62L shedding with simultaneous prevention of increased CD11b expression (5). *In vitro* flow chamber assays revealed that targeting the α_2-adrenoceptor in HUVECs, but not the neutrophils, decreased transendothelial migration of neutrophils (5). These data indicate that both leukocytes and the endothelium are important targets for

TABLE 1 | Effects of hormonal signals on leukocyte trafficking.

	Duration of stimulus	Receptor	Compound	Cell	Effect	References
Catecholamines	Acute (2 h *in vivo*)	Not assessed	A	Rat CD62L neg. monocytes	Increased numbers in blood	(20)
	Acute (2 h *in vivo*)	Not assessed	A, NA, NA+A	Rat monocytes	Increased numbers in blood, decreased CD62L expression	(20)
	Acute (2 h *in vivo*)	Not assessed	A, NA, NA+A	Rat CD62L neg. neutrophils	Increased numbers in blood	(20)
	Acute (2 h *in vivo*)	Not assessed	A, NA, NA+A	Rat CD62L pos. neutrophils	Increased numbers in blood	(20)
	Acute (2 h *in vivo*)	Not assessed	A	Rat CD62L neg. T, NK cells	Decreased numbers in blood	(20)
	Acute (2 h *in vivo*)	Not assessed	NA+A	Rat CD62L pos T, NK cells	Decreased numbers in blood	(20)
	Acute (2 h *in vivo*)	Not assessed	A, NA, NA+A	Rat NK cells	Decreased CD62L expression	(20)
	Acute (2 h *in vivo*)	Not assessed	A, NA+A	rat lymphocytes	Decreased numbers in blood, decreased CD62L expression	(20)
	Acute (2 h *in vivo*)	Not assessed	A, NA+A	Rat cytotoxic T cells	Decreased numbers in blood	(20)
	Acute (2 h *in vivo*)	Not assessed	NA	Rat CD62L neg. B cells	Decreased numbers in blood	(20)
	Acute (2 h *in vivo*)	Not assessed	A, NA, NA+A	Rat CD62L pos. B cells	Decreased numbers in blood, CD62L expression unaffected	(20)
	Acute (2 h *in vivo*)	Not assessed	NA	Rat B cells	Decreased numbers in blood	(20)
	Acute (2 h *in vivo*)	Not assessed	A, NA+A	Rat B cells	Decreased numbers in blood	(20)
	Acute (4 h *in vitro*)	Not assessed	A	Human neutrophils and monocytes	Increased CD11b expression; suppression of LPS-induced CD11b and CD18 expression	(4)
	Acute (4 h *in vitro*)	not assessed	A	Human white blood cells	Dose-dependent increase in IL-8 levels; suppression of LPS-induced production of IL-1β, IL-8, and CCL2	(4)
	Acute (90 min *in vitro*)	β-AR	A, NA, Isoprenaline	Human PMNs	Reduced fMLP-induced migration, CD11b/CD18 expression and ROS production	(21)
	Acute (30 min pre-treatment *in vitro*)	Not assessed	A	Human neutrophils	Reduced adhesion to HUVECs by down-modulation of EC adhesion molecule expression	(6)
	Acute (30 min *in vitro*)	Not assessed	A, NA	Mouse macrophages/neutrophils	Dose-dependent activation of NFκB, decrease of IκBα levels	(9)
	Acute (4 h *in vitro*)	Not assessed	A, NA	Mouse macrophages	Dose-dependent activation of NFκB, release of TNFα, IL-1β, IL-6, CXCL2	(9)
	Chronic (8 days *in vivo*)	β2-AR	A	Mouse macrophages	Production of IL-6, leading to persistent neutrophil trafficking	(22)
	None (endogenous)	β2-AR	Endogenous	Human/mouse macrophages	Changes in VCAM-1 expression levels	(8)
	None (endogenous)	β2-AR	Endogenous	Mouse lymphocytes	Inhibition of egress from lymph node through CCR7 and CXCR4	(10, 11)
	Acute (20 min *in vitro*)	α2-AR	Xylazine, UK14304	Human neutrophils	Reduced trafficking without affecting CD62L and CD11b expression	(5)
	Acute (6 h *in vitro*)	α2-AR	Xylazine, UK14304	Human endothelial cells	Decreased transendothelial migration of neutrophils	(5)
	Chronic (5 days *in vivo*)	β3-AR	BRL37344	Mouse endothelial cells	Upregulation of VCAM-1, P- and E-selectin expression, more BM homing	(7)
Glucocorticoids	Acute (6 h *in vivo*)	GR	Dexamethasone	Human granulocytes	Increased numbers in blood; detached more easily in *ex vivo* assay	(3)
	Acute (2 h *in vitro*)	GR	Dexamethasone	Human granulocytes	Detached more easily in *in vitro* assay	(3)
	Chronic (7 days *in vivo*)	GR	Mifepristone (RU486)*	Rat neutrophils	Increased numbers in blood; CD62L expression increased in blood, decreased in BM	(13)
	Int. (24 h and 2 h *in vivo*)	GR	Mifepristone (RU486)*	Mouse neutrophils	Decreased annexin A1, altered neutrophil maturation and homing	(12)

(Continued)

TABLE 1 | Continued

Duration of Stimulus	Receptor	Compound	Cell	Effect	References
Acute (10 h *in vivo*)	GR	Mifepristone (RU486)*	Human T cells	Increased CXCR4 expression in behavioral rest phase, decreased in active phase (inverse to blood numbers)	(15)
None (endogenous)	GR	Endogenous	Mouse T cells	When T cell GR is disrupted, CXCR4 expression is reduced and homing impaired in active phase	(23)
Acute (8 h *in vivo*)	MR	Fludrocortisone	Human naïve T cells	Agonism decreased circulating numbers, increased CXCR4 expression (*in vivo*)	(24)
Acute (2–4 h *in vitro*)	MR	Spironolactone*/ Fludrocortisone	Human naïve T cells	Agonism increased CXCR4 and CD62L expression, antagonism decreased CD62L and CCR7 expression	(24)
Acute (1 h pre-treatment *in vivo*)	GR	Dexamethasone	Mouse leukocytes	Reduced LPS-induced adhesion	(14)
Int. (18 h and 1 h pre-treatment *in vivo*)	GR	Mifepristone (RU486)*	Mouse leukocytes	Increased LPS-induced adhesion, but reduced endothelial VCAM-1 expression	(14)
Acute (1 h pre-treatment *in vivo*)	GR	Dexamethasone	Mouse neutrophils	Inhibited LPS-induced neutrophil migration into lungs if administered during rest phase, but not during active phase	(16, 17)
Chronic (trait assessments)	Not assessed	Endogenous	Macaque leukocytes	Positive correlation of cortisol and neutrophil numbers in blood in low-nervous animals, no association in high nervous animals	(25)
Chronic (16 months) + acute (2 h)	GR	Endogenous (stress) + dexamethasone	Macaque leukocytes	Stressed animals show reduced sensitivity to dexamethasone-induced reduction of circulating lymphocytes	(26)

Summary of main effects of catecholamines and glucocorticoids upon leukocyte migration as described in the literature.
**Denotes antagonist; A, adrenaline; AR, adrenoceptor; BM, bone marrow; GR, glucocorticoid receptor; MR, mineralocorticoid receptor; NA, noradrenaline.*

catecholaminergic signaling in the regulation of leukocyte trafficking. However, the exact mechanisms in different cell types and the interplay of systemic and local factors remain to be identified.

Whereas most studies have investigated the effects of systemic administration of catecholamines and thereby mimicking a stress response, other reports focused on the ablation of catecholaminergic signaling and thus the endogenous role these hormones play in steady state. One study examined the consequence of unilateral surgical ablation of local nerves in mice upon leukocyte adhesion to innervated tissues such as bone marrow and skeletal muscle. Whilst leukocyte adhesion in nerve-intact organs showed a diurnal rhythm (high at night onset, lower during the day), this was abolished in denervated tissues. This pattern corresponded to a rhythmic expression pattern of ICAM-1 in mouse vascular endothelial cells, which was flattened after denervation (7). Rhythms in adherent leukocyte cell numbers were equally lost in mice lacking β_2- or β_3-adrenoceptors, indicating that rhythmic adhesion requires local delivery of adrenergic signals by nerves and that the microenvironment is an important regulator of leukocyte trafficking and target site of stress hormones. In murine lymph nodes, activation of β_2-adrenoceptors leads to the retention of lymphocytes and therefore affects the extent of adaptive immune responses (11). Under steady state conditions, lymphocyte numbers in lymph nodes peak at night (11, 23, 29), which coincides with peak levels of noradrenaline in these tissues (11). After functional depletion of adrenergic nerves using a sympathetic neurotoxin (6-OHDA), restricted lymphocyte egress from the lymph node in the active phase of the animals was observed. The same group had previously demonstrated the physical interaction of β_2-adrenoceptors with the chemokine receptors CCR7 and CXCR4, which are critically involved in lymphocyte homing to and their retention in murine lymph nodes (10). These data therefore provide evidence for an important time-of-day-dependent regulation of migratory factors on leukocytes and non-hematopoietic cells by β_2-adrenergic signaling under homeostatic conditions.

Lack-of-function assays are also suited to tease apart the complex interplay of signaling pathways involved in the hormonal regulation of leukocyte migration. Previous data reported that adrenergic signaling through β_3-adrenoceptors

promotes rhythmic egress of hematopoietic stem cells from the mouse bone marrow via downregulation of the retention factor CXCL12 (2). Activation of β_3-adrenoceptors during the day promotes egress from bone marrow, yet activation of β_2- or β_3-adrenoceptors at night promotes homing of murine leukocytes to tissues (2, 7). This apparent paradox was recently investigated in the context of cholinergic signaling, which is a potent inhibitor of endothelial activation in inflammatory scenarios (30) and part of the inflammatory reflex pathway (31, 32). Using mice with decreased cholinergic tone, García-García et al. found that during the day, acetylcholine inhibits vascular adhesion while noradrenergic signals promote egress via β_3-adrenoceptors, providing complementary effects which increase leukocyte content in blood. At night, the higher circulating adrenaline levels preferentially stimulate β_2-adrenoceptors while at the same time sympathetic cholinergic signals downregulate β_3-adrenoceptor expression, promoting nocturnal homing (33). This series of studies highlights the complex interactions between different signaling pathways *in vivo* and the importance of considering neuroendocrine regulation of leukocyte trafficking in an integrative manner.

GLUCOCORTICOIDS

The adrenal-derived steroid hormones (glucocorticoids and mineralocorticoids) are another significant class of stress hormones which influence leukocyte migration. Produced in the adrenal cortex, these hormones bind to their cognate receptors [glucocorticoid receptor (GR) and mineralocorticoid receptor (MR)] but with significant overlap. Whilst mineralocorticoids such as aldosterone can only bind MR, endogenous glucocorticoids such as cortisol (humans) and corticosterone (rodents) can bind both receptors. However, due to the higher affinity of endogenous glucocorticoids for MR, this receptor is favored at lower glucocorticoid concentrations and signaling via GR emerges at higher concentrations (34, 35). Appropriate balance between the MR/GR pathways is regulated by the 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes. 11β-HSD2 converts cortisol and corticosterone into inactive forms, effectively restricting MR signaling to mineralocorticoids in tissues where it is highly expressed, such as the kidney. On the other hand, 11β-HSD1, highly expressed in the liver, can "reactivate" these inactive compounds and locally increase glucocorticoid signaling [see (36) for a review of 11β-HSD functions]. With the DNA-binding domains of human GR and MR showing 94% identity (37), there is also a degree of commonality in their target genes and effects. Innate and adaptive immune cell populations express both MR [reviewed in (38)] and GR, although some sex-specific differences in GR expression levels and isoform distribution are reported in human cells (39). The use of specific, synthetic compounds is therefore more commonly employed to allow more refined investigations into the relative contributions of these pathways, as synthetic glucocorticoids such as dexamethasone show much higher affinity for GR than endogenous ligands (approx. 5-fold) and remain a major class of anti-inflammatory agents in clinical use.

Recently, Fay et al. investigated the influence of glucocorticoid administration on leukocyte demargination and found similar effects to that of catecholamines. Dexamethasone led to increased leukocyte numbers in the bloodstream of patients. *In vitro* experiments showed that dexamethasone increased granulocyte demargination independently of changes in vascular adhesion molecule expression. Although not to the same extent as adrenaline, *in vitro* dexamethasone treatment also induced changes to the actin cytoskeleton, leading to softening of granulocytes and enabling their detachment (3). In addition to effects on biophysical properties of leukocytes, glucocorticoids modulate expression of key receptors on leukocytes to influence maturation, homing, and egress. Neutrophil maturation is accelerated in rats treated with a GR antagonist (mifepristone/RU486) (13), an effect which may be attributable to reduced expression of Annexin A1. Annexin A1 is up-regulated by glucocorticoids, and circulating neutrophils from Annexin A1-deficient mice express higher levels of CXCR4, representing an 'aged' phenotype (12). Annexin $A1^{-/-}$ neutrophils did not migrate as efficiently as wild-type cells to CXCL12 *in vitro*, and stromal cells from Annexin $A1^{-/-}$ mice also produced less CXCL12 *in vivo*. The accelerated maturation and inability to home leads to persistent neutrophilia in these mice, and may be a route through which GR antagonism exerts its effects (12). Recent work has also shown this pathway to be involved in the redistribution of T cells (15, 23). In humans, GR antagonism using mifepristone affected T cell CXCR4 expression in a manner dependent on circulating cortisol levels. Using timed administration of mifepristone it was revealed that when endogenous cortisol was low, the GR antagonist increases CXCR4 expression on $CD4^+$ and $CD8^+$ subsets through a partial agonist effect, whereas administration when cortisol was high led to reduced CXCR4 expression by traditional antagonism (15). This axis has been more extensively investigated in mice, where GR agonism was shown to increase expression of the IL-7 receptor, which then drove increased CXCR4 expression when circulating glucocorticoids were high. Significantly fewer memory $CD4^+$ T cells were observed in spleen, lymph node, and lungs of mice lacking GR in T cells than in wild type controls, suggesting that cell-intrinsic GR signaling enhances survival of this population and promotes migration to peripheral lymphoid tissues (23). Furthermore, MR signaling also increases CXCR4 expression on naïve human T cells but does so along with CD62L and CCR7, suggesting that MR activation facilitates homing to lymph nodes whereas GR activation preferentially drives cells toward the bone marrow (24). In an inflammatory scenario, glucocorticoid administration generally inhibits the immune response, as GR activation decreases expression of many pro-inflammatory cytokines. In a mouse model of LPS-induced inflammation, dexamethasone treatment also resulted in reduced leukocyte rolling flux, adhesion and emigration, along with reduced circulating leukocyte counts, whereas mifepristone treatment increased adhesion and emigration (14). These data show that GR agonism attenuates interactions between leukocytes and the endothelium in this model, consistent with dexamethasone-induced inhibition of ICAM-1 and VCAM-1 expression on the inflamed endothelium. Interestingly,

the blockade of endogenous GR signaling by mifepristone resulted in a counter-intuitive decrease in VCAM-1 expression, suggesting that there may be a difference between endogenous and exogenous glucocorticoids and their effects on leukocyte-endothelium interactions (14). It will be interesting to see whether further studies can dissect the relative contributions of endogenous or exogenous glucocorticoids and their signaling through GR and/or MR.

In addition to sensitivity of adrenergic and glucocorticoid signaling to acute environmental signals and stressors, these signals are also regulated on a longer time scale by the circadian rhythm [see (40) for a review of circadian regulation of immune function]. Circulating glucocorticoids and adrenergic tone both increase at the start of an organism's behavioral active phase, providing a rhythmic signal promoting redistribution of leukocytes across the body. The influence of such rhythmic signal is seen in the results of Besedovsky et al. (15), where the diurnal oscillation in endogenous cortisol significantly influenced the ability of GR antagonism to elicit changes in human T cell CXCR4 expression. Shimba et al. (23) also addressed the role of GR in a rhythmic manner, supporting data by other groups (11, 29) and providing an additional mechanism to regulate leukocyte trafficking in a daily cycle. In inflammatory scenarios a rhythmic glucocorticoid signal is known to modulate chemokine signaling and neutrophil trafficking to the mouse lung via time-of-day dependent inhibition of epithelial CXCL5 production (16, 17), providing an additional layer of fine-tuning the inflammatory response. Under chronic stress conditions, however, elevated glucocorticoid levels are associated with a reduction in cellular sensitivity to these hormones. Experiments using rhesus macaques have illustrated a link between both nervous temperament and social stress and impaired leukocyte trafficking patterns (25, 26). Whilst control animals showed glucocorticoid-induced redistribution of circulating leukocytes, those exposed to social stress showed a reduced correlation between cortisol concentration and blood lymphocyte content (26). In further experiments without social manipulation but with analysis of behavior and temperament, the expected correlation between cortisol and blood neutrophil counts was found at a population level, but this was significantly attenuated in nervous macaques (25). These results have interesting implications for human scenarios of disrupted neuroendocrine functions, stress, and anxiety. In these situations, a disconnection appears between circulating hormone levels and inflammatory cell responsiveness, which may explain the lack of efficacy of glucocorticoid treatment in some patients. Furthermore, there may even be a cycle of inflammatory exacerbation due to the effects of stress upon monocyte trafficking and microglial activation, whereby reactive endothelium and enhanced trafficking of cells to the brain releases cytokines and reinforces stress- and anxiety-like signaling [reviewed in (41, 42)]. Similar to catecholamines, glucocorticoids are key systemic orchestrators of immune cell migration. Yet, due to the complexity of the interlocking signaling cascades in different leukocyte subsets and tissues, the precise effects in different sites of the body remain elusive.

CONCLUSION

In summary, the hormones adrenaline, noradrenaline and glucocorticoids, typically associated with a stress response, exert diverse effects on leukocyte migration under both steady-state and stimulated conditions. These effects are dependent not only on the responding cell type but also on location, duration and source of the stress/hormone signal, inflammatory context, and even time of day. Whereas adrenaline increases circulating neutrophil numbers, it reduces lymphocyte numbers in blood. Noradrenaline, on the other hand, increases both neutrophil and B cell numbers with distinct temporal profiles. Glucocorticoids can act to redistribute T cells from the bloodstream into organs at their endogenous peak levels, but synthetic agonists are widely used in inflammatory scenarios to inhibit chemokine production and disrupt excessive inflammatory responses. This potential divergence between the function of endogenous hormones and their clinical counterparts should be explored further, particularly with respect to cell-specific differences in receptor expression and diurnal rhythms in endogenous hormone concentrations. To achieve this, analyses using lineage specific ablation of hormone receptors will be needed in combination with well-controlled *in vitro* and *in vivo* studies to dissect their complex and highly interwoven signaling pathways and functions.

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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REVIEW
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Molecular Players in Hematologic Tumor Cell Trafficking

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The trafficking of neoplastic cells represents a key process that contributes to progression of hematologic malignancies. Diapedesis of neoplastic cells across endothelium and perivascular cells is facilitated by adhesion molecules and chemokines, which act in concert to tightly regulate directional motility. Intravital microscopy provides spatio-temporal views of neoplastic cell trafficking, and is crucial for testing and developing therapies against hematologic cancers. Multiple myeloma (MM), chronic lymphocytic leukemia (CLL), and acute lymphoblastic leukemia (ALL) are hematologic malignancies characterized by continuous neoplastic cell trafficking during disease progression. A common feature of these neoplasias is the homing and infiltration of blood cancer cells into the bone marrow (BM), which favors growth and survival of the malignant cells. MM cells traffic between different BM niches and egress from BM at late disease stages. Besides the BM, CLL cells commonly home to lymph nodes (LNs) and spleen. Likewise, ALL cells also infiltrate extramedullary organs, such as the central nervous system, spleen, liver, and testicles. The α4β1 integrin and the chemokine receptor CXCR4 are key molecules for MM, ALL, and CLL cell trafficking into and out of the BM. In addition, the chemokine receptor CCR7 controls CLL cell homing to LNs, and CXCR4, CCR7, and CXCR3 contribute to ALL cell migration across endothelia and the blood brain barrier. Some of these receptors are used as diagnostic markers for relapse and survival in ALL patients, and their level of expression allows clinicians to choose the appropriate treatments. In CLL, elevated α4β1 expression is an established adverse prognostic marker, reinforcing its role in the disease expansion. Combining current chemotherapies with inhibitors of malignant cell trafficking could represent a useful therapy against these neoplasias. Moreover, immunotherapy using humanized antibodies, CAR-T cells, or immune check-point inhibitors together with agents targeting the migration of tumor cells could also restrict their survival. In this review, we provide a view of the molecular players that regulate the trafficking of neoplastic cells during development and progression of MM, CLL, and ALL, together with current therapies that target the malignant cells.

Keywords: hematological cancer, cell trafficking, adhesion molecule, chemokines (CK), immunotherapy

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INTRODUCTION

The trafficking of hematologic malignant cells follows trajectories that are governed by the functional involvement of adhesion and chemokine receptors expressed by these cells, and their ligands exposed at specific homing sites. The lodging of neoplastic cells at these sites starts with cancer cell diapedesis across endothelia and perivascular cell layers. This is followed by cell migration in response to cell-bound and extracellular matrix (ECM)-bound chemokines as well as to ECM proteins, which guide the tumor cells to permissive niches. Among the various hematologic malignancies, we will focus on three lymphoproliferative disorders: multiple myeloma (MM), chronic lymphocytic leukemia (CLL), and acute lymphocytic leukemia (ALL). A common trafficking step during progression of these malignancies is the neoplastic cell migration into and out of the bone marrow (BM). In addition, CLL cells travel to lymph nodes (LNs), whereas ALL cells infiltrate extramedullary organs such as the central nervous system (CNS). Localization in these niches is beneficial to the malignant cells, as they receive survival and proliferative signals, which contribute to progression of the disease.

MM is the second most common hematologic malignancy and is characterized by the accumulation of malignant plasma cells at multiple sites in the BM. This causes the characteristic multifocal lesions that highlight MM cell ability to traffic into and out of different niches in the BM (1–3). In most cases, MM is preceded by an asymptomatic pre-malignant condition, monoclonal gammopathy of undetermined significance, followed by another asymptomatic phase called smoldering myeloma (1, 2, 4, 5). The latest MM phases are characterized by the egress of MM cells from the BM to the bloodstream, once they become independent from growth and survival signals provided by the BM, a condition named extramedullary disease. MM cells in circulation can subsequently colonize different organs, or develop plasma cell leukemia (6). Alkylating agents, proteasome inhibitors, steroids, autologous stem cell transplantation, and immunomodulatory drugs are the most frequent protocols in MM treatment (7–10). Furthermore, immunotherapy protocols with monoclonal antibodies and CAR-T cells are entering a new era of MM treatment. Yet, although substantial improvement in patient survival has been achieved in recent years, MM remains mostly incurable. In addition, resistance responses to proteasome inhibitors and immunomodulatory agents represent important clinical challenges in MM treatment (7).

CLL, the most common leukemia in Western countries, is characterized by the accumulation of mature $CD5^+$ B lymphocytes in the peripheral blood (PB) and the progressive infiltration of lymphoid organs by these cells (11, 12). The traffic of CLL cells between PB and lymphoid organs, as well as the malignant cell retention in these tissues, is regulated by adhesive and migratory molecules and contributes to CLL progression (13, 14). Clinically, CLL is a heterogeneous malignancy, with good or poor prognosis mostly determined by the presence of specific markers, particularly mutated (M-CLL) or unmutated (U-CLL) immunoglobulin heavy-chain variable region (IGVH) (11, 12). Differences in adhesion/migration pathways between M-CLL and U-CLL have also been demonstrated by proteomic analyses, which showed that U-CLL cells have a less migratory and more adhesive protein pattern than M-CLL cells (15). This fact could favor their retention in lymphoid tissues and the presence of lymphadenopathy, as observed in U-CLL patients. Current therapies for CLL include the combination fludarabine-cyclophosphamide-rituximab, as well as the newer compounds ibrutinib (Bruton's tyrosine kinase [BTK] inhibitor), idelalisib (phosphatidylinositol 3-kinase δ [PI3-Kδ] inhibitor), and venetoclax (Bcl-2 inhibitor) (12, 16). Although many patients respond to treatment and some achieve remission, CLL remains an incurable disease.

ALL is the most frequent pediatric cancer and accounts for 20% of adult leukemia (17). ALL leukemic cells can originate from B-cell lymphoblasts (B-ALL, 85% of ALL) or T-cell progenitors (T-ALL, 15% of ALL). T-ALL is relatively rare and characterized by an inferior treatment outcome than B-ALL. Clinically, the standard-risk ALL comprises those patients between 1 and 10 years old (y.o.), hyperdiploidy and the translocation t(12;21) ETV6/RUNX1. In contrast, high-risk includes those patients younger than 1 y.o. or elder than 10 y.o., an initial leukocyte count higher than 50,000 per cubic millimeter, hypodiploidy, and other genomic alterations (18, 19). Currently, standard induction therapy includes several anti-tumor drugs such as prednisone, dexamethasone or vincristine, with or without prophylactic intrathecal therapy. At the end of the induction, the complete remission or the presence of MRD is evaluated, as patients with MRD after chemotherapy present higher risk of relapse and death (18).

ALL cells use similar molecular mechanisms than normal lymphocytes to migrate across physical barriers (20). ALL initiates either in the BM or in the thymus, and leukemic cells may remain in these organs or egress, entering the circulation and infiltrating other tissues such as the spleen, CNS, and testes. ALL cells located in the BM or migrating through other tissues interact with highly complex microenvironments composed of ECM proteins (collagens, fibronectin, laminin, proteoglycans), soluble molecules (cytokines, chemokines, and growth factors), and other cell types (stromal cells, osteoblasts, endothelial cells, and macrophages) (21). Recent evidence, based on the use of an *in vitro* 3D microfluidic system that includes stromal cells, osteoblasts, and B-ALL cells, supports the notion that biophysical properties, such as the matrix stiffness drive ALL progression and dissemination (22).

Integrins are the main adhesion receptors facilitating the trafficking of neoplastic cells. Integrins are heterodimers of α and β subunits that mediate cell-cell and cell-ECM interactions, and connect the ECM with the actin cytoskeleton (23, 24). Additionally, integrin-dependent cell adhesion triggers intracellular signaling that contributes to the control of cell growth and survival (23, 25). Integrins adopt different conformations, which determine their state of activation linked to their ability to bind ligands with high-affinity and to induce subsequent intracellular signaling (26–29). Integrin activation is a dynamic process that can be achieved by several stimuli from outside (outside-in) or inside (inside-out) the cell, a property

that highlights the integrin role as main connectors between the cancer cells and their environment (24).

Chemokines are chemotactic cytokines that promote cell migration and activation under homeostatic and inflammatory conditions, and play critical roles during hematopoiesis, immune surveillance and inflammation, morphogenesis, and neovascularization, as well as in the trafficking of hematologic tumor cells (30–32). Chemokines bind to seven transmembrane-spanning receptors coupled to heterotrimeric guanine nucleotide-binding (G) proteins, which transmit intracellular signals for cell adhesion, migration, and survival (30, 33–35). Ligand binding by chemokine receptors involves the receptor N-terminal domain and three extracellular loops, whereas the intracellular loops and the C-terminal region are coupled to receptor internalization and to heterotrimeric G proteins, respectively (35). The conserved DRY motif is located intracellularly, and is critical for coupling the chemokine receptor to G proteins and for transmitting downstream signaling. Several atypical receptors, including CXCR7 and DARC, lack the DRY motif and are unable to associate with G proteins (36) and induce signaling, therefore acting as scavengers for chemokines (37). Besides binding to these receptors, chemokines also interact with glycosaminoglycans (GAGs), and this contributes to chemokine retention on the surface of endothelial cells (38).

Selectins have also been implicated in the initial adhesion steps of the trafficking of hematologic tumor cells. Selectins are a family of C-type lectin receptors divided according to their expression in leukocytes (L-selectin), platelets (P-selectin), or endothelial cells (E- and P-selectins) (39, 40). The roles of these cell surface receptors and their glycosylated ligands have been extensively explored in leukocyte recruitment, granular secretion, and placental development (40, 41). Selectins and their ligands are crucial in multiple physiological and pathological situations, including those related to cancer and immune response (39). Of note, cancer cells present changes in cell-surface glycosylation that are recognized by selectins, galectins, and siglecs (42). For this reason, targeting selectin-ligand interactions has clinical relevance for cancer immunotherapies.

Matrix metalloproteinases (MMPs) are a large family of Zn^{2+}-dependent proteases that facilitate cell migration by degrading basement membranes and ECM, as well as by releasing matrix-bound chemokines and growth factors (43). In depth proteomic analyses have demonstrated that MMPs can degrade many other substrates, including cytoskeletal proteins and signaling molecules (44, 45). Additionally, it is now well-established that many MMPs also display non-catalytic activities, which mostly rely on their localization at the cell surface, either *via* their transmembrane domain (MT-MMPs), or by binding to specific cell surface receptors (46). MMP-9 (gelatinase-B) is the most relevant MMP regulating the migration and other functions of lymphocytes.

In this review we summarize the most relevant molecules involved in MM, CLL, and ALL cell trafficking, indicating their function, interconnection, and possible use as therapeutic targets.

INTEGRINS IN HEMATOLOGIC TUMOR CELL TRAFFICKING

The α4β1 Integrin in MM, CLL, and ALL

Compelling evidence has clearly established that the α4β1 integrin (CD49d/CD29, very late antigen-4, VLA-4) is a key molecule involved in hematopoietic cell trafficking. α4β1 interacts with the IgG domains 1 and 4 of vascular cell adhesion molecule-1 (VCAM-1, CD106) and with the CS-1 site (EILDV sequence) in fibronectin (47). In addition, α4β1 is a receptor for MMP-9 in CLL cells and recognizes the specific sequence VPLDTHDVFQ, located in blade 4 of the MMP-9 hemopexin domain (48, 49). Besides contributing to lymphocyte trafficking to sites of injury and infection, α4β1 plays key roles during lymphopoiesis and myelopoiesis in the BM (50).

The attachment of MM cells to the α4β1 ligands VCAM-1 and fibronectin, which are present in the BM microenvironment (**Figure 1**), was recognized early (51, 52) and later shown to contribute to MM progression in *in vivo* models (53, 54). We recently demonstrated by intravital imaging a key role of α4β1 in MM and CLL cell attachment to the BM microvasculature (55) (**Figures 1**, **2**). Furthermore, *in vivo* experiments have demonstrated that blocking α4β1 function with specific antibodies abolishes homing of CLL (55–57) and primary B-ALL cells (58–60) to BM and LNs (**Figures 2**, **3**). The α4β1 integrin is expressed in ∼40% of CLL cases, and $\alpha4^+$ cells show increased migratory capacity when tested *in vitro* (61). Indeed, BM infiltration by CLL cells directly correlates with the levels of α4β1 expression (57, 62), and high α4β1 levels correlate with early development of lymphadenopathy (56, 63, 64). Likewise, minimal residual disease tumor cells from myeloma BM samples have high α4β1 expression (65), whereas the levels of this integrin are much lower in circulating MM cells (66). This evidence reveals that α4β1 plays a crucial functional role in the engraftment and progression of these hematologic malignancies.

Because CLL expansion relies on the ability to home and locate in lymphoid tissues, α4β1 constitutes a robust, independent prognostic marker, and its high expression (>30% positive cells) adversely correlates with overall and progression-free survival (67–69). In line with this, α4β1 was shown to be overexpressed in CLL cases with trisomy 12, a genetic alteration associated with higher cell proliferation and disease progression (70). Additionally, *in vitro* and *in vivo* phenotypic studies have shown that transendothelial migration regulates α4β1 expression, as LN-derived CLL cells have higher α4β1 levels than PB-CLL cells (71, 72). α4β1 is also regulated by the B-cell receptor (BCR), and targeting this receptor constitutes a therapeutic option in CLL (see below). α4β1 is also an independent risk factor in pediatric B-ALL, where high α4β1 expression associates with worse probabilities of relapse-free and overall survival (58, 73). Interestingly, low α4β1 levels correlate with adverse survival in a cohort of adult patients with T- and B-ALL (74), suggesting that α4 expression associates with poor outcome only in pediatric patients.

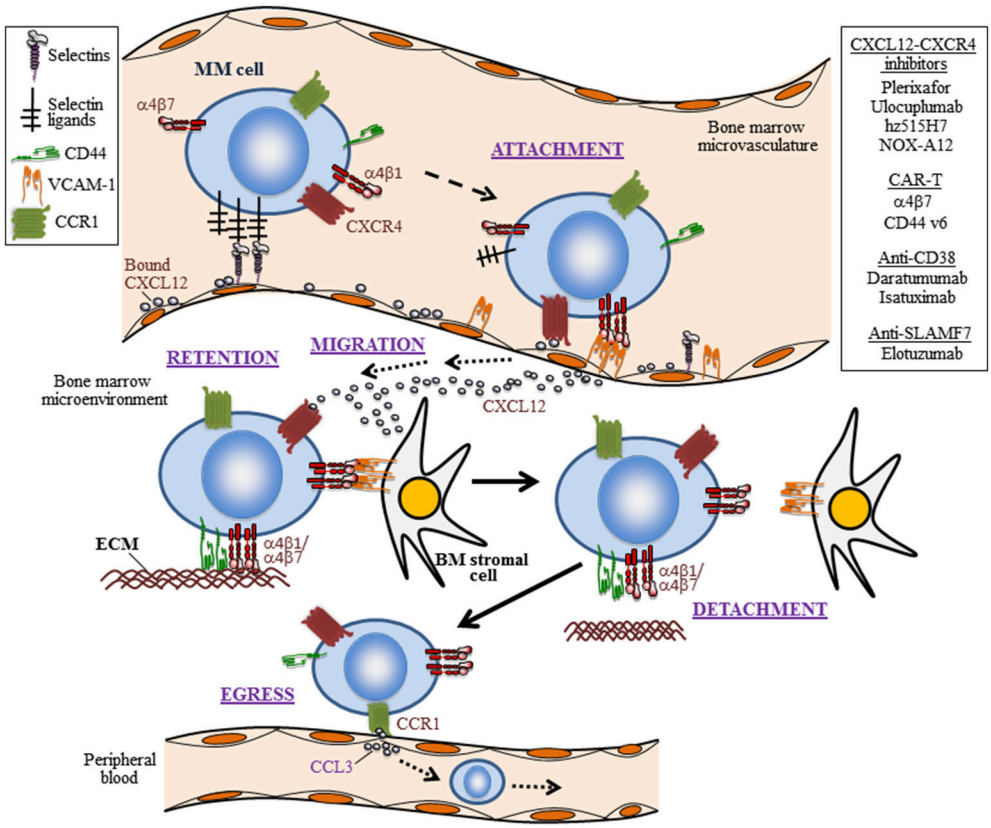

FIGURE 1 | The trafficking life of MM cells. Depicted are five steps (attachment, migration, retention, detachment, and egress) in the trafficking of myeloma cells into the BM and their escape to the periphery, as well as some of the involved trafficking receptors and their ligands. The initial attachment of MM cells to the BM microvasculature is controlled by selectins, the CXCL12-CXCR4 axis, and the α4β1 integrin interaction with VCAM-1. Importantly, CXCL12-CXCR4 leads to upregulation of α4β1-dependent MM cell adhesion. The migration and retention of MM cells in the BM is contributed by the CXCL12-CXCR4 chemoattraction module, by adhesion mediated by the integrins α4β1 and α4β7, as well as by CD44. Ligands for these adhesion receptors are components of the extracellular matrix (ECM), such as fibronectin and hialuronic acid. Weakening or disrupting these adhesive interactions causes MM cell detachment from the BM microenvironment and egress to peripheral blood. The homing of MM cells to the BM can be inhibited by the CXCL12 inhibitor NOX-A12, and neutralizing CXCL12 binding to CXCR4 with the plerixafor blocks MM cell interaction with the BM microenvironment. A putative active cell egress mechanism is proposed as depicted by the CCL3-CCR1 interaction. Not shown is the trafficking of circulating MM cells to extramedullary colonization sites. In addition to therapies targeting the CXCL12-CXCR4 axis, the use of CAR T cells addressing α4β7 and CD44, and humanized monoclonal antibodies against CD38 and SLAMF7, which are currently being tested in MM, are shown.

Besides its key role in cell migration, the interaction of α4β1 with its ligands favors proliferation, survival, and chemoresistance in hematologic malignancies. For example, the α4β1-dependent CLL cell adhesion regulates proteins of the Bcl-2 family and induces cell survival signaling (75–79). Cell adhesion-mediated drug resistance (CAM-DR) is a process involved in chemoresistance and operates in B-ALL (80) and in the response of MM cells to the proteasome inhibitor bortezomib (BTZ) (81–83). It was also demonstrated that MM cell attachment to BM stroma involving α4β1 activates the MAP kinase and NF-κB pathways, increases cell cycle regulatory and anti-apoptotic proteins and induces IL-6 secretion, overall stimulating MM cell growth, survival, and migration (1). In other studies, β1 integrins, likely α4β1, were shown to cooperate with IL-6 to induce STAT3 signaling and Pyk2 phosphorylation (84, 85), and to contribute to MM cell survival. The α4β1/VCAM-1 interactions also contribute to the defective production of normal blood cells and to the tumor-associated osteolysis in the BM of aggressive B-ALL (86). The crucial role of α4β1 in MM, CLL and ALL cell trafficking, proliferation, survival, and chemoresistance is schematically depicted in **Figures 1–3**.

Other β1 Integrins in Hematologic Malignancies

Leukemic cells also express other members of the β1 integrin subfamily. The α3β1 integrin is widely expressed in CLL cells, and its combined expression with L-selectin (CD62L) and ICAM-1 (CD54) constitutes a good prognostic marker for the disease (87). The only function described so far for α3β1 in CLL is the ability to mediate cell migration on laminin-332, a protein present in LNs (88). The consistent expression of α3β1 integrin in CLL cells is intriguing and deserves further studies. The α2β1 integrin was shown to regulate adhesion and pro-survival signals of T-ALL cells (89). Additionally, the α5β1 integrin is highly

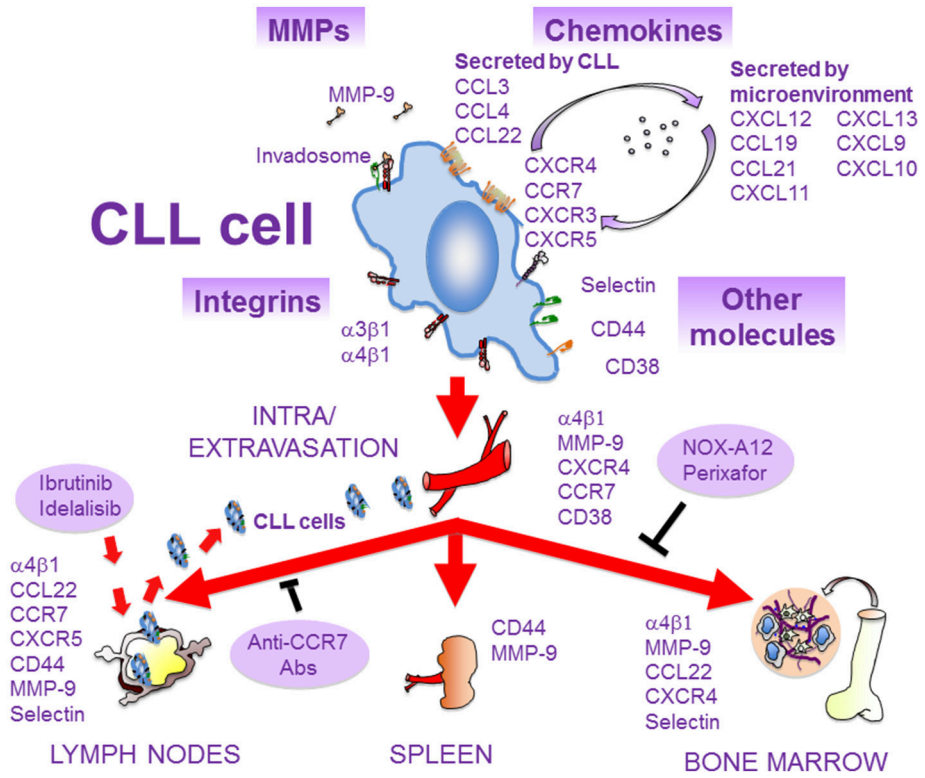

FIGURE 2 | Molecules involved in the migration of CLL cells**.** As CLL progresses, malignant cells extravasate and infiltrate the BM and secondary lymphoid organs. This process is mediated by several molecules, expressed and/or secreted by CLL cells or by the microenvironment of lymphoid tissues. Some of these molecules interact physically and/or functionally and may constitute the CLL cell invadosome. The α4β1 integrin is critical for CLL cell homing to BM and LNs, and blocking BCR signaling with ibrutinib or idelalisib inhibits α4β1 function and results in lymphocytosis. The chemokine receptors CCR7 and CXCR4 are crucial for CLL cell traffic to LNs and BM, respectively. These receptors can be blocked by specific antibodies or inhibitors (NOX-A12, perixafor). MMP-9, CD44, and CD38 are also important for CLL cell migration and localization in lymphoid niches. Adhesive interactions with stromal cells in these niches, mediated by some of the depicted molecules, favor survival and chemoresistance of CLL cells and contribute to disease progression.

expressed in Ph^+ (Philadelphia chromosome positive) B-ALL cells and controls their adhesion and engraftment in xenograft mice models (90). These β1 integrins may therefore be considered potential targets to control leukemic cell dissemination and organ infiltration.

The β7 Integrins in MM

Another integrin expressed on MM cells and which shares with α4β1 the α4 subunit is the lymphocyte homing receptor α4β7 (91) (**Figure 1**). The α4β7 integrin interacts with mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1) and with fibronectin (92–94), both expressed in the BM microenvironment (95). Similar to α4β1, the activity of α4β7 can be regulated by chemokines, including CXCL12 (96). Recent data indicate that α4β7 might constitute a useful target for chimeric antigen receptor (CAR) T cells in MM (91) (see below). The β7 subunit can also associate with the αE subunit to form the αEβ7 integrin, which mediates cell adhesion to E-cadherin (97, 98). Since BM stromal cells express E-cadherin (99), MM cells use both αEβ7 and α4β7 integrins to attach to BM stroma (100, 101), and these interactions contribute to MM cell lodging in the BM (100). The expression of β7 integrins on MM cells is driven by the oncogene c-maf (101), and correlates with poor survival outcome (100). Interestingly, hypoxia in MM leads to decreased expression of E-cadherin, thus reducing MM cell attachment to BM stroma and enhancing egress of these cells to the circulation (102).

The αLβ2 Integrin in CLL and ALL

The αLβ2 integrin (CD11a/CD18, leukocyte function-associated antigen-1, LFA-1) is a key adhesion receptor for leukocyte transendothelial migration into lymphoid tissues and sites of inflammation, and interacts with its ligands intercellular cell adhesion molecule (ICAM)-1, -2, or -3 (103). αLβ2 is expressed in many CLL cases, but its function is not fully understood. Compared to normal B cells, several groups have demonstrated defective signaling mechanisms in response to chemokines that leads to deficient αLβ2 activation in CLL (104, 105). The impaired CLL cell motility and transendothelial migration could be overcome by further stimulation of αLβ2 as well as α4β1 with VEGF (104). αLβ2 does not seem to be required for *in vivo* homing of CLL cells to LN or BM, or for CLL cell adhesion to stromal cells, at difference with α4β1 (63, 106). Further studies are needed to clarify the role of αLβ2 in CLL and its contribution to CLL pathology.

In contrast to CLL, the blockade of αLβ2 was shown to affect T-ALL cell adhesion and survival on BM stromal cells (107). Additionally, B-ALL cells with high αLβ2 levels are able to promote leukemia and CNS infiltration in mouse xenograft models (108). On the other hand, CD7 was shown to upregulate the β2 integrin subunit in the B-ALL cell line Tanoue, impacting in the adhesion and extramedullary invasiveness of these cells (109). The apparently different role of αLβ2 in ALL and CLL may suggest that distinct molecular mechanisms or kinetics orchestrate cell migration in these malignancies.

CHEMOKINES AND THEIR RECEPTORS IN HEMATOLOGIC TUMOR CELL TRAFFICKING

The CXCL12-CXCR4 Axis in Hematologic Malignancies

CXCR4 is a main chemokine receptor expressed by MM, CLL and ALL cells (**Figures 1**–**3**). Interaction of CXCR4 with its ligand CXCL12 plays a critical role in the trafficking of these neoplastic cells to the BM (13, 110, 111). The CXCL12 chemokine is highly expressed in active MM by BM stromal and endothelial cells, as well as by MM cells (112–114), and its expression is associated with BM areas with high MM cell infiltration (115). CXCL12 expression can be regulated by TGF-β1 and HIF-2α, with important functional adhesive and migratory consequences (116, 117). Previous studies using the myeloma 5TMM mouse model demonstrated the involvement of the CXCL12/CXCR4 axis in the homing and progression of MM (118), and revealed the reduction of both processes by *in vivo* neutralization of CXCL12 with olaptesed-pegol (NOX-A12) (115). Furthermore, blocking CXCL12 binding to CXCR4 with the plerixafor (AMD3100) inhibitor disrupts MM cell interaction with the BM microenvironment (119), causing MM cell mobilization into the circulation (120) (**Figure 1**). Plerixafor was also shown to prevent B- and T-ALL cell transendothelial migration and homing into the BM (**Figure 2**), as well as their extramedullary infiltration in liver, lung and CNS (121–123), highlighting the key role of the CXCL12-CXCR4 module in malignant cell trafficking.

The expression of CXCR4 varies during the course of MM, CLL, and ALL. It was recently reported that MRD subclones in MM express high levels of CXCR4 (65). Increased CXCR4 amounts correlates with the acquisition of an epithelial-mesenchymal transition phenotype, favoring MM cell invasion and bone metastasis (124). In CLL cells, CXCL12 binding induces CXCR4 endocytosis, thus reducing the surface expression of this

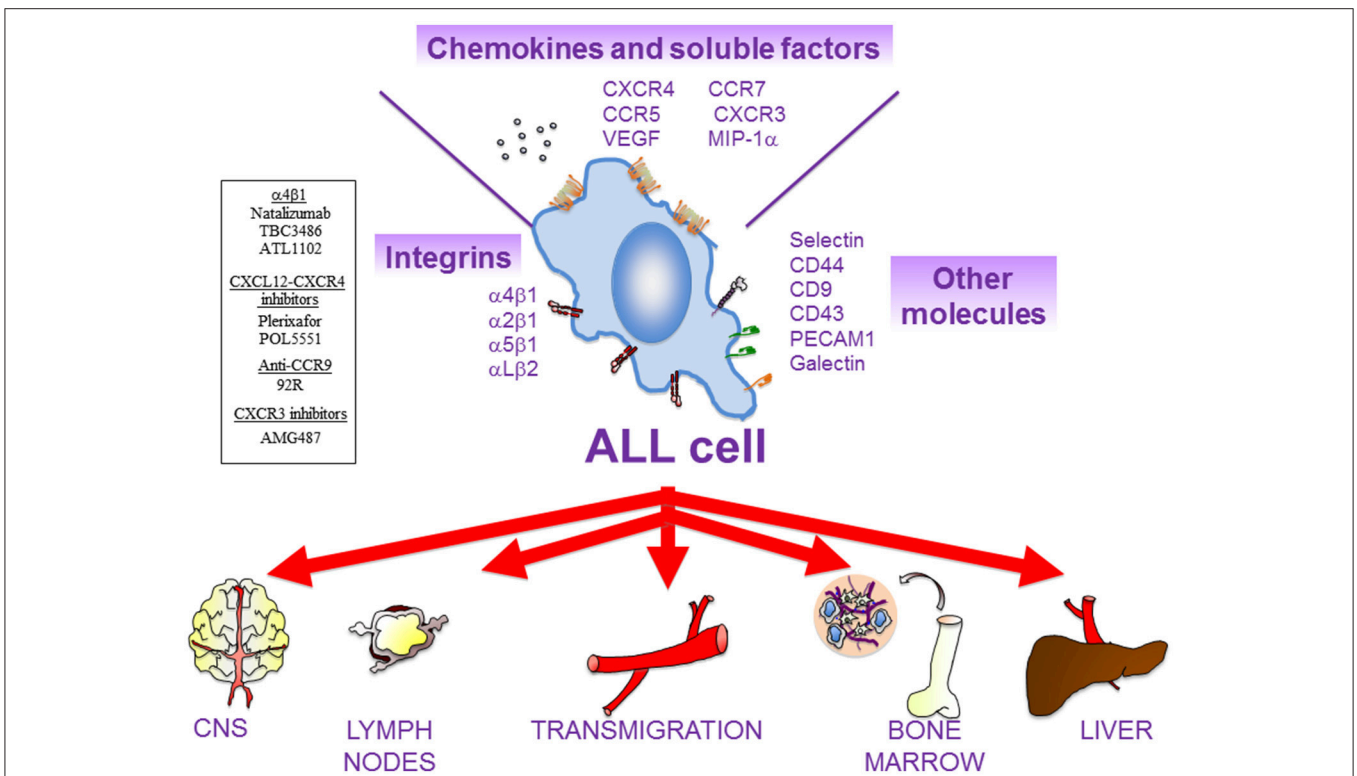

FIGURE 3 | Main actors of ALL cell migration. There are multiple cell surface receptors and soluble molecules that drive ALL cell movement. ALL cells might remain in the BM or colonize multiple extramedullary organs, such as central nervous system (CNS), lymph nodes (LNs), liver, and testes. In general, β1 integrins (α4β1, α2β1, α5β1) and the integrin αLβ2 are critical for the homing of ALL cells into the BM and for their infiltration in most of the extramedullary organs. The chemokine receptor CXCR4 mediates ALL cell infiltration into BM, liver, lung, and CNS, whilst CCR7 controls ALL infiltration into CNS and LNs. CXCR3 is also critical for CNS infiltration. Other molecules that might contribute to ALL migration includes MIP-1α, VEGF, PECAM-1, VE-Cadherin (for CNS infiltration); IL-7 (for LNs); and CD44 (for BM). Several compounds targeting molecular players in ALL cell migration that are currently being tested in preclinical trials are shown on the left.

receptor (125). This fact serves to distinguish peripheral blood CXCR4^{high} CLL cells from CLL cells derived from lymphoid tissues, which are CXCR4^{low}. CXCR4 is the most abundant chemokine receptor expressed by B- and T-ALL cells (126), and its elevated expression has been extensively correlated with poor prognosis in ALL patients (86).

The mechanisms that regulate the expression of CXCR4 in hematologic malignancies have therefore been the focus of intense investigations. These studies have shown that hypoxia in the BM leads to increased CXCR4 expression in MM cells, resulting in enhanced migration and homing of circulating MM cells to new BM niches (102, 127). CXCR4 expression can also be stimulated by Notch signaling, and blockade of this signaling leads to reduced MM cell infiltration in the BM (128). On the other hand, using a mouse MM model, it has been reported that treatment with BTZ reduces CXCR4 expression, which might favor MM cell egress from the BM milieu and promote extramedullary disease (129). In CLL, CXCR4 expression is regulated by BTK and its downstream target PIM, and both kinases phosphorylate CXCR4 at Ser 339 (130, 131). Using the Eμ-TCL1 murine model of CLL (132), as well as human CLL cells, Chen et al. (130) showed that BTK inhibition by ibrutinib decreased CXCR4 membrane expression along with a rapid release of CLL cells from spleen and LNs to the circulation. In addition, inhibition of PIM by the small molecule SEL24-B489 also blocked CLL cell migration by reducing CXCR4 surface expression and CXCR4-dependent mTOR activation (131). For acute leukemias, it has been reported that calcineurin signaling promotes the expression of cortactin in T-ALL cells, which in turn controls the levels of CXCR4 at the surface of these tumor cells (133, 134).

CXCR4 can also be regulated by tetraspanins, a family of proteins with four transmembrane domains that play important roles in molecular trafficking and in cell adhesion mediated by integrins (135). For instance, the tetraspanin CD63 interacts with CXCR4 on activated B cells and downregulates this receptor (136). Using *in vivo* models and the B-ALL cell lines REH and Nalm-6, the tetraspanin CD9 was shown to modulate CXCR4-mediated cell migration involving Rac1 signaling, as well as tumor cell survival and homing into the BM and testes (137).

CXCL12 signaling *via* CXCR4 is critical for the regulation of hematologic tumor cell adhesion. The tight functional links in signaling between α4β1 and CXCR4 in MM were revealed by the demonstration that CXCL12 rapidly and transiently stimulates α4β1-dependent MM cell adhesion (138), involving RhoA and Rac1 activities (139, 140). In addition, talin and kindlin-3 positively regulate CXCL12-stimulated, α4β1-dependent MM cell attachment, whereas ICAP-1 (Integrin Cytoplasmic domain-Associated Protein-1) negatively controls this adhesion (55). Moreover, sphingosine-1-phosphate (S1P) stimulates CXCL12-promoted MM cell adhesion to α4β1 ligands (141), and targeting S1P with FTY720 reduces CXCR4 cell-surface levels and inhibits *in vitro* and *in vivo* MM cell migration toward CXCL12 (142). Spatially, CXCL12-dependent α4β1 activation has been shown to directly correlate with restricted lateral diffusion and integrin immobilization in T cells (143), and hence it might also represent a mechanism for spatial regulation of α4β1 by CXCL12 in MM cells. Therefore, the functional links between the CXCL12-CXCR4 axis and the α4β1-mediated cell adhesion provide an essential contribution to blood cancer cell homing to and retention in the BM.

In addition, *via* promoting cell adhesion and migration, the CXCL12-CXCR4 interaction also stimulates cell survival. Thus, besides decreasing CXCR4 expression and altering CLL cell trafficking, ibrutinib, and SEL24-B489 also inhibited CXCL12/CXCR4-mediated CLL cell-tumor microenvironment cross-talk signaling, such as induction of cell survival and CD20 upregulation (131, 144). Similarly, the CXCL12-CXCR4 axis was shown to be critical for leukemia-initiating cell activity and disease progression in primary xenografts of T-ALL cells (133, 134).

CXCR7, a chemokine receptor that may function as a non-signaling scavenger for CXCL12, is expressed in active MM, and inhibition of both CXCR4 and CXCR7 with plerixafor and NOX-A12 functionally interfered with MM cell chemotaxis to the BM, and re-sensitized these cells to proteasome inhibitors (145). Similar to MM, CXCR7 is highly expressed in T-ALL cells compared to normal lymphocytes, and contributes to CXCL12-mediated cell migration (146). Additional *in vivo* studies using relevant mouse models are needed to assess the functional implications and relevance of CXCR7 in MM and T-ALL.

Macrophage migration inhibitory factor (MIF) is a CD74 ligand that can also bind CXCR4 and CXCR7 (147). Interestingly, high MIF levels were detected in MM bone marrow in association with poor survival, and MIF silencing downregulated MM cell adhesion to BM stroma and led to extramedullary spread of the MM cells in SCID mice (148). The implication of MIF in MM progression deserves further studies.

Role of CCL3, CCL4, CCL22, and CXCL8 in Hematologic Tumor Cell Trafficking

CLL cells secrete several chemokines upon stimulation, mainly CCL3, CCL4, CCL22, and IL-8 (13, 149) (**Figure 2**). CCL3 and CCL4 are upregulated and secreted after BCR stimulation or when co-cultured with nurse-like cells, indicating that interactions of CLL cells with the microenvironment increase the expression of these chemokines (13, 149). Because CLL-derived monocyte-macrophages, as well as T cells, express CCR1 and CCR5, the receptors for CCL3 and CCL4, respectively, their secretion by CLL cells may serve to attract macrophages and other cells to CLL niches. Therefore, CCL3 and CCL4 may play a role in the regulation of CLL cell interactions with other cells in lymphoid tissues. These interactions contribute to CLL cell survival, and a recent study has demonstrated that macrophages induce survival signals in CLL *via* CCR1-dependent upregulation of the anti-apoptotic protein Mcl-1 (150). Consistent with the above observations, the plasma levels of CCL3/CCL4 are elevated in CLL patients with adverse prognosis. Additionally, Zucchetto et al. (151) showed that CCL3 and CCL4 are overexpressed in CD49d$^+$/CD38$^+$ CLL cells (poor prognosis), compared to CD49d$^-$/CD38$^-$ CLL cells, and that ligand engagement of CD38 upregulated both chemokines in the double positive cells.

CCR1 is also expressed in MM cells (152, 153). High CCR1 expression confers poor prognosis in newly diagnosed MM patients, and is associated with increased circulating MM cells (127). Interestingly, CCL3 abrogated MM cell migration toward CXCL12, raising the possibility that the CCL3/CCR1 axis might actively promote MM cell egress from the BM, perhaps competing with retention signals from the CXCR4-α4β1 axis (**Figure 1**). This active migration from the BM could be similar to the alterations in chemokine receptor expression in lymphocytes egressing from the LNs (154).

Ghia et al. (155) reported an attracting role for the chemokine CCL22 in CLL. They showed that CLL cells from LNs or BM, but not from PB, constitutively express and secrete CCL22. Upon CD40 ligation, PB CLL cells also expressed and secreted CCL22 to the culture media. Indeed, these media induced the migration of $FoxP3^+$ regulatory T cells, characterized by high expression of CCR4, the receptor for CCL22. Interaction of CLL cells with Treg in lymphoid tissues through CD40-CD40L may provide survival signals to CLL cells, such as the upregulation of anti-apoptotic proteins (156).

CLL cells stimulated *via* CD40 or CD74 were also shown to secrete CXCL8 (IL-8) (157), and CXCL8 plasma levels correlated with CLL survival, suggesting a possible prognostic value for this chemokine (158). However, a recent study (159) demonstrated that highly purified CLL cells do not produce CXCL8, either constitutively and upon activation, do not express the CXCL8 receptors CXCR1 or CXCR2, and therefore, do not respond to this chemokine. Instead, CXCL8 was released by a small amount of contaminating monocytes present in the culture. The role of CXCL8 in CLL therefore needs to be re-evaluated. In the case of acute leukemias, it has been reported that CXCL8 enhances B-ALL cell adhesion to BM mesenchymal stem cells (160).

CCR7, CXCR3, and CXCR5 in Hematologic Tumor Cell Trafficking

CCR7 is the receptor for CCL19 and CCL21, two chemokines expressed by high endothelial venules or within lymph nodes that drive lymphocyte homing to LNs (56, 149) (**Figure 2**). Like in the case of the α4β1 integrin, CCR7 overexpression in CLL cells correlates with the presence of lymphadenopathy, and an anti-CCR7 monoclonal antibody inhibits *in vitro* CLL cell migration and induces complement-dependent cytotoxicity against CLL cells (56, 161, 162). The same antibody also drastically reduced tumor burden and dissemination in xenograft models of human mantle cell lymphoma (163), further supporting its potential therapeutic use in both malignancies. Additionally, we have shown that the CCL21/CCR7 axis upregulates MMP-9 involving ERK1/2 activation, thus suggesting a role for MMP-9 in LN infiltration by CLL cells (164) (**Figure 2**). The expression of the CCR7 chemokine receptor is higher in T- than B-ALL cells, which strongly correlates with CNS infiltration (165). Furthermore, CCR7 is also critical for the infiltration of T-ALL cells into LNs (166).

CXCR3 is the receptor for the CXCL9, CXCL10, and CXCL11 chemokines, which are present in the serum of CLL patients, with higher levels in U-CLL compared to M-CLL cases (167). The cell surface expression of CXCR3 is variable in CLL, and low expression correlates with advanced disease and other prognosis markers (149, 168). In a more recent study, CXCR3 levels were also shown to inversely correlate with the activation status of CLL cells, that is, with their proliferative capacity (167). These authors showed that the combined expression of CXCR3 and CXCR4 has prognostic value in CLL, and that the $CXCR3^{low}/CXCR4^{high}$ pattern correlated with shorter time to the first treatment. They also demonstrated that CXCR3 engagement specifically diminished both CXCR4/CXCL12-directed chemotaxis and α4β1 integrin-mediated cell tethering, thus having a negative regulatory role on the activity of these migration and adhesion receptors. Additional detailed mechanistic studies should help establish the significance of CXCR3 in CLL progression.

The CXCR5 chemokine receptor regulates CLL cell homing to LNs and its main function is the positioning of B cells within lymphoid follicles (169). CXCR5 is the receptor for CXCL13, a chemokine constitutively secreted by stromal cells in these follicles (170). CXCL13 binding to CLL cells induces CXCR5 endocytosis, actin polymerization and activation of ERK1/2 kinases (170). CXCR5 is overexpressed in CLL cells, particularly in cases with nodal involvement, but its expression levels are similar to those in normal $CD5^+$ B cells (170, 171). The crucial role for CXCR5 in CLL cell migration and expansion was demonstrated using the Eμ-TCL1 mouse model and intravital imaging (172). The authors showed that CXCR5 guided leukemic cells to splenic B-cell follicles, where they interacted with resident dendritic cells favoring proliferation and disease progression. Additionally, CLL cells stimulated stromal cells *via* lymphotoxin-β-receptor activation, leading to CXCL13 release. Moreover, targeting CXCL13/CXCR5 and lymphotoxin-β-receptor signaling abrogated the proliferative and survival advantage of CLL cells in these niches and retarded disease progression.

MATRIX METALLOPROTEINASES

MMP-9 is the main MMP expressed by CLL cells (173–175), and elevated MMP-9 intracellular levels correlate with advanced disease and poor patient survival (174). Correlation of MMP-9 as well as MMP-14 levels with survival, but not with peripheral organ infiltration, was also observed in relapsed pediatric patients with B- and T-ALL (176). In contrast, MMP-2 expression correlates with an invasive B-ALL phenotype in adult but not in pediatric patients (177).

Although MMP-9 is a secreted protein found in the serum of CLL patients and in CLL cell culture supernatants, it is also consistently detected at the CLL cell surface (48, 174). This localization involves binding to a docking complex formed by α4β1 integrin and 190 kDa CD44v (48) (**Figure 2**), and may serve to concentrate the MMP-9 catalytic activity at the cell periphery and facilitate cell migration and invasion. However, localization of MMP-9 to the cell membrane also has other important consequences that contribute to CLL pathology. For example, we have demonstrated that MMP-9 binding to α4β1 in CLL cells induces a Lyn/STAT3/Mcl-1 signaling survival pathway, and this

function does not require the MMP-9 catalytic activity (78). *In vitro* and *in vivo* experiments have also shown that CLL cell migration requires optimal MMP-9 expression, and that above these optimal levels migration is inhibited (178, 179). This effect is partly due to the downregulation of migration regulatory pathways, such as those involving the GTPase RhoA, Akt, ERK, and FAK, as well as to the upregulation of p190RhoGAP and PTEN, and also implicates catalytic and non-catalytic MMP-9 activities (178, 179). Whether MMP-9 also regulates other molecules with migratory functions in CLL deserves further studies. Importantly, the dual regulatory role of MMP-9 operates *in vivo,* since contact with stroma increases cell-bound MMP-9, and CLL cells from lymphoid tissues express more MMP-9 than their PB counterparts. Elevation of cell-bound MMP-9 at these sites may help the retention of malignant cells in tissues, therefore contributing to disease progression.

CD44 AND CD38

CD44 was originally defined as a homing receptor and its role in the expansion of many solid tumors has been widely documented (180). The involvement of CD44 variants in the *in vivo* MM cell homing to the BM was early shown using the 5T33MM mouse model (181, 182). CD44 may play an important role in the retention of MM cells in the BM (**Figure 1**), since MRD subclones in the BM of MM patients express high levels of CD44 (65). The potential contribution of CD44 in MM cell trafficking was also reported for MM extramedullary disease, as samples from liver and pleural effusions displayed high CD44 expression (183, 184), indicating that CD44 likely facilitates MM cell attachment at multiple steps of the MM cell trafficking life.

Elevated levels of soluble CD44 are found in the serum of many CLL patients, in correlation with advanced disease (185). CLL cells express CD44s and CD44v and their expression increases upon cell activation (185). Using murine models and human samples, a major role for CD44, particularly CD44v6, in CLL cell homing, engraftment and proliferation in the spleen has been demonstrated (185). CD44v6 is also involved in ALL cell infiltration and altered BM localization (186) (**Figure 3**). As a functional co-receptor for MMP-9 (48, 187), CD44v likely contributes to CLL cell retention in lymphoid organs, where MMP-9 concentration is high. CD44 may thus function as a migration stop-signal in CLL (**Figure 2**). In agreement with this, activation of CLL cells by T cells in lymphoid organs induced high avidity CD44-hyaluronan interactions, mostly involving CD44v6, which impaired migration and induced CLL cell arrest on immobilized hyaluronic acid (185).

As in the case of the α4β1 integrin, high expression of CD38 (>30% cells) constitutes a marker for poor prognosis in CLL (188–190). Accordingly, $CD38^+$ CLL cells show increased migration, not only in response to chemokines but also in their absence, due to enhanced basal cell spreading and migration (191, 192) (**Figure 2**). The mechanism involved in the CD38 action was recently shown to involve Ca^{2+}-induced activation of the GTPase Rap1, thus providing a novel role for this GTPase in CLL aggressiveness (192). Besides its role in cell migration, CD38 may constitute a marker for activated or/and recently born CLL cell subsets, as CD38 expression is modulated by the microenvironment and has been associated with CLL cell proliferation (193). In line with this, $CD38^+$ CLL cells also show enhanced signaling induced by BCR or by anti-IgM/IgD antibody crosslinking, as well as a survival and proliferation advantage, compared to $CD38^-$ clones of the same individual (191).

SELECTINS

The P-selectin glycoprotein ligand-1 (PSGL-1) is highly expressed on the surface of MM cells (194, 195). *In vitro* and *in vivo* studies have shown that PSGL-1 contributes to MM cell interaction with the BM microvasculature (195), by facilitating the rolling of these neoplasic cells on P-selectin on the endothelium (55). The important roles of the selectins in the initial steps of MM cell homing to the BM were further supported by the demonstration that sialyltransferase ST3Gal-6, an enzyme critical for the generation of E-selectin ligands, is involved in MM cell homing to the BM (196). Moreover, using intravital microscopy, we have demonstrated that antibodies to P- and E-selectin inhibit the rolling and subsequent firm arrest of MM and CLL cells to the BM microvasculature (55). Therefore, both P- and E-selectin seem to control the initial steps of MM and CLL cell homing (**Figures 1**, **2**). With respect to acute leukemias, it has been reported that T-ALL cells adhere to IL-1β-stimulated HUVEC in an E-selectin-dependent manner (197) (**Figure 3**). In contrast to normal leukocytes and MM cells, where PSGL-1 is the major ligand of P- and E-selectin, B-ALL cells express low levels of PSGL-1 and their rolling and adhesion is mainly mediated through CD43. Of note, CD43 downregulation impairs tissue engraftment in a B-ALL xenograft mouse model (198).

In vivo studies of the initial interactions with high endothelial venules have demonstrated a crucial role for L-selectin (CD62L) in CLL cell homing to LNs (199) (**Figure 2**). This work revealed a higher rolling fraction of cells with high L-selectin expression albeit the rolling velocity was decreased, a fact that may facilitate CLL cell retention in LNs. In line with this, the PI3-Kδ inhibitor idelalisib diminished L-selectin expression, increased rolling velocity and reduced CLL cell entry into LNs (199). BCR engagement was also shown to downregulate CD62L as well as CXCR4, and to favor cell arrest in LNs, in this case by inducing an adhesive phenotype (200, 201).

FUNCTIONAL MOLECULAR COMPLEXES: THE CLL CELL INVADOSOME

Functional and/or physical associations among molecules involved in CLL cell trafficking have been well-documented. We have shown that ligand engagement of the α4β1 integrin upregulates MMP-9 expression and induce its localization in podosomes, where MMP-9 degrades extracellular matrix and facilitates cell migration (175). Likewise, the CXCL12/CXCR4 or CCL21/CCR7 axes also upregulated MMP-9, involving different pathways than those upregulating α4β1, and increased CLL cell

migration (164, 175). Additionally, MMP-9 binds to a CLL cell-specific functional complex formed by α4β1 and CD44v, and this association is important for CLL cell migration and survival (48, 78). Similar to α4β1 and to the chemokine receptors CXCR4 and CCR7, CD38 signaling was also shown to upregulate MMP-9 expression and function in CLL (202). CD38 also synergized with the chemotactic function of CXCL12 and enhanced the adhesive and signaling activity of α4β1 in CLL (202, 203). Further analyses by these same authors demonstrated a physical association of CD38 with CXCR4 and α4β1 at the CLL cell membrane, which provides a possible explanation for the observed functional cross-talk between these proteins (202, 203). In agreement with these studies, Buggins et al. (204) reported the presence of the MMP-9/α4β1/CD44/CD38 macromolecular complex in CLL cases with poor prognosis. CLL cell trafficking between PB and lymphoid tissues therefore involves multiple molecules, which seem to interact physically and/or functionally to form macromolecular complexes. While these complexes may represent the CLL invadosome, as previously suggested (205) (**Figure 2**), certain issues regarding their dynamic formation, regulation by the microenvironment, turnover, differential composition, etc., still need to be resolved. The fact that the MMP-9/α4β1/CD44/CD38 complex was mainly observed in poor prognosis cases reinforces the importance of molecules regulating migration as contributors to CLL progression.

TRAFFICKING MOLECULES AS THERAPEUTIC TARGETS IN HEMATOLOGIC TUMORS

Because MM, CLL, and ALL progression involves critical infiltration and retention of malignant cells in lymphoid tissues, targeting the molecules that control the cancer cell traffic appears as an efficient therapeutic approach to treat these diseases. Strategies aimed to target α4β1 integrin have proven difficult due to the essential physiological function of this integrin in lymphocyte development and traffic. A recombinant humanized anti-α4 monoclonal antibody (natalizumab) was shown to block stroma-dependent MM cell proliferation, to inhibit *in vivo* tumor growth, and to chemosensitize MM cells to BTZ (206). However, treatment with natalizumab entails several side complications, such as the activation of the John Cunningham (JC) virus and associated progressive multifocal leukoencephalopathy (207), as well as the unwanted egress of normal hematopoietic stem cells linked to inhibition of α4β1 function (208). For these reasons, treatment of hematologic malignancies with natalizumab has been discontinued.

Targeting the signaling pathways that control the α4β1 integrin may be a more suitable therapeutic approach. An important regulator of α4β1 is the BCR, and several studies have demonstrated that inhibiting the BCR-target BTK with ibrutinib abolishes the CLL adhesive and migratory function of α4β1 in tissues, a fact that correlates with the observed ibrutinib-induced lymphocytosis (209, 210) (**Figure 2**). A more recent report has shown that BCR activates α4β1 integrin and that ibrutinib only partially reduces this activation, since ibrutinib-treated cells were still able to activate α4β1 in response to anti-IgM stimuli (211). These authors suggested that CLL cells with high levels of α4β1 are more likely to be retained in tissues, and that analysis of α4β1 expression may help identify patients which would benefit from ibrutinib therapy

In ALL, the small molecule inhibitor of the α4 subunit TBC3486 blocks ALL cell adhesion, reduces α4 expression, sensitizes B-ALL cells for death *in vitro* and extends survival time in a B-ALL xenograft model (212). In addition, ATL1102, an α4 antisense oligonucleotide developed to treat multiple sclerosis, downregulates the expression of α4 and β1 subunits in the B-ALL Kasumi-2 cell line *in vitro* (213). Unfortunately, this antisense oligonucleotide fails to downregulate α4 expression and to improve survival in a mouse model of B-ALL (213), indicating that antisense drugs must be improved for clinical application in this malignancy. Interestingly, as there is a functional link between α4β1 and the histone methyltransferase G9a, which regulates migration of Jurkat (T-ALL) cells (214), the G9a inhibitor CM-272 has been reported to block cell proliferation and infiltration in an *in vivo* xenograft model (215).

Similar to the high-affinity conformations of the integrins α4β1 and LFA-1, the α4β7 integrin can also display activated conformations that can be recognized by specific antibodies (216). α4β7 has been recently the subject of therapeutic studies in MM using α4β7-based chimeric antigen receptor (CAR) T cells (91). An epitope in the N-terminal region of the β7 subunit (MMG49) is accessible in the α4β7 active conformation but inaccessible in the resting integrin conformer. The study showed that T cells transduced with MMG49-derived CAR exerted anti-MM effects without damaging normal hematopoietic cells, indicating that MMG49 CAR T cell therapy might be promising for MM treatment (91).

The CXCR4-CXCL12 axis has been also the subject of therapeutic studies in MM, CLL and ALL. Thus, *in vivo* CXCL12 inhibition with NOX-A12 led to a tumor microenvironment less receptive for MM cells, causing reduced MM cell growth and disease progression (115) (**Figure 1**). Furthermore, NOX-A12 increases the anti-MM capability of combined BTZ and dexamethasone (217). In CLL, treatment with NOX-A12 led to inhibition of CLL cell chemotaxis and stroma-mediated drug resistance (**Figure 2**) (218).

In addition to promoting $CD34^+$ mobilization in MM treatments (219), plerixafor augments the sensitivity of MM cells to multiple therapeutic agents (120). Several anti-CXCR4 monoclonal antibodies are being tested in different clinical trials for MM, including ulocuplumab (BMS-936564/MDX1338) and hz515H7 (220, 221) (**Figure 1**), supporting the relevance of targeting MM cell trafficking molecules to inhibit disease progression. Besides ulocuplumab and plerixafor, other monoclonal antibodies or small molecule antagonists to CXCR4, such as BL-8040 and PF-06747143 have been shown to inhibit the CXCL12/CXCR4-mediated migration of CLL cells (222) (**Figure 2**). Since CXCR4 is also an important survival factor in these cells, some of these antagonists also induce cell apoptosis. Thus, plerixafor and NOX-A12, in combination with rituximab, lenalidomide or bendamustine, are in the initial phases of clinical trials for CLL (222). In the case of ALL, the CXCR4 antagonist

POL5551 has been proposed as a possible therapeutic agent in high-risk B- and T-ALL patients (223).

Other chemokines receptors, such as CCR7, CCR9, or CXCR3, have also been considered potential therapeutic targets in CLL and ALL, and the focus of several investigations. Thus, anti-CCR7 mAbs may have potential use to prevent CLL cell traffic to LNs, although they are still in preclinical phases of study (162). In addition, it has been recently described that immunotherapy based on antibodies against CCR9 has anti-tumor potential, without affecting the chemotactic response of T-ALL cells to CCL25 (224). Furthermore, treatment with AMG487, a specific CXCR3 inhibitor, impairs B-ALL infiltration into BM, spleen and brain in *in vivo* models (126).

As mentioned above, CD44 represents an important cell surface molecule for blood cancer cell trafficking. In MM, CD44 has been the focus of CAR T cell studies. T cells targeted to CD44v6 using a CAR construction elicited a potent anti-tumor effect against primary MM and acute myeloid leukemia, while sparing normal hematopoietic stem cells and CD44v6-expressing keratinocytes (225). On the other hand, the humanized anti-CD44 mAb RG7356 is in phase 1 for acute myeloid leukemia and has also shown preclinical activity in CLL (162, 226). Two antibodies against CD38, daratumumab (FDA-approved) and isatuximab (SAR650984; clinical trial not yet completed), have been developed (10, 227), and shown to have anti-MM activity, especially when combined with lenalidomide (228). Likewise, isatuximab is in phase 1/2 clinical trial for $CD38^+$ hematologic malignancies, including CLL (227). Additionally, daratumumab has shown preclinical anti-tumor activity in a CLL mouse model, as it eliminates cells from infiltrated organs and inhibits CLL cell homing to spleen (162, 227, 229).

CD19 and the SLAMF7/CS1 receptors are also the subject of therapeutic studies aimed at hematologic malignancies. For instance, CAR T cell therapy directed to $CD19^+$ cells is undergoing clinical trials in CLL with promising results (230, 231). Combination of this therapy with approaches addressed to inhibit cell migration may constitute an efficient treatment for CLL. Moreover, CD19-targeted CAR T-cell therapeutics for B-ALL are being developed (232). SLAMF7/CS1 is a putative adhesion molecule mediating MM cell attachment to BM stromal cells (233). A humanized anti-CS1 antibody, elotuzumab (HuLuc63), exerted significant *in vivo* anti-MM activity *via* NK-mediated antibody-dependent cellular citotoxicity (234). In a recent phase 3 study, it was reported that the combination of elotuzumab, lenalidomide, and dexamethasone led to significant reduction in the risk of disease progression or death (235). Furthermore, CAR-engineered NK cells specific for CS1 exhibited significant anti-MM activity both *in vitro* and *in vivo* (236). Collectively, these data indicate that agents targeting adhesion and migration receptors represent promising therapeutic protocols to hamper the progression of blood cancers, including MM, CLL, and ALL.

CONCLUSION

Tumor cell migration is a critical process that contributes to the development and progression of hematologic malignancies. Homing and retention of neoplastic cells in tissues involves multiple adhesive and migratory mechanisms and favors survival and chemoresistance of the malignant cells. We have focused this review on the molecular components that regulate malignant cell traffic in three common hematologic tumors, namely MM, CLL, and ALL. These components include integrins, chemokines and chemokine receptors, selectins, metalloproteinases, and other molecules such as CD44 and CD38. These molecules may act in concert at different steps of the trafficking process. Numerous *in vivo* and *in vitro* studies have established the crucial role of the α4β1 integrin and the CXCR4 chemokine receptor in controlling the migration and retention of MM, CLL, and ALL cells in tissues. The role of other molecules (CD44, CD38, MMP-9, selectins) differs among the three malignancies, but they are also important for interactions with the tissue microenvironment. Indeed, most of the migration regulatory molecules also provide survival signaling upon binding to their ligands, thus contributing to progression of the disease. Due to this dual effect, targeting the molecules involved in malignant cell traffic or the signaling pathways that regulate their functions may constitute an efficient therapeutic approach for these diseases. Initial pre-clinical and/or clinical trials with inhibitors for some of these molecules are promising, although the fact that normal lymphocytes use similar migratory mechanisms adds serious difficulties to these approaches. Clearly, more specific targets or signaling pathways need to be identified and tested. Additionally, the development of improved monoclonal antibodies targeting molecules involved in the trafficking of MM, CLL, and ALL cells, such as CXCR4, CD44, SLAMF7/CS1, and CCR9 might open new opportunities for clinical treatments against these hematologic malignancies. Finally, further work on CAR T cell technology, as well as combined therapy using immune check-point inhibitors and agents targeting neoplastic cell trafficking will provide important tools to restrict the progression of these diseases. Additional analyses of the mechanisms involved in the recruitment of neoplastic cells from the bloodstream into different organs using imaging techniques could provide new insights on the migration and tissue-induced survival of malignant cells.

AUTHOR CONTRIBUTIONS

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Beta2-Integrins and Interacting Proteins in Leukocyte Trafficking, Immune Suppression, and Immunodeficiency Disease

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Beta2-integrins are complex leukocyte-specific adhesion molecules that are essential for leukocyte (e.g., neutrophil, lymphocyte) trafficking, as well as for other immunological processes such as neutrophil phagocytosis and ROS production, and T cell activation. Intriguingly, however, they have also been found to negatively regulate cytokine responses, maturation, and migratory responses in myeloid cells such as macrophages and dendritic cells, revealing new, and unexpected roles of these molecules in immunity. Because of their essential role in leukocyte function, a lack of expression or function of beta2-integrins causes rare immunodeficiency syndromes, Leukocyte adhesion deficiency type I, and type III (LAD-I and LAD-III). LAD-I is caused by reduced or lost expression of beta2-integrins, whilst in LAD-III, beta2-integrins are expressed but dysfunctional because a major integrin cytoplasmic regulator, kindlin-3, is mutated. Interestingly, some LAD-related phenotypes such as periodontitis have recently been shown to be due to an uncontrolled inflammatory response rather than to an uncontrolled infection, as was previously thought. This review will focus on the recent advances concerning the regulation and functions of beta2-integrins in leukocyte trafficking, immune suppression, and immune deficiency disease.

Keywords: integrin, trafficking, kindlin-3, leukocyte adhesion deficiency, leukocyte adhesion cascade

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INTEGRINS AND INTEGRIN REGULATION

Integrins are heterodimeric type I transmembrane proteins consisting of alpha and beta subunits (1). Integrins are expressed in all nucleated cells and play a key role in adhesion, cell communication, and migration. They mediate adhesion to the extracellular matrix, by binding to the RGD motif of fibronectin, collagen, and laminin, among others (2). Integrins in leukocytes also bind to soluble ligands such as the complement component iC3b, and to other cells, by binding to ICAMs (Intercellular adhesion molecules) and VCAM-1 (Vascular cell adhesion molecule) (3, 4). Additionally, integrins link to the actin cytoskeleton inside the cell and thereby connect the inside of the cell with the outside.

Integrins have large extracellular domains which contain the ligand-binding sites, and short cytoplasmic domains which are important for integrin regulation. The ability of the integrin to

bind to ligands is regulated through conformational changes as well as by integrin clustering. Integrins can be found in three main conformational states: inactive (bent-closed), intermediate (extended-closed), and active state (extended-open) (5). The predominant state seems to be the inactive (bent-closed) state based on affinity and thermodynamics studies with K562 cells (alpha5beta1-integrins, bent-closed: 99.75%; extended-closed: 0.10%; extended-open: 0.15%) (6). The active conformation (extended-open) has a 4,000-fold increase in ligand affinity compared to the other two states (7). Also on resting peripheral T cells the vast majority of LFA-1 (Lymphocyte function-associated antigen-1, alphaLbeta2-integrin) appear to be in the inactive conformation, as stabilizing the active conformation leads to a 1,000-fold increase in affinity of the integrin (8). The LFA-1 conformational change (integrin extension) on the surface of migrating T cells has recently been directly measured by super-resolution microscopy [interferometric photoactivation, and localization microscopy (iPALM)] (9).

Integrin activation takes place upon cell stimulation through various cell surface receptors such as chemokine receptors or the T cell receptor. Cell stimulation triggers an inside-out signaling pathway that ultimately recruits cytoplasmic factors such as talin and kindlin to the NPxY motifs of the cytoplasmic tail of the integrin's beta-chain, which causes the cytoplasmic tails of the integrin subunits to separate (10) and switches the integrin to the active (extended-open) conformation (11, 12). Kindlin and talin connect the integrin to the actin cytoskeleton and stabilize the extended-open conformation of the integrin through actin cytoskeleton exerted tensile force (6, 13). In addition, many other proteins, such as 14-3-3 proteins, alpha-actinin, coronin 1A, cytohesin 1, filamin A, and Dok1 can interact directly with the integrin beta-chain and modulate integrin function (14–16). These interactions are often regulated by phosphorylation of the integrin beta-chain cytoplasmic domain (15–18).

In addition to their ability to respond to the environment through inside-out signaling, integrins can take part in a variety of signaling cascades following ligand binding (outside-in signaling). Integrins take part in the formation of adhesion complexes and focal adhesions in cells such as fibroblasts, modulation of actin cytoskeleton dynamics, cell migration, differentiation, proliferation, angiogenesis, and apoptosis (19).

BETA2-INTEGRINS IN LEUKOCYTE TRAFFICKING

Beta2-integrins (CD11a/CD18, alphaLbeta2, LFA-1; CD11b/CD18, alphaMbeta2, Mac-1, CR3; CD11c/CD18, alphaXbeta2, p150.95, CR4; and CD11d/CD18, alphaDbeta2) are a subgroup of integrins which share a common beta2- or CD18-chain but have different alpha-chains and ligands. Beta2-integrins are expressed exclusively in leukocytes, but the different members have their own distinct expression pattern. CD11a/CD18 is expressed on all leukocytes, while CD11b/CD18, CD11c/CD18, and CD11d/CD18 are mainly expressed on myeloid cells, but at varying levels (19, 20). CD11a/CD18 has a more restricted ligand binding capacity than the other beta2-integrins, and binds ligands such as ICAM-1-5 found on the surface of other cells. In contrast, CD11b/CD18 is a very promiscuous integrin with more than 40 reported ligands, including ICAMs, iC3b, fibrinogen, RAGE (receptor for advanced glycation end products), and CD40L (20). Interestingly, ligand-specific blockade of CD11b/CD18 has recently been shown to protect against bacterial sepsis, while blocking all CD11b/CD18 functions potentiates it, showing that CD11b/CD18 indeed has very complicated roles in immunity due to its many ligands (21). In addition to leukocytes, beta2-integrins are also found in extracellular vesicles (EVs), and integrins in EVs may play novel roles in development of pathogenic conditions such as sepsis (22).

It is undisputed that beta2-integrins are of fundamental importance for leukocyte trafficking. This is because they are required for the firm adhesion to the endothelial layer surrounding the blood vessels under conditions of shear flow (blood flow) and for leukocyte extravasation into tissues (23). The leukocyte adhesion cascade (**Figure 1**) is a multistep process involving rolling, firm adhesion or arrest, spreading/crawling, and finally extravasation (24). This complex process is accomplished by several proteins acting in parallel and succession, as the leukocyte proceeds to its destination. Initially contacts between the leukocyte and the endothelial cells allows selectins and ICAM-1 on endothelial cells to mediate leukocyte rolling on the endothelium. The close contact between the cells during rolling allows the leukocyte to sense chemokines present on the endothelium. In neutrophils, both selectins and chemokine receptors activate beta2-integrins via a signaling pathway involving the small GTPase Rap1a and phosphatidylinositol-4-phosphate 5-kinase (PIP5Kγ90). The activation of beta2-integrins involves conversion into the intermediate affinity state that mediates slow rolling, followed by conversion into the high affinity state, which mediates leukocyte arrest (25). Both selectins and integrins can form slip bonds, whose lifetime is shortened by applied shear force, as well as catch bonds, which strengthen under shear force (26–28), inducing further changes downstream of the integrins. During these leukocyte-endothelial contacts numerous integrin-ligand bonds are continuously broken and formed and further reinforced by the recruitment of more integrins and downstream cytoskeletal proteins such as talin, kindlin-3, focal adhesion kinase, and paxillin to form adhesion complexes which strengthen cell adhesion and induce actin reorganization and cell spreading (26). Following adhesion, cells crawl along the endothelium looking for a suitable extravasation site, a process critically dependent on the beta2-integrin CD11b/CD18 (29). As integrins act as mechanosensors in cells (30), it is likely that integrins are also central for the subsequent steps of probing the endothelium for suitable points of exit, either through a paracellular or transcellular route.

Talin has long been known to be indispensable for leukocyte trafficking (31–34). More recently, also kindlin-3 and its interaction with the beta2-integrin tail has been shown to be vital for neutrophil and effector T cell firm adhesion under shear flow and for neutrophil and T cell trafficking *in vivo* (35–38), and for homing of progenitor T cells to the vascularized thymus

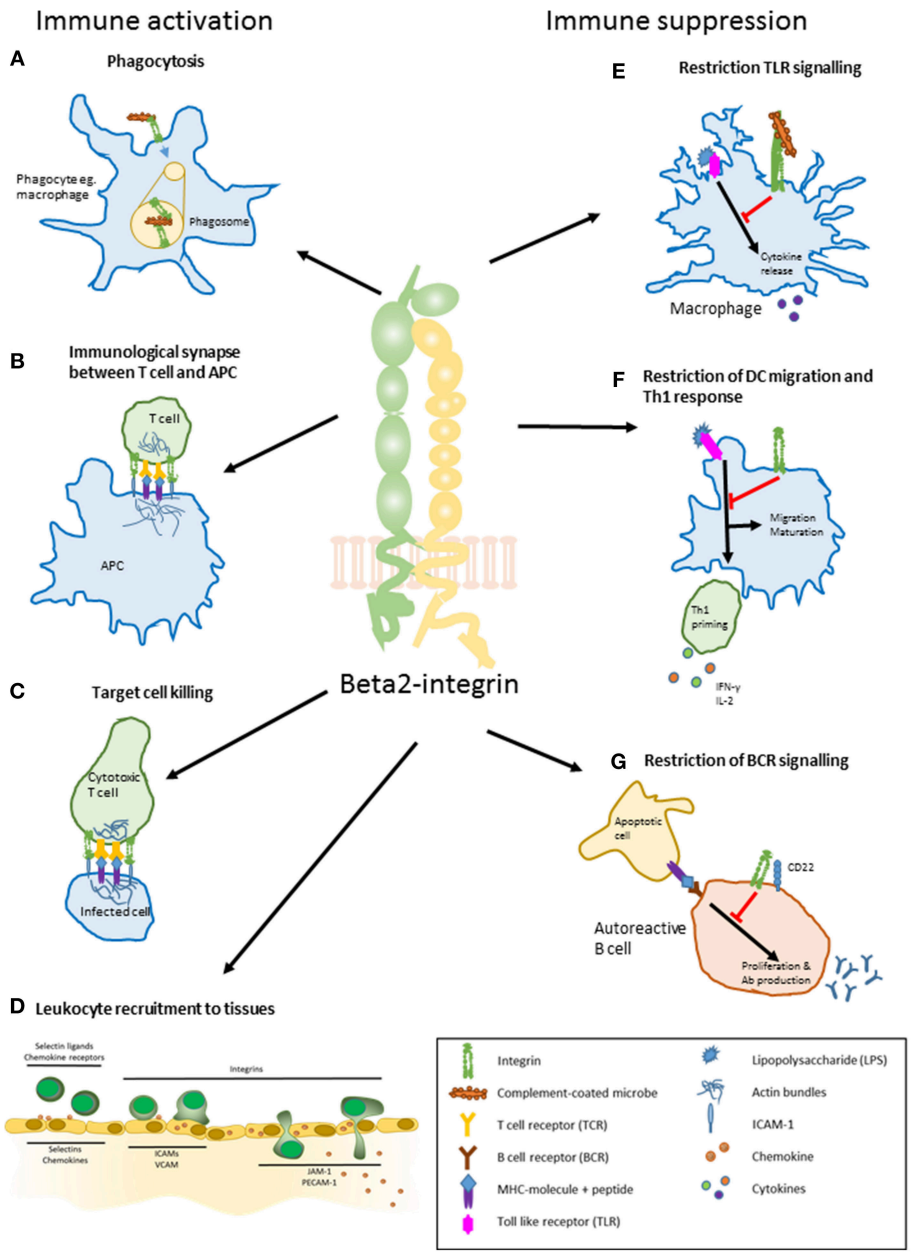

FIGURE 1 | The main roles of beta2-integrins in immune activation and suppression. **(A)** phagocytosis. Beta2-integrins mediate phagocytosis by binding to iC3b on the surface of complement-coated bacteria. **(B)** regulation of T cell activation by a dendritic cell. Beta2-integrins participate in the formation of an immunological synapse between a T cell and an antigen presenting cell such as DC. The synapse stabilizes the interaction between and regulates signaling in the two participating cells. **(C)** target cell killing. Beta2-integrins participate in forming and maintaining an immunological synapse between a cytotoxic T cell and an infected cell. **(D)** leukocyte recruitment to tissues. Leukocytes are activated by selectins and chemokines on the surface of activated endothelial cells close to a site of inflammation. This slows down the leukocyte speed and induces integrins to change their conformation through inside-out signaling, allowing them to bind ICAMs on the endothelium. Beta2-integrins are essential in the slow rolling and firm adhesion of a leukocyte, after which the cells transmigrate to the inflamed tissue. Leukocytes use the chemokine gradient to navigate toward the site of inflammation. **(E)** regulation of TLR-signaling. Beta2-integrin CD11b/CD18 restrains macrophage activation and cytokine production upon TLR (Toll-like receptor) activation by LPS (lipopolysaccharide). **(F)** restriction of dendritic cell migration, maturation, and Th1 priming. Proper beta2-integrin—cytoskeleton linkage controls DC maturation toward a migratory phenotype and restricts priming of Th1 cells. **(G)** restriction of B cell receptor signaling. Interaction of CD11b/CD18 and CD22 on the surface of an autoreactive B cell leads to constraint in BCR signaling. This decreases auto-reactive B cell proliferation and antibody production.

(39). However, talin and kindlin-3 regulate different aspects of leukocyte trafficking. Talin is required for the conformational change of the integrin to the extended, intermediate affinity conformation which mediates slow rolling. In contrast, both talin and kindlin-3 are required for the induction of the high-affinity conformation, full integrin activation and neutrophil arrest (33,

38, 40). Recently, Src kinase-associated phosphoprotein 2 (Skap2) has been shown to be essential for the recruitment of talin and kindlin-3 to the beta2-integrin tail, and for neutrophil trafficking *in vivo* (41). Interestingly, a bent-open conformation of beta2-integrins has been reported on neutrophils, which limits neutrophil recruitment by binding to ICAM-1 *in cis*, but the molecular mechanisms regulating this process are currently unknown (42).

In contrast to talin and kindlin-3, filamin A has been suggested to negatively regulate integrin functions *in vitro* (15, 43, 44). However, it has also been reported to be required for platelet shear flow adhesion because it stabilizes the links between the plasma membrane and the underlying actin cytoskeleton (45). Recent studies utilizing T cell-specific filamin A-deficient mice have shown that filamin A is required for the optimal firm adhesion of T cells under shear flow conditions, trafficking of T cells into lymph nodes, and to the inflamed skin (46). These results demonstrate that in T cells, filamin A does not function as an integrin inhibitor but rather is required for cell trafficking *in vivo*. However, filamin A is not required for neutrophil adhesion under shear flow conditions, but instead filamin A-deficient neutrophils display enhanced adhesion, spreading, and defects in uropod retraction, thereby revealing cell-type specific functions of this integrin interacting protein (47, 48).

In contrast to leukocyte trafficking from blood stream to lymph nodes and tissues, leukocyte trafficking within tissues (e.g., in a confined 3D environment in the absence of shear flow) is a mechanistically different process that can occur even in the absence of integrins (49). In lymph nodes, integrins, and chemokine receptors contribute partly to naïve T cell migration speed (50). In this environment the integrin CD11a/CD18 (LFA-1) is required as a frictional interface with the substrate (the so called "integrin clutch") by generating traction forces, but does not mediate substantial adhesion to the substrate (50). In some cases, integrins can even *restrict* leukocyte migration in tissues. Indeed, the beta2-integrin-kindlin-3 interaction negatively regulates DC migration to lymph nodes both under steady state and inflammatory conditions (36, 51). beta2-integrins restrict DC migration through a downstream mechanism which involves regulation of the transcriptional program and migratory phenotype of these cells (**Figure 1**).

BETA2-INTEGRINS IN OTHER IMMUNE-RELATED FUNCTIONS

In addition to their fundamentally important role in leukocyte trafficking, beta2-integrins also mediate other cell-cell contacts that are essential for immunological processes (**Figure 1**). Beta2-integrins (e.g., CD11a/CD18-integrin; LFA-1) are central components of the immunological synapse which forms between an antigen presenting cell (APC) and a T cell [reviewed in Dustin (52)], between a B cell and a T cell (53) and between an NK cell and its target cell (54). In brief, the cell-cell interactions mediated by CD11a/CD18 on the T cell enables T cell activation, by binding to ICAM-1 on the APC. T cells sample antigens on dendritic cells in lymph nodes via short term contacts, termed kinapses (52). When antigen is found, T cells stop migrating and form an immunological synapse with the dendritic cell (52). LFA-1 on the T cell binding to ICAM-1 on the DC play a crucial role in this structure. LFA-1, together with talin, kindlin-3, and Rap1, is positioned in the p-SMAC (peripheral supramolecular activation cluster), thereby stabilizing the interaction between the T cell receptor and peptide-MHC II at the center of the contact (c-SMAC) (52, 55). Optimal T cell activation *in vivo* requires talin and kindlin-3 to bind to LFA-1 (32, 56). Upon activation, LFA-1 can the signal into the T cell and thereby contribute to T cell activation and polarization of the T cell response (57). For example, LFA-1 ligation in T cells has been shown to promote Th1 polarization through a pathway involving Erk and Akt-mediated GSK3beta-inhibition, in turn leading to activation of the Notch pathway (58), and LFA-1 can also be regulated by, and engage in crosstalk with TGF-beta signaling in T cells (59, 60). In addition, a role for an intracellular pool of beta2-integrins in T cell activation and differentiation has recently been reported (61).

In addition to T cell activation, CD11a/CD18 is involved in the killing of infected target cells by cytotoxic T cells, by stabilizing the contact between the T cell and the target cell, and by sealing the contact zone so that cytolytic granules cannot escape (57). LFA-1 furthermore plays a role in the generation of T cell memory (57), survival of T follicular helper cells (62) and regulatory T cells (63) and B cell-mediated antibody production, by mediating cell-cell contacts, but also by initiating intracellular signaling cascades (57, 64). LFA-1 is important for CD8+ T cell trafficking (65) and for Th2 (but not Th1) homing, as well as Th2-induced allergic lung disease (66). Interestingly, certain CD11a polymorphisms critically influence Th2 homing (67).

In myeloid cells such as macrophages, beta2-integrins can initiate intracellular signaling pathways leading to cytokine secretion, either by themselves or together with Toll like receptors (TLRs) (21, 68, 69). In addition, many neutrophil functions such as cytokine release and oxidative burst are dependent on beta2-integrins (70–73). CD11b/CD18 and CD11c/CD18 are receptors for complement component iC3b and are essential for phagocytosis of opsonized pathogens in neutrophils and other phagocytic cells, where they induce a RhoA-dependent phagocytic pathway (74–76). The differential roles of these two highly similar integrins have been studied *in vivo* in $CD11b^{-/-}$ and $CD11c^{-/-}$ mice. The results indicate that CD11b/CD18 is involved in neutrophil functions and in the anti-inflammatory functions of macrophages, whereas CD11c/CD18 is more relevant in the regulation of macrophage inflammatory functions (77). Recently, beta2-integrins has been shown to be required for recruitment of monocytes, as well as hematopoiesis of these cells during Schistosome infection, and a low expression of beta2-integrins correlates with increased parasite burden in a murine model of the disease (78).

BETA2-INTEGRINS IN IMMUNE SUPPRESSION

In addition to their well-characterized role in mediating cellular interactions and promoting pro-inflammatory

signaling, beta2-integrins have also been associated with many immunosuppressive functions (20) (**Figure 1**). Beta2-integrins can inhibit TLR signaling in macrophages through negative feedback loops, either directly or indirectly, through the anti-inflammatory cytokine IL-10 (79, 80). TLR stimulation leads to PI(3)K- and RapL-mediated inside-out activation of CD11b/CD18. Integrin outside-in signaling activates Src/Syk, leading ultimately to degradation of the important TLR signaling transducers MyD88 and TRIF and downregulation of TLR signaling (80). The mechanism of CD11b/CD18-dependent modulation of TLR responses has been shown to involve inhibition of the NF-κB pathway and activation of the p38 MAPK pathway (81). Beta2-integrins have been found to repress DC-mediated T cell activation (82–84), and the presence of CD11b/CD18 on APCs has been demonstrated to suppress Th17 differentiation and lead to immune tolerance (85, 86). Recently, CD11b/CD18-expressing neutrophils have been shown to suppress T cell-dependent influenza pathology *in vivo* by limiting T cell proliferation (87). The immunoregulatory role of leukocyte integrins may be taken advantage of by the macrophage-infecting bacterium *Francisella tularensis*, which is phagocytosed in a CD11b-dependent manner and uses the CD11b-driven inhibition of inflammasome activation to evade the innate immune system (88). In addition to opsonized bacteria, CD11b/CD18, and CD11c/CD18 also recognize iC3b-opsonized apoptotic cells, which leads to inhibition of proinflammatory cytokine production through NF-κB inhibition (89).

A series of important findings of the immunoregulatory roles of beta2-integrins has been produced using mice where the kindlin-3 binding site in the CD18-chain has been mutated, leading to expressed but inactive integrins on the surface of immune cells (TTT/AAA-beta2-integrin KI mice) (35). DCs from these mice mature toward a migratory phenotype, accumulate in lymphoid organs, and induce increased Th1 immune responses *in vivo* (51). In addition, functional integrins are essential for restricting the accumulation of mast cells in inflamed skin and mast cell responses *in vitro*, and inflammatory cytokine production in the inflamed skin *in vivo* (36). In the context of obesity-associated inflammation, mice on a high fat diet display increased numbers of neutrophils in white adipose tissue, increased insulin resistance and elevated inflammatory profile (90). However, the total deletion of an individual beta2-integrin, e.g., CD11b in mice led to increased weight gain on a high fat diet and lowered insulin sensitivity but to decreased inflammatory gene expression compared to WT mice *in vivo*, suggesting that the CD11b-integrin specifically is pro-inflammatory under diet-induced obesity conditions (91).

Interestingly, variations at the ITGAM gene, which encodes for CD11b, is one of the strongest genetic risk factors for systemic lupus erythematosus (SLE). These nucleotide polymorphisms confer amino acid changes in the CD11b protein, leading to deficient ligand binding, and a reduced ability to restrict cellular cytokine expression (92–94). Interestingly, activation of CD11b/CD18 with a CD11b agonist LA1 is able to overcome the effects of CD11b/CD18 malfunction in the carriers of the SLE-associated polymorphisms (95).

While most of the findings concerning the immunoregulatory role of beta2 integrins have been made in myeloid cells, also some lymphocyte subgroups express CD11b/CD18. Indeed, in B cells, CD11b/CD18 has been shown to negatively regulate B cell receptor signaling to maintain autoreactive B cell tolerance (96). Together, these results show that, while it is clear that beta2-integrins are important for immune cell activation and function, beta2 integrins (especially CD11b) have an equally significant role in repressing the body's reactions against self. Therefore, manipulating integrin activation pharmacologically could be an efficient therapeutic approach in treating certain inflammatory or autoimmune diseases.

LEUKOCYTE ADHESION DEFICIENCIES

The importance of beta2-integrins in immunity is highlighted by the rare genetic diseases known as Leukocyte adhesion deficiencies type I and type III (LAD-I and LAD-III) (**Table 1**). LAD syndromes are a group of congenital autosomal-recessive diseases with immune deficiency condition resulting in impaired leukocyte adhesion and migration. In LAD-I, the expression of CD18 (the beta2-integrin-chain) is either diminished or abolished. In LAD-III, mutations in kindlin-3 prevents it from activating beta2-integrins. Both conditions present partly with similar symptoms, which include leukocytosis and a lack of neutrophil extravasation from the blood stream into tissues. Consequently, the patients end up suffering from recurrent life-threatening infections, unless they receive a hematopoietic stem cell transplant (HSCT) (97). LAD-II is a selectin- (rather than integrin) related disease which is caused by a failure in selectin ligand expression (98) and will not be discussed further here.

LAD I—Over 200 mutations have been identified in LAD-I patients which cause decreased expression of CD18. The severity of the disease varies according to the functionality of the beta2-integrin (99). LAD-I patients suffer from life threatening bacterial and fungal infections early in life, and especially neutrophil trafficking is reduced into the inflamed tissue. In a recent (2018) review of all published LAD cases before 2017 (323 cases) (100) it was reported that the most common infections in severe LAD-I (<2% CD18 expression) were respiratory tract infections (including pneumonia), sepsis, and otitis media whilst in LAD-I with moderate CD18 expression the most common infections were periodontal infection, otitis media and sepsis. Perianal skin infections and necrotic skin ulcers were reported in both groups. Delayed umbilical cord detachment is common. In addition, patients suffer from symptoms such as delayed wound healing.

For severe LAD-I, survival beyond 2 years of age was only 39%, showing that severe LAD-I remains a life-threatening condition (100). The prognosis for LAD-I with moderate CD18 expression is much better, with survival over 2 years and beyond (to adulthood) for over 90% of cases with >4% CD18 expression (100, 101).

HSCT remains the only cure for patients expressing very low (<1–2 %) levels of CD18 protein in leukocytes, but unfortunately transplant-related mortality remains high (19% for all groups in LAD-I) (100).

TABLE 1 | Beta2-integrins in immunodeficiency and inflammatory disease.

Disease	Symptoms	Beta2-integrin defects	Impaired immune functions
LAD I	Bacterial and fungal infections in skin and other tissues; Delayed wound healing. Periodontitis, Leukocytosis, Candidiasis	Mutation in CD18 chain leading to decreased or non-existent expression of beta2-integrins	Decreased neutrophil trafficking to the site of inflammation. Defective adaptive immune responses (especially in T cells) Impaired restriction of inflammatory responses (e.g., cytokine release)
LAD III	Same as Lad I but also Glanzmann thrombasthenia. Osteopetrosis	Mutation in kindlin-3 protein, leading to incorrect activation of betal-, beta2-, and beta3-integrins	In addition to LAD I functions: Impaired platelet activation and blood clotting Impaired osteoclast function
SLE (Systemic Lupus Erythematosus)	Severe fatigue, Joint pain and swelling, Headaches, Rashes on cheeks and nose, Hair loss, Anemia, Blood-clotting problems	R77H, P1146S and A858V substitutions in CDIIb	Impaired ligand binding and phagocytosis(R77H) Increased adhesion, spreading, and migration (P1146S) Increased pro-inflammatory cytokine release (R77H and P1146S)

Beta2-integrin deficient mice have similar immune defects as LAD-I patients (102). These mice have been useful to investigate the role of beta2-integrins and their function in different leukocytes (102, 103).

LAD-III—LAD-III is a much rarer disease than LAD-I, with <40 patients reported worldwide (104). Patients suffer from similar symptoms as LAD-I patients, e.g., recurrent bacterial infections including bacteremias, pulmonary infections, omphalitis, and other soft tissue infections. Also fungal infections have been reported. However, unlike LAD-I patients, LAD-III patients additionally have Glanzmann type thrombasthenia, a bleeding disorder. Transfusions have been performed in >90% of cases as bleeding is a hallmark of the disease and remains a serious complication (105). In addition, recombinant factor VIIa has been used successfully in LAD-III to treat bleeding events (105). Furthermore, patients can suffer from osteopetrosis, due to deficient integrin-mediated osteoclast bone resorption.

LAD-III patients have normal integrin expression but carry mutations in the FERMT3 gene encoding kindlin-3 protein (106). Since kindlin-3 binds to beta1, beta2, and beta3-integrins and regulates their function, patients display more complex symptoms compared to LAD-I patients. In platelets kindlin-3 is required for αIIbβ3-integrin-mediated formation of blood clots. Kindlin-3 further regulates normal bone regeneration by several integrins. As for LAD-I, the only curative treatment for LAD-III is HSCT, and HSCT-related mortality remains high [22%, (105)].

Kindlin-3 has a central role in immunity which is shown by the phenotype of the kindlin-3 deficient mice (12, 38). These mice die early after birth because of excessive bleeding. These mice, as well as mice carrying a mutation in the kindlin-3 binding site in beta2-integrin cytoplasmic tail (TTT/AAA-beta2-integrin KI mice) have shown a crucial role of kindlin-3 and beta2-integrins in the regulation of immune cells (35, 36, 51, 56).

LAD AND INFLAMMATION

Many of the symptoms in LAD patients are thought to be caused by defective leukocyte (especially neutrophil) trafficking into inflamed tissue. However, not all symptoms in LAD-I are due to defective leukocyte-mediated immune surveillance. Instead, periodontitis and associated bone loss in LAD-I has recently been shown to be associated with an increased inflammatory response, with excessive production of IL-17 and related cytokines (107), and blocking the IL-17 cytokine response reduces symptoms in a LAD-I patient (108). In addition, particular inflammatory disorders (e.g., colitis) have been reported in LAD-patients (109–111). This indicates that at least some pathological symptoms in LAD-I patients are caused by dysregulated inflammatory responses. The increased IL-17 production in LAD-I patients may be—at least in part—due to defective neutrophil recruitment into tissues e.g., dysregulation of the so called "neutrostat," which senses and regulates neutrophil numbers *in vivo* (107). However, beta2-integrins have been shown to *directly* restrict cytokine responses in many types of immune cells, such as macrophages (80), DCs (51), and mast cells (36), and to restrict Th1 (51) and Th17 (85) polarization *in vivo*. In addition, functional beta2-integrins restrict expression of cytokines in a skin inflammation model, although neutrophil trafficking is relatively normal in this model (36). Dysregulated cytokine responses may therefore contribute to the paradoxical increase in inflammation (periodontitis, colitis) in LAD-I patients (107, 109, 110, 112).

THERAPEUTIC TARGETING OF BETA2-INTEGRINS

Because of the crucial role of beta2-integrins in leukocyte functions such as leukocyte recruitment, beta2-integrins have been considered attractive targets in inflammatory disease such as psoriasis, arthritis, and multiple sclerosis [reviewed in Mitroulis et al. (113)]. Indeed, an antibody against alphaL-integrin chain, efalizumab, has previously been in clinical use in psoriasis (113). However, the drug was withdrawn from the market in 2009 because it was associated with serious side effects, e.g., reactivation of latent John Cunningham (JC) virus infection and resulting progressive multifocal leukoenphalopathy (PML). Therefore, therapeutic blocking of beta2-integrins in disease may be difficult because these molecules play such multifaceted roles in central immune reactions.

CONCLUSIONS AND FUTURE PERSPECTIVES

Beta2-integrins are of crucial importance for leukocyte trafficking and immune cell activation, but interestingly play a role in immune suppression as well. Consequently, dysfunctional or absent integrins are linked not only to immune deficiency disease but also to inflammatory disease, thereby contributing to both ends of the spectrum of immune-related diseases. A better understanding of the disease processes where dysfunctional beta2-integrins are involved may provide novel drug targets for immunodeficiency and inflammatory disease symptoms (95, 108).

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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Leukocyte Trafficking and Regulation of Murine Hematopoietic Stem Cells and Their Niches

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Hematopoietic stem cells (HSC) are the most powerful type of adult stem cell found in the body. Hematopoietic stem cells are multipotent and capable of giving rise to all other types of hematopoietic cells found in the organism. A single HSC is capable of regenerating a functional hematopoietic system when transplanted into a recipient. Hematopoietic stem cells reside in the bone marrow in specific multicellular structures called niches. These niches are indispensable for maintaining and regulating HSC numbers and function. It has become increasingly clearer that HSC and their niches can also be regulated by migrating leukocytes. Here we will discuss the composition of murine bone marrow niches and how HSC and their niches are regulated by different types of leukocytes that traffic between the periphery and the niche. Unless otherwise indicated all the studies discussed below were performed in mouse models.

Keywords: hematopoietic stem cell, niches, leukocyte trafficking, neutrophils, Tregs

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ORGANIZATION OF THE MURINE HSC NICHE

Bone marrow (BM) Hematopoietic stem cells niches are very complex structures in which different cell types with overlapping and unique functions cooperate to regulate HSC maintenance, self-renewal, trafficking, and differentiation. Loss of niche cells or niche-derived signals inevitably leads to loss of HSC. A scheme showing the overall structure of the murine BM niche is shown in **Figure 1**. Key niche components are:

Endothelial Cells

The BM is enclosed by bone but blood vessels are the main structure that defines and organizes the BM cavity. Arterioles enter the BM through the bone before giving rise to a dense sinusoidal network that drains through a central vein (1). Imaging studies have revealed that all HSC are intimately associated with the vasculature. Multiple independent approaches confirmed the role of endothelial cells as critical components of the murine niche. The cytokines Cxcl12 and stem cell factor (Scf) are key regulators of HSC trafficking and self-renewal. The Morrison and Link's groups demonstrated that conditional *in vivo* deletion of Cxcl12 or Scf in BM endothelial cells (using *Tie2-cre* mice) was sufficient to cause loss of HSC (2–4). Similarly the Butler group showed that conditional *Jagged1 in vivo* deletion in endothelial cells (using *Ve-cadherin-cre* mice) led to HSC exhaustion (5). In the BM E-selectin is only expressed by endothelial cells (6), the Levesque group showed that HSC in E-selectin knockout mice have reduced cell cycling indicating that endothelial E-selectin promoted HSC proliferation (6). These studies formally demonstrated that endothelial cells are bona-fide niche cells with different functions in regulating steady-state HSC.

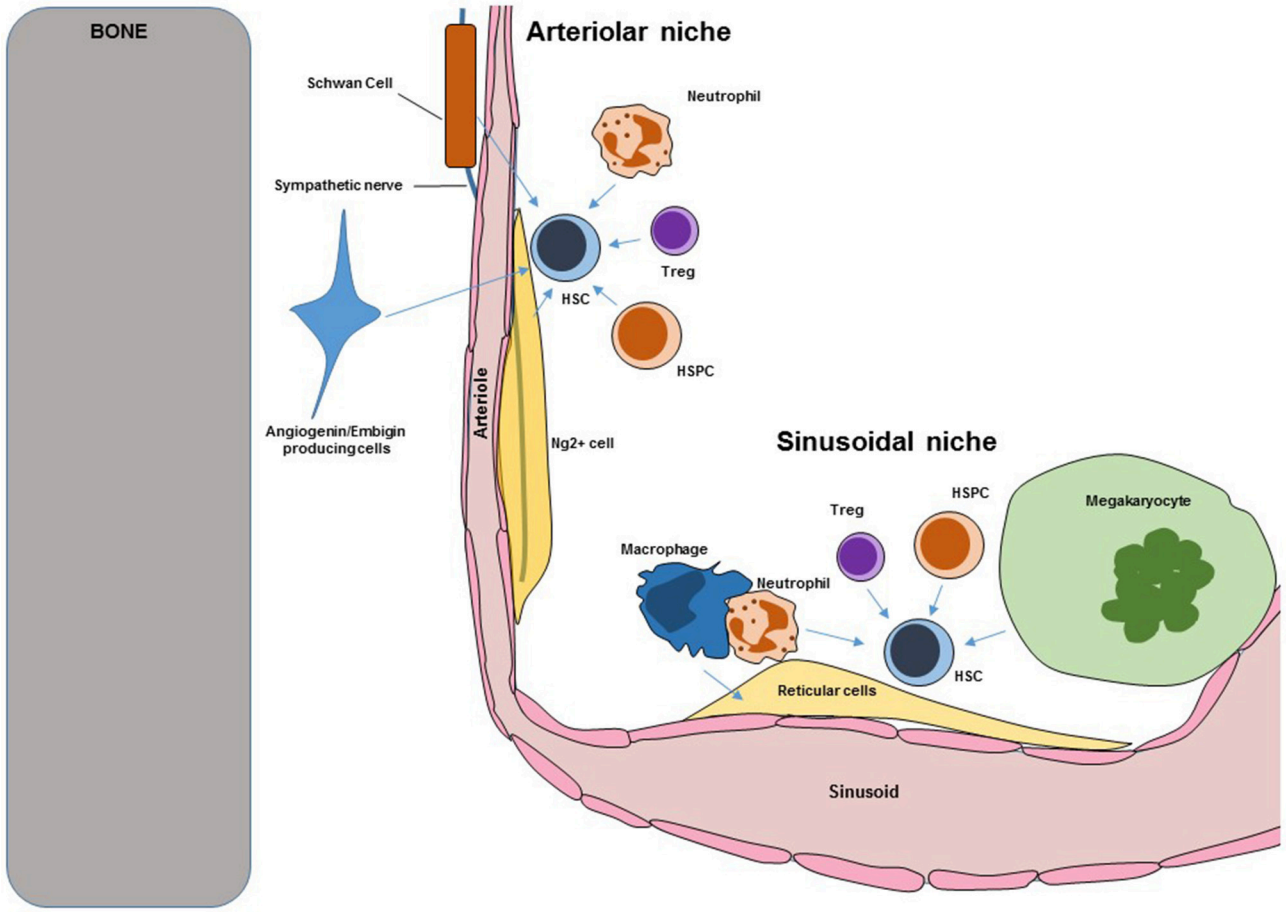

FIGURE 1 | Scheme showing key components of murine BM HSC niches. Blue arrows indicate regulation. HSC, hematopoietic stem cells; HSPC, hematopoietic stem and progenitor cells.

In addition *in vivo* conditional overexpression of the Notch1 intracellular domain (that leads to increased Notch signaling) in endothelial cells led to increased angiogenesis and HSC numbers (7). In contrast, conditional deletion of *Rbpj* (which is required for transcription of Notch regulated genes) led to deficits in BM endothelial cells (8). These indicate that endothelial cells not only regulate HSC directly but also regulate the number of niches.

The most dramatic example of the importance of endothelial cells in hematopoiesis is during regeneration. Myeloablation (the chemotherapy and/or radiation treatments used to condition the BM prior HSC transplantation) also leads to the almost complete disappearance of BM sinusoids (1, 9). Restoration of a functional sinusoidal network is the limiting step in reestablishing normal hematopoiesis (9). This is not only due to restoration of their homeostatic functions but because endothelial cells upregulate molecules like Jagged2 and Pleiotrophin that promote HSC engraftment and hematopoietic regeneration (10, 11). The precise mechanisms through which sinusoids are restored are not well established although it is known that the *Vegf, Tnfa, Nfκ B,* and *Angiopoietin1* pathways have different contributions during regeneration (9, 12–14).

Stromal Cells

Reticular Stromal Cells

The BM is crisscrossed by a network of reticular stromal cells that also associates with blood vessels. These stromal cells produce Cxcl12, Scf, Pleiotrophin, and other cytokines that maintain and regulate HSC (15). These cells receive different names depending on the method used to isolate them. For example CAR stands for Cxcl12-abundant reticular cells and are isolated using *Cxcl12-gfp* reporter mice, *Nestin-GFP^{dim}* cells are isolated using *Nestin-gfp* reporter mice and LepR^{+} cells are detected using *LepR-cre:Tomato* mice (15). Because the *LepR-cre* mouse also allows genetic manipulation of the reticular stromal cells it is quickly becoming the method of choice to label these cells (3). Reticular cells have osteoprogenitor and adipogenic potential *in vivo*, have mesenchymal stem cell activity (i.e., can differentiate to osteoblasts, adipocytes, and chondrocytes) *in vitro*, and upon transplantation in ossicles can generate an ectopic niche that supports extramedullary hematopoiesis (16, 17). Conditional deletion of *Cxcl12*, *Scf*, *Pleiotrophin* and other genes in these reticular cells results in loss of HSC (2–4, 11). Taken together these studies formally demonstrate that reticular stromal cells are a niche for HSC in the BM.

Periarteriolar Ng2 $^{+}$ Stromal Cells

These are an extremely rare population of stromal cells that ensheathes the arterioles in the BM. These cells can be labeled as Nestin-GFP^{bright} cells using *nestin-gfp* mice or as Ng2^{+} cells using *Ng2-cre^{ERT2}:gfp mice* (1). Even though they are very rare they are key regulators of HSC function. Imaging analyses showed that ∼30% of BM HSC localized to arterioles and that this association was closer than expected from random suggesting that these cells might be niche components. Conditional depletion of Ng2^{+} cells using *Ng2-Cre^{ERT2}:iDTR* mice led to HSC loss (1). In a follow up experiment the Frenette group showed that Ng2^{+} cells are the major source of CXCL12 in the BM and that conditional *Cxcl12* deletion in these cells led to loss of quiescent HSC (18). These results demonstrate that Ng2^{+} periarteriolar stromal cells are a key component of the HSC niche.

Non-myelinating Schwann Cells

These are very rare cells that ensheath the sympathetic nerves that enter the BM via arterioles. Because of this they are intimately associated with arterioles and HSC. In a seminal study the Nakauchi lab showed that these glia cells are the main source of activated Tgfβ in the BM and that sympathetic denervation led to the loss of these cells and concomitant HSC loss (19). Despite their role in HSC maintenance the function and regulation of these cells is not well-studied. This is because it has not been yet possible to purify these cells for more detailed analyses.

Embigin and Angiogenin-Producing Cells

A fraction of HSC localizes close to the endosteal surface of the bone. By purifying and comparing the expression profile of endosteal cells that were proximal or distal to HSC after transplantation the Scadden and Hu laboratories identified embigin and angiogenin as candidate HSC "niche" factors. Conditional angiogenin deletion in reticular stromal cells and Ng2^{+} periarteriolar cells led to increased numbers of HSC in the BM due to increased proliferation but these HSC were deficient in engraftment potential indicating that angiogenin is necessary to maintain HSC function (20). In addition they found that angiogenin deletion in Osterix^{+} osteoprogenitors also caused loss of HSC indicating that these cells also function as an HSC niche (20). The mechanism of action of angiogenin is especially interesting. Angiogenin is a ribonuclease secreted by stromal cells that is then imported into HSC where it modulates endogenous RNAs. This causes reductions in protein synthesis and increases in HSC function (21). Antibody blockade of embigin leads to HSC proliferation and accumulation in the BM. Embigin producing cells could be isolated as col2.3-GFP^{+}Embigin^{+}VCAM^{+} cells (using *col2.3-gfp* reporter mice) and were also enriched for CXCL12. These experiments demonstrated that these cells are a novel component of the niche (20).

Hematopoietic Cells

Megakaryocytes

These are very large multinucleated cells that localize, exclusively, to the sinusoids where they release platelets to the circulation. They are hematopoietic cells and were the first hematopoietic cells shown to directly regulate HSC. The role of megakaryocytes in the niche was independently discovered by the Frenette', Li', and Suda's groups (22–25). Imaging analyses revealed that most sinusoidal HSC are also in contact or within 5 μm of megakaryocytes. Megakaryocyte depletion using *Cxcl4-cre:iDTR* or *Cxcl4-cre:Mos-iCsp3* mice induced a 10-fold increase in BM HSC due to hyperproliferation that was followed by HSC loss due to exhaustion (22–25). These results indicated that megakaryocytes maintain HSC numbers and fitness by restricting proliferation. Megakaryocytes are the main source of the cytokine CXCL4 and *Cxcl4$^{-/-}$* mice had fewer functional HSC (22). Megakaryocytes are also a major source of TGFβ and administration of these cytokine into megakaryocyte-depleted mice rescued the HSC phenotype (25). Bone marrow megakaryocytes also produce thrombopoietin which is known to regulate HSC quiescence. Deletion of the C-type lectin like receptor-2 in megakaryocytes using *Cxcl4-cre:Clec2$^{flox/flox}$* mice led to impaired thrombopoietin production by megakaryocytes and fewer megakaryocyte and HSC numbers (23, 24). These results indicate that megakaryocytes regulate HSC numbers and function by secreting Cxcl4, Tgfβ, and Thrombopoietin (22–25).

Hematopoietic Progenitors

Hematopoietic stem cells and progenitors can also regulate each other. Because E-selectin induces HSC proliferation (6) the Hidalgo lab examined whether expression of the E selectin ligand Esl1 in HSC might mediate this regulation. Unexpectedly they found reduced HSC numbers and proliferation in Esl1-knockout mice. Further when Esl1-deficient hematopoietic stem and progenitor cells (HSPC) were cotransplanted together with WT HSPC into WT recipients the WT HSPC also showed reduced numbers and proliferation. These indicated that Esl1 expression regulated HSPC proliferation in a non-autonomous manner (26). This is likely mediated via two different effects. The first one is the observation that hematopoietic Esl1 deficiency leads to reductions in a key component of the murine niche: reticular stromal cells (26). The second is modulation of Tgfβ activity as blockade of this pathway in cocultures of WT and Esl1-deficient HSPC rescued the proliferation defect in both genotypes (26). This study shows that HSC and their immediate offspring regulate each other and reticular stromal cells in the niche.

Leukocytes

These are mature hematopoietic cells that were thought to have no role in regulating hematopoiesis. However, a series of studies in the last decade have demonstrated that mature leukocytes (macrophages, neutrophils, and T-cells) are critical regulators of HSC and niche function and that leukocyte trafficking also impacts HSC. How migrating leukocytes function in the niche is the focus of the second part of the review.

Other Candidate Niche Cells

In addition to the cell types described above, other stromal cells (osteoblasts, osteocytes, osteoclasts, and adipocytes) have been proposed to be components of the HSC niche in some studies while other studies have shown no role for these cells in HSC regulation. The evidence for and against the role of each of these

cells in the niche was reviewed recently (27). Additional studies are needed to precisely clarify the role of these cells in the HSC niche and in the BM microenvironment.

Distal Regulation of Bone Marrow HSC by Other Organs

All the cell types described above reside in the BM and most of them are intimately associated with HSC. Imaging of HSC location and interaction with candidate niche cells remains one of the most powerful tools to identify new components of the niche. However, an emerging concept in the field is that HSC and their niches can be regulated (directly or indirectly), long-distance, by different organs.

The nervous system is the best characterized organ(s) that regulates HSC distally. The initial discovery showed that sympathetic innervation of the BM is necessary for HSC release from their niches into the circulation (28). Follow up studies showed that the sympathetic nervous system orchestrates daily oscillations of Cxcl12 production by the niche and thus controls HSC trafficking (16, 29), regulates the regeneration of the niche after myeloablation (30) and even controls niche remodeling during hematopoietic malignancies (31, 32) and aging (33).

HSC can also be regulated by hormones; parathyroid hormone acts on stromal cells (likely reticular stromal cells) increasing their number and thus leading to increased HSC numbers (34). In female mice, estrogen acts directly on HSC to drive their proliferation (35). Ovariectomy suppressed this effect indicating that ovaries are the source of estrogen that regulates HSC (35). Pituitary glucocorticoids act directly on HSC to impair their mobilization in response to granulocyte colony-stimulating factor (G-CSF) (36). A series of studies in zebrafish and mouse models and with human cells showed that prostaglandins positively regulate HSC numbers under homeostasis and can be used to promote regeneration and HSC engraftment after *in vivo* and *ex vivo* treatments and to mobilize stem cells from the bone marrow to the circulation where they can be harvested for transplantation (37–41).

Two recent studies demonstrated that the liver and the intestine also regulate BM HSC. Thrombopoietin has long been known to regulate HSC quiescence but the source of this cytokine remained unknown. Using elegant conditional deletion experiments, the Ding lab suggested that BM sources of thrombopoietin did not regulate HSC (42). Instead they found that deletion of thrombopoietin from hepatocytes results in loss of HSC quiescence and subsequent exhaustion (42). Although very interesting, this study raises two important questions. The first one is that megakaryocyte-derived thrombopoietin was reported to regulate HSC quiescence (23, 24). The second is that thrombopoietin is also required for megakaryocyte maturation and megakaryocytes regulate HSC quiescence (22–25). It will be interesting to dissect the contribution of megakaryocyte- and hepatocyte-derived thrombopoietin to HSC maintenance and to determine whether they function by acting directly on HSC or indirectly by regulating megakaryocyte numbers. The intestine also regulates BM HSC distally; the Hidalgo lab showed that intestinal macrophages regulate the activity of the niche by modulating G-CSF production (43). Because these macrophages are regulated by trafficking leukocytes their function and regulation are discussed in detail in the next section.

FUNCTIONAL AND SPATIAL HETEROGENEITY IN THE NICHE

In the last 5 years it has become increasingly clear that HSC are not a homogeneous population and can be fractionated (based on expression of different markers) into subsets with different *in vivo* potential. Examples of this heterogeneity are the use of *Hdc-GFP* reporter mice to identify Hdc-GFP$^+$ myeloid biased HSC (44); the use of von Willebrand factor-reporter mice to identify HSC biased toward megakaryocyte production (45); different levels of reactive oxygen species (ROS) (46); and differences in cell cycle status (1). The mechanisms underlying this heterogeneity are not known but it is likely that this is mediated by interactions with components of the niche in arteriolar and sinusoidal locations. Several lines of evidence support this. Ng2$^+$ cells localize, exclusively, to arterioles where they are intimately connected with endothelial cells, sympathetic axons, and GFAP$^+$ Schawnn cells (1). In contrast, sinusoids are surrounded by a network of reticular cells and are the site where megakaryocytes localize (3, 22). Imaging analyses (defining HSC as Lin$^-$CD48$^-$CD41$^-$CD150$^+$ cells) showed that most HSC associate with sinusoids with a smaller fraction that localizes close to arterioles (1, 46). The large majority of BM HSC (80%) are quiescent with a smaller fraction (20% of all HSC) actively cycling. Using Ki67 to detect cycling and non-cycling HSC the Frenette lab found a statistically significant difference in the localization of Ki67$^+$ and Ki67$^-$ HSC with the latter group localizing farther away from arterioles (1). Ablation of Ng2$^+$ periarteriolar cells using *Ng2-cre^{ERTM}:iDTR* mice lead to reductions in HSC numbers, loss of quiescence, and relocalization of HSC away from arteries (1). These results suggest that arterioles are a niche that maintains a subset of HSC that associate with them, and that subset is enriched in quiescent HSC. In agreement with these results the Lapidot lab showed that HSC could be fractionated according to the level of reactive oxygen species (ROS) by *in vivo* injection of hydroethidine (46). Previous studies had shown that ROS cause HSC proliferation and migration in the bone marrow (47, 48). The Lapidot lab found that HSC that localized to arterioles were uniformly ROS^{low} (and presumably quiescent) whereas HSC that localized to sinusoids could be ROS^{high} or ROS^{low} (46). They also found that conditional deletion of *Fgfr1* and *Fgfr2* in endothelial cells caused increased vascular permeability which in turn caused ROS accumulation in the stem cells and reductions in HSC numbers. This was due to the increases in ROS levels as treatment with a ROS scavenger rescued the HSC defect in the *Fgfr1/2* conditional knockouts (46). In agreement with this, the Frenette group recently reported that conditional SCF deletion in arteriolar, but not sinusoidal, endothelial cells (using *Bmx1-cre* as an arteriole-specific cre) caused HSC loss (49).

The studies above support the concept that arterioles maintain a subset of HSC that is enriched for cells in a quiescent/low

metabolic status. However, sinusoids also maintain a subset of HSC while promoting quiescence. Megakaryocytes localize to the sinusoids and ROS^{low} HSC in the sinusoids colocalize with megakaryocytes (46): loss of megakaryocytes or megakaryocyte-derived molecules like Cxcl4, Tgfβ, and Thrombopoietin lead to HSC proliferation and exhaustion (22–25). However, megakaryocyte ablation did not impact the localization of the HSC subset close to arterioles suggesting that arteriolar and sinusoidal niches were functionally independent (22). The Jacobsen and Nerlov labs showed that von Willebrand factor-reporter mice can be used to identify vWF-eGFP$^+$ HSC that were biased toward a myeloid and megakaryocytic fate whereas vWF-eGFP- HSC were lymphoid biased. Using imaging analyses the Frenette lab reported that vWF-eGFP$^+$ HSC localized to sinusoidal megakaryocytes whereas vWF-eGFP$^-$ HSC localized to arterioles (50). Megakaryocyte depletion using *CD169:iDTR* mice caused exhaustion of myeloid-biased vWF-eGFP$^+$ HSC but not lymphoid-biased vWF-eGFP$^-$ HSC (50). Ng2$^+$ cell depletion using *NG2-cre^{ERTM}:iDTR* mice caused loss of lymphoid-biased but not myeloid-biased HSC (50).

Arteriolar endothelial cells, Schawnn cells, sympathetic nerves, and Ng2$^+$ periarteriolar cells are intimately associated (1). Similarly, sinusoidal endothelial cells are tightly associated with reticular stromal cells and megakaryocytes (3, 22). These suggest that different types of niche components associate form spatially and functionally independent niches that maintain different subsets of HSC by promoting quiescence. They also suggest that the subset of cycling/metabolically active HSC (20% of all HSC) localizes to the sinusoids where they are maintained in a megakaryocyte-independent manner. However, controversies remain. For example the Frenette group recently reported that Cxcl12 deletion in Ng2$^+$ periarteriolar cells but not LepR$^+$ reticular stromal cells caused BM HSC loss (18). This challenges two manuscripts by the Morrison's and Link's groups showing that reticular stromal cells are the major source of Cxcl12 that maintains HSC numbers (2, 4). Another controversy is that while imaging analyses using Lin$^-$CD48$^-$CD41$^-$CD150$^+$ to identify HSC showed a clear association between a subset of HSC and arterioles (1, 46) imaging HSC as α-catulin-GFP$^+$c-kit$^+$ cells in *α-catulin-gfp* reporter mice did not find a specific association of HSC with arterioles (51). Instead they found that all HSC preferentially associated with sinusoids and LepR$^+$ perivascular cells (51). Thus, more detailed analyses are needed to reconcile these results.

REGULATION OF HSC AND THEIR NICHES BY LEUKOCYTES. ROLE OF LEUKOCYTE TRAFFICKING

HSC and their niches are also regulated by different types of leukocytes. This adds two layers of complexity to HSC regulation. All leukocytes are the offspring of stem and progenitor cells. When leukocytes impact the number and function of the HSC they will, ultimately, affect their own production which in turn might further affect HSC function. This is further complicated because many of the pathways that regulate HSC retention in the niche and trafficking into the circulation like Cxcl12 (**Figure 2**), S1P (52), Ccr2 (53), and Cxcr2 (54) signaling also regulate leukocyte trafficking. In this section we discuss recent advances showing how leukocytes regulate HSC and their niches and how leukocyte migration impacts these regulatory mechanisms.

Macrophages

Three independent studies showed a critical role for BM macrophages in retaining HSC in the niche. While studying the mechanisms of G-CSF-induced HSC mobilization the Levesque lab noticed that G-CSF caused loss of a population of macrophages that was intimately associated with endosteal cells and that they named "osteomacs." Selective depletion of myeloid cells using MAFIA mice or phagocyte depletion with clodronate-loaded liposomes caused osteomac loss and HSC mobilization. This correlated with downregulation of Cxcl12 and SCF in stromal cells purified from the endosteum (55). The Link laboratory had previously shown that G-CSF acts on a hematopoietic cell to induce mobilization but the identity of these cells remained unknown (56). In a follow up study they generated *CD68-G-CSFR* mice in which the G-CSF receptor is expressed, exclusively, in monocytes and macrophages (57). G-CSF-induced HSC mobilization in this model was as potent as in wild-type mice and caused loss of monocytes/macrophages in the BM. Since G-CSF functions by downregulating Cxcl12 in the niche these experiments demonstrated that monocytes/macrophages modulated niche activity in response to G-CSF (57). The Frenette lab hypothesized that BM macrophages might regulate Cxcl12-producing reticular cells in the niche. Using three models of monocyte/macrophage ablation (*CD11b-DTR*, MAFIA and clodronate-loaded liposomes) they demonstrated that monocyte/macrophage ablation was sufficient to downregulate *Cxcl12* production in BM reticular stromal cells. They then used *CD169-DTR* mice, which depletes macrophages but not monocytes, to demonstrate that macrophages controlled niche function (58). These studies showed that macrophages crosstalk with niche components to control the production of Cxcl12 that retains HSC in the niche. It is also possible that macrophages might regulate HSC directly. The Lapidot lab found rare αSMA1$^+$COX2$^+$ macrophages that colocalized with HSC. These cells could promote HSC survival *in vitro* in a COX2-dependent manner (likely via prostaglandin E2 production by COX2). *In vivo* pharmaceutical COX2 inhibition led to HSC depletion suggesting that these αSMA1$^+$ macrophages maintain HSC (59).

Macrophages also modulate HSC engraftment after transplantation. Kaur et al. used *Csf1r-gfp* reporter mice to demonstrate that CD169$^+$ BM macrophages survived irradiation and regenerated autonomously (i.e., independently of donor HSC). Depletion of these macrophages using *CD169-DTR* mice completely blocked HSC engraftment demonstrating a critical role for macrophages during regeneration (60).

Neutrophils

These are short-lived cells that are indispensable to maintain innate immunity. They are produced in large numbers in the BM and enter the circulation and tissues before being cleared a few hours later in the BM, liver, and spleen. The

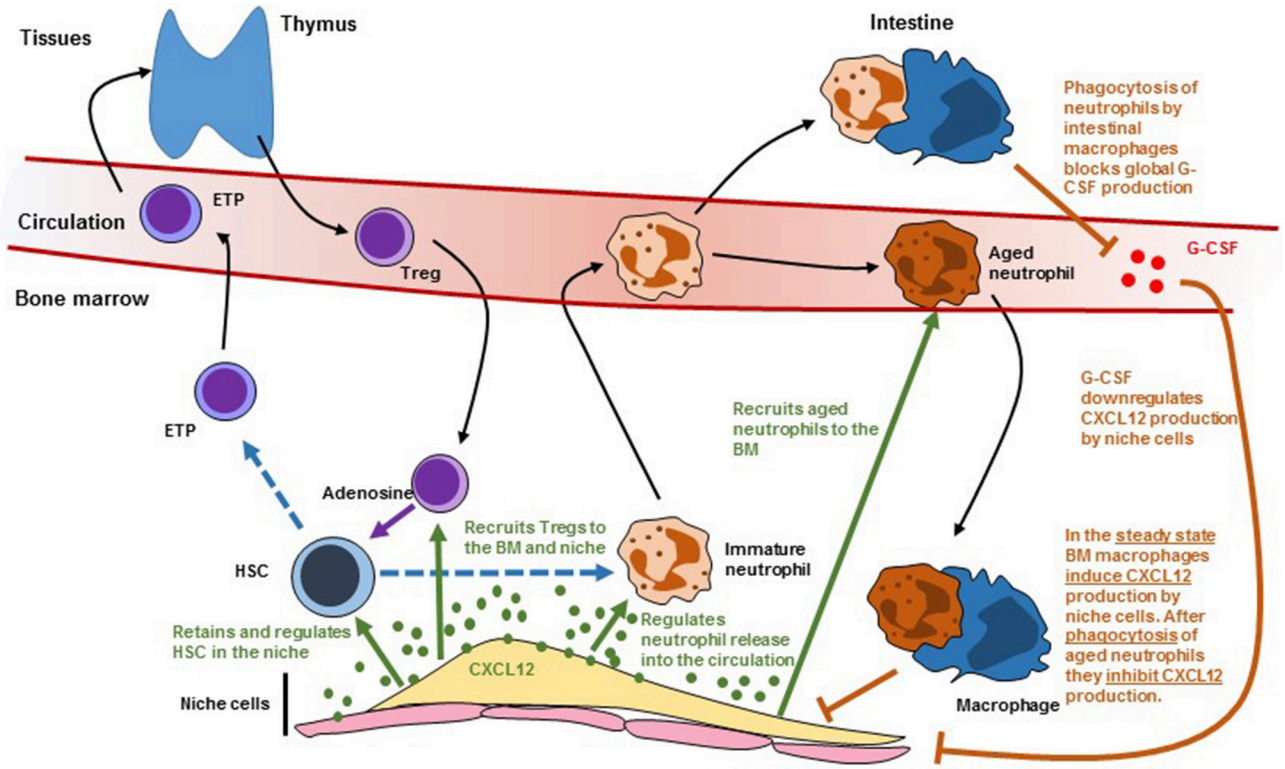

FIGURE 2 | Scheme showing how leukocyte trafficking regulates (and is regulated by) niche cells. Dashed lines indicate differentiation. Solid black arrows indicate migration. Solid green arrows indicate regulation by CXCL12. Solid orange arrows indicate regulation by macrophages. HSC, hematopoietic stem cells**;** ETP, early T cell progenitor; G-CSF, granulocyte colony-stimulating factor.

Hidalgo lab found that aged neutrophils can be defined as $CD62L^{lo}Cxcr4^{hi}$ cells and discovered that the number of these aged neutrophils in the blood oscillated following a circadian pattern (61). Because previous studies showed that circadian oscillations in sympathetic activity regulated Cxcl12 production in the niche (16) they investigated whether neutrophils could also impact the niche. They found that circadian oscillations in the number of Cxcl12-producing reticular cells in the BM were controlled by neutrophil trafficking; neutrophil depletion, or blocking recruitment of aged neutrophils to the BM (by deleting Cxcr4 in neutrophils) led to increases in reticular stromal cells and reduced HSC release into the circulation (61). These phenotypes required that BM macrophages phagocytosed the aged neutrophils and was dependent on expression of LXR receptors in the macrophages (61). This study was the first demonstration of a mature cell regulating niche size. In a follow up study the Hidalgo lab found that neutrophil trafficking into the intestine also controlled HSC niche activity in the BM. Mice deficient in FUT7 have neutrophils with limited ability to extravasate into tissues. These mice also showed reduced numbers of Cxcl12-producing reticular niche cells in the bone marrow and constitutive HSC release in the circulation (43). These phenotypes can be rescued by parabiosis (joining the circulation) with WT mice indicating that neutrophil extravasation regulates niche activity (43). Surprisingly, the niche and HSC trafficking phenotype could also be rescued when $Fut7^{-/-}$ mice were parabiosed with $Mrp8\text{-}cre;Cxcr4^{fl/fl}$ mice in which neutrophil recruitment to the BM is completely abolished (43). This indicated that the niche defect in $Fut7^{-/-}$ mice was independent of the recruitment of aged neutrophil to the BM described above (61). When analyzing the fate of extravasated neutrophils they found that only intestinal macrophages failed to engulf $Fut7^{-/-}$ neutrophils. They found that neutrophil phagocytosis by intestinal macrophages inhibited IL23 production by these cells; this in turn led to lower global levels of G-CSF which led to reduced HSC release from BM niches (43). In the $Fut7^{-/-}$ mice there were increased IL23 and G-CSF levels and antibody blockade of either molecule was sufficient to correct HSC release from the niche (43). These two studies highlight how neutrophil trafficking and phagocytosis by macrophages in different tissues controls niche activity. It is also likely that neutrophils also regulate HSC function directly. Using histidine decarboxylase-GFP (*Hdc-GFP)* reporter mice, the Wang lab found that around 10% of HSC were $Hdc\text{-}GFP^{+}$. In transplantation experiments these $Hdc\text{-}GFP^{+}$ HSC produced higher numbers of myeloid cells than the $Hdc\text{-}GFP^{-}$ HSC indicating that they were myeloid biased (44). They also found that $Hdc\text{-}GFP^{+}$ HSC in the marrow of $Hdc^{-/-}$ mice cycled faster which in turn led to exhaustion and loss of myeloid biased HSC, indicating that histamine maintains $Hdc\text{-}GFP^{+}$ HSC by restricting their proliferation (44). The major source of histamine in the bone marrow are

neutrophils and imaging analyses showed that myeloid biased Hdc-GFP$^+$ HSC were in contact with HDC-GFP$^+$ neutrophils. In contrast there was no specific association between HDdc-GFP$^+$ neutrophils and Hdc-GFP$^-$ HSC. These results indicate that histamine producing cells (likely neutrophils) regulate myeloid biased HSC (44). A second possibility is that Hdc-GFP$^+$ HSC might regulate themselves via histamine secretion in an autocrine loop. Neutrophils also control the regeneration of endothelial cells in the niche. After myeloablation, immature BM neutrophils are recruited to injured vessels where they promote vessel and hematopoietic regeneration via TNFα secretion (12).

Regulatory T Cells

The Lin laboratory found that, after allogeneic transplantation of HLA-mismatched HSC, the donor stem cells survived in the recipients without any type of immunosuppression indicating that BM niches were immune privileged (62). Imaging analyses showed that these allogeneic HSC were surrounded by Foxp3$^+$ regulatory T cells. Depletion of Tregs by using *FoxP3-DTR* mice led to loss of the allogeneic HSC. Transfer of WT but not IL10$^{-/-}$ Tregs prevented allogeneic HSC loss after transplantation (62). These results demonstrated that Tregs confer immune privilege to the niche via IL10 signaling. Tregs also regulate HSC in the steady-state. Tregs in *FoxP3-cre;Cxcr4$^{fl/fl}$* mice have reduced trafficking to the BM. These causes a ∼2-fold increase in HSC and was mediated by increased reactive oxygen species (ROS) in HSC as antioxidant treatment rescued the HSC expansion (63). Imaging analyses showed that Tregs localized close to *Cxcl12*-producing reticular cells and sinusoids and that a subset of Tregs that expressed high levels of CD150 associated with HSC (63). These CD150$^+$ Tregs regulate HSC via adenosine as *FoxP3-cre;CD39$^{fl/fl}$* mice, in which Tregs are deficient in adenosine production, or wild-type mice treated with adenosine receptor antagonists, also showed increased HSC numbers (63). These studies show that Treg recruitment to the niche regulates BM HSC metabolism.

CONCLUSIONS AND OPEN QUESTIONS

Different types of leukocytes traffic between the periphery and the BM where they regulate the numbers and function of HSC and their niches. These regulatory pathways likely crosstalk at multiple different levels (**Figure 2**). For example, Treg recruitment to the niche is mediated by Cxcl12/Cxcr4 signaling and LepR$^+$ reticular stromal cells (63). Macrophages regulate Cxcl12 production in the niche after being activated by phagocytosing aged neutrophils (61). These neutrophils are also recruited to the BM via Cxcl12/Cxcr4. The same signals regulate immature neutrophil release to the circulation which might impact the ability of intestinal macrophages to regulate systemic G-CSF which will further impact niche function (43). The Cxcl12/Cxcr4 pathway is not the only signal that regulates both HSC and leukocyte trafficking. *Cxcr2*$^{-/-}$ mice have increased HSC numbers but these stem cells have impaired function in transplant assays (54). This study indicated that Cxcr2 signaling regulates HSC. However, Cxcr2 is a critical regulator of neutrophil trafficking and *Cxcr2*$^{-/-}$ neutrophils are retained in the bone marrow (64). This neutrophil trafficking defect presumably will alter niche function through the mechanisms described in the previous section. Sphingosine 1-phosphate (S1P) regulates HSC trafficking from blood to tissues and lymph (52). However, S1P also triggers Cxcl12 release by reticular stromal cells (65) and inhibits Treg differentiation (66). Loss of adhesion molecules like selectins and integrins (α4, β1, or β2) all impact both HSC and leukocyte trafficking. Teasing apart direct effects on stem cells from those mediated indirectly by alterations in leukocyte migration is necessary to gain a better understanding of how these pathways regulate normal and diseased hematopoiesis.

The crosstalk between leukocytes and HSC niches likely functions as a biological rheostat through which the BM monitors the periphery. Leukocyte numbers and trafficking are altered after inflammation or infection, and in many hematological diseases (for example acute myeloid leukemia or myelodysplastic syndromes). It is likely that alterations in leukocyte trafficking direct bone marrow hematopoietic output and contribute to the disease phenotypes. This is an area of great interest for future investigations.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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REVIEW
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Capturing the Fantastic Voyage of Monocytes Through Time and Space

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Monocytes are a subset of cells that are categorized together with dendritic cells (DCs) and macrophages in the mononuclear phagocyte system (MPS). Despite sharing several phenotypic and functional characteristics with MPS cells, monocytes are unique cells with the ability to function as both precursor and effector cells in their own right. Before the development of hematopoietic stem cells (HSCs) *in utero*, monocytes are derived from erythro-myeloid precursors (EMPs) in the fetal liver that are important for populating the majority of tissue resident macrophages. After birth, monocytes arise from bone marrow (BM)-derived HSCs and are released into the circulation upon their maturation, where they survey peripheral tissues and maintain endothelial integrity. Upon sensing of microbial breaches or inflammatory stimuli, monocytes migrate into tissues where their plasticity allows them to differentiate into cells that resemble macrophages or DCs according to the environmental niche. Alternatively, they may also migrate into tissues in the absence of inflammation and remain in an undifferentiated state where they perform homeostatic roles. As monocytes are typically on the move, the availability of intravital imaging approaches has provided further insights into their trafficking patterns in distinct tissue compartments. In this review, we outline the importance of understanding their functional behavior in the context of tissue compartments, and how these studies may contribute towards improved vaccine and future therapeutic strategies.

Keywords: monocytes, marginal pool, bone marrow, spleen, CXCR4 = chemokine receptor 4, inflammation, steady-state, intravital 2P microscopy

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INTRODUCTION

When agent Grant was traveling through the blood vessels of Dr Jan Benes in the science fiction movie "*Fantastic Voyage*," he might have noticed a large white blood cell with abundant cytoplasm and a hefty eccentrically placed kidney bean-shaped nucleus. This cell measured approximately 20 μm in diameter and was the largest of all circulating leukocytes. Known as the monocyte, this cell is renowned for its phagocytic activity and constitutes about 5–10% of total blood leukocytes.

For half a century, monocytes were touted to be an intermediate cell type with the sole purpose of replenishing tissue macrophages (1, 2). This dogma was based on Van Furth and Cohen's findings in the mid twentieth century (3, 4) and has been a subject of intense research and debate in the past decade. While genetic fate-mapping experiments have since revealed embryonic progenitors as the precursors of most tissue macrophages (5–7), it is increasingly apparent that these original

theories are not entirely incorrect either. Instead, it is now proposed that monocytes have the ability to reconstitute the macrophage pool, in a temporal and spatial manner (8, 9), with competition for a restricted number of niches as the main driving factor (10).

With monocytes no longer functioning solely as steady-state macrophage precursors, it remains unclear what tasks they may perform in immunity and host defense. Monocytes are heterogeneous and consist of a classical population ($Ly6C^{hi}$ in mice; $CD14^{++}CD16^{-}$ in humans) and a non-classical population ($Ly6C^{lo}$ in mice; $CD14^{+}CD16^{+}$ in humans) (7, 11, 12) with distinct functional roles (13). Interestingly, amidst the flurry of excitement in examining macrophage ontogeny by genomics/epigenomics approaches, the understanding of monocyte function in the context of spatial distribution and tissue niche was also steadily emerging as a key focus area. Together with the development in molecular and cell biological studies (14), the advent of imaging techniques such as two-photon intravital microscopy (2P-IVM), which allows direct visualization of immune cells using fluorescent reporter-tagged mice *in vivo* and *in situ* (15, 16), has helped to uncover a wide array of imperative monocyte biology. Nevertheless, monocyte behavior is highly distinct in each tissue compartment due to their plasticity and sensitivity to niche signals (17). Therefore, it is extremely vital that we consider their functional role in a dynamic and spatiotemporal manner. In this mini-review, we will provide insights on the trafficking patterns of monocytes and how their behavior in distinct tissue compartments governs their function in immune responses (**Figure 1**).

TRAVELING BACK IN TIME: RECOGNITION OF THE FETAL MONOCYTE

When van Furth and Cohen's proposal of ontogeny of tissue macrophages arising solely from monocytes (3) was challenged in the early twenty-first century, scientists postulated that adult tissue macrophages were derived from embryonic precursors before birth instead (6, 7, 18). In mice, these embryonic precursors emerged before the development of hematopoietic stem cell (HSC) progenitors and comprised of erythro-myeloid precursors (EMPs) that appear in the yolk-sac blood islands of the embryo at around E7.0 of gestation (19, 20). Importantly, these EMPs could bypass the monocyte stage and give rise directly to primitive macrophages that would seed the organs of the growing embryo (6, 21, 22). However, it was later discovered that upon establishment of the blood circulation, these EMPs migrate and seed the fetal liver at E9.5 of gestation (19, 23, 24), giving rise to multiple myeloid lineage cells, including a very important cell type—the fetal monocyte (25–27).

Abbreviations: BM, bone marrow; cMop, common monocyte progenitor; CSF-1, colony stimulating factor-1; DC, dendritic cell; EMP, erythro-myeloid precursor; HSC, hematopoietic stem cell; IL, interleukin; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MHC, major histocompatibility complex; PLVAP, plasmalemma vesicle-associated protein; S1PR5, sphingosine-1-phosphate receptor 5; 2P-IVM, two-photon intravital imaging; TNF, tumor necrosis factor; TpMo, transitional pre-monocyte; ZT, Zeitgeber.

In mice, fetal monocytes were first reported by Naito et al. and were shown to emerge in the fetal liver around E12.5 before being released into the blood from E13.5 onwards (27, 28). Despite primitive macrophages already occupying the tissue niches at this stage, fetal monocytes were discovered to colonize the remaining open niches of every tissue at E14.5 with the exception of the brain (26, 29–32) (**Figure 1A**). To date, little is known about the trafficking mechanisms that are adopted by fetal monocytes. Nevertheless, fetal monocyte migration into tissues is independent of the CCR2-CCL2 axis (26) while their egress from the fetal liver is dependent on plasmalemma vesicle-associated protein (PLVAP), which is an endothelium-specific molecule that forms diaphragm-like structures in the fenestrae of the liver sinusoidal endothelium (33) (**Figure 1A**). Functionally, fetal monocytes share many common traits with adult BM-derived monocytes but have reduced expression of antigen presentation and pathogen recognition-associated genes (26). In contrast to adult monocytes, fetal monocytes also retain a high proliferative capacity in tissues that is CSF-1 receptor independent (29), thereby allowing fetal monocytes to harbor a competitive advantage in replenishing tissue macrophages (34). Further investigations would be required to comprehend how fetal monocytes traffic into tissues and what signals affect their retention in their respective niches as they differentiate into macrophages.

MONOCYTES IN-WAITING: THE BONE MARROW AND SPLEEN

Unlike fetal monocytes that are derived from late EMPs in the fetal liver, adult monocytes originate from HSC progenitors in the BM after birth (7, 35, 36). It was initially thought that $Ly6C^{hi}$ monocytes originated directly from the common monocyte progenitor (cMop) and are poised to leave the BM upon maturing beyond the cMop stage (35). However, contrary to this assumption, recent findings by Chong et al. have demonstrated that cMops undergo an additional step of maturation into a transitional precursor before the ensuing mature monocytes (37). This transitional precursor was termed "transitional pre-monocytes" (TpMos), and was discovered when BM $Ly6C^{hi}$ monocytes were found to contain two distinct subpopulations: (1) the $CXCR4^{hi}$ subpopulation, which constitutes TpMos derived directly from cMops and are immobilized in the BM where they proliferate rapidly to replenish mature monocytes; (2) the $CXCR4^{lo}$ subpopulation, which consists of *bona fide* mature $Ly6C^{hi}$ monocytes that have exited the cell cycle and are readily mobilized from the BM (37) (**Figure 1B**i). Since TpMos are highly proliferative and immobilized in the BM under regular circumstances, their presence likely serves as a regulatory checkpoint for the rapid replenishment and prevention of an uncontrolled release of BM monocytes.

In comparison to other myeloid cells (38), monocytes transit quickly through the BM and are released rapidly into the circulation after their last division (39). Their egress and retention in the BM is critically dependent on CCR2-signaling

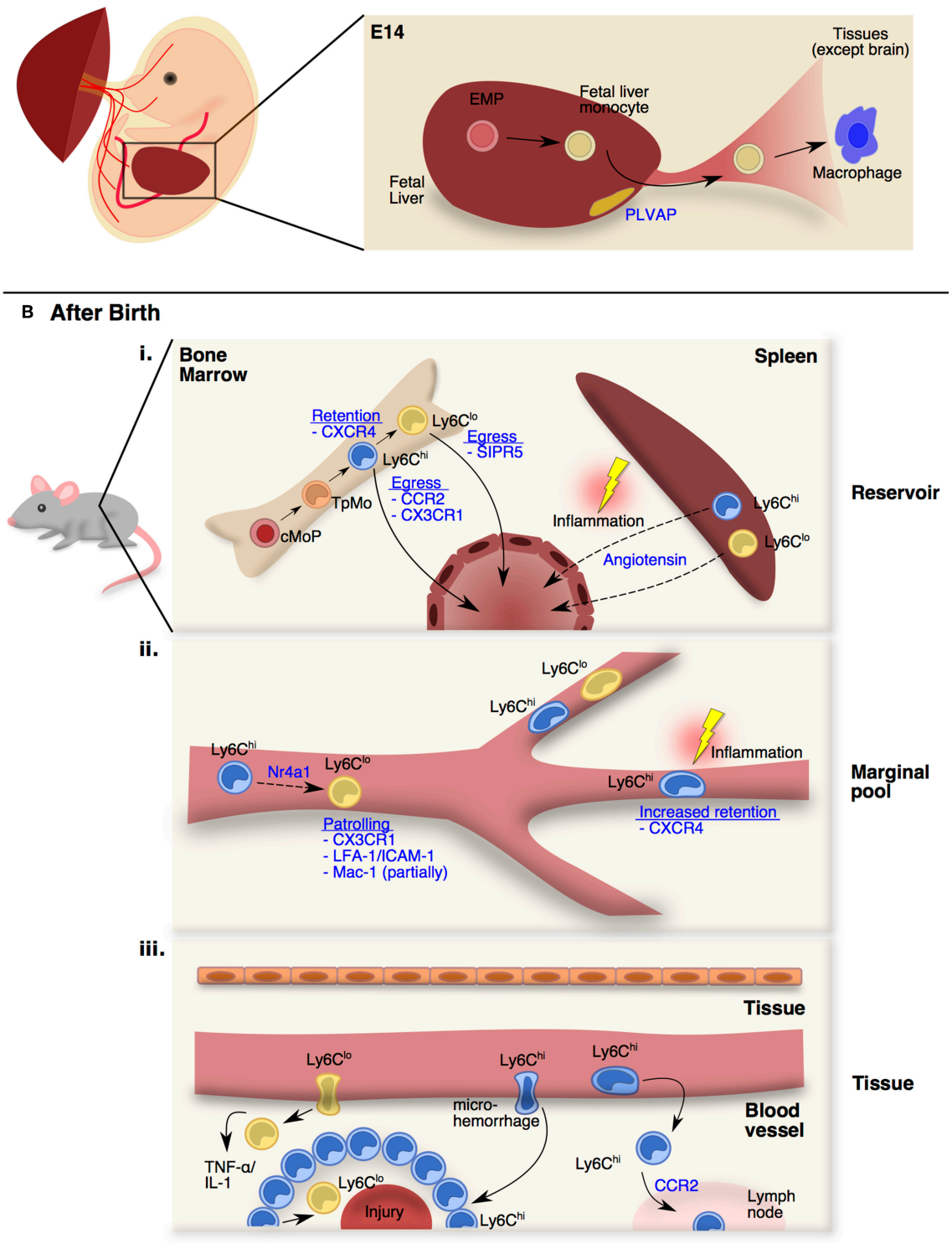

FIGURE 1 | Monocyte trafficking and function in distinct stages and peripheral sites. **(A)** From E13.5 onwards, fetal monocytes derived from erythro-myeloid precursors (EMPs) in the fetal liver can be released into the circulation in a plasmalemma vesicle-associated protein (PLVAP) dependent manner. At E14.5, these fetal monocytes will colonize the open niches of every tissues as fetal monocyte-derived macrophages except the brain. **(B)** After birth (i) Adult monocytes originate from the common monocyte progenitors (cMoPs) that give rise to Ly6C^{hi} monocytes through a transitional precursor called transitional pre-monocytes (TpMos). Ly6C^{hi}

(Continued)

FIGURE 1 | monocytes are released into the circulation upon their last division, and differentiate into Ly6C^{lo} monocytes. The retention and egress of Ly6C^{hi} monocytes are dependent on CXCR4- and CCR2-signaling respectively, whereas Ly6C^{lo} monocytes egress is dependent on S1PR5-signaling. At steady state, circulating monocytes enter the spleen as a secondary reservoir. During inflammation, splenic Ly6C^{hi} and Ly6C^{lo} monocytes are mobilized into the circulation via Angiotensin-II/AGTR1A-signaling. (ii) Upon entering the circulation, short-lived Ly6C^{hi} monocytes gradually differentiate into longer-lived Ly6C^{lo} monocytes via Nr4a1-signaling. Ly6C^{lo} monocytes patrol the vessels partially via Mac-1, but significantly via CX3CR1-signaling and LFA-1/ICAM-1 interaction with the endothelial cells. At steady state, Ly6C^{hi} monocytes do not interact closely with the endothelium except in the vascular beds of distinct peripheral organs. CXCR4 regulates steady state monocyte margination in the lung. During inflammation, Ly6C^{hi} monocytes increased their transit time, resulting in increased retention in the microvasculature. (iii) At steady state, Ly6C^{hi} monocytes survey the tissue environment for antigens to transport into draining lymph nodes. During injury, Ly6C^{lo} monocytes infiltrate rapidly into inflamed site to provide TNF-α and IL-1. Besides the classical rolling and migration steps, a proportion of Ly6C^{hi} monocytes utilizes microhemorrhages to extravasate and enter inflammatory sites rapidly and form a ring-like structure before differentiating into Ly6C^{lo} monocytes for tissue repair.

(40, 41) and CXCR4-signaling (37, 42, 43), respectively. Unlike vascular monocytes that are highly motile, Ly6C^{hi} monocytes in the BM parenchyma are comparatively sessile, displaying slow random displacements (44) while being juxtaposed to Nestin^{+} stromal cells (42, 45). Upon sensing of inflammatory stimuli like LPS through Toll-like receptor 4 (45), Nestin^{+} stromal cells express CCL2 (42), which causes BM Ly6C^{hi} monocytes to increase their velocity and displacement (46). This CCL2 exposure also leads to desensitization of monocyte response to CXCL12 (ligand of CXCR4) possibly through internalization of CCR2-CXCR4 complexes, which weakens the CXCR4 anchoring signal and results in their eventual egress (42). Furthermore, only mature Ly6C^{hi} monocytes, and not TpMos, were able to leave the BM under subclinical doses of LPS because TpMos were unable to respond to CCL2 as efficiently as mature Ly6C^{hi} monocytes (37). CX3CR1 was also discovered to regulate Ly6C^{hi} monocyte numbers in the BM after cyclophosphamide-induced myeloablation although their effect is less pronounced than CCR2-signaling (47). While signals governing the release of BM Ly6C^{hi} monocytes are well-documented, mechanisms regulating Ly6C^{lo} monocyte egress are less defined. Nevertheless, it was discovered that Ly6C^{lo} monocytes have very low levels of the CCR2 receptor and thus their egress is more likely to depend on S1PR5 (48).

Besides the BM, monocytes have also been found to reside in the subcapsular red pulp of the spleen as a secondary reservoir (49). In contrast to the BM whose main function lies in immune cell generation from HSC progenitors, the spleen functions mainly as a lymphatic organ (50). Therefore, the steady state monocyte reservoir is not generated in the spleen itself, but derived from circulating monocytes that have entered the spleen (49). Exceptions to this rule, however, do occur in the case of extramedullary hematopoiesis, when monocyte progenitors were found to expand in the spleen during inflammation, contributing to the monocyte reservoir *in situ* (51, 52). More importantly, splenic monocytes can increase their motility and exit into the blood during myocardial infarction via Angiotensin II-signaling and this process is independent of CCR2-signaling (49) (**Figure 1B**i). Interestingly, Angiotensin II-dependent recruitment of monocytes into the infarct (a localized area of dead tissue resulting from failure of blood supply) is strictly mediated from the spleen and peripheral circulation, but not from the BM (51). Splenic monocytes were also found to be mobilized to the ovaries where they enhance ovulatory processes (53). Notably, the spleen is also a key site for an alternative source of monocytes in cardiovascular diseases (52, 54, 55), tumor progression (56) and lung ischemia (57). These findings hence suggest that the spleen fulfills the urgent demand of monocytes during inflammation by providing an emergency source, which extends time for the BM to generate more monocytes concurrently.

MONOCYTES ON-THE-GO: NAVIGATING THROUGH THE CIRCULATORY HIGHWAYS

Upon entering the circulation, monocytes rely heavily on the circulatory system for transportation to peripheral compartments. Ly6C^{hi} monocytes have a half-life of approximately 20–24 h in the peripheral blood before gradually differentiating into Ly6C^{lo} monocytes (half-life of 48 h in mice; 7 days in humans) via *Nr4a1*-signaling (58–61). Unlike classical Ly6C^{hi} monocytes that roll along vessels, CX3CR1^{high} non-classical Ly6C^{lo} monocytes in mice (62) and their human counterparts (CD14^{+}CD16^{+} monocytes) (63) patrol vessels by crawling at a speed of 12 μm/min. Their patrolling behavior is partially mediated by Mac-1 and is highly dependent on CX3CR1-signaling and LFA-1/ICAM-1 or ICAM2 interaction with endothelial cells (62, 64, 65). Furthermore, this patrolling activity is critical for micro-scavenging the luminal surface of vessels and maintaining endothelial integrity (64) (**Figure 1B**ii). Notably, an increase in atherosclerotic endothelial apoptosis (66), amyloid deposition (67) and tumor metastasis (68) was observed when Ly6C^{lo} monocytes were absent in *Nr4a1*$^{-/-}$ mice. Because of their close interaction with vessels, Ly6C^{lo} monocytes orchestrate the recruitment and activation of neutrophils upon sensing a breach in vascular integrity through TLR7-signaling, which subsequently leads to their retention in the capillaries (64, 69).

In contrast to Ly6C^{lo} monocytes that patrol vessels, it is commonly recognized that Ly6C^{hi} monocytes do not interact closely with the endothelium in the steady-state (70). However, exceptions to this rule do occur in vascular beds of distinct peripheral organs. These vascular beds consist of multiple small-caliber microvessels (<5 μm in diameter), which necessitate larger leukocytes (6–8 μm) to deform and physically interact with the endothelium for their transit (71). This phenomenon results in substantial leukocyte retention and the formation of a "marginal pool." In particular, the lungs represent a major site of leukocyte margination, and classical Ly6C^{hi} monocytes were discovered to form close interactions with the lung vasculature

under resting state (37, 72, 73). Ly6C^{hi} monocytes are highly adherent upon contact with surfaces and can be seen to extend their pseudopods upon movement (**Figure 2A**). Notably, we discovered that CXCR4 regulates steady-state monocyte margination in the lung (37) (**Figure 1B**ii). Upon endotoxin sensing, classical Ly6C^{hi} monocytes increased their lung transit time (74) by adhering to the endothelium, resulting in increased predisposition towards lung injury that can be reversed with CXCR4 inhibition (37). Apart from the lung, intravital imaging of monocytes in vascular beds of the kidney (75, 76) and liver (77) revealed increased retention of monocytes in the microvasculature during inflammation. Increased adhesion of Ly6C^{hi} monocytes, but not neutrophils, in the brain microvasculature during cerebral malaria is also associated with progressive worsening of clinical symptoms (78). Additionally, the BM was discovered to contain a CX3CR1-dependent marginal pool of monocytes that can be rapidly deployed to the peritoneum (79).

Since the BM is constantly releasing monocytes into the circulation, it is conceivable that a counterbalancing mechanism exists to ensure that circulating monocyte numbers return to homeostasis. Indeed, CXCR4-signaling keeps this homeostasis in check by influencing the spatiotemporal localization of monocytes between the circulation and peripheral compartments (**Figure 2B**). Notably, circulating monocytes were found to return at a constant rate to the BM and spleen parenchyma in a CXCR4-dependent manner (37). More importantly, the number of circulating monocytes compared to the numbers in the peripheral compartments were found to vary according to circadian rhythmic oscillations, with more monocytes present in the circulation at Zeitgeber 5 (ZT5) than ZT13 in mice (where ZT0 refers to lights on and ZT12 to lights off) (37, 80). This diurnal oscillation of monocyte numbers is regulated by the circadian gene, *Bma1* (80), and also corresponds with diurnal fluctuations in CXCR4 levels on mature monocytes (37), such that absence of CXCR4 also abolishes the diurnal oscillation in monocyte numbers.

MONOCYTES EXITING THE HIGHWAYS: EXPLORING TISSUES

The entry of monocytes into tissues is critical for pathogen clearance and wound healing. Furthermore, it is typically acknowledged that their time of entry dictates their function, as ingress of monocytes in the early phase of inflammation is associated with a pro-inflammatory phenotype, while their presence in the later phase corresponds to an anti-inflammatory function (81, 82) (**Figure 1B**iii). Mediators that attract circulating monocytes into tissues include chemokines, complement components, and products of tissue matrix degradation (83). Since patrolling Ly6C^{lo} monocytes interact closer with the endothelium compared to Ly6C^{hi} monocytes, it is conceived that their migratory dynamics into tissues are quicker than Ly6C^{hi} monocytes. Indeed, Ly6C^{lo} monocytes infiltrate within an hour into inflamed tissues induced by aseptic wounding, irritants or *Listeria monocytogenes* to provide the initial sources of TNF-α and IL-1 (62). In contrast, Ly6C^{hi} monocyte recruitment into tissues typically occurs 24–48 h after injury (84). Their entry into tissues involves vascular rolling, adhesion, and transendothelial migration that has been well-documented (14, 83, 85). Nevertheless, a proportion of Ly6C^{hi} monocytes have also been shown to utilize microhemorrhages to exit blood vessels and enter inflammatory sites rapidly (86). This allows Ly6C^{hi} monocytes to enter the injury site as quickly as neutrophils, where they were found to scout the wound bed randomly before progressively slowing down over a study period of 2.5 h (86). While it is unclear what causes this behavioral change, it is likely that this may be associated with the conversion of Ly6C^{hi} into Ly6C^{lo} monocytes that is critical for wound healing. Indeed, Ly6C^{hi} monocytes entered the injury site and formed a ring-like structure around the injured foci that persisted for 48 h in a model of sterile hepatic injury (77). These Ly6C^{hi} monocytes subsequently differentiated into Ly6C^{lo} monocytes after sensing IL-4 and IL-10 within the ring-like structure. Notably, this phenotypic conversion was critical for monocytes to move further into the injury area and to initiate optimal repair. These findings further highlight the plasticity of monocytes in their functional reprogramming by switching from an inflammatory phenotype to a profile that facilitates wound repair.

Upon entering tissues, infiltrating monocytes progressively alter their phenotype by adopting macrophage characteristics while losing monocyte features, and this gradual differentiation process is known as the classical "monocyte waterfall" effect (8, 87, 88). Besides replacing certain residential macrophages in the steady-state (6, 18), monocytes may also differentiate into TNF/iNOS-producing DCs (Tip-DCs) (89), wound-associated macrophages (WAMs) (90) or tumor-induced myeloid suppressor cells (91). However, *bona fide* classical monocytes have also been found to remain undifferentiated in the tissue at resting state (92). These monocytes extravasated constitutively into tissues and lymph nodes in a CCR2-dependent manner and retained most of their existing monocyte transcriptional profile. Nevertheless, these Ly6C^{hi} monocytes increased their expression of MHCII, co-stimulatory molecules and CCR7, suggesting that these cells survey the tissue environment for antigens to transport to draining lymph nodes in the steady state. Since monocyte extravasation into tissues in the steady-state was found to be microbiota-independent (92), it would be interesting to determine the specific mechanisms that dictate their migration into tissues and the factors that preserve their profile in these circumstances.

CONCLUSION AND FUTURE PERSPECTIVES

Despite being described in many important studies in the last century, our comprehension of monocyte biology has only taken a substantial leap in the past decade upon the advent of highly sophisticated imaging techniques that complement the current use of biochemistry, cell biology and genetic tools. More importantly, 2P-IVM has unveiled critical trafficking

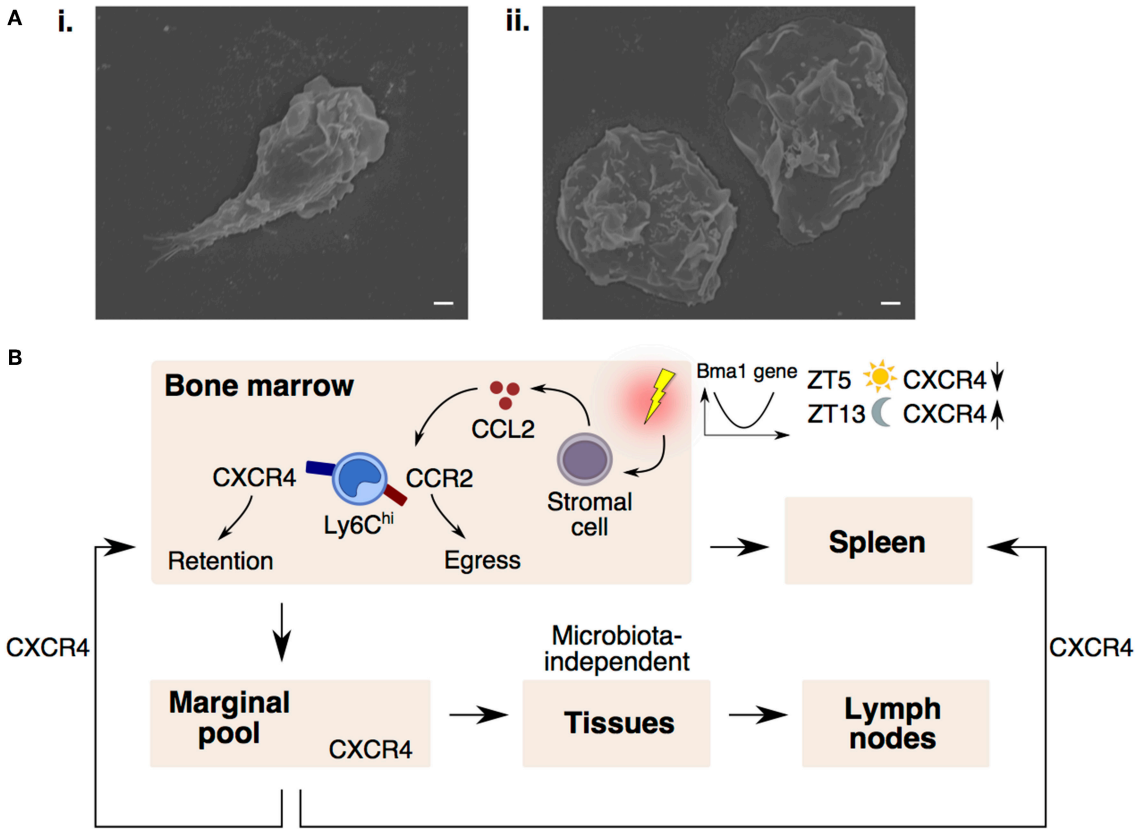

FIGURE 2 | CXCR4 controls monocyte trafficking into different peripheral compartments. **(A)** Scanning electron microscopy images of a Ly6C^{hi} monocyte (i) protruding its pseudopod upon adhering to coverslip and (ii) extending their cytoplasmic membrane when fully adhered to the coverslip. Bars, 1 μm. **(B)** Monocyte egress and retention in the bone marrow is dependent on CCR2-signaling and CXCR4-signaling. Upon sensing inflammatory stimuli, stromal cells release CCL2, desensitizing monocyte response to CXCL12 (CXCR4 ligand), resulting in monocyte entry into the circulation and spleen. In the circulation, CXCR4 regulates steady-state monocyte margination in tissue marginal pools. Monocytes may also extravasate into tissues and lymph nodes in a microbiota-independent manner. CXCR4-signaling also regulates the homing of circulating monocytes back to the bone marrow and spleen. Monocyte numbers display diurnal oscillation that is regulated by the circadian gene, *Bma1*. Lower CXCR4 levels at ZT5 (lights on period) results in more circulating monocytes, whereas higher CXCR4 levels at ZT13 (lights off period) results in higher monocyte retention in the bone marrow.

mechanisms that may have important implications for future vaccine designs/therapeutic strategies. In particular, the specific kinetics of monocyte trafficking in different tissue compartments and their interaction with other immune cells will allow scientists to optimize their drug administration and design according to these dynamics. For example, clinicians who aim to reduce tissue inflammation may take advantage of the knowledge that non-classical monocytes recruit neutrophils in the early stages of inflammation (64). Therefore, selecting specific drugs that target molecules only on non-classical monocytes, instead of both monocyte subsets, may help to reduce the likelihood of any off-target effects and secondary infections during long periods of therapy. While 2P-IVM has provided valuable insight, major technical bottlenecks still exist against gaining a global understanding of these cells in chronic disease states. These issues are due to the highly plastic nature of monocytes, which may include the loss of fluorescence signal as they differentiate into monocyte-derived cells. Furthermore, their differentiated phenotypes are distinct in various chronic disease settings (13, 93). In this regard, a combination of tools that would enable researchers to identify monocyte-derived cells with greater spatiotemporal specificity would be beneficial in addressing these issues. In particular, multiplex immunofluorescence techniques (94, 95) in a histo-cytometry setting (96, 97) that involves optically cleared large tissue samples (98) would provide a global view of their localization and interaction with other immune cells. Furthermore, refining image analysis methods that deal with large volumes of data, such as using a hue-saturation-brightness-based surface creation to streamline multi-channel image cytometry for three-dimensional images (99), would allow us to uncover new markers on monocyte-derived cells that can be used to generate improved fluorescent-tagged mice. Importantly, while transcriptomic studies have shown mouse and human monocytes to be homologous, a reverse pattern in certain genes such as TREM-1, CD36, CXCR4, and CD9 was also discovered (12). Therefore, future work adopting humanized mice for 2P-IVM studies, is warranted to verify if trafficking mechanisms of mouse monocytes are similar to that

in humans. Taken together, we believe that the combination of these state-of-the-art imaging tools in future studies will provide further insight into the temporal and spatial landscape of monocytes that could hold the key for future biomarker and therapeutic discoveries.

AUTHOR CONTRIBUTIONS

YCT, JLD, LGN and SZC wrote and conceptualized the manuscript. YCT did the figures.

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