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ARREST CHEMOKINES

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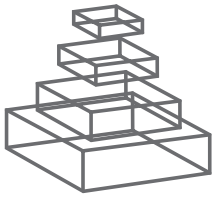

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ARREST CHEMOKINES

Topic Editor:
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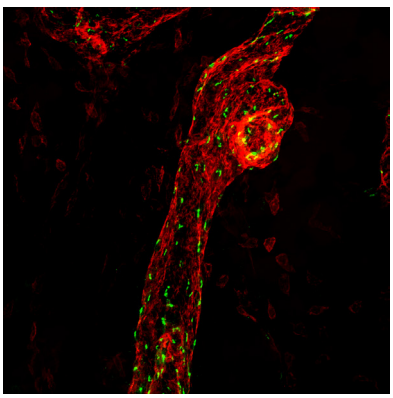

CCL21 expression (green) in lymphatics (stained with LYVE-1, red) of mouse intestine. Whole mount image taken on Zeiss 780 microscope, courtesy of Z. Mikulski, K. Park and C.C. Hedrick

Arrest chemokines are a small group of chemokines that promote leukocyte arrest from rolling by triggering rapid integrin activation. Arrest chemokines have been described for neutrophils, monocytes, eosinophils, naïve lymphocytes and effector memory T cells. Most arrest chemokines are immobilized on the endothelial surface by binding to heparan sulfate proteoglycans. Whether soluble chemokines can promote integrin activation and arrest is controversial. Many aspects of the signaling pathway from the GPCR chemokine receptor to integrin activation are the subject of active investigation. Leukocyte adhesion deficiency III is a human disease in which chemokine-triggered integrin activation is defective because of a mutation in the cytoskeletal protein kindlin-3. About 10 different such mutations have been described. The defects seen in patients with LAD-III elucidate the importance of rapid integrin activation for host defense in humans. Here we present a series of ten reports that help clarify this crucial first step in the process of leukocyte transendothelial migration.

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Arrest chemokines

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Chemokines are a large family (~50 members) of chemoattractants that bind to cognate chemokine receptors (~25 known). Leukocytes roll along the vascular endothelium through selectins interacting with their glycoprotein ligands until they encounter a chemokine that stops them in their tracks (1, 2). The fact that chemokines can induce arrest of rolling leukocytes and make them adhere was discovered in the 1990s (3–6), and the term "arrest chemokines" was coined in 2003 (7). Many chemokines including CXCL1, 2, 8, 9, 10, 12, CCL 3, 5, 11, 19, 21, and CX3CL1 have been shown to activate leukocyte integrins and induce arrest, but other chemokines may also have this ability and simply have not been tested in rolling-to-arrest assays. In this Research Topic, 26 authors have contributed 9 articles touching on many of the known arrest chemokines. This Research Topic is aimed at covering the structure, expression, and physiological function of arrest chemokines, the biophysical processes associated with leukocyte arrest, and the molecular mechanisms of rapid leukocyte integrin activation responsible for arrest.

Bongrand's group has pioneered the study of the biomechanics of cell adhesion for the past 30 years (8). In their contribution to this Research Topic (9), they discuss the finite time required for integrin activation, the nanoscale dynamics of the arrest process, and the contribution of local membrane deformation. They apply this knowledge of the biomechanics of leukocyte arrest to the study of the leukocyte arrest defect seen in patients with leukocyte adhesion deficiency (LAD) type III. In this disorder, the cytoskeletal protein kindlin-3 is not expressed and integrin activation is impaired.

Once rolling leukocytes encounter immobilized or soluble chemokine, a series of signaling events is triggered that ultimately results in integrin activation by conformational extension, affinity increase, and clustering. The proximal signaling is clear: the chemokine binds its G-protein coupled receptor and the Gα subunit dissociates from Gβγ. The distal signaling is also fairly clear: both talin-1 and kindlin-3 bind to the cytoplasmic domain of the β chain of the leukocyte integrin responsible for arrest. But what links the two processes is an area of active investigation. Laudanna and colleagues focus on the roles Rap1 and RhoA, two of many small G proteins found in leukocytes (10).

Another signaling paper in this Research Topic focuses on calcium. Intracellular free calcium rises rapidly when a chemokine binds its receptor, because the dissociated Gβγ subunit of chemokine receptors can trigger calcium release from intracellular stores by activating phospholipase C (PLC)β. It has long been known that arrest is associated with a rise in intracellular free calcium (11), but it is not known whether this is required and if so, for which step in the signaling cascade. Scott Simon's group has worked on calcium signaling induced by selectin-mediated leukocyte interactions (12). In their contribution to the Research Topic, Simon's group focuses on the calcium rise that occurs after arrest (13). Their work suggests that elevated intracellular free calcium is required to induce a migratory phenotype in arrested neutrophils.

Rolling leukocytes do not always stop, but may instead slow down considerably. This slower rolling is associated with partial integrin activation to a state that is known as extended. Talin-1 binding to integrin appears to be sufficient for this. However, for arrest to occur, integrin extension appears necessary, but not sufficient: a high affinity conformation of integrin is needed. This last step can be induced by chemokines and requires kindlin-3 (14). Lefort and Ley suggest that talin-1 is required for both integrin extension and high affinity, and kindlin-3 is only required for inducing the high affinity conformation. A competing hypothesis is that kindlin-3 may be involved in integrin clustering (15). More direct evidence in primary leukocytes will be needed to distinguish between these two competing models.

Chigaev and Sklar have pioneered the use of small fluorescent peptides to report the activation of integrins. In their contribution to the Research Topic, they review the insights obtained by this approach with a focus on the αLβ2 integrin LFA-1 expressed by all leukocytes and α4β1 integrin expressed by monocytes and lymphocytes (16).

Among the ~50 chemokines known, only a handful functions as arrest chemokines. One requirement seems to be binding to the endothelial surface, but not all chemokines that bind to the endothelial surface induce arrest. Weber's group was among the first to describe arrest chemokines (17). In their contribution to this Research Topic, Weber's group reviews human chemokines and the therapeutic potential of modulating their function (18).

Macrophage inhibitory factor (MIF) is not a classical chemokine, but signals through the chemokine receptor CXCR2 and can activate LFA-1 (19). Bernhagen's group proposes that MIF binding to CXCR2 initiates a "motility program" in leukocytes. Because CXCR2 is one of the most efficient chemokine receptors triggering arrest, and because it has at least eight known ligands, a separate review in this Research Topic is focused on this one receptor (20). CXCR2 has been targeted by small allosteric inhibitors, and some of these show promise in clinical trials, which is the focus of the contribution by Zarbock's group (20).

Some chemokine receptors do not signal through dissociation of Gα from Gβγ. Initially, these receptors including Duffy antigen receptor for chemokines (DARC) and D6 were called decoy receptors, because they were thought to sequester chemokines and prevent them from having effects. In recent years, it has become clear that these receptors have important functions in transporting chemokines across endothelial cells. In their contribution to this Research Topic, Antal Rot's group focuses on the role of DARC in this process. In fact, DARC may be a receptor that positions chemokines correctly on the endothelial surface to fulfill their arrest function (21).

Although progress on arrest chemokine function over the last 20 years has been remarkable, many aspects still require more work. It is controversial whether arrest chemokines and their receptors are monomers, homodimers, or heterodimers. It remains unknown how calcium signaling may be involved in integrin activation. We can expect that the exact function of talin-1 and kindlin-3 in integrin activation will be discovered through novel structure–function and live cell imaging approaches. An exciting prospect of more research aimed at understanding arrest chemokines is that their manipulation may have therapeutic potential in inflammatory diseases.

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Biophysical description of multiple events contributing blood leukocyte arrest on endothelium

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Blood leukocytes have a remarkable capacity to bind to and stop on specific blood vessel areas. Many studies have disclosed a key role of integrin structural changes following the interaction of rolling leukocytes with surface-bound chemoattractants. However, the functional significance of structural data and mechanisms of cell arrest are incompletely understood. Recent experiments revealed the unexpected complexity of several key steps of cell-surface interaction: (i) ligand-receptor binding requires a minimum amount of time to proceed and this is influenced by forces. (ii) Also, molecular interactions at interfaces are not fully accounted for by the interaction properties of soluble molecules. (iii) Cell arrest depends on nanoscale topography and mechanical properties of the cell membrane, and these properties are highly dynamic. Here, we summarize these results and we discuss their relevance to recent functional studies of integrin-receptor association in cells from a patient with type III leukocyte adhesion deficiency. It is concluded that an accurate understanding of all physical events listed in this review is needed to unravel the precise role of the multiple molecules and biochemical pathway involved in arrest triggering.

Keywords: adhesion, ligand-receptor interaction, bond strength, integrin, clustering, avidity, dynamics, LAD-III

INTRODUCTION

Immune cells such as lymphocytes or phagocytes can bind to specific blood vessel areas and further migrate toward peripheral tissues. This allows memory lymphocyte patrolling throughout the organism to detect invading foreign material. Also, this allows endothelial cells of inflamed areas to trigger the arrest of blood leukocytes that are flowing in a resting state. Basic mechanisms have been elucidated during the early nineties (Lawrence and Springer, 1991; von Andrian et al., 1991; Springer, 1994), leading to a general paradigm that remains valid (Ley et al., 2007): leukocytes move with a velocity of several millimeters/second imposed by the blood flow (Atherton and Born, 1972). The earliest event is cell-surface tethering by specialized membrane receptors (Lawrence and Springer, 1994) such as P-selectin (CD62-P) on stimulated endothelial cells or L-selectin that is concentrated on the tip of leukocyte microvilli. Cells then display a somewhat jerky displacement (5–10 $\mu m\ s^{-1}$) called rolling. This is due to the rapid formation and dissociation of bonds such as are formed between endothelial E- and P-selectins and lymphocyte-associated ligands comprising P-selectin glycoprotein ligand 1 (PSGL-1), E-selectin ligand 1 (ESL-1), and the hyaluronan receptor CD44 (Hidalgo et al., 2007). Tethering and rolling may also be driven by the interaction between vascular cell adhesion molecule 1 (VCAM-1) expressed on properly stimulated endothelial cells and $\alpha 4\beta 1$ (VLA-4, CD29dCD49) expressed on some leukocyte populations (Alon et al., 1995). A key property of bonds mediating rolling is their capacity to resist hydrodynamic forces of several tens of piconewtons for several tenths of a second (Evans et al., 2001, 2004). Rolling does not require any active cell participation since it may be reproduced with fixed cells (Lawrence and Springer, 1993) or with cell-free systems (Brunk et al., 1996). A likely explanation of rolling jerkiness is that at a given time a leukocyte is bound by a few or even a single bond and each bond rupture event results in a discrete forward displacement. Indeed, rolling velocity is strongly correlated to the bond dissociation rate (Alon et al., 1997).

The initial step of rapid rolling may be followed by an intermediate phase of "slow rolling" with more than twofold velocity decrease. This may result from a partial activation of lymphocyte function associated 1 integrin (LFA-1, CD11aCD18) enabling it to interact with intercellular cell adhesion molecule 1 (ICAM-1, CD54) expressed by endothelial cells (Jung et al., 1998). LFA-1 activation may be induced by E-selectin interaction with PSGL-1 (Kuwano et al., 2010) or CD44 (Yago et al., 2010).

Other phenomena were found to contribute the following arrest phase: the pulling force applied on cell-surface receptors may generate membrane tubes of up to 40 μm length (Schmidtke and Diamond, 2000), thus decreasing the force applied on bonds as explained below. Also, it was recently shown that the tethers formed on neutrophils could wrap around rolling cells and display a "stepwise peeling" through patches of PSGL-1 molecules interacting with substrate P-selectin (Sundd et al., 2012). The authors suggested that this particular behavior might be responsible for the neutrophil capacity to roll at extremely high shear rates.

Arrest is mainly triggered by the complete activation of leukocyte integrins such as LFA-1 or VLA-4, enabling them to firmly bind endothelial ligands such as ICAM-1 or VCAM-1 respectively,

as reviewed in this research topic (Chigaev and Sklar, 2012; Lefort and Ley, 2012). Subsecond integrin activation (Grabovksy et al., 2000; Alon and Dustin, 2007) is triggered by endothelium-bound chemoattractants that often belong to the chemokine family (Zlotnik and Yoshie, 2012). Thus, the same receptor family may be involved in directing cell locomotion and triggering arrest under shear flow (Campbell et al., 1998). The specificity of leukocyte species and arrest location is imparted by a particular combination of chemokines, adhesion molecules, and stimulation pathway (Rot and von Andrian, 2004). Following arrest, leukocytes may start crawling toward endothelial junctions and transmigrate toward surrounding tissues (Schenkel et al., 2004).

A current challenge is to understand the role of all involved molecules and signaling pathways. Here we shall describe the elementary physical events contributing the transition from rolling motion to LFA-1-mediated firm arrest. Indeed, a detailed understanding of physical constraints should help us understand the rationale of all cell processes contributing arrest. General concepts will be illustrated by addressing a specific problem: relating kindlin-3 deficiency to functional defects in LAD-III patients.

A prerequisite for assessing the use and significance of elementary events such as integrin clustering or membrane topographical reorganization is to build a quantitative scheme of the arrest phenomenon as a physical process.

PHYSICAL BACKGROUND

To estimate the intensity and effect of forces applied on leukocytes under flow, we need a simple model of cells as physical objects.

MECHANICAL AND GEOMETRICAL PROPERTIES OF BLOOD LEUKOCYTES

Micrometer-scale leukocyte rheological properties were studied by monitoring the deformation of cells sucked into micropipettes with controlled pressure (Evans and Yeung, 1989). Neutrophils behaved as viscous liquid spherical droplets (about 10^{-5} Pa.s viscosity and 8 μm diameter) surrounded by a membrane under tension ($\sim 3.5 \times 10^{-5}$ N m^{-1}). This is a minimal model (Herant et al., 2003). First, cells are composite objects. Thus, nuclear and cytoplasmic properties may be widely different. Secondly, applying mechanical forces may initiate active mechanical responses (Horoyan et al., 1990). However, this model may be relevant to the initial phase of leukocyte arrest under flow.

The structural basis of cell mechanical properties was studied with electron microscopy. Leukocytes are surrounded by a fairly inextensible lipid bilayer with numerous folds appearing as finger-like structures called microvilli or ridge-like folds (Bruehl et al., 1996; Shao et al., 1998). The average length is ∼0.3 μm and diameter or thickness is ∼0.2 μm. When pulling at microbeads bound to neutrophil microvilli, Shao et al. (1998) found that forces lower than 34 pN triggered elongation with a proportionality Hook parameter of 43 pN μm^{-1}, while forces higher than 61 pN separated the plasma membrane from underlying cytoskeleton, thus generating tethers with an elongation rate proportional to the applied force. More recently, based on the brownian motion of microspheres bound to the tips of microvilli, Yao and Shao (2007) estimated the flexural stiffness at 7 pN μm^{-1}.

Thus, membrane unfolding is required for a spherical cell to spread on a surface. The maximum increase of apparent cell area after complete unfolding is ∼50–100% (Evans and Yeung, 1989; Bruehl et al., 1996). Further area increase may require fusion of intracellular vesicles with plasma membranes, which may occur a few minutes after the onset of spreading (Gauthier et al., 2009).

We shall use this information to estimate the constraints experienced by a blood leukocyte made to stop in a specific area in blood vessels.

EFFECT OF FLOW ON BLOOD LEUKOCYTES

Blood flow is very different in millimeter diameter arteries and micrometer-diameter capillary vessels. Here, we shall focus on postcapillary venules with a diameter of several tens of micrometers, since they are a typical region of leukocyte arrest. As recalled on **Figure 1A**, the blood velocity near the vessel wall at any point M is parallel to the vessel axis and close to $G.z$, where z is the distance between M and the wall, and G (in second^{-1}) is called the wall shear rate. Typical wall shear rates of a few hundreds of s^{-1} are found in postcapillary venules (Atherton and Born, 1973). The contact time between microvillus receptors and endothelium is thus lower than 1 ms (Zhao et al., 2001). This is the time allowed for initial tethering of cells to the endothelial surface. What happens then?

The force applied on a 8 μm diameter leukocyte when the shear rate is 200 s^{-1} is ∼102 pN (**Figure 1B**). The force on a P-selectin-PSGL-1 couple of 80 nm length may be sevenfold higher than the force on the cell (**Figure 1C**; Pierres et al., 1995). If the bond is located at the tip of a protrusion of 0.3 μm length, the force will be 3.7-fold higher than the force on the cell. This may induce tether formation if the receptor is not firmly anchored to the cell cytoskeleton. This was actually observed (Schmidtke and Diamond, 2000; Sundd et al., 2010, 2012). Thus a few bonds located at the tip of microvilli may not be sufficient to immobilize a leukocyte. Repeated bond formation and rupture will generate

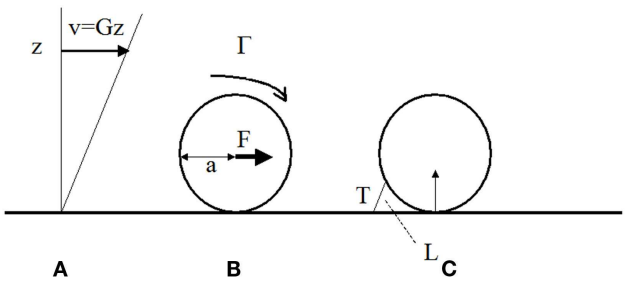

FIGURE 1 | Hydrodynamic forces on cells bound to blood vessel walls. **(A)** In a laminar viscous shear flow near a plane, the blood velocity at any point near the wall is parallel to the plane and equal to the distance z to the wall times the wall shear rate G (in s-1). The shear stress is the shear rate times the fluid viscosity μ (μ ∼ 0.001 Pa.s in aqueous medium). It represents the viscous force applied by the fluid on an unit area on the wall. **(B)** The fluid exerts on a sphere of radius bound to the wall a total force $F \sim 32\,\mu a^2 G$ and a torque $G \sim 11.9\,\mu a^3 G$ (Goldman et al., 1967). **(C)** if the sphere is maintained at rest by a single bond of length L and the contact between the surface and the wall is assumed to be frictionless, the tension T on the bond is $\sim 31\,\mu a^2 G\,(a/L)^{1/2}$ (Pierres et al., 1995).

a rolling motion. Molecular contacts between leukocyte receptors and endothelial ligands may then last several tens of milliseconds rather than milliseconds for freely flowing cells. This may permit integrin-mediated attachments.

Thus, stopping a leukocyte on the blood vessels will need to resist local pulling forces between 100 and 700 pN. We need know how many adhesion receptors are needed to fulfill this task. Results accumulated during the last two decades may provide a clear answer to this question.

NEW METHODS AND CONCEPTS PROVIDE US WITH QUANTITATIVE INFORMATION ON THE PROPERTIES OF BOND FORMATION AND DISSOCIATION BETWEEN SURFACE-ATTACHED MOLECULES

Inability of the conventional framework to account for interactions between surface-attached molecules

As previously reviewed (Bongrand, 1999; Zhu et al., 2002; Robert et al., 2007), the interaction between two molecules A and B in solution is well accounted for by two numbers, the association rate k_{on} and dissociation rate k_{off}:

$$A + B \underset{k_{off}}{\overset{k_{on}}{\rightleftarrows}} AB \quad (1)$$

$d[AB]/dt = k_{on}\ [A][B] - k_{off}\ [AB]$, the ratio k_{on}/k_{off} is the affinity constant K_a.

However, this conventional framework could not account for interactions between membrane-bound receptors and ligands: Firstly, bonds formed between surface-bound molecules are often subjected to external forces, and until recently no information was available on the effect of forces on bond lifetime. Secondly, as emphasized earlier, even the dimension of association rate between surface-bound molecules (corresponding to so-called 2D conditions) is different from the dimension of conventional (3D) association rates as defined in Eq. 1 (Pierres et al., 2001). Thirdly, 2D conditions impose special constraints on multivalent associations. We shall address these points sequentially.

Rupture of bonds between surface-attached molecules

During the last two decades, experiments based on laminar flow chambers (Kaplanski et al., 1993; Pierres et al., 1995), atomic force microscopes (Florin et al., 1994), the biomembrane force probe (Merkel et al., 1999), or optical tweezers (Nishizaka et al., 1995) allowed us to study single-bond formation and rupture between surface-attached molecules subjected to controlled forces. Bond rupture under force often followed a simple formula (Chen and Springer, 2001; Evans et al., 2010) previously suggested by Bell (1978):

$$k_{off}(F) = k_{off}(0)\ \exp\left(F/F^0\right) \quad (2)$$

where $k_{off}(F)$ is the rupture frequency (in s^{-1}) of a single-bond subjected to a distractive force F. A simple interpretation of this formula can be obtained by viewing bond rupture as the passage of a molecular complex AB from a bound state at zero separation to a free state that is reached by crossing an energy barrier of height W at separation distance d (**Figure 2**). According to Boltzmann's law, the probability of barrier-crossing should be proportional to $\exp(-W/k_BT)$, where k_B is Boltzmann's constant and T is the absolute temperature. Applying a force F will decrease W by the product Fd (**Figure 2**) thus multiplying the rupture frequency k_{off} by $\exp(Fd/k_BT)$. Bell estimated at 0.5 nm the order of magnitude of parameter d for an antigen-antibody interaction corresponding to the depth of an antibody binding site, leading to an estimate of ∼8 pN for parameter $F^\circ = k_BT/d$. More detailed discussion may be found in a number of papers following Eyring's (1935) and Kramer's (1940) seminal papers (Hänggi et al., 1990; Evans and Ritchie, 1997; Dudko et al., 2008). The rupture frequency and force coefficient F° for a number of receptors including selectins, integrins, cadherins, or antibodies were often on the order of 1–100 pN and 0.01–10 s^{-1}. Depending on molecule conformation, the force-free rupture frequency of LFA-1/ICAM-1 bond varied between 0.008 and 2 s^{-1}, with a force coefficient of 7–10 pN (Evans et al., 2010). However, the above results are only an approximation and single molecule studies confirmed that bond rupture is a complex process requiring multiple barrier-crossing events (Pierres et al., 1995; Merkel et al., 1999; Derenyi et al., 2004).

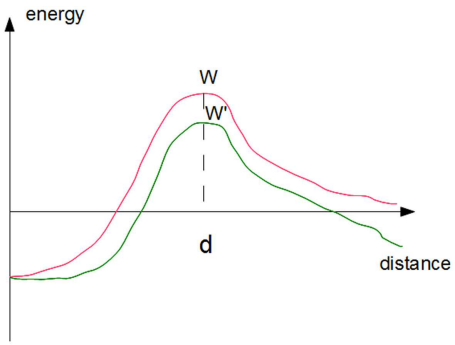

FIGURE 2 | Effect of forces on the kinetics of bond rupture. The simplest approximation consists of representing the free energy of a ligand-receptor complex as a simple function of the distance between ligand and receptor surfaces (red curve). Rupture requires the crossing of an energy barrier of height *W*. The rupture rate may be viewed as the product of the frequency of attempts at crossing times the success probability that is proportional to Boltzmann's factor $\exp(-W/k_BT)$. Applying a force will decrease the barrier height by the product *F.d*, i.e., the force times the distance between the barrier and the equilibrium distance, thus multiplying the escape frequency by $\exp(Fd/k_BT)$.

The catch-bond phenomenon, which is highly relevant to leukocyte-endothelium interaction, was predicted on the basis of thermodynamical reasoning by noticing that a disruptive force might decrease bond rupture frequency k_{off}, although it had to decrease binding affinity k_{on}/k_{off}. Bonds displaying such a strange behavior were dubbed "catch bond," in contrast with "ordinary" bonds that were called "slip bonds," responding to disruptive forces with increased rupture frequency (Dembo et al., 1988). A few years later, it was reported that L-selectin-mediated rolling required a minimal shear level, suggesting that L-selectin might form catch bonds (Finger et al., 1996). More recently, it was demonstrated with flow chambers that a lectin-like bacterial adhesin formed catch bonds (Thomas et al., 2002), and a similar property was demonstrated on P-selectin/PSGL-1 interaction with both flow

chamber and atomic force microscopy (Marshall et al., 2003): Bond lifetime displayed a fairly sharp maximum in presence of a pulling force close to 30 pN. P-selectin/PSGL-1 thus displayed catch-bond behavior in presence of a force ranging between 0 and 30 pN. Theoretical studies led to the conclusion that actual biomolecules interactions are much more complex that sketched on **Figure 2**. Thus, a catch-bond behavior might be accounted for by the existence of two dissociation pathways (Pereverzev et al., 2005).

Formation of bonds between surface-attached molecules

The rate of bond formation between two surfaces bearing known receptors and ligands cannot be derived from a "2-dimensional on-rate constant" since it is dependent on a number of parameters that are extrinsic to the receptor and ligand, including distance between surfaces, lateral mobility of receptors and ligands, length and flexibility of the links between binding sites and membranes, and behavior of surrounding molecules. First, it was suggested that the 3D k_{on} (a number expressed in μm^2 molecule^{-1} s^{-1}) had to be replaced with a function $k_{on}(d)$ representing the frequency (in s^{-1}) of bond formation between a ligand and a receptor molecules maintained at distance d (Pierres et al., 1996). The function $k_{on}(d)$ could in principle be derived experimentally by simultaneous determination of the binding frequency of receptor-bearing microspheres and ligand-coated surfaces and microsphere-to-surface distance (Pierres et al., 1998). However, other experiments show that this seemingly straightforward method may be difficult to use. Indeed, robust receptor-ligand association may not be immediate, and require a non-negligible amount of time for progressive crossing of barriers from less stable to more stable binding states (Pierres et al., 1995; Marshall et al., 2005; Pincet and Husson, 2005). This point was addressed experimentally in a model system (Robert et al., 2009): The formation of an ICAM-anti-ICAM-1 bond required a minimal contact time of about 10 ms to resist a disruptive force of order of 100 pN during at least 200 ms. This challenges the current framework used to describe bond formation (Eq. 1): the probability of bond formation between a ligand and a receptor is not proportional to the contact time. It is 0 if contact is shorter than some threshold, and 1 above this threshold. The threshold is dependent on the sensitivity of bond detection. More experiments are needed to check the relevance of these results to integrin-ligand associations. This is made more difficult to study experimentally by the dependence of integrin conformation on interactions with underlying membranes. However, since antigen-antibody association is very rapid, it is likely that kinetic effects may be found on most biological systems.

Difficulty of relating multivalent interactions to monovalent interactions when surface-bound molecules are considered

Theoretical studies (Seifert, 2000) have long revealed the difficulty of relating the properties of multivalent attachments to single bonds. This difficulty is a consequence of two important processes: force-sharing and rebinding. This point can be made easier to grasp by comparing the lifetime of attachments mediated by one or two identical bonds.

First, let us consider the effect of an external force F: if the force is equally shared between both bonds, the lifetime of each bond will be divided by $\exp(F/2F^\circ)$, where F° is the aforementioned force constant. After the rupture of a first bond, the whole force will be applied on the remaining one, thus inducing rapid failure. The dissociation rate of the divalent attachment will thus vary as $\exp(F/2F^\circ)$. In absence of force-sharing, the force is expected to divide attachment lifetime by $\exp(F/F^\circ)$. As a numerical example, a disrupting force of 40 pN is expected to reduce the lifetime of a single-bond attachment mediated by an integrin of force constant $F^\circ \sim 7$ pN by 300, while the lifetime of a force-sharing divalent attachment will be reduced by only 17.

The importance of rebinding may be still more impressive. Let us consider an attachment involving high affinity receptors such that bond formation is much more frequent than bond rupture. If a bond has a high probability to reform after spontaneous rupture, provided a second bond maintains surfaces in close contact, the lifetime of a divalent attachment may be nearly infinite, and in any case much higher than that of a monovalent attachment.

Recently, this point was addressed experimentally by comparing the lifetime of monovalent and divalent attachments formed between ICAM-1-coated surfaces and anti-ICAM-1-coated microspheres (LoSchiavo et al., 2012): the proportion of divalent attachments resisting a force of 30 pN for at least 5 s was 3.7-fold higher than that of monovalent attachments. This was due to a combination of force-sharing, bond maturation and rebinding. Importantly, rebinding requires a tight proximity between receptors.

Remarkably, clustering has been recognized by many authors as a key feature of integrin function (Cambi et al., 2006; Selhuber-Unkel et al., 2008; van Zanten et al., 2009), although other experiments were compatible with the assumption that conformational activation of individual molecules might suffice to initiate adhesion in absence of any significant modulation of clustering (Kim et al., 2004).

The physical background we summarized will help us understand the mechanisms of integrin-mediated leukocyte arrest.

CELL ACTIONS REQUIRED FOR INTEGRIN-MEDIATED ARREST

Selectins seem unable to induce durable cell arrests (Lawrence and Springer, 1991) and integrins are required for this purpose. Key events following chemokine-mediated activation (Montresor et al., 2012) are depicted on **Figures 3A,B** as described below.

INTEGRIN FUNCTIONAL ACTIVATION

Chemokines were shown to induce *within seconds* an extension of previously bent integrins and opening of binding sites resulting in an affinity increase (Montresor et al., 2012) as a consequence of both increase of binding rate (Vitte et al., 2004; Zhang et al., 2005) and bond lifetime. The binding of immobilized ligands may result in further activation (Alon and Dustin, 2007).

MEMBRANE ALIGNMENT

Membrane deformation is required to allow contact between integrin molecules and ICAM-1 ligand. Indeed, the length of the ICAM-1 + LFA-1 couple is about 40 nm, less than the size of the longest microvilli, and LFA-1 is not concentrated on the tip of microvilli in a resting cell (Erlandsen et al., 1993). Molecular contact may thus require at least one of three processes: (i) forces applied on the tip of microvilli may cause significant enlargement and shortening (Sundd et al., 2010). (ii) Chemokines may

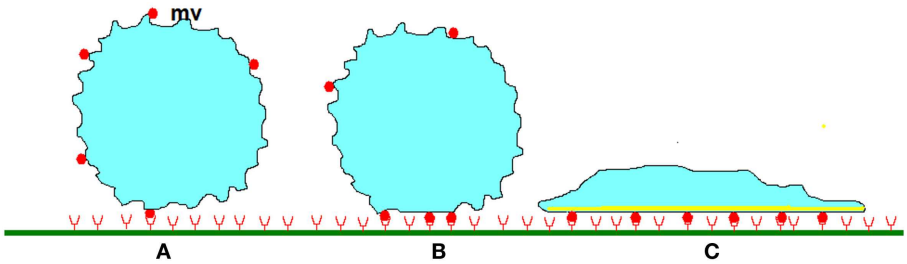

FIGURE 3 | Stabilization of leukocyte attachment to the blood vessels. **(A)** When a cell studded with protrusions (mv) of several hundreds of nm length encounters a plane surface, contact between membrane receptors such as selectins or integrins (red disks) and their ligands (red half circles) of total length lower than 50–100 nm can only occur on the tip of protrusions, allowing formation of a low number of bonds. As indicated in text, very high association rates are needed to tether freely flowing leukocytes within a molecular contact shorter than a few milliseconds and initiate rolling. **(B)** Within the following tens of seconds, rolling cells undergo (i) micrometer-scale flattening similarly to liquid droplets encountering a wettable surface, (ii) submicrometer-scale smoothing of microvilli, first due to compressive forces, and possibly later to intracellular signaling triggered by chemokines. (iii) lateral diffusion of membrane receptors that are trapped into the contact area. At some moment, these phenomena induce cell arrest. **(C)** Further attachment strengthening may involve a more extensive increase of contact area as a consequence of spreading, increase of membrane stiffness due to local cytoskeleton reinforcement, and possibly increase of the strength of membrane receptors attachment to underlying cytoskeleton, thus preventing further lateral displacement.

trigger within seconds ezrin-radixin-moesin dephosphorylation resulting within tens of seconds in microvillus disruption and membrane release (Brown et al., 2003). (iii) Membrane release may enhance transverse membrane undulations as reported at interfaces between glass coverslips and immune cells microscopy (Zidovska and Sackmann, 2006; Pierres et al., 2008; Crétel et al., 2011). Early reports done with electron microscopy (Foa et al., 1988) or fluorescence microscopy (Dustin, 1997) demonstrated submicrometer membrane alignment within minutes (Foa et al., 1988) or even tens of seconds (Dustin, 1997) following cell-surface contact. More recent studies done with interference reflection microscopy showed that the initial attachment of monocytic cells to adhesive surfaces was followed within a minute by progressive interaction tightening that might be interpreted as a nanometer scale alignment of interacting surfaces (Pierres et al., 2002, 2008).

LATERAL REDISTRIBUTION OF INTEGRINS

Integrin alignment with ligands on opposing surfaces

Integrins likely need lateral mobility to align along ligands on opposing surfaces, and the mobility requirement may be higher as lower ligand density (Chan et al., 1991). A positive correlation between lymphocyte adhesiveness to ICAM-1-coated surfaces and LFA-1 membrane mobility was reported (Kucik et al., 1996). More recently, Bakker et al. (2012) concluded that monocytes required a mobile population of surface integrins to adhere to ICAM-1-coated surfaces under static or flow conditions.

The difficulty of relating integrin-cytoskeletal association to cell adhesiveness (Lub et al., 1997) may be due to (i) heterogeneity of mobilities of LFA-1 molecules on a given cell, (ii) contradictory need for mobility (to allow ligand-receptor contact) and integrin-cytoskeleton attachment (to ensure mechanical strength), (iii) dependence of integrin-cytoskeleton interaction on cell differentiation and activation status (Cairo et al., 2006).

Integrin clustering

Since the lifetime of a newly formed LFA-1/ICAM-1 bond may be quite short if full activation has not been triggered, a pre-clustering of LFA-1 molecules might strongly enhance the duration of initial attachment and allow for the formation of additional bonds. This may be less necessary if integrins are in a fully activated state. Note that the precise chronology of clustering remains controversial. Studies made on phagocytes strongly suggest that integrin clustering preceded ligand binding (Detmers et al., 1987; Cambi et al., 2006; van Zanten et al., 2009). Other authors concluded that the binding of multivalent ligands was required to induce a clustering of lymphocyte integrins (Kim et al., 2004).

In conclusion, integrin-mediated cell arrest likely results from a combination of conformational changes (within seconds), nanotopographical membrane rearrangement to allow contact with ligand-bearing surfaces (within tens of seconds), and lateral diffusion of integrin molecules to align along ligands, form clusters, or both. However, other experiments suggest that initial arrest is followed within minutes by an attachment strengthening phase including several actions, as described below.

MEMBRANE STRENGTHENING AND ACTIVE SPREADING

Arrest stabilization may include at least three concomitant processes (**Figure 3C**) That we shall describe separately.

Reinforcement of cell stiffness in contact area

Monitoring cell-surface detachment under flow revealed significant cell deformation during detachment (Mège et al., 1986; Cao et al., 1998). Flow induced detachment is a peeling process, with a sequential detachment of membrane stripes involving a few bonds. The rupture force therefore increases in parallel with membrane stiffness (Evans, 1985). This reasoning is supported by experimental evidence (Rees et al., 1977; Badley et al., 1981). This supports the functional importance of microfilament concentration in cell-surface contact areas (André et al., 1991).

Reinforcement of integrin anchoring to underlying cytoskeleton

Strong integrin-mediated cell attachment requires that integrins be strongly attached to the cell-surface. Microfilaments indeed

enhanced integrin-mediated attachment in some experiments (Lub et al., 1997). Thus, integrin-cytoskeleton interaction may be a multiphasic, time-dependent process: initial integrin release should favor alignment with ligand and clustering, binding of ligand-attached integrins to cytoskeletal elements would then strengthen overall attachment. This is consistent with the multiplicity of integrin states (free versus immobile, isolated versus clustered) on the cell membrane (Cairo et al., 2006).

Increase of cell-surface interaction area through active spreading

Spreading may follow and markedly stabilize cell adhesion when this is mediated by suitable receptors (Pierres et al., 2002). A frequent consequence of integrin-ligand association is the generation of signaling cascades (this is outside-in signaling) inducing rapid spreading (Abram and Lowell, 2009). This was demonstrated not only with LFA-1 (Feng et al., 2012) but also β1 (Zeller et al., 2010) or β3 (Kasirer-Friede et al., 2007) integrins.

In conclusion, LFA-1-mediated leukocyte arrest on endothelial cells is a key step of inflammation. This strongly depends on a combination of integrin-mediated processes that are likely to involve a network of tens of proteins and hundreds of interactions (Ley et al., 2007; Zaidel-Bar et al., 2007). A possible way of understanding the functions of these networks is to analyze the perturbation (Ku et al., 2012) generated by the deficiency of a specific component. A recently characterized defect of *FERMT3* gene resulting in abnormal or absent kindlin-3 protein provides a good example.

LEUKOCYTE ADHESION DEFICIENCY TYPE III EXEMPLIFIES THE CONSEQUENCES OF A SPECIFIC INTEGRIN DEFICIENCY

Leukocyte adhesion deficiency (LAD) type I was identified three decades ago as a syndrome caused by a partial or complete defect of integrin β2 chain expression. Symptoms included sensitivity to bacterial infection, leukocytosis, and absence of pus formation. In 1997, a functional β2-integrin deficiency associated with a bleeding tendency and abnormal platelet spreading was reported in a patient suffering symptoms resembling but somewhat milder than LAD-I (Kuijpers et al., 1997). This was called LAD-I/variant or LAD-III (Alon and Etzioni, 2003). It was ascribed to a defective expression of kindlin-3 (Kuijpers et al., 2009). Kindlin-3 is expressed on hematopoietic cells and binds the C-terminal NXXY/F site of integrin β2 chain, thus stabilizing active conformations together with talin (Abram and Lowell, 2009). Kindlin-3 overexpression induced integrin clustering (Feng et al., 2012). Also, Kindlin-3 participates integrin-mediated cell spreading, which is considered as a consequence of outside-in signaling (Abram and Lowell, 2009; Meves et al., 2009).

Analyzing the function of kindlin-3-defective cells might give valuable information on both the role of kindlin-3 in integrin function and the interrelation of the physical events described in this review. We used the availability of a LAD-III patient to investigate neutrophils and T lymphocytes: we quantified three steps of the arrest sequence triggered by several integrin activators (Robert et al., 2011): (i) cell adhesion to ICAM-1-coated surfaces was monitored in a low shear flow ($20\,s^{-1}$). Under these conditions a single molecular bond could induce a detectable arrest (Pierres et al., 2008a), and the *total arrest frequency* should thus reflect the presence of extended integrins. (ii) The *frequency of durable arrests* (2 min or more) should account for a combination of integrin clustering and high affinity state acquisition. (iii) Finally, the *molecular contact area* between leukocytes and ICAM-1-coated surfaces after 15 min interaction, was used as a reporter of membrane-surface alignment and *spreading*. Cells were stimulated with Mn^{++}, which stabilizes active conformations without involving intracellular cascades, or chemotactic peptide fMLF, phorbol myristate acetate (PMA), and calcium ionophore ionomycin that are know to activate cells by triggering signaling cascades. The following conclusions were obtained:

(i) A clear hierarchy of measured parameters was obtained: spreading could not be normal if durable arrest frequency was normal, and durable arrest could no be stimulated in patients' cells if total arrests were lacking.

(ii) As expected, Mn^{++}-induced arrests were normal in patients cells, validating the possibility of detecting individual interactions provided integrin unbending was correctly induced.

(iii) Total arrest frequency was normal, but durable arrest frequency was decreased in fMLF-stimulated neutrophils, confirming the importance of active cell functions to stabilize arrests in contrast with short-term molecular interactions (Pierres et al., 1994). This is consistent with the hypothesis that the integrin extension induced by fMLF (Diamond and Springer, 1993; El Azreq et al., 2011) might be obtained in absence of kindlin-3. It was not feasible to ascribe the arrest stabilization and spreading defects to incomplete integrin activation or defect of fMLF-induced clustering (Detmers et al., 1987). Interestingly, Lefort et al. (2012) used a murine model to compare the consequences of talin and kindlin-3 deficiencies: they concluded that talin was sufficient to trigger integrin extension and enable slow rolling, but synergy with kindlin-3 was required to induce high affinity conformation and cell arrest under flow.

(iv) Phorbol myristate acetate (PMA) was reported to induce both mobility changes (Kucik et al., 1996) and at least partial affinity increase (Lollo et al., 1993) in stimulated leukocytes. PMA treatment triggered normal arrest frequency and duration in patients's neutrophils while T lymphocytes were markedly defective for both parameters. This is in line with a previous finding that a same pharmacological treatment had opposing effects on lymphocyte and neutrophil integrins (Marwali et al., 2003; Solomkin et al., 2007; Abram and Lowell, 2009).

(v) Surprisingly, T lymphocytes from LAD-III patients displayed abnormal spreading on anti-CD3-coated surfaces. Interestingly, LFA-1 was recently found to be involved in T lymphocyte activation by anti-CD3-coated surfaces, even in absence of ICAM-1 (Li et al., 2009) and kindlin-3 was found to lower the threshold for NK cell activation (Gruda et al., 2012). This suggests additional roles for kindlin-3. In contrast to our results, Feigelson et al. (2011) found normal ICAM-1-independent spreading of T lymphocytes from another LAD-III patient. That different kindlin-3-defective cell populations might display different deficiencies is in line with a report showing that only one of two

LAD-III siblings suffered osteopetrosis (Jurk et al., 2010). Gene-gene interactions may provide a likely explanation for phenotypic differences between two subjects or cell population sharing a common genetic deficiency (Cordell, 2009).

CONCLUSION

The recent expansion of molecular biology techniques allowing high throughput analysis of gene sequence and expression in individuals makes it a prominent goal to define with maximum accuracy the function of newly characterized genes and proteins. This is a formidable task due to the complexity of molecular networks driving cell functions. The current challenge is to find tractable ways of analyzing these networks. A possible way of approaching this goal may consist of determining the functional consequences of a well defined network perturbation (Ku et al., 2012). As shown in this review, recent studies provided a quantitative description of the elementary physical processes contributing cell functions (e.g., molecule clustering, molecular interactions, conformational change all contribute integrin-mediated adhesion). This should allow us to draw networks connecting these elementary physical events. These "function networks" should be simpler than the huge networks involving hundreds of molecular components and interactions that are presently available (Zaidel-Bar et al., 2007). The analysis of well defined genetic defects found in patients may be very informative in this domain.

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Chemokines and the signaling modules regulating integrin affinity

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Integrin-mediated adhesion is a general concept referring to a series of adhesive phenomena including tethering–rolling, affinity, valency, and binding stabilization altogether controlling cell avidity (adhesiveness) for the substrate. Arrest chemokines modulate each aspect of integrin activation, although integrin affinity regulation has been recognized as the prominent event in rapid leukocyte arrest induced by chemokines. A variety of inside-out and outside-in signaling mechanisms have been related to the process of integrin-mediated adhesion in different cellular models, but only few of them have been clearly contextualized to rapid integrin affinity modulation by arrest chemokines in primary leukocytes. Complex signaling processes triggered by arrest chemokines and controlling leukocyte integrin activation have been described for ras-related rap and for rho-related small GTPases. We summarize the role of rap and rho small GTPases in the regulation of rapid integrin affinity in primary leukocytes and provide a modular view of these pro-adhesive signaling events. A potential, albeit still speculative, mechanism of rho-mediated regulation of cytoskeletal proteins controlling the last step of integrin activation is also discussed. We also discuss data suggesting a functional integration between the rho- and rap-modules of integrin activation. Finally we examine the universality of signaling mechanisms regulating integrin triggering by arrest chemokines.

Keywords: signal transduction, chemokine, integrin activation, integrin affinity, leukocyte recruitment, adhesion, rho small GTPases, rap small GTPases

INTRODUCTION

Leukocytes spend the majority of their life circulating into blood and lymphoid vessels until local environmental cues claim their presence into sites of immune response. The capability to resist to extreme hemodynamic stress and turbulence within high diameter vessels and to avoid cell–cell aggregation in the circulation are prerequisites to allow leukocytes to embrace blood and lymph vessels like highways leading to the widest possible distribution of the immune system in the organism. However, there are adverse implications. Indeed, nothing can be more difficult for a cell than trying to stop its motion under the frantic flow conditions generated by the circulation. These conditions are important to regulate cell trafficking, by imposing physical thresholds to leukocyte recruitment, with the flow itself providing mechano-chemical signals regulating leukocyte trafficking (Zhu et al., 2000; McEver and Zhu, 2010). But at the end, to fulfill their duties, leukocytes must be able to counteract the pushing force generated by the flow, arresting on the surface of endothelial cells and transmigrating into tissues. Everything must be done within few second or less to cope with the timing imposed by flow dynamics. In the past two decades consistent efforts have been made to understand the physiology and molecular bases of the leukocyte recruitment process and a general, widely validated, model describing the entire process has been generated (Laudanna and Alon, 2006; Ley et al., 2007; Alon and Shulman, 2011). A critical step in this process is the transition from rolling to stable arrest, which is the moment when leukocytes become fully resistant to the flow and definitively stop on the vessel wall. This critical phase is mediated by a family of leukocyte-expressed cytoskeleton-regulated adhesive receptors, called integrins (Takada et al., 2007). Integrins are capable of establishing sudden and very stable adhesive interactions with endothelial ligands expressed on the inner surface of the vessels and belonging to the immunoglobulin superfamily. The strong adhesive interaction between integrins and their ligands supporting arrest of circulating leukocytes is, *de facto*, the primum movens of the immune response.

Integrins basally interact with the ligand with rather low affinity. To increase binding efficiency integrins must undergo dramatic structural and topological modifications consisting of extensive conformational changes leading to increased affinity for the ligand, along with concurrent spatial rearrangement on the cell plasma membrane. This phenomenon is globally indicated as integrin activation and is mandatory to rapid arrest of circulating cells. The step of integrin activation is finely regulated to allow diversity of leukocyte recruitment, but its most distinguishing property is the speediness. This implies the existence of environmental factors capable of activating integrins with corresponding dynamics. The original discovery of a role for a PTX-sensitive Gai-protein linked signaling in regulation of lymphocyte homing (Bargatze and Butcher, 1993) prompted the search for microvessel-presented agonists capable of triggering integrin-dependent arrest within seconds. It is now established that arrest chemokines, a subgroup of chemotactic cytokines capable of rapid integrin activation (Rot and von Andrian, 2004;

Bromley et al., 2008), are the most potent physiological activator of leukocyte adhesiveness. They do that by triggering complex signaling transduction mechanisms leading to extremely rapid activation of integrins and, ultimately, of adhesion. In this context, the most studied and best-known signaling events are represented by the signaling networks regulated by the small GTP binding proteins of the ras-like rap and rho family (Caron, 2003; Scheele et al., 2007; Tybulewicz and Henderson, 2009). More than 670 interacting proteins belong to these signaling networks, including upstream regulators and downstream effectors (see http://www.pathwaycommons.org/pc/), with specific sub-sets of these interactions devoted to modulation of leukocyte integrin activation and dependent adhesion. Here we will summarize the available data about the signaling mechanisms triggered by arrest chemokines and controlling rapid integrin affinity transitions critical to leukocyte arrest.

INTEGRIN-DEPENDENT ADHESION: AN OVERVIEW

At least two distinct modalities of integrin activation are known, namely conformational changes, leading to increased affinity, and lateral mobility leading to increased valency, both concurrently enhancing cell avidity (adhesiveness; Arnaout et al., 2005). The most detailed information about integrin structural rearrangement during affinity up-regulation in leukocytes comes from studies of LFA-1. Recent structural and biophysical data predict that LFA-1 exists in at least three conformational states, which differ both in their overall extension over the plasma membrane as well as in the arrangement of their headpiece (Carman and Springer, 2003; Luo et al., 2007; Springer and Dustin, 2011; **Figure 1**). Inside-out signaling events trigger integrins to undergo a dramatic transition from a bent low-affinity conformation to an extended intermediate-affinity to a high-affinity conformation, characterized by a complete opening of the ligand-binding pocket (Nishida et al., 2006; Luo et al., 2007). Extended β2 integrin conformations with high topographical availability of the ligand-binding headpiece but low affinity for the ligand have been also postulated (Salas et al., 2002, 2006). This extended but low/intermediate-affinity conformation may increase the capability of LFA-1 to mediate rolling on ICAM-1 upon selectin triggering (Chesnutt et al., 2006; Zarbock et al., 2007; Miner et al., 2008).

It is important to emphasize that low-, intermediate-, and high-affinity integrins likely represent discrete, reversible, states in a continuum of integrin conformational changes (**Figure 1**). Thus, the correct interpretation of the integrin activation process is a dynamic equilibrium between different conformers, corresponding to inside-out triggered conformational changes of the heterodimer displaying increasing affinity (binding energy) for the ligand (Carman and Springer, 2003; Shamri et al., 2005). Importantly, it has been demonstrated that LFA-1 integrin conformational changes are critical to the *in vivo* arrest of lymphocytes on

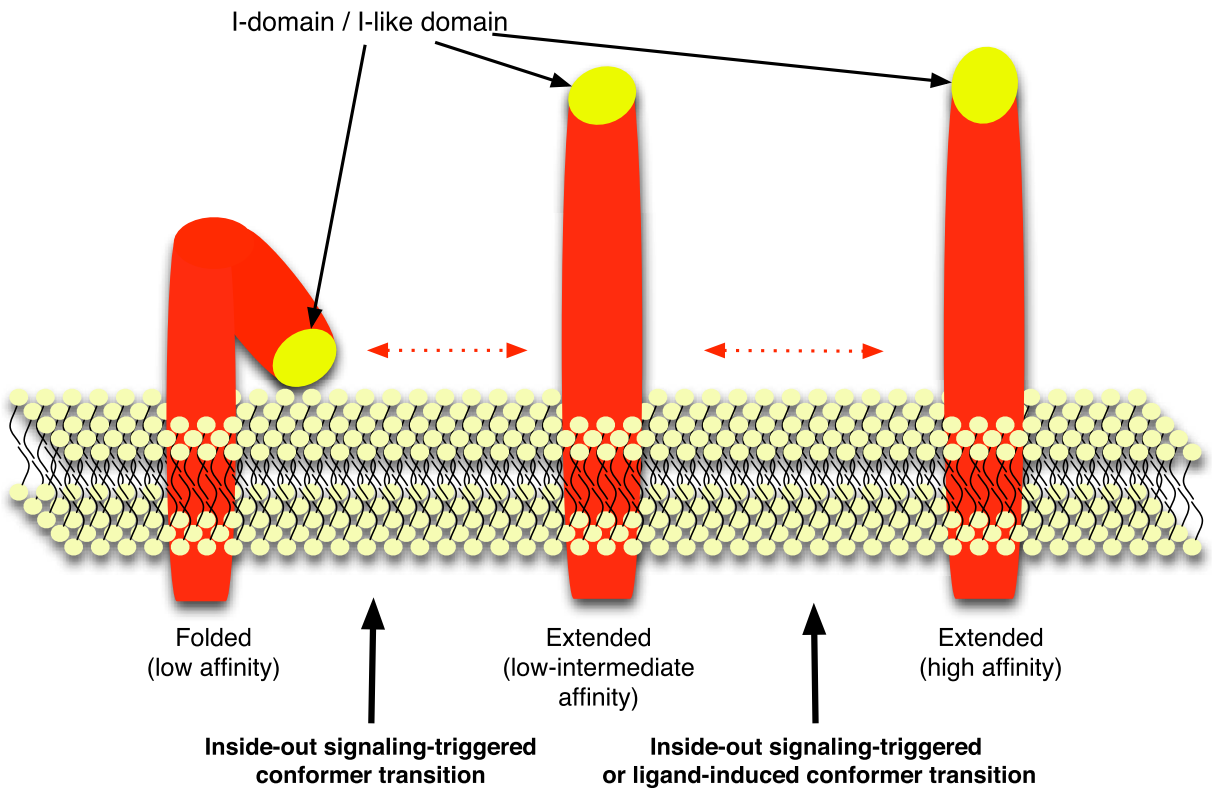

FIGURE 1 | Integrin affinity triggering. The diagram refers to the LFA-1 affinity-triggering model. Shown is the dynamic equilibrium between three conformers displaying low-, low/intermediate-, and high-affinity for ICAM-1. The progressive extension of the heterodimer is accompanied by increasing topological availability of the I-domain and I-like domain (in yellow), which are involved in ligand binding with increasing affinity.

the high endothelial venules (HEV) of secondary lymphoid organs (Giagulli et al., 2004). Thus, modulation of integrin affinity is now recognized as the critical step to leukocyte arrest *in vivo*.

Notably, integrins may undergo not only rapid inside-out regulation of affinity but also structural stabilization transmitted to the cytosolic tail by the bound ligand. The two processes are believed to cooperate to enhance integrin-mediated adhesiveness in a rapid and reversible manner. For instance, the induction of conformational changes transmitted to the cytosolic tail of the LFA-1 heterodimer upon ICAM-1 binding (Kim et al., 2003) may have a role in stabilizing leukocytes arrest under flow. However, this bi-directional regulation of integrin binding may be insufficient to support prolonged shear-resistant firm adhesions, and so post ligand occupancy events leading to integrin anchorage to the cytoskeleton are apparently required to further increase mechanical stability of individual ligand-occupied integrins (Cairo et al., 2006). Notably, these phenomena can be concurrent to the other modality of integrin activation, that is valency up-regulation. Heterodimer lateral mobility and valency increase is also regulated by interaction with the cytoskeleton, which could behave as mobility restrain (Stewart et al., 1998; van Kooyk and Figdor, 2000; Svensson et al., 2010; Bakker et al., 2012). Thus, it seems that, to stabilize the adhesion, a shuttling between restraining and stabilizing cytoskeletal proteins must occur. Valency up-regulation can be directly triggered by chemokine signaling leading to formation of multivalent complex on the plasma membrane. This may have a role under specific conditions by facilitating the encountering of activated mobile integrins with the immobilized ligand (Constantin et al., 2000). Furthermore, increase of integrin valency may also contribute to the initiation of outside-in signaling cascades, leading to the efficient recruitment of protein tyrosine kinases (PTKs; Berton et al., 2005) and the initiation of the full repertoire of outside-in signaling pathways leading to adhesion stabilization. Notably, it has been shown that lacking of integrin signaling capability leads to accelerated leukocyte detachment (Giagulli et al., 2006). Thus, chemoattractant-triggered inside-out and integrin-initiated outside-in signaling events concurrently cooperate to increase integrin affinity for the ligand and to stabilize and prolong the arrest of circulating leukocytes.

THE ROLE OF rap AND rho IN INTEGRIN AFFINITY MODULATION

A plethora of signaling events have been implicated in the regulation of various kinetic aspects of integrin-mediated adhesion. Overall, at least 65 signaling proteins are possibly involved in the regulation of integrin-mediated adhesion by chemoattractants and other agonists (**Table 1**). However, it is important to emphasize that only a subset of this group of signaling molecules has been validated under physiological conditions significant to chemokine-modulated rapid arrest of circulating leukocytes. These conditions, which we may call "*the four criteria*," should include: (a) evaluation of signaling events in primary leukocytes; (b) evaluation of adhesion under flow conditions; (c) measurement of rapid kinetics of adhesion triggering (seconds or less); and (d) direct detection of heterodimer conformational changes. The rationale for adopting such criteria is based of the following considerations: (a) Signaling studies should be always accurately contextualized, by first focusing on physiological, standard, conditions followed by comparative analysis in more specific contexts. For instance, neoplastic leukocyte cell lines are not appropriate models of physiologic leukocyte adhesion since the neoplastic transformation may alter the signaling machinery with respect to normal primary cells, thus affecting response to the agonists and data interpretation. (b) Flow is the natural condition during cell recruitment by generating a shear stress, which imposes a mechanistic threshold to adhesion activation; thus, the efficacy of signaling events in regulating leukocyte arrest should be always challenged by applying flow conditions. (c) Integrin activation under-flow occurs in the range of seconds or less (likely milliseconds); thus, to correlate signaling events to integrin activation relevant to leukocyte recruitment, the kinetics of the two events must be coherent. Such kinetics cannot be studied in static assays. (d) As stated above, integrin-mediated adhesion is a general concept. To precisely assess whether a signaling event specifically regulates integrin conformational changes leading to affinity increase it is necessary to be able to directly detect integrin structural rearrangements by means of soluble ligand-binding assays or reporter monoclonal antibodies detecting activation epitopes. *These four criteria should be always satisfied in order to correlate signaling events to affinity triggering controlling leukocyte rapid arrest in physiological contexts.* Unfortunately, these experimental criteria are not systematically applied in the literature and this may affect the correct interpretation of the regulatory role of pro-adhesive signal transduction events.

The intracellular signaling cascade from arrest chemokines, such as CCL19, CCL21, or CXCL12, to integrin affinity modulation is still incompletely understood. The two most studied and validated signaling mechanisms activated by arrest chemokines and leading to up-regulation of integrin affinity, especially in the context of the β2 integrin LFA-1, are related to signaling delivered by the small GTP binding proteins of the rap and rho family. Both rap and rho are capable of activating a variety of downstream effectors and are, in turn, activated by several upstream regulators (Caron, 2003; Scheele et al., 2007; Tybulewicz and Henderson, 2009). However, in the specific context of integrin activation by arrest chemokines, rap and rho regulate restricted signaling modules devoted to the specific control of integrin affinity.

The role of the rap isoform Rap1A in integrin activation by arrest chemokines has been extensively studied and it is now clearly demonstrated that Rap1A regulates rapid integrin-dependent adhesion either in the context of the β2 integrin LFA-1 as well as β1 integrin VLA-4 (Duchniewicz et al., 2006). Rap1A was shown to control arrest of rolling lymphocytes as well as *in vivo* homing to secondary lymphoid organs (Ebisuno et al., 2010). Rap1A is activated by arrest chemokines by means of an upstream signaling mechanism involving phospholipase C (PLC). Indeed, ligation of chemokine receptors, and more in general of all chemoattractants Gαi-protein coupled receptors (GPCRs), results in rapid intracellular calcium influx and activation of PLC (particularly of the β isoforms), which leads to generation of inositol-1,4,5-trisphosphate (IP_3, which further increases intracellular calcium from intracellular stores) and diacylglycerol (DAG). Calcium and DAG are, in turn, critical to activate the rap-specific guanine-nucleotide-exchange factor (GEF) calcium- and DAG-regulated

Table 1 | The signaling molecules involved in the regulation of integrin-dependent adhesion in leukocytes.

HGNC-ID	GI-ID	Aliases	Approved names	Chromosome
ACTN1	87		Actinin, alpha 1	14q24.1
APBB1IP	54518	INAG1, RIAM	Amyloid beta (A4) precursor protein-binding, family B, member 1 interacting protein	10p12.1
ARF1	375		ADP-ribosylation factor 1	1q42.13
ARF6	382		ADP-ribosylation factor 6	14q21.3
CDC42	998	G25K, CDC42Hs	Cell division cycle 42 (GTP binding protein, 25 kD)	1p36.1
CYTH1	9267	B2-1, D17S811E, PSCD1	Cytohesin-1; pleckstrin homology, Sec7 and coiled-coil domains 1	17q25
DOCK2	1794	KIAA0209	Dedicator of cytokinesis 2	5q35.1
FERMT3	83706	URP2, KIND3, MIG2B, MGC10966, MIG-2, UNC112C	Kindlin-3	11q13.1
FGR	2268	c-fgr, p55c-fgr	Gardner-Rasheed feline sarcoma viral (v-fgr) oncogene homolog	1p36.2-p36.1
FYB	2533	SLAP-130, ADAP	FYN binding protein (FYB-120/130)	5p13.1
HCK	3055	JTK9	Hemopoietic cell kinase	20q11-q12
HRAS	3265		v-Ha-ras Harvey rat sarcoma viral oncogene homolog	11p15.5
ILK	3611		Integrin-linked kinase	11p15.4
PIK3AP1	118788	BCAP, FLJ35564	Phosphoinositide-3-kinase adaptor protein 1	10q24.2
PIK3C2A	5286	PI3K-C2alpha	Phosphoinositide-3-kinase, class 2, alpha polypeptide	11p15.5-p14
PIK3C2B	5287	C2-PI3K, PI3K-C2beta	Phosphoinositide-3-kinase, class 2, beta polypeptide	1q32
PIK3C2G	5288		Phosphoinositide-3-kinase, class 2, gamma polypeptide	12p12
PIK3C3	5289	Vps34	Phosphoinositide-3-kinase, class 3	18q12.3
PIK3CA	5290		Phosphoinositide-3-kinase, catalytic, alpha polypeptide	3q26.3
PIK3CB	5291		Phosphoinositide-3-kinase, catalytic, beta polypeptide	3q21-qter
PIK3CD	5293	p110D	Phosphoinositide-3-kinase, catalytic, delta polypeptide	1p36.2
PIK3CG	5294		Phosphoinositide-3-kinase, catalytic, gamma polypeptide	7q
PIK3R1	5295	GRB1, p85-ALPHA	Phosphoinositide-3-kinase, regulatory subunit 1 (p85 alpha)	5q13.1
PIK3R2	5296	P85B	Phosphoinositide-3-kinase, regulatory subunit 2 (p85 beta)	19q13.2-q13.4
PIK3R3	8503		Phosphoinositide-3-kinase, regulatory subunit 3 (p55, gamma)	1p34.1
PIK3R4	30849	VPS15, P150	Phosphoinositide-3-kinase, regulatory subunit 4, p150	3q22.1
PIK3R5	23533	P101-PI3K	Phosphoinositide-3-kinase, regulatory subunit 5, p101	17p13.1
PIP5K1C	23396	PIP5Kgamma, KIAA0589	Phosphatidylinositol-4-phosphate 5-kinase, type I, gamma, 87 kD isoform	19
PIP5K1C	23396	PIP5Kgamma	Phosphatidylinositol-4-phosphate 5-kinase, type II, gamma, 90 kD isoform	19
PKD1	5310	PBP	Polycystic kidney disease 1 (autosomal dominant)	16p13.3
PLCB1	23236	KIAA0581	Phospholipase C, beta 1 (phosphoinositide-specific)	20p12
PLCB2	5330		Phospholipase C, beta 2	15q15
PLCB3	5331		Phospholipase C, beta 3 (phosphatidylinositol-specific)	11q13
PLCB4	5332		Phospholipase C, beta 4	20p12
PLCE1	51196	KIAA1516, PLCE	Phospholipase C, epsilon 1	10q23
PLCG1	5335	PLC148, PLC-II, PLCgamma1	Phospholipase C, gamma 1	20q12-q13.1
PLCG2	5336		Phospholipase C, gamma 2 (phosphatidylinositol-specific)	16q24.1
PLD1	5337		Phospholipase D1, phosphatidylcholine-specific	3q26
PRKAA1	5562	AMPKa1	Protein kinase, AMP-activated, alpha1 catalytic subunit	5p12
PRKAA2	5563	AMPK	Protein kinase, AMP-activated, alpha2 catalytic subunit	1p31
PRKAB1	5564	AMPK beta 1	Protein kinase, AMP-activated, beta 1 non-catalytic subunit	12q24.1-24.3
PRKAB2	5565	AMPK beta 2	Protein kinase, AMP-activated, beta 2 non-catalytic subunit	1q21.2
PRKACA	5566		Protein kinase, cAMP-dependent, catalytic, alpha	19p13.1
PRKACB	5567		Protein kinase, cAMP-dependent, catalytic, beta	1p36.1
PRKAG1	5571		Protein kinase, AMP-activated, gamma 1 non-catalytic subunit	12q12-q14

(Continued)

Table 1 | Continued

HGNC-ID	GI-ID	Aliases	Approved names	Chromosome
PRKAG2	51422	AAKG, AAKG2	Protein kinase, AMP-activated, gamma 2 non-catalytic subunit	7q35-q36
PRKAG3	53632		Protein kinase, AMP-activated, gamma 3 non-catalytic subunit	2
PRKAR1A	5573		Protein kinase, cAMP-dependent, regulatory, type I, alpha (tissue specific extinguisher 1)	17q23-q24
PRKAR1B	5575		Protein kinase, cAMP-dependent, regulatory, type I, beta	7pter-p22
PRKAR2A	5576		Protein kinase, cAMP-dependent, regulatory, type II, alpha	3p21.3-p21.2
PRKAR2B	5577		Protein kinase, cAMP-dependent, regulatory, type II, beta	7q31-qter
PRKCZ	5590	PKC2	Protein kinase C, zeta	1p36.33-p36.2
RAC1	5879	TC-25, p21-Rac1	Ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)	7p22
RAP1A	5906	KREV-1, SMGP21	RAP1A, member of RAS oncogene family	1p13.3
RASGRP1	10125	CalDAG-GEFII, RASGRP, V	RAS guanyl releasing protein 1 (calcium and DAG-regulated)	15q15
RASSF5	83593	RAPL, Maxp1, NORE1, MGC10823,	Ras association (RalGDS/AF-6) domain family 5	1q31
RHOA	387	RhoA, Rho12, RHOH12	Ras homolog gene family, member A	3p21.3
RHOH	399	RhoH, TTF	Ras homolog gene family, member H	4p13
SRC	6714	ASV, c-src	v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)	20q12-q13
SKAP1	8631	SKAP55	src kinase associated phosphoprotein 1	17q21.32
STK4	6789	MST1, KRS2, YSK3	Serine/threonine kinase 4	20q11.2-q13.2
SYK	6850		Spleen tyrosine kinase	9q22
SWAP70	23075	KIAA0640, SWAP-70, HSPC321	SWAP switching B cell complex 70 kD subunit	11p15
TLN1	7094	ILWEQ	Talin-1	9p23-p21
VAV1	7409		vav 1 oncogene	19p13.2

The table lists the 65 signaling proteins reported to date to be involved in regulation of integrin-mediated adhesion in different experimental contexts. Notably, few of them have been validated under experimental conditions satisfying the four criteria (see text). Shown are HGNC and Gi protein IDs, alias, full names, and chromosome localization.

GEF (CALDAG–GEF), also known as RAPGEF2; Crittenden et al., 2004; Bergmeier et al., 2007), which, ultimately, activates Rap1A. The role of other rap GEFs, such as C3G (RAPGEF1) and EPAC (RAPGEF3), in the context of chemokine signaling leading to integrin affinity regulation, is still not addressed. Once activated, Rap1A transmits downstream signals through different effectors, including RAPL (RASSF5; Ebisuno et al., 2010) RIAM (APBB1IP; Lafuente and Boussiotis, 2006), MST1 (STK4; Katagiri et al., 2006), SKAP55 (SKAP1), and ADAP (FYB; Menasche et al., 2007; see below). Although these signaling events lead to pro-adhesive events, the role of these signaling molecules in chemokine-triggered integrin affinity regulation mediating leukocyte arrest is still not clarified. Notably, in monocytes, PLC-mediated calcium signaling is required for induction of high-affinity α_4-integrin ligation and monocyte arrest (Hyduk et al., 2007). However, an important recent study showed that Rap1A is, *de facto*, unable to modulate LFA-1 affinity conformeric transitions, thus apparently implicating Rap1A and its effectors in adhesive events other than integrin affinity regulation (Ebisuno et al., 2010), such as removal of cytosolic constrains or post-binding stabilization (Ebisuno et al., 2010).

A role for the rho isoform RhoA in chemoattractant-induced rapid integrin activation was originally suggested in the context of the β1 integrin VLA-4 (Laudanna et al., 1996), although those studies did not fully satisfied the *four criteria* described above. However, more recently it was clearly demonstrated that, under physiological conditions, RhoA and Rac1 mediate LFA-1 affinity triggering by arrest chemokines (Giagulli et al., 2004; Bolomini-Vittori et al., 2009). The role of rho-specific GEF in mediating RhoA and/or Rac1 activation by arrest chemokines is still not fully clarified, although recent data show that DOCK2 (dedicator of cytokinesis 2; Garcia-Bernal et al., 2006) and VAV1 (Gakidis et al., 2004; Garcia-Bernal et al., 2005) may participate to leukocyte integrin affinity modulation. In other studies, however, VAV1 seems to have a negative regulatory role on VLA-4 affinity regulation (Garcia-Bernal et al., 2009). Once activated, RhoA and Rac1 activate a variety of downstream effectors, but only few of them have been tested as effectors to integrin affinity modulation by chemokines. In this context, PLD1 and the 87 kD isoform of PIP5K1C have been demonstrated to play a critical role in LFA-1 affinity modulation by chemokines. Thus, RhoA- and Rac1-activated PLD1 was shown to control chemokine triggering of LFA-1 extensions corresponding to both intermediate and high affinity states. Moreover, by leading to plasma membrane accumulation of phosphatidic acid, PLD1 mediates the activation of PIP5K1C. However, PIP5K1C was shown to control LFA-1 affinity triggering by chemokines in a conformer-selective manner, with transition from intermediate to high affinity, but not

from low to intermediate affinity, states controlled by the kinase activity of PIP5K1C (Bolomini-Vittori et al., 2009). These findings have important implication for our comprehension of LFA-1 affinity modulation. Indeed, these data show that the complete LFA-1 conformeric transition from a bent to a fully extended structure is accurately controlled at the level of inside-out signal transduction, even in absence of ICAM-1 interaction with LFA-1 in extended intermediate affinity state (Bolomini-Vittori et al., 2009). Thus, arrest chemokines are fully competent to trigger a complete LFA-1 affinity transition supporting arrest of rolling leukocytes. In contrast, in the context of rho-mediated LFA-1 affinity modulation, CDC42 was shown to negatively regulate LFA-1 affinity triggering by chemokines, thus establishing a sharp dichotomy with respect to the other two most homologous rho small GTPases (Bolomini-Vittori et al., 2009). CDC42 seems to affect LFA-1-mediated adhesion by blocking PIP5K1C activation (Bolomini-Vittori et al., 2009). Moreover, it was recently shown that activated CDC42 also inhibits Rap1A activation by chemoattractants (Kempf et al., 2011), thus highlighting a complex negative regulatory role for CDC42 in adhesion-de-adhesion cycling. Overall, arrest chemokines regulate integrin-dependent rapid adhesion by means of two main signaling modules: (a) the rap-module, likely including PLC, CALDAG–GEF, Rap1A, RAPL, RIAM, STK4, SKAP55, and ADAP; (b) the rho-module likely including at least DOCK2, VAV1, RhoA, Rac1, CDC42, PLD1, and PIP5K1C.

DEEP IN THE MODULES: THE VERY DOWNSTREAM EVENTS

Several cytosolic proteins, either or not of cytoskeletal nature, have been shown to directly interact with the integrin cytoplasmic tails of both alpha and beta chains and to regulate integrin functionality (Alon, 2010; Hogg et al., 2011). The most proximal to the heterodimer (downstream) signaling event leading to integrin affinity triggering is likely represented by interaction with actin-binding proteins. Among them, Talin-1 (TLN1) is the most studied actin-binding protein implicated in triggering integrin affinity up-regulation. Talin-1 is an anti-parallel homodimer. The F3 region of the head domain interacts with the cytoplasmic tail of the β chain of platelet gpIIb/IIIa ($\alpha_{IIb}\beta_3$-integrin) and triggers the transition to an increased affinity state (Tadokoro et al., 2003). The idea that the head of Talin-1 wedges between the α and β cytoplasmic tails of integrins (Tadokoro et al., 2003) is consistent with the observation that the α and β tails move apart during LFA-1 activation (Kim et al., 2003). At present, it is not clear whether Talin-1 controls the triggering of LFA-1 to its intermediate- or high-affinity state. Other actin-binding proteins, such as α-actin in and L-plastin have been also suggested to mediate LFA-1 affinity transition (Jones et al., 1998; Sampath et al., 1998). More recently, Kindlin-3 (FERMT3) and Cytohesin-1 (CYTH1), a GEF for ADP-ribosylation factor 6 (Arf6), have been suggested to mediate LFA-1 affinity activation (Weber et al., 2001; Manevich-Mendelson et al., 2009; Moser et al., 2009; Lefort et al., 2012), although not always under fully physiological conditions. Furthermore, the Rap1A effectors RAPL and RIAM have been shown to behave as direct integrin-binding proteins regulating integrin activation. RAPL was shown to directly bind the cytosolic tail of LFA-1 alpha chain (CD11a), but this was related to stabilization events and not to affinity triggering (Ebisuno et al., 2010). On the other hand, RIAM was suggested to directly bind Talin-1, thus potentially bridging Rap1A to Talin-1-controlled integrin affinity triggering. However, the role of these Rap1A effectors in affinity modulation by chemokines in conditions satisfying the *four criteria* is still not described. Thus, at present, is not clear how the rap-module may control the last steps of integrin activation. A link between the rho-module and the very downstream events of integrin affinity activation demonstrated under conditions satisfying the *four criteria* is also lacking. However, it is possible to speculate. Indeed, Talin-1, Kindlin-3, Cytohesin-1, and RIAM possess FERM and PH domains critical to support their interaction with plasma membrane phospholipids. Thus, it is likely that the lipid kinase activity of rho/PLD1-activated PIP5K1C, by increasing the local membrane concentration of phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2), may trigger the membrane translocation, activation, and direct integrin interaction of these regulatory proteins. Notably, a functional link between Cytohesin-1 and RhoA in the context of LFA-1 affinity triggering was recently described (Quast et al., 2009). Theoretically, since the 90 kD isoform of PIP5K1C (also activated by RhoA) directly interacts with Talin-1 (Di Paolo et al., 2002), it is also possible that this PIP5K1C isoform physically bridges directly RhoA to integrin affinity activation, independently of PtdIns(4,5)P2 lipid kinase activity. However, a recent report, although obtained under condition not satisfying the *four criteria*, seems to challenge this hypothesis (Wernimont et al., 2010).

DO rap AND rho TALK EACH OTHER?

Overall, in the context of signal transduction networks controlling chemokine-triggered integrin activation, rap and rho small GTPases may orchestrate the signaling activity of at least 18 signaling proteins and including PLC, CALDAG–GEF, Rap1A, RAPL, RIAM, STK4, SKAP55, ADAP, DOCK2, VAV1, RhoA, Rac1, CDC42, PLD1, PIP5K1C, Talin-1, Kindlin-3, and Cytohesin-1. It is quite likely that other signaling players will be discovered, especially accounting for context-specificity (see below). Moreover, we still need testing the role of some of these molecules under physiological condition, fully satisfying the *four criteria* proposed above. However, it is unquestionable that rap- and rho-modules represent, at present, our best paradigm of integrin affinity regulation by chemokines. An obvious question is whether these two signaling modules display concurrency and if they work in parallel of serially. The concept of "concurrency" is derived from computer science, where computation of contemporary events often occurs (D'Ambrosio et al., 2004). Thus, in a concurrent model of integrin activation, the final state of the system (fully extended conformation leading to high affinity for the ligand) is achieved only if the regulatory signaling events are delivered simultaneously and integrated at quantitative level. The simple fact that chemokines trigger simultaneously the activation of rap and rho small GTPases with kinetics consistent with rapid integrin activation, clearly suggests that the system displays concurrency (although we are still very far from a quantitative view of the process). This conclusion is supported by recent findings showing cooperation between rap and rho in controlling integrin activation (Vielkind et al., 2005; Kim et al., 2012; Li et al., 2012). An even more interesting question concerns the possibility that rap

and rho not only cooperate but also directly influence each other biochemical activity, which corresponds to ask whether rap and rho act in parallel or serially. Although not yet verified in conditions satisfying the *four criteria*, data from the literature may suggest interesting possibilities. Indeed, RhoA and Rac1 activate PLC isoforms, including the PLCβ, PLCγ, and PLCε (Thodeti et al., 2002; Illenberger et al., 2003; Wing et al., 2003; Piechulek et al., 2005; Seifert et al., 2008; Walliser et al., 2008; Guo et al., 2010) which, in turn, could determine the activation of Rap1A through CALDAG–GEF. Notably, the lipid kinase activity of PIP5K1C itself may contribute to Rap1A activation by increasing the local concentration of PtdIns(4,5)P2, which, in turn, is substrate of PLCs leading to activation of CALDAG–GEF and, ultimately, of Rap1A. Furthermore, it was recently shown that PLD1 activity is required to Rap1A plasma membrane translocation and activation (Mor et al., 2009), thus establishing a strong functional link between rho signaling activity and rap activation. Finally, as reported above, CDC42 was recently shown to inhibit Rap1A activation (measured as GTP bound state) (Kempf et al., 2011). Altogether, these data suggest that Rap1A signaling activity may be directly influenced by rho, thus controlling a critical arm of the global module of integrin activation, possibly devoted to aspects of integrin-dependent adhesion other than conformational changes and affinity up-regulation (**Figure 2**).

IS CHEMOKINE SIGNALING TO INTEGRIN AFFINITY MODULATION UNIVERSAL?

The complexity of pro-adhesive signaling event triggered by arrest chemokines also imposes more general questions: is the mechanism of integrin affinity regulation conserved among leukocyte subpopulations? Are there universal mechanism of integrin activation or, at least, common relevant proteins activated by chemokines? Some recently published data might provide answers to these questions. For instance, PLD1 does not seem to be crucial to VLA-4 activation (Garcia-Bernal et al., 2009). Moreover, DOCK2 involvement in integrin activation seems cell-specific (Nombela-Arrieta et al., 2004). A chemokine-selective role for RhoA involvement in LFA-1 affinity regulation was also recently suggested (Pasvolsky et al., 2008). Furthermore, surprising data come from a recent study in B cell chronic lymphocytic leukemia (B-CLL; Montresor et al., 2009), a lymphoproliferative disorder characterized by accumulation of immune incompetent B-lymphocytes in the blood, bone marrow, lymph nodes and spleen. In human normal B-lymphocytes, the CXCL12-triggered rho-module of LFA-1 affinity triggering is functionally conserved, with no differences with respect to normal T lymphocytes. However, and in sharp contrast, in B-CLL cells the CXCL12-triggered rho-module of LFA-1 affinity triggering appears no longer fully operational. Specifically, RhoA and PLD1 are fully

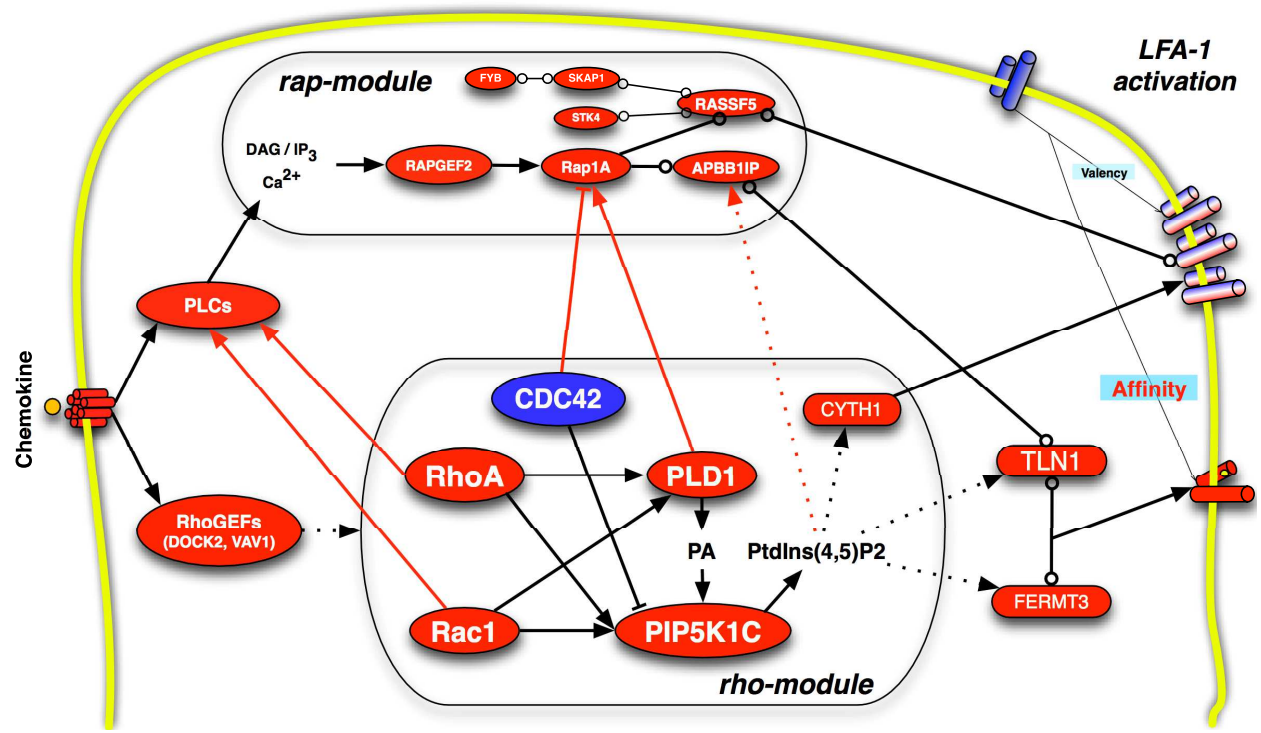

FIGURE 2 | The rap- and rho-modules of integrin affinity modulation by chemokines. The diagram mainly reports data regarding LFA-1 affinity triggering. Dotted lines refer to hypothetic effects. Red lines emphasize the functional relationships between rap and rho modules. Arrowed lines indicate activation. Flat ending lines indicate inhibition. Circle ending lines indicate docking activity. Positive regulators of adhesion are represented as red elliptic shapes; negative regulators are in blue. Notably, although the physical and functional interactions between the different signaling molecules have been demonstrated, the functional role of these interactions in the specific context of integrin affinity regulation under experimental conditions satisfying the *four criteria* is still not always validated.

activated and involved in LFA-1 affinity regulation also in B-CLL cells. In contrast, Rac1 and CDC42 are variably involved in LFA-1 affinity modulation, depending on the studied B-CLL patients, who could be grouped in two cohorts, either showing conserved or absent regulatory role for Rac1 and CDC42 in LFA-1 affinity modulation by CXCL12. Even more surprisingly, PIP5K1C emerges as totally irrelevant to LFA-1 affinity triggering in all studied B-CLL patients. Thus, the neoplastic transformation and progression may completely bypass the role of PIP5K1C and variably affect the Rac1 and CDC42 roles. Since the capability of CXCL12 to trigger LFA-1 affinity states is always fully conserved, altogether these findings show that other proteins regulate the inside-out signaling in leukemic cells, thus highlighting the relative, not universal, nature of the rho-module. Overall, these observations show that universal signaling mechanisms of LFA-1 (and more in general integrin) affinity triggering likely do not exist. An accurate definition of these mechanisms in several different cell-, agonist-, and disease-specific experimental contexts, with particular attention to the *four criteria* proposed herein, will be mandatory to fully understand the mechanisms by which arrest chemokines regulate cell trafficking.

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Chemokines, selectins and intracellular calcium flux: temporal and spatial cues for leukocyte arrest

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Leukocyte trafficking to acute sites of injury or infection requires spatial and temporal cues that fine tune precise sites of firm adhesion and guide migration to endothelial junctions where they undergo diapedesis to sites of insult. Many detailed studies on the location and gradient of chemokines such as IL-8 and other CXCR ligands reveal that their recognition shortly after selectin-mediated capture and rolling exerts acute effects on integrin activation and subsequent binding to their ligands on the endothelium, which directs firm adhesion, adhesion strengthening, and downstream migration. In this process, G-protein coupled receptor (GPCR) signaling has been found to play an integral role in activating and mobilizing intracellular stores of calcium, GTPases such as Rap-1 and Rho and cytokeletal proteins such as Talin and F-actin to facilitate cell polarity and directional pseudopod formation. A critical question remaining is how intracellular Ca^{2+} flux from CRAC channels such as Orai1 synergizes with cytosolic stores to mediate a rapid flux which is critical to the onset of PMN arrest and polarization. Our review will highlight a specific role for calcium as a signaling messenger in activating focal clusters of integrins bound to the cytoskeleton which allows the cell to attain a migratory phenotype. The precise interplay between chemokines, selectins, and integrins binding under the ubiquitous presence of shear stress from blood flow provides an essential cooperative signaling mechanism for effective leukocyte recruitment.

Keywords: calcium, chemokine, cytoskeletal proteins, inflammation, integrin affinity, LFA-1, neutrophils, Orai1

TRIGGERING LEUKOCYTE ADHESION AT VASCULAR SITES OF INFLAMMATION

Leukocyte recruitment to sites of inflammatory insult has been described as a multi-step process governed by chemokines, selectins, and integrins that engage in a step-wise manner to initiate intracellular signals and adhesive bond formation (Campbell et al., 1998; Ley, 2002; Simon and Green, 2005). β_2-integrins are key adhesion receptors in this process as they perform both adhesion and signaling functions. In the circulation, β_2-integrins are expressed on the membrane at low numbers and in a low affinity state that rapidly shift to high affinity and increase in number, and surface density as they make contact with endothelium at sites of inflammation. Affinity is regulated via allosteric changes in integrin structure that in turn modulate their adhesion potential. Following selectin dependent capture and rolling, an upshift occurs from a low affinity bent conformation to an extended conformation associated with intermediate affinity that can bind to endothelial ligands and effect deceleration of rolling leukocytes. Chemokines play a key role in signaling a shift in integrin conformation from intermediate to high affinity that is associated with adhesive stabilization, such that the leukocyte becomes resistant to tensile and shear repulsive forces of blood flow. In fact, it is control of the number and density of high affinity integrins and endothelial presentation of their cognate ligands that determines when and where leukocytes are recruited to emigrate during inflammation (Constantin et al., 2000; Beals et al., 2001; Kim et al., 2004; Sarantos et al., 2005; Bachmann et al., 2006; Green et al., 2006). Chemokines can induce a conformational switch in the CD11a/CD18 or LFA-1 subunit within a second of contact as demonstrated using an allosteric antibody that reports on the high affinity ligand binding states (Shamri et al., 2005; Green et al., 2006). Neutrophil receptors for chemokine binding such as CXCR1 and CXCR2 are linked to G-protein coupled receptor (GPCR) pathways that activate both CD11b/CD18 or Mac-1 and LFA-1 β_2-integrins to initiate firm arrest and subsequent migration (Zarbock et al., 2007a). A detailed understanding of how GPCR activation cooperates with signaling via E-selectin ligands on rolling and arresting PMNs is only now emerging (Simon et al., 2000a; Zarbock et al., 2007b). These integrins once activated to a high affinity state can bind ligand and themselves initiate outside-in signals to remodel the cytoskeleton facilitating the next step in the process of pseudopod extension and transendothelial migration (Alon and Ley, 2008).

ENGAGEMENT OF SELECTINS AND GPCRS COOPERATE IN MEDIATING STABLE ADHESION OF PMN

Engagement of GPCRs activates Phospholipase C (PLC), which then mobilizes Inositol-1,4,5 triphosphate (IP_3) and Diacylglycerol (DAG) that triggers an elevation in intracellular calcium level through release of PLC dependent ER stores

(Hellberg et al., 1996; Kinashi, 2005). Pharmacological inhibition of PLC in neutrophils, monocytes, and platelets completely abrogates integrin activation downstream of GPCR signaling (Schaff et al., 2008; Graham et al., 2007; Hyduk et al., 2007; Zarbock et al., 2007a). Immediate effector molecules downstream of GPCR and PLC signaling are the Rho GTPases, Rap-1 and cytoskeletal modulators including Talin1, all of which regulate integrin affinity and clustering following ligand binding (Calderwood et al., 1999; Boettner and Van Aelst, 2009). Signaling through GPCRs and DAG activates a Guanine Exchange Factor (GEF), that is denoted CalDAG-GEF1, which in turn activates Rap-1 and modulates Talin1-β_2 integrin association (Shimonaka et al., 2003; Kinashi et al., 2004; Ghandour et al., 2007; Pasvolsky et al., 2007; Lim et al., 2010). Upon binding of the Talin1 head domain to cytoplasmic sites of the β-integrin tail, a conformational shift is induced that allows the α and β subunits of LFA-1 to move apart and shift to an extended conformation (Calderwood et al., 1999; Kim et al., 2003). A second event significant to converting a rolling PMN to arrest is Rap-1 mediated recruitment of another effector molecule, RapL, to the α cytodomain that facilitates clustering of high affinity LFA-1 (Katagiri et al., 2003).

Activation of integrins can also be achieved by engagement and rolling on selectins, which facilitates the initial capture of leukocytes on the endothelial surface (Ley, 2002; Simon et al., 2000a). Specifically, E-selectin, P-selectin, and L-selectin are critical to leukocyte and lymphocyte capture and rolling through PSGL-1 and other glycosylated ligands. While E and P-selectin are expressed on the endothelium, L-selectin is expressed only on leukocytes and is involved in secondary capture of neutrophils during recruitment (Taylor et al., 1996; Dwir et al., 2001). Selectins form adhesive catch bonds with their glycosylated ligands with high on and off rates and require a threshold level of hydrodynamic shear stress to support rolling and subsequent signaling (Thomas et al., 2002; McDonough et al., 2004; Zhu and McEver, 2005). E-selectin binding to PSGL-1 activates tyrosine kinase Syk and MAPK, which together signal a shift in LFA-1 conformation to an extended and intermediate affinity state (Simon et al., 2000b; Zarbock et al., 2007a). This intermediate affinity state in LFA-1 facilitates deceleration of neutrophil rolling on the endothelium and can trigger firm arrest in the presence of a sufficient density of ICAM-1 (McDonough et al., 2004; Green et al., 2006). Rolling on E-selectin is synergistic with signaling via GPCRs in activation of integrin dependent arrest. The mechanism is not completely elucidated, but may involve calcium acting as a secondary messenger to amplify conversion of additional integrins to a high affinity state and facilitate their formation into focal clusters (Campbell et al., 1998; Alon and Feigelson, 2002; Green et al., 2006; Schaff et al., 2008). Recent studies suggest that E-selectin mediated slow rolling and β_2 integrin activation in neutrophils is dependent on PLCγ2 and PI3Kγ, which are critical regulators of intracellular calcium release (Mueller et al., 2010). These investigations highlight the cooperativity between chemokines, selectins, and the presence of hydrodynamic shear force for optimum activation of integrins through bi-directional signaling to support a migratory cell phenotype (Simon and Green, 2005).

CALCIUM: A TEMPORAL AND SPATIAL CUE FOR PMN ADHESIVE FUNCTIONS

Calcium (Ca^{2+}) is a versatile signaling molecule that is critical to synchronizing rolling, arrest and polarization events during leukocyte migration. Ca^{2+} transients are spatially and temporally regulated by communication between the calcium stores in the endoplasmic reticulum (ER) and membrane distributed calcium channels activated through GPCR signaling and integrin engagement with their ligands on the endothelium. We have mentioned above how chemokine activation through GPCRs is followed by an intracellular Ca^{2+} burst mediated through PLCs that is necessary to trigger integrin activation and leukocyte arrest. This Ca^{2+} flux serves to activate downstream messengers that include calpain, calmodulin, GTPases, and Talin1, some of which also regulate superoxide production, and exocytosis of secretory granules containing additional integrins and proteolytic enzymes (Truneh et al., 1985; Ginis and Tauber, 1990; Smith et al., 1990; Franco et al., 2004; Brechard et al., 2008). Engagement of Mac-1 and LFA-1 can themselves trigger Ca^{2+} transients in the cytosol and activate downstream Ca^{2+} dependent kinases that recruit cytoskeletal proteins necessary for migratory function (Marks and Maxfield, 1990; Jaconi et al., 1991; Hellberg et al., 1995, 1996; Pettit and Hallett, 1997). For example, Ca^{2+} transients are required for neutrophil migration on fibrinogen and fibronectin through Mac-1 engagement and are also important for cell adhesion of platelets, lymphocytes, fibroblasts, and endothelial cells (Su et al., 2000; Schaff et al., 2008).

Studies employing multi-channel fluorescence microscopy have provided insight into the spatial and temporal regulation of Ca^{2+} bursts that facilitate cell migration. Using fast confocal laser scanning technology, global cytosolic waves of Ca^{2+} have been reported as "puffs" that are initiated at a submicron scale in response to GPCR engagement (Hillson and Hallett, 2007). Imaging Ca^{2+} dynamics using real-time fluorescence microscopy allows detection of calcium regulation during integrin engagement and its role in leukocyte migration. There are two components of the Ca^{2+} flux signal; a rapid release from ER stores in response to GPCR activation, followed by a slower entry of Ca^{2+} via calcium release activated channels (CRACs) that is mediated by both transient receptor potential (TRP) channels and Orai1, 2, and, 3 that control store operated calcium entry (SOCE; **Figure 1**). Human neutrophils possess TRPC 1,3 4, and 6, while only TRPC 6 mediates SOCE following E-selectin and GPCR engagement (Heiner et al., 2003; Itagaki et al., 2004; McMeekin et al., 2006). Orai1 CRAC appears to cooperate with these TRPC's to activate calcium influx in human neutrophils (Brechard et al., 2008). The coupling between ER and plasma membrane CRAC to modulate SOCE has recently been shown to involve STIM and Orai proteins (Luik et al., 2006; Brandman et al., 2007; Parvez et al., 2007). STIM1 is a single spanning membrane protein with an unpaired Ca^{2+} binding EF-hand domain that functions as a sensor of ER luminal Ca^{2+}, and dynamically redistributes to position the ER proximal to Orai1 spanning the plasma membrane. The association between STIM1 and Orai1 in sensing ER depletion and communicating with the CRAC channel has been elegantly demonstrated using tools that include siRNA mediated knockdown, real time FRET and

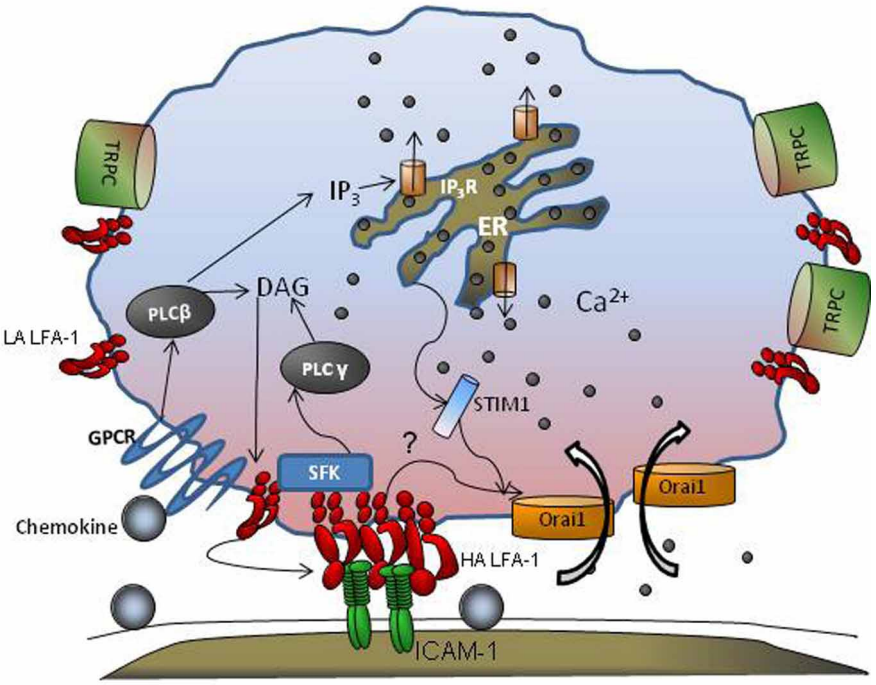

FIGURE 1 | GPCR and CRAC cooperate during leukocyte adhesion. Engagement of GPCRs by chemokines activates PLCβ that is cleaved into DAG and IP_3. While DAG remains membrane bound, IP_3 is released into the cytosol that then binds to IP_3 receptors in the endoplasmic reticulum (ER) liberating stores and leading to a rise in cytosolic Ca^{2+} concentration. Integrin receptors shift from low affinity (LA) to high affinity (HA) in response to GPCR and increase their mobility in the membrane. STIM1 senses ER store depletion, binds to the ER membrane, and provides an anchor for transmembrane Orai1 and TRPC channels that cluster and facilitate local Ca^{2+} influx at the plasma membrane. Src family Kinases (SFKs) are recruited to nascent HA LFA-1, which promote clustering, and in turn activate PLCγ which elicits additional ER dependent Ca^{2+} release. Tension on focal clusters of LFA-1/ICAM-1 bonds may also engage cytoskeletal adaptor proteins that activate CRAC mediated calcium influx further promoting integrin clustering and bond formation within a region of adhesive contact we denote the *inflammatory synapse*.

immunofluorescence imaging (Roos et al., 2005; Brandman et al., 2007; Brechard et al., 2008). STIM1 thus facilitates organized clustering, and conformational changes in TRP and Orai1 to allow Ca^{2+} entry through these channels (Zhang et al., 2005; Wu et al., 2006; Navarro-Borelly et al., 2008). Orai1 is uniformly distributed throughout the plasma membrane in unactivated cells and is the predominant CRAC channel that colocalizes with STIM1 upon ER store depletion (Luik et al., 2006; Wu et al., 2006). Orai1 mediated Ca^{2+} flux was first shown to be critical for T cell function and formation of the immunological synapse, and subsequently its role in Ca^{2+} regulation was identified in B cells, mast cells, and neutrophils (Hoth and Penner, 1992; Feske et al., 2006; Gwack et al., 2008; Schaff et al., 2009). In the context of neutrophil recruitment, we have reported that Orai1 is the predominant CRAC that synchronizes the transition from cell rolling to arrest by cooperating with IP_3 gated channels downstream of PLC activation (**Figure 1**). Orai1 CRAC cooperates with other TRP channels on the membrane to mediate Ca^{2+} entry in neutrophils (Brechard et al., 2008). Orai1 mediated Ca^{2+} influx is emerging as a mechanism for signal transduction via mechanical force as tension is transduced intracellularly by high affinity LFA-1 bond clusters during neutrophil arrest. It is reported that tensile force actively stabilizes high affinity LFA-1 bonds during the transition from rolling to arrest (Green et al., 2006; Alon and Dustin, 2007; Schaff et al., 2008). A putative mechanism is one in which Orai1 and high affinity LFA-1 become colocalized during bond formation with ICAM-1 (Dixit et al., 2011). In this manner, integrin mediated local Ca^{2+} flux enhances integrin contact with the endothelium by promoting cytoskeletal redistribution that engage and anchor integrin cytodomains (Cinamon et al., 2001; Dixit et al., 2011). Local Ca^{2+} at these sites reinforces adhesion by recruiting additional clusters of LFA-1. This process may explain why high affinity LFA-1 clusters bound to ICAM-1 are observed both at the uropod and at the base of newly forming pseudopods as PMN adopt a polarized morphology and migrate perpendicular to the direction of blood flow (Dixit et al., 2011). F-actin is also found enriched at these sites of high PMN traction (Smith et al., 2007; Schaff et al., 2009; Dixit et al., 2011). In the absence of the shift from intermediate to high affinity LFA-1, there is insufficient localization and recruitment of Orai1 to LFA-1 sites leading to decreased intracellular Ca^{2+} flux (Dixit et al., 2011). In the absence of stable high affinity LFA-1/ICAM-1 bonds, impaired F-actin polymerization is also observed and the processes of neutrophil polarization and transmigration are abolished. The role of local Ca^{2+} flux in the timing of cell arrest-polarization-migration

has been elucidated by real-time imaging of Ca^{2+} flux. Using flash lamp-based excitation, it was shown that Ca^{2+} transients cycling at 6 μs intervals were associated with Myosin-II activation during uropod retraction (Clark and Petty, 2008). In addition, we have shown that engagement of high affinity LFA-1 clusters and shear stress are critical to initiation of Ca^{2+} influx during arrest (Dixit et al., 2011). Taken together, these data suggest that neutrophils rolling to arrest utilize focal adhesions as mechanosensors that convert shear stress mediated tensile force into local bursts of Ca^{2+} influx that promotes cytoskeletal engagement, and an adhesion strengthened and migratory phenotype.

CYTOSKELETAL ORGANIZATION AT INTEGRIN CYTODOMAINS

An important question that has emerged is what are the cytodomain linkages that transduce force intracellularly from the high affinity bonds between β_2-integrin and ICAM-1? Furthermore, how does neutrophil polarity and directional migration become responsive to the magnitude and direction of shear stress? The earliest steps in neutrophil recruitment are chemokine activation of GPCRs that triggers integrin activation and initiates linkage to the cytoskeleton at relatively low levels of cytosolic Ca^{2+} (i.e., ∼100 nM; Lum et al., 2002; Green et al., 2006). Cytoplasmic adaptor proteins including Kindlin-3 and Talin1 build up at the integrin cytodomain, as high affinity clusters of integrins accumulate on a rolling neutrophil, even before integrin bonds form focal adhesions at the inflammatory substrate (Lefort et al., 2012). In the case of LFA-1, we have observed that a high affinity state and engagement to ICAM-1 homodimers results in bonds that last ∼10-fold longer and transmit 100-fold higher force as compared to monomeric LFA-1/ICAM-1 bonds (Sarantos et al., 2005; Evans et al., 2010). The valence and conformation of the integrin bond in this case can influence the bond lifetime and amount of force that is transduced across the membrane. In this manner, LFA-1 clusters can form adhesion-strengthened complexes that are resistant to dissociation as they link to the nascent cytoskeleton leading up to migration (Astrof et al., 2006; Alon and Dustin, 2007; Puklin-Faucher and Sheetz, 2009). The β tail of integrins acts as a scaffold for binding cytoskeletal adaptor proteins, as well as tyrosine kinases such as Src Family Kinases (SFKs) including Src and Syk that signal to activate and cluster more integrins at the contact site (Obergfell et al., 2002). SFKs associate rapidly with the LFA-1 cytodomain and can regulate integrin affinity, avidity, and subsequent signaling to the cytoskeleton to initiate cell spreading (Roskoski, 2004; Arias-Salgado et al., 2005; Sarantos et al., 2008). Genetic deletion and inhibition of SFKs in neutrophils abrogates rearrangement of high affinity LFA-1 clusters along the uropod-pseudopod axis and impairs co-clustering of high affinity CD18 with F-actin during polarization (Piccardoni et al., 2004; Sarantos et al., 2008). Not only is slow rolling on E-selectin abolished in Syk deficient bone marrow chimeric mice, but these mice also exhibited impaired integrin mediated signaling, defective respiratory burst, degranulation, and spreading in response to inflammatory stimuli (Mocsai et al., 2002; Zarbock et al., 2007a). Thus, Src and Syk tyrosine kinases appear to function in events both leading up to LFA-1 ligand engagement and signaling of subsequent effector functions. More research on their discrete functions during PMN migratory responses is needed.

There is much recent interest in the roles of Talin1 and Kindlin-3 as key cytoskeletal adaptor proteins in the regulation of integrin affinity and clustering during the transition from neutrophil rolling to arrest and shape polarization as it navigates to sites of transmigration on inflamed endothelium (Sarantos et al., 2008; Puklin-Faucher and Sheetz, 2009; Lefort et al., 2012). How these cytoskeletal proteins bind to the LFA-1 cytodomain as it engages ICAM-1 and transduce signals to guide neutrophil migration under shear flow is beginning to come to light. Talin1 associates with the β2 tail of LFA-1, unclasping the α and β chains to allow a conformational upshift to its ligand-binding state as reported in neutrophils and T cells (Calderwood et al., 1999; Simonson et al., 2006). Talin clusters with LFA-1 at the immunological synapse and also localizes at focal adhesions with LFA-1 in leukocytes along with paxillin, which provides its main linkage to F-actin during assembly in response to a local cytosolic gradient of Ca^{2+} (Lum et al., 2002). Kindlin-3 also binds to integrin β-tails and has been shown to play a role in GPCR activated upshift in integrin affinity and subsequent leukocyte adhesion on the endothelium (Moser et al., 2009; Svensson et al., 2009). This protein was recently identified as the key molecule defective in leukocyte adhesion deficiency III (Mory et al., 2008). Talin and Kindlin-3 recognize two distinct binding sites on the LFA-1 cytoplasmic tail and cooperative binding may be requisite for assembly of a high affinity LFA-1 that is competent to form multivalent bond clusters with ICAM-1 (see **Figure 2**) (Moser et al., 2009). Talin and Kindlin-3 are critical for adhesion strengthening and cell spreading under shear stress at a step downstream than affinity regulation as shown in studies of β_1 integrin (Feigelson et al., 2011; Hyduk et al., 2011). Kindlin-3 has been carefully studied in T-cell receptor mediated outside-in stabilization of chemokine activated LFA-1 bond formation with ICAM-1. It was shown to associate with RACK1 at the cytodomain in order to effect clustering of LFA-1 (Feigelson et al., 2011; Feng et al., 2012). The observation that $\alpha_{II}\beta_3$ receptors on Kindlin-3 deficient platelets activated to high affinity by Mn^{2+} can bind to fibrinogen coated substrates, but downstream cell spreading is severely impaired, indicates that integrin mediated cytoskeletal rearrangement through outside-in signaling is defective (Moser et al., 2008). Thus, Kindlin-3 is critical for stabilization and downstream transduction events necessary for adhesion strengthening through β_3 subunits. In the case of β_2-integrin, Kindlin-3 association precedes recruitment of Talin to the β-subunit of LFA-1 in a pathway that involves GPCR activation, Ca^{2+} flux, Rap-1 recruitment, and Phosphatidylinositol 4,5 bisphosphate (PIP_2) activation (Di Paolo et al., 2002; Puklin-Faucher and Sheetz, 2009; Lefort et al., 2012). However, the significance of Ca^{2+} influx through CRAC and cooperation with GPCR release of ER stores for initial Kindlin-3 association is yet to be elucidated. Our laboratory has been examining the role of Kindlin-3 in PMN arrest and adhesion strengthening in shear flow. We observe that Kindin-3 binding to the β-subunit of LFA-1 is critical for adhesion strengthening of arrested PMN at high shear stress and facilitates rapid clustering of LFA-1 at focal sites that engage ICAM-1. This data thus supports previous studies highlighting Kindlin-3

as a key player in mediating adhesion strengthening through β_1 integrins and its dispensable role in GPCR mediated integrin affinity upshift (Hyduk et al., 2011). Transmission of tensile force provided by blood flow to the cytodomain of LFA-1 activates a local burst of Ca^{2+} via Orai1 CRAC that cooperates with ER stores to effect a local burst in Ca^{2+} concentration (**Figure 2**). Kindlin-3 appears to be critical at this step, since knockdown of Kindlin-3 expression using lentiviral transfection of shRNA abrogates its physical association with high affinity LFA-1 and Orai1. Thus, Kindlin-3 may serve a critical role as an adaptor molecule whose binding to the LFA-1 cytodomain requires a force sensitive allosteric step that allows binding directly or in a complex with as yet unidentified proteins to open proximal Orai1 channels. This linkage between high affinity LFA-1/ICAM-1 bonds and Orai1 via Kindlin-3 begins to explain how it serves as an adaptor in mediating focal clustering of LFA-1 that supports adhesion strengthening (Dixit et al., 2011 **Figure 2**). Precisely how Kindlin-3 communicates with Orai1 and what the role of other cytoskeletal proteins such as Talin, paxillin and vinculin associated with focal LFA-1 clusters in this process are under study in our laboratory.

LEUKOCYTE SIGNALING IN DISEASE

Remarkable is the high frequency of immunodeficiency diseases that are associated with mutations in the effector molecules that directly influence affinity modulation and clustering of integrins. These include Orai1, Kindlin-3, WASp, CalDAG-GEF1, and Vav1, all of which have been identified in leukocyte adhesion deficiencies. Moreover, all of these components cooperate with Ca^{2+} mediated signaling of adhesion stabilization and integrin outside-in signaling (Marks and Maxfield, 1990; Sjaastad and Nelson, 1996). CRAC channels and their crosstalk with ER stores of Ca^{2+} are critical to facilitating F-actin polymerization and integrin polarity during migration (Schaff et al., 2009; Dixit et al., 2011). Recently, a point mutation in the Orai1 gene at the R91W locus was discovered to be associated with a severe immunodeficiency in patients. This mutation is clinically manifested by infections in childhood, ectodermal dysplasia, and congenital myopathy (Feske et al., 2006). These symptoms were similar to those observed in SCID (severe combined immunodeficiency disease) patients except that total lymphocyte counts were normal in Orai1 deficient patients as compared to SCID (Feske, 2009). This missense mutation in Orai1 did not interfere with interactions between Orai1 and STIM1, which suggest that the immunodeficiency is derived from defective Orai1 driven Ca^{2+} flux. Blocking SOCE with CRAC channel inhibitors, using siRNA, or genetic deletion to knockdown Orai1 expression all result in impaired neutrophil arrest, polarization, and abrogation of directional migration under shear flow (Schaff et al., 2009; Dixit et al., 2011). Furthermore, Ca^{2+} entry through Orai1 and STIM1 drives focal adhesion turnover through Ras and Rac1 and together play a vital role in tumor metastasis (Yang et al., 2009). This critical role of calcium in regulating cellular adhesive processes makes it an attractive therapeutic target to reduce pro-inflammatory responses in specific leukocyte subsets.

Immunodeficiencies have also been linked to impaired GTPases, GEFs, and cytoskeletal protein signaling. Leukocyte adhesion deficiency I, II, and III occur due to defects in β_2 integrin structure, mutations in the fucosyl transporter gene required for producing sialyl-Lewis^x selectin ligands that support leukocyte rolling on the endothelium, and a general defect in integrin activation of β_1, β_2, and β_3 integrins, respectively (Abram and Lowell, 2009). Cytoskeletal proteins such as Talin1 and Kindlin-3 provide activation and stabilization signals when bound to cytoplasmic domains of integrins (Zhang et al., 2008; Hyduk et al., 2011). Upstream of these proteins, integrin activation is controlled by GTPases such as Rap-1 and its GEF, CalDAG-GEF1 which function downstream of GPCR activation (Pasvolsky et al., 2007; Mory et al., 2008). Mutations in Kindlin-3 are responsible for LAD III related integrin activation defects contributing to recurrent bacterial infections, impaired healing of wounds, defects in platelet activation and severe bleeding tendencies (Abram and Lowell, 2009). Mutations in CalDAG-GEF1 were also found present in a subset of LADIII patients and re-expression of CalDAG-GEF1 was unable to rescue the LADIII phenotypic defects (Svensson et al., 2009). In comparison, re-expression of the Kindlin-3 protein in immortalized lymphoblast cell lines derived from patients restored their adhesive and migratory defects (Abram and Lowell, 2009; Malinin et al., 2009; Svensson et al., 2009). This implicates Kindlin-3 as the key defective protein underlying LADIII manifestation.

Similar to Kindlin-3, WASp also connects the actin cytoskeleton to integrin cytodomains to facilitate leukocyte migration via control of integrin adhesion functions. A crucial effector of Rho GTPases and an important activator of the Arp2/3 cytoskeletal complex, WASp deficiency leads to Wiskott-Aldrich syndrome that is characterized by increased susceptibility to infections (Thrasher, 2002). We reported that a defect in WASp in both mice and human is associated with impaired clustering of β_2-integrins and severely impaired adhesion and migration of neutrophils on inflamed endothelium (Zhang et al., 2006). WASp deficiency contributes to defective T cell trafficking toward a chemokine gradient, revealing its profound role in signaling through GPCR pathways and guiding leukocyte migration (Snapper et al., 2005). Many other signaling proteins associating with integrins such as Rho family of GTPases, P21 activated kinases (PAKs) and their effector molecules are now emerging as significant contributors to inflammatory disorders and cancer progression (Ahn et al., 2011; Yoon et al., 2011). These molecules are all activated downstream of GPCR engagement and assist in strengthening integrin bond clusters required for leukocyte pseudopod extension and eventual recruitment to sites of insult.

CONCLUSION AND PERSPECTIVES

With each heartbeat, leukocytes make a fateful decision when they encounter vascular sites of inflamed endothelium; to arrest or not to arrest. This singular event multiplied by millions of encounters can determine the intensity of the neutrophilic response to infectious or autoimmune tissue insults. Assisting in this decision process is the relative density of chemokines and selectins expressed on inflamed endothelium that facilitate neutrophil activation by ligating their respective cognate receptors on the tethered cell. In this review, we detailed how cytosolic release of Ca^{2+} converges with influx through CRAC, thereby providing a

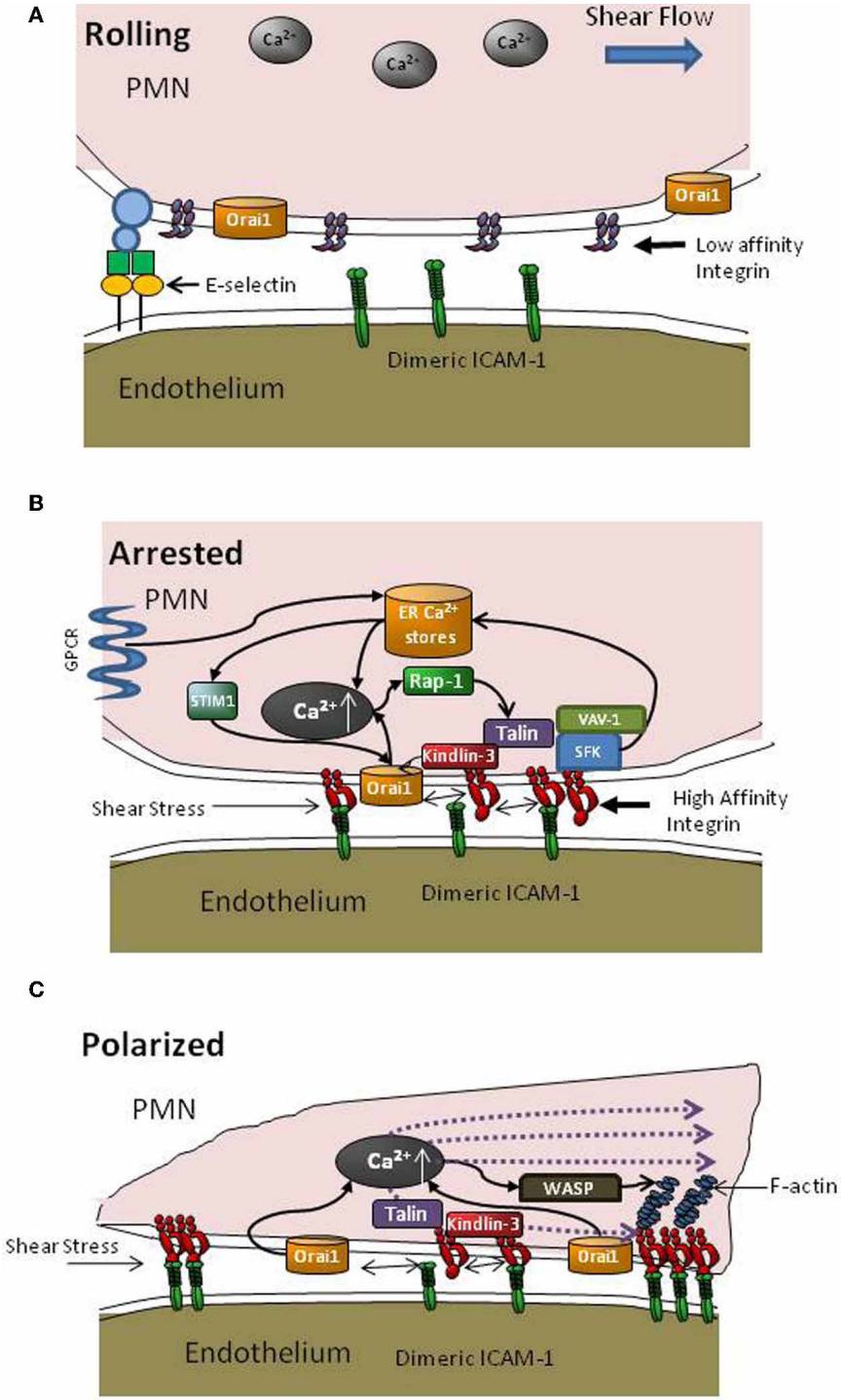

FIGURE 2 | Intracellular signaling events supporting PMN recruitment. **(A)** During PMN capture and rolling on inflamed endothelium, β_2-integrins are randomly distributed on the plasma membrane predominantly at low affinity and a low basal level of intracellular Ca^{2+} is maintained. **(B)** Transition from rolling to arrest involves activation via GPCR signaling that elicits Ca^{2+} release via DAG (see **Figure 1**) and an upshift in LFA-1 to a high affinity state, which promotes bond formation with ICAM-1 on inflamed endothelium. Depletion of ER stores leads to communication with Orai1 CRAC at the membrane via STIM1 proteins. As LFA-1/ICAM-1 bonds take up tensile forces they recruit Kindlin-3 and colocalize with Orai1 to facilitate cooperation with PLC mediated Ca^{2+} flux, which in turn catalyzes recruitment of Rap-1 GTPases and cytoskeletal elements such as Talin to LFA-1 cytodomains to initiate F-actin recruitment and pseudopod projection. **(C)** New pseudopod projection and cell polarization is oriented by the dynamic redistribution of LFA-1/ICAM-1 into macro-clusters, Orai1 mediated Ca^{2+} influx, and assembly of the F-actin cytoskeleton that guides migration in a manner dependent upon direction of shear stress and cytoskeletal force distribution.

means to dynamically modulate the number and location of integrin bonds and subsequent migration. This is accomplished by shear stress mediated tensile force transmission, which requires bond formation at sites in which β_2-integrins are engaged at sufficient bond strength and density where their survival is ensured. At these locations, high affinity LFA-1 associates with cytosolic Kindlin-3 thus enabling association of a complex with Orai1 that together transduce a local increase in Ca^{2+}. This in turn activates membrane diffusion of additional high affinity LFA-1 to bond with available endothelial ICAM-1. Further, local cytosolic release of Ca^{2+} promotes the assembly of cytoskeletal elements including Talin and F-actin to the integrin tail in a complex that provides the machinery for adopting a polarized elongated shape as a neutrophil extends pseudopods and initiate transendothelial migration. In this manner, mechano-transduction through integrins provides a means for sensing the direction and magnitude of shear force via a complex that involves at a minimum LFA-1, Orai1, Kindlin-3, Talin1, Vav-1, and WASp. These molecules enable neutrophils to efficiently navigate the journey from the blood stream to inflammatory sites that is critical for host defense.

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Neutrophil arrest by LFA-1 activation

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Lymphocyte function-associated antigen-1 (LFA-1) is a heterodimeric integrin consisting of α_L (gene name, *Itgal*) and β_2 (gene name, *Itgb2*) subunits expressed in all leukocytes. LFA-1 is essential for neutrophil recruitment to inflamed tissue. Activation of LFA-1 by chemokines allows neutrophils and other leukocytes to undergo arrest, resulting in firm adhesion on endothelia expressing intercellular adhesion molecules (ICAMs). In mice, CXCR2 is the primary chemokine receptor involved in triggering neutrophil arrest, and it does so through "inside-out" activation of LFA-1. CXCR2 signaling induces changes in LFA-1 conformation that are coupled to affinity upregulation of the ligand-binding headpiece (extended with open I domain). Unlike naïve lymphocytes, engagement of P-selectin glycoprotein ligand-1 (PSGL-1) on neutrophils stimulates a slow rolling behavior that is mediated by LFA-1 in a distinct activation state (extended with closed I domain). How inside-out signaling cascades regulate the structure and function of LFA-1 is being studied using flow chambers, intravital microscopy, and flow cytometry for ligand and reporter antibody binding. Here, we review how LFA-1 activation is regulated by cellular signaling and ligand binding. Two FERM domain-containing proteins, talin-1 and Kindlin-3, are critical integrin co-activators and have distinct roles in the induction of LFA-1 conformational rearrangements. This review integrates these new results into existing models of LFA-1 activation.

Keywords: neutrophil, chemokine, integrin, LFA-1, inflammation

LFA-1 STRUCTURE, FUNCTION, AND INSIDE-OUT ACTIVATION

Twenty-four different integrins are expressed in humans, each composed of non-covalently associated α and β chains (Hynes, 2002). Integrins have large extracellular domains, single-pass transmembrane segments, and short intracellular tails. Lacking enzymatic activity, the short integrin cytoplasmic domains serve as scaffolds for signaling and structural proteins that allow integrins to be a conduit of bidirectional communication between the cytoplasm and extracellular ligands (Legate and Fassler, 2009; Moser et al., 2009b) [**Box 1**]. The integrin ligand recognition site spans the β subunit inserted-like (βI) domain and α subunit β-propeller domain or, for about half of the integrin family, resides entirely within the α subunit I (αI) domain (Lu et al., 2001; Luo et al., 2007). All β_2 integrins, including LFA-1 ($\alpha_L\beta_2$ or CD11a/CD18) [**Box 2**], contain this extra αI domain within the headpiece. The structural features of the various domains of LFA-1 have been reviewed in great detail elsewhere (Luo et al., 2007).

The macromolecular structure of integrins is coupled to the accessibility of the ligand-binding pocket and to its ligand-binding affinity. Therefore, the regulation of integrin conformation is critical for their adhesive and signaling function (Luo et al., 2007). Studies employing crystallography (Shimaoka et al., 2003b), nuclear magnetic resonance (Huth et al., 2000; Legge et al., 2000), electron microscopy (Nishida et al., 2006), and molecular dynamics simulation (Jin et al., 2004) have suggested that LFA-1 can assume at least three distinct conformational states (**Figure 1**). In its inactive state, the LFA-1 extracellular domain has a bent structure shaped like an inverted V (Nishida et al., 2006) with the low affinity headpiece closely approaching the plasma membrane (Larson et al., 2005; Nishida et al., 2006), similar to what has been shown for other integrins (Xiong et al., 2001; Chigaev et al., 2003; Zhu et al., 2008; Xie et al., 2010). The inactive state is also characterized by inter-domain contacts between the N-terminal headpiece and membrane-proximal lower legs, between the lower legs of the α and β chains, and between the α and β transmembrane domains (TMDs; Takagi et al., 2002; Li et al., 2005; Luo et al., 2005; Partridge et al., 2005; Nishida et al., 2006). Based primarily on mutational studies, it is thought that close association of TMDs stabilizes the inactive state and disruption of this association leads to integrin activation (Hughes et al., 1996; Lu and Springer, 1997; Vinogradova et al., 2002; Luo et al., 2005).

In contrast to the compact structure of inactive LFA-1, active LFA-1 conformations exhibit an extended extracellular domain with the ligand-binding headpiece situated more than 20 nm above the membrane (Nishida et al., 2006). Extended structures of LFA-1 (**Figure 1**) differ in the conformation of the headpiece and αI domain, the angle between the βI domain and hybrid domain into which the βI is inserted, and the distance between the α_L and β_2 TMDs in the plane of the plasma membrane. The extended ectodomain with a closed headpiece has also been termed the "intermediate affinity state" integrin (Luo et al., 2007). It is thought that the spatial orientation of the headpiece alone in the extended intermediate affinity structure may account for enhanced recognition of ligands, as steric hinderance may prevent large, immobilized ligands from accessing the αI domain in the bent, inactive LFA-1 conformer. This idea is supported by data showing that the isolated LFA-1 αI domain alone, in its basal state, can mediate transient interactions with intercellular

BOX 1 | "Inside-out" and "outside-in" signaling.

Inside-out signaling refers to a process by which a cellular stimulus, for example, a chemokine binding to its receptor, leads to integrin activation by intracellular signaling pathways. Inside-out signaling leads to vast conformational changes in integrins, but not directly to clustering (redistribution of integrin heterodimers in the plane of the plasma membrane). By contrast, outside-in activation refers to integrin activation induced by ligand binding. Although the terminology is often confused, outside-in *signaling*, distinct from outside-in *activation*, refers to a process whereby the cell receives signals from the extracellular environment through integrins that have already been activated and are now bound to immobilized ligands on another cell or in the extracellular matrix. Adhesion strengthening, or stabilization, is a process following integrin activation and initial ligand binding. Adhesion strengthening most likely involves integrin clustering and is required to keep arrested leukocytes from being washed away.

BOX 2 | β_2 integrin family.

The β_2 subfamily of integrins comprises four heterodimers. $\alpha_L\beta_2$ (LFA-1, CD11a/CD18) is expressed on all leukocytes; $\alpha_M\beta_2$ (Mac-1, CD11b/CD18) is expressed on granulocytes, monocytes, macrophages, and subsets of activated lymphocytes; $\alpha_X\beta_2$ (P150,95, CD11c/CD18) is expressed on dendritic cells, macrophages, and small subsets of blood monocytes; and $\alpha_D\beta_2$ (CD11d/CD18) is expressed on tissue-specific subsets of macrophages.

adhesion molecule-1 (ICAM-1) that result in a rolling phenotype (Knorr and Dustin, 1997; Eniola et al., 2005), whereas the full-length LFA-1 molecule does not support interaction under flow conditions (Salas et al., 2002). However, in a series of disulfide-stabilized crystal structures of the α_L I domain, distinct conformations with low, intermediate, and high affinity were identified, where the transition from low to intermediate affinity was mainly driven by an increase in the bimolecular association rate (k_{on}) and the transition from intermediate to high affinity was mainly driven by lower k_{off} (slower release of bound ligand; Shimaoka et al., 2003b). Notably, the αI metal ion-dependent adhesion site (MIDAS) that recognizes ligand retains the same coordination and conformation in the putative αI low and intermediate affinity conformations. Therefore, in addition to the macromolecular integrin structures, it is apparent that the αI domain may exist in three distinct conformations regulated by the relative position of the α7 helix. There is ample evidence, as will be discussed below, that extended LFA-1 mediates neutrophil slow rolling by transiently binding to ICAM-1. During slow rolling, the α_L I domain is most likely not in the high affinity conformation.

In the "high affinity state" structure, swingout of the hybrid domain away from the α subunit by approximately 60° is coupled to opening of the headpiece through the downward movement of the αI domain α7 helix that connects to the βI domain as an internal ligand for the βI MIDAS (Alonso et al., 2002; Xiao et al., 2004; Luo et al., 2007). This shift in the α7 helix is coupled to rearrangement of the αI domain, enhancing its affinity for ligand (Alonso et al., 2002; Yang et al., 2004). It is the high affinity LFA-1 conformer that mediates arrest of leukocytes on the endothelium (Constantin et al., 2000; Giagulli et al., 2004), and *in vitro* studies demonstrate that the open headpiece of LFA-1 is necessary and sufficient to mediate cell arrest under flow conditions (Salas et al., 2002, 2004).

The structure and affinity of integrin receptors is thought to be regulated by cells in an "inside-out" manner [**Box 1**] through signaling cascades that impinge upon the integrin cytoplasmic tails (Dustin and Springer, 1989; O'Toole et al., 1990; Sims et al., 1991). Two intracellular protein families have been implicated in the final events of integrin activation. Talins and Kindlins both contain a

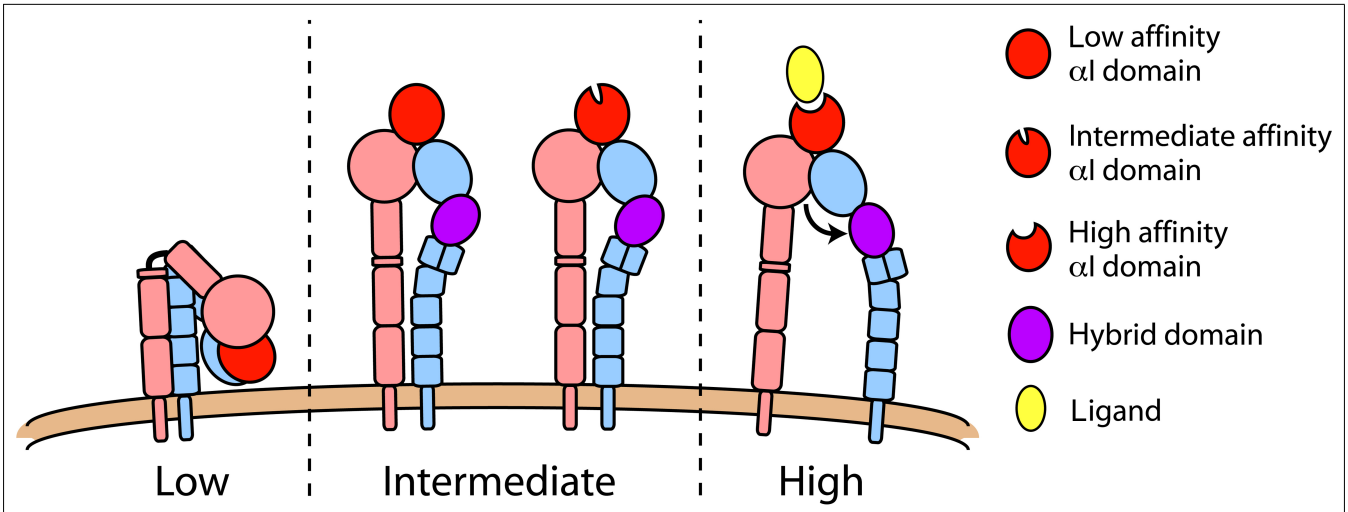

FIGURE 1 | LFA-1 conformations. Integrin function is structurally regulated and three distinct conformations have been demonstrated. In its low affinity state (left), LFA-1 is compact with a sharp bend at the "genu" and headpiece closely approaching the plasma membrane. Extended conformations (middle) differ in the orientation of their hybrid domain (purple), with swingout of this domain representing a conversion from intermediate to high affinity state (right). Data suggest that the extended/closed conformation (middle) can have either a low or intermediate affinity αI domain.

band 4.1/ezrin/radixin/moesin (FERM) domain with four independently folded subdomains (F0–F3; Moser et al., 2009b). While the FERM domain comprises the talin head that is sufficient for activating integrins (Calderwood et al., 1999; Ye et al., 2010), talins also have a flexible rod domain that contains actin-binding sequences and mediates cytoskeletal association (Critchley, 2009). Although many of the structural and functional studies that have dissected the role of talin in integrin activation have employed experimental systems where the talin head domain alone is used, the full-length molecule exists in a basal autoinhibited state that is mediated by contact between the integrin-binding F3 subdomain and rod domain (Goksoy et al., 2008). Binding to phospholipids, such as phosphatidylinositol 4,5-bisphosphate (PIP_2), relieves this autoinhibition and activates talin for integrin binding (Martel et al., 2001; Goksoy et al., 2008). The ability of talins and Kindlins to activate integrins depends on their binding to membrane phospholipids. For talin, this occurs through positively charged surfaces within the F1, F2, and F3 subdomains (Anthis et al., 2009; Goult et al., 2010). Kindlins have an analogous surface within the F1 subdomain (Bouaouina et al., 2012) as well as an additional pleckstrin homology domain that is inserted within its F2 subdomain (Tu et al., 2003; Qu et al., 2011), both of which bind to membrane phospholipids and are important for integrin activation.

Talins and Kindlins mediate integrin activation by binding directly to separate NXX(Y/F) motifs within the integrin β chain short cytoplasmic tail (Tadokoro et al., 2003; Harburger et al., 2009). Multiple lines of evidence demonstrate that talins contribute to the separation of the integrin TMDs (Kim et al., 2003; Wegener et al., 2007), but the mechanisms by which Kindlins are involved in regulating integrin conformation are largely unknown (Moser et al., 2009b; Shattil et al., 2010). Mutations of Kindlin-3, the Kindlin isoform expressed in hematopoietic cells, were found to underlie the leukocyte adhesion deficiency type III (LAD-III) pathology (Malinin et al., 2009; Moser et al., 2009a; Svensson et al., 2009) [**Box 3**]. Kindlin-3-deficient leukocytes are unable to arrest on inflamed endothelium, but their selectin-mediated rolling capacity is normal, indicating an important role for Kindlin-3 in LFA-1 activation (Moser et al., 2009a). The roles of talin-1 and Kindlin-3 in regulating LFA-1 in neutrophils are discussed in further detail below.

BOX 3 | Leukocyte adhesion deficiency type III.

In the human disease leukocyte adhesion deficiency type III (LAD-III), activation of β_2, β_3, and some β_1 integrins is defective. LAD-III was discovered in 1997 and initially called LAD-I variant (Kuijpers et al., 1997). Kuijpers and colleagues recognized that expression of β_2 integrins was normal but their activation was defective in these patients (Alon et al., 2003; Alon and Etzioni, 2003; Malinin et al., 2009; Manevich-Mendelson et al., 2009; Svensson et al., 2009; Jurk et al., 2010; McDowall et al., 2010; Robert et al., 2011). Alon and colleagues (Pasvolsky et al., 2007) reported a point mutation in *RASGRP2*, the gene encoding CalDAG-GEFI, and thought that defective CalDAG-GEFI caused LAD-III. This idea was supported by the leukocyte and platelet adhesion deficiencies exhibited by CalDAG-GEFI knockout mice (Bergmeier et al., 2007). However, it was later found that mutation of the *FERMT3* gene encoding Kindlin-3 that causes the pathology of LAD-III (Kuijpers et al., 2009; Svensson et al., 2009), dominates the phenotype of the subset of LAD-III patients with mutations in both *FERMT3* and *RASGRP2*. Humans deficient in CalDAG-GEFI, but not Kindlin-3, have not been described. There is now consensus that mutations in the *FERMT3* gene encoding kindlin-3 cause all known LAD-III cases (Abram and Lowell, 2009).

MODELS OF LFA-1 ACTIVATION

There are several models of integrin activation by inside-out signaling. The dynamic equilibrium that exists between the various integrin conformational states (**Figure 1**) appears to vary between the 24 different integrin subtypes and even amongst the four different β_2 integrins (Nishida et al., 2006). Therefore, aside from the most basic structural commonalities, integrin activation mechanisms may exhibit significant variability.

The switchblade model of integrin activation stems from the delineation of the three distinct affinity states described above, and was formulated based on crystallography (Xiong et al., 2001; Xiao et al., 2004) and studies of isolated integrins by electron microscopy (Takagi et al., 2002; Nishida et al., 2006). The switchblade model postulates that only the closed, unliganded integrin headpiece can stably exist in the overall bent structure. The switchblade-like extension of the extracellular domain occurs as a result of disruption of headpiece-lower leg and α/β lower leg stabilizing contacts, either by separation of the TMDs or by ligand-induced hybrid domain swingout (Luo et al., 2007). The switchblade model received support from the recent finding that single, full-length $\alpha_{IIb}\beta_3$ integrin embedded in membrane has the same compact structure observed in isolated extracellular domains and can undergo ectodomain extension in the presence of talin head domain bound to the integrin cytoplasmic tail (Ye et al., 2010). Most of the activated structures observed in this study, however, resembled the intermediate rather than the high affinity conformation. As discussed below, an important unresolved question is whether inside-out signaling alone (that is, in the absence of ligand binding) induces the high affinity structure with an open headpiece.

The deadbolt model of integrin activation was proposed (Xiong et al., 2003) based on the observation that crystals of the unbound (Xiong et al., 2001) and ligand-occupied (Xiong et al., 2002) integrin $\alpha_V\beta_3$ exhibited the same compact, bent structure. The crystal structure of ligand-occupied, bent $\alpha_V\beta_3$ was further supported by electron microscopy studies (Adair et al., 2005). In addition, FRET studies have shown that small ligand binding to $\alpha_4\beta_1$ integrin (VLA-4) on the living cell surface can occur in the absence of ectodomain extension (Chigaev et al., 2007). The deadbolt model posits that a loop within the β subunit extracellular membrane-proximal β-tail domain contacts the α7 helix of the βI domain that regulates its conformation and affinity (Xiong et al., 2003). During activation, movement of the TMDs would disrupt the deadbolt and allow headpiece opening in the bent structure (Xiong et al., 2003). However, elimination of the β-tail domain loop that was proposed to form the deadbolt did not enhance ligand binding (Zhu et al., 2007).

More recent work suggests that inside-out integrin activation is more complicated than the switchblade and deadbolt models

imply. For example, in kinetic studies of VLA-4 on live cells, binding of antibodies that recognize epitopes selectively exposed in extended integrin structures was minimal after triggering two different physiologic inside-out activation pathways, but was robust in the presence of a small ligand even without cell stimulation (Chigaev et al., 2009). The same group recently performed similar experiments with a fluorescent ligand probe of LFA-1 and found that small ligand binding to LFA-1 in the absence of inside-out activation was very slow (Chigaev et al., 2011). These findings indicate a greater restraint on inactive LFA-1 compared to VLA-4 with respect to ligand binding. Inside-out stimulation via chemokine or formyl peptide receptors [both G-protein coupled receptors (GPCRs)] rapidly enhanced LFA-1 small ligand binding, suggesting affinity upregulation of the headpiece. These studies suggest that mechanisms of integrin conformational regulation, including inside-out and ligand-induced outside-in activation, have both shared and distinct components between integrin subtypes and activating stimuli.

It is clear that inside-out signal transduction pathways, such as those elicited by chemokine receptor engagement, stimulate an increase in the affinity of integrins. The ability of LFA-1 to bind ICAM-1 in its soluble, rather than immobilized, form has been utilized as a sensitive assay that specifically reports the high affinity state (Constantin et al., 2000; Shimaoka et al., 2003a,b). Further support for this assay as a means to discriminate between extended/closed and extended/open conformations of LFA-1 was provided by Laudanna and colleagues (Bolomini-Vittori et al., 2009) by showing that a signaling defect in headpiece opening due to silencing of phosphatidylinositol-4-phosphate 5-kinase type I gamma (PIP5KC) blocks chemokine-induced soluble ICAM-1 binding to T lymphocytes despite the ability of LFA-1 to become extended (as indicated by a reporter mAb) under the same conditions. Many studies have thus demonstrated that chemokines induce a high affinity state of LFA-1 that is competent for soluble ICAM-1 binding and that mediates T lymphocyte arrest (Constantin et al., 2000; Giagulli et al., 2004; Bolomini-Vittori et al., 2009).

Despite these extensive studies, it is not clear whether chemokine stimulation alone, in the absence of ligand, triggers opening of the LFA-1 headpiece. Chemokines induce the binding of epitope-specific reporter antibodies, such as mAb 24 (Dransfield et al., 1992) and 327C mAb (Beals et al., 2001), that specifically recognize the extended/open high affinity conformation of LFA-1 (Bolomini-Vittori et al., 2009). However, these reporter antibodies also recognize ICAM-1-bound LFA-1 in the absence of cellular stimulation (Beals et al., 2001). Therefore, it remains possible that chemokines enhance the affinity of LFA-1 for ICAM-1 and permit a transition to the high affinity state, but do not directly induce opening of the headpiece associated with the high affinity αI domain. Rather, ligand engagement and a force pulling on the engaged integrin may subsequently be needed for LFA-1 to achieve its high affinity state. Affinity measurements of LFA-1 on the cell surface show that inside-out signaling in response to stromal cell-derived factor-1 (SDF-1, also known as chemokine CXCL12) or PMA, an activator of protein kinase C, enhanced binding of soluble monovalent ICAM-1, indicating affinity upregulation (Schurpf and Springer, 2011). However, artificially stabilizing specific LFA-1 structures resulted in ICAM-1 binding affinities of the intermediate affinity state that were similar to those achieved by inside-out activation, and of the high affinity state that were much higher. In a novel assay in which antibodies that specifically recognize extended LFA-1 conformers (KIM127 mAb and NKI-L16 mAb) or the putative high affinity state of LFA-1 (327C mAb) were co-immobilized with the chemokine CXCL12, it was found that peripheral blood lymphocytes bound only KIM127 and NKI-L16, but not 327C (Shamri et al., 2005). These data all suggest that chemokines trigger LFA-1 extension, but cannot induce the full affinity of the ligand-binding headpiece.

If inside-out activation does not shift integrin affinity to its highest state, how then does ligand binding do so? The demonstration of a catch bond between LFA-1 and ICAM-1 provides insight into a possible mechanism and also suggests an important role for force in affinity maturation of the αI domain (Chen et al., 2010). Using a biomembrane probe to ligate single LFA-1 molecules and measure bond kinetics in the absence or presence of a pulling force, Zhu and colleagues (Chen et al., 2010) demonstrate that ICAM-1-occupied LFA-1 passes through three distinct bond lifetime regimes with increasing force. Pulling on the bond first enhanced LFA-1 affinity (catch bond) and then, for larger forces, resulted in a decrease in bond lifetime (slip bond). They propose that pulling on the αI domain results in its movement away from the βI domain, causing the anchored (to the βI MIDAS) α7 helix to experience a relative shift downward (as discussed above) and resulting in affinity upregulation of the αI domain (Chen et al., 2010). Thus, in the context of physiologic leukocyte–endothelial interactions in which LFA-1 bonds experience a force from blood flow acting on rolling or adherent cells, chemokines may stimulate LFA-1 extension while enhancing affinity of the headpiece only moderately. Upon ICAM-1 engagement, the bond would then undergo further affinity maturation and transition into the high affinity conformation supporting long-lived bonds. This model of force-mediated LFA-1 affinity maturation also suggests a role for cytoskeletal anchorage of the integrin tail (Alon and Dustin, 2007; Zhu et al., 2008). Talin represents the best candidate for mediating such actin linkage through sequences in its rod domain (Critchley, 2009), though a specific role for talin-mediated actin filament association in LFA-1 activation is yet to be described. A potential role for Kindlins in mediating indirect cytoskeletal anchorage is also discussed below in further detail.

REGULATION OF LFA-1 ACTIVATION IN NEUTROPHILS

Neutrophils play a central role in immunity as the first leukocyte subset to enter tissues in response to infection or injury. Inflammatory cues produced locally in the tissue are relayed to neutrophils through the expression of adhesion molecules, such as E-selectin and ICAM-1, on the luminal surface of endothelial cells. This induced expression of adhesion molecules initiates a cascade of progressive interactions between leukocytes and the vascular wall that precede transmigration across the endothelial barrier and into the affected tissue (Ley et al., 2007).

Members of the β_2 integrin family [**Box 2**], whose expression is restricted to leukocytes and leukocyte-derived tissue cells, are

involved at several steps of the leukocyte adhesion cascade. Initial capture and rolling of neutrophils is mediated primarily by endothelial E- and P-selectins binding to P-selectin glycoprotein ligand-1 (PSGL-1) and other selectin ligands on the surface of neutrophils. In addition to its function as an adhesion receptor, PSGL-1 transduces an intracellular signal that partially activates LFA-1 (Zarbock et al., 2007b; Miner et al., 2008; Kuwano et al., 2010; **Figure 2**). Although other leukocyte subsets, including some memory T lymphocytes, express functional PSGL-1, it seems that ligation of PSGL-1 triggers LFA-1 extension only in myeloid cells. It may be that the lymphoid homologs of one or more molecules downstream of PSGL-1 do not participate in this signaling pathway (Alon and Ley, 2008). The molecular details of the signal transduction pathway triggered by PSGL-1 engagement and leading to LFA-1 activation in neutrophils has been the subject of recent reviews (Zarbock et al., 2009, 2011), and new details on this signaling cascade continue to emerge (Stadtmann et al., 2011; Block et al., 2012; Lefort et al., 2012; Shao et al., 2012; Spertini et al., 2012). During neutrophil rolling interactions with P- and E-selectin, PSGL-1 signaling results in the conversion of LFA-1 from an inactive state to an extended conformation that then interacts with ICAM-1 to reduce neutrophil rolling velocity (Chesnutt et al., 2006; Zarbock et al., 2007b; Kuwano et al., 2010). Two main pieces of evidence demonstrate that the extended LFA-1 conformer with a closed headpiece mediates slow rolling in both murine and human neutrophils. First, an LFA-1 allosteric antagonist that binds to the βI domain and blocks structural communication with the αI domain, thus preventing headpiece opening, completely abrogates soluble ICAM-1 binding (Shimaoka et al., 2003a) and neutrophil arrest, but does not impair LFA-1-dependent slow rolling (Zarbock et al., 2007b). Second, the KIM127 mAb (Robinson et al., 1992) and NKI-L16 mAb (van Kooyk et al., 1991) epitopes that report extension of the β_2 and α_L legs, respectively, are exposed in human neutrophils rolling on E-selectin in flow chambers (Kuwano et al., 2010). In the same assay, the mAb 24 (Dransfield et al., 1992) recognition site that is only accessible in the high affinity state remains buried unless neutrophils are activated by Mn^{2+} (Kuwano et al., 2010). Thus, ligation of PSGL-1 induces LFA-1 extension, but not (full) opening of the headpiece.

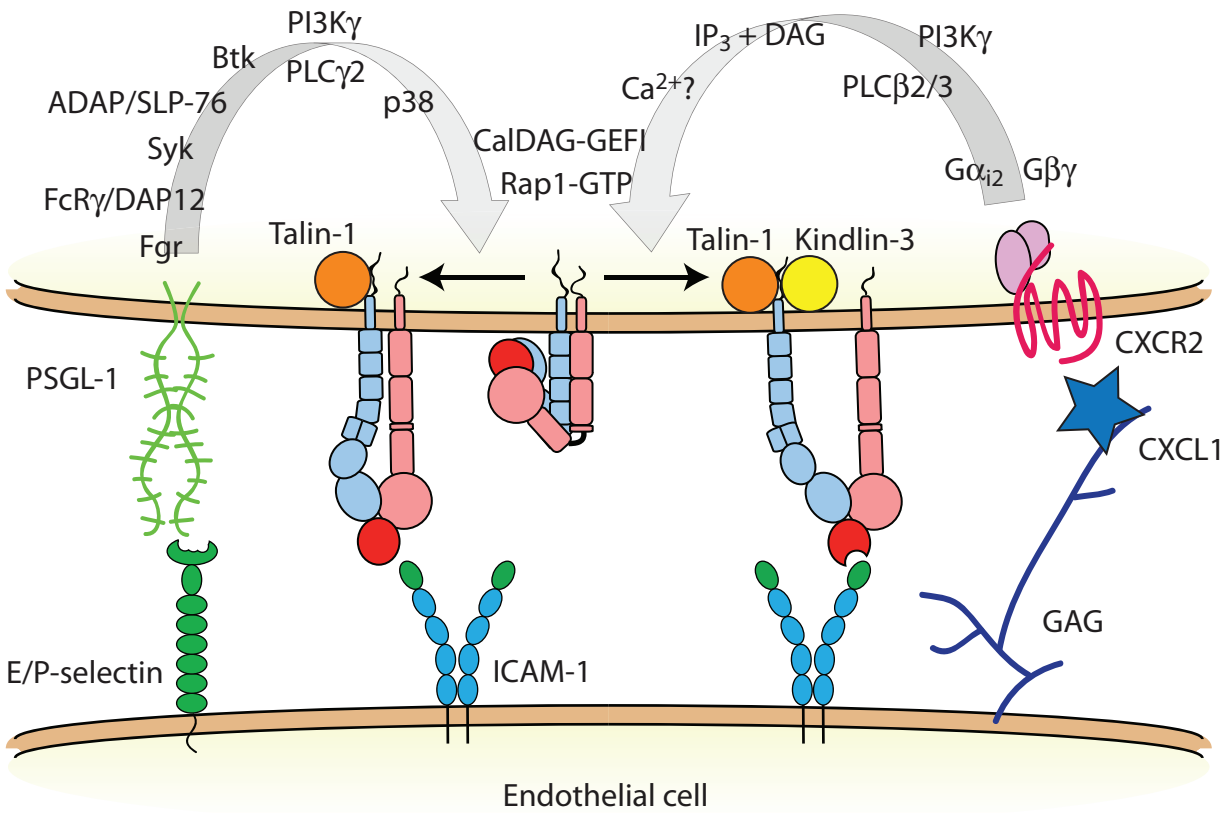

FIGURE 2 | Neutrophil inside-out activation of LFA-1. Two distinct signal transduction pathways modulate LFA-1 activation in neutrophils: (1) selectin binding to PSGL-1, and (2) chemokine receptor engagement. PSGL-1 signals talin-1-dependent LFA-1 extension, whereas chemokine GPCRs signal LFA-1 activation to a high affinity state that requires both talin-1 and Kindlin-3. Some of these signaling molecules may exist in preformed modules to facilitate rapid integrin activation. Abbreviations: Fgr, feline Gardner–Rasheed sarcoma kinase; FcRγ, immunoglobulin gamma Fc receptor; DAP12, DNAX activating protein of 12 kDa; Syk, spleen tyrosine kinase; ADAP, adhesion and degranulation promoting adaptor protein; SLP-76, SH2 domain-containing leukocyte phosphoprotein of 76 kDa; Btk, Bruton tyrosine kinase; PI3K, phosphoinositide 3-kinase; PLC, phospholipase C; p38, p38 mitogen activated protein kinase; IP_3, inositol trisphosphate; DAG, diacylglycerol; CalDAG-GEFI, calcium- and diacylglycerol-regulated guanine nucleotide exchange factor I; PSGL-1, P-selectin glycoprotein ligand-1; ICAM-1, intercellular adhesion molecule-1; GAG, glycosaminoglycan.

During inflammation, neutrophils encounter immobilized chemokines as they roll on the vascular wall. LFA-1 is the primary integrin receptor involved in leukocyte arrest on inflamed endothelium, with little or no detectable contribution of Mac-1 (Ding et al., 1999; Ley et al., 2007). SDF-1α (a ligand for CXCR4) and chemokine ligands for CXCR2 trigger soluble ICAM-1 binding and rapid lymphocyte arrest on immobilized ICAM-1 (Constantin et al., 2000; Giagulli et al., 2004; Shamri et al., 2005; Bolomini-Vittori et al., 2009). This has been interpreted to mean that high affinity LFA-1 is required to mediate leukocyte arrest. Intravital microscopy analysis of the mouse cremaster muscle, a thin tissue that envelops the testes, after intravenous injection of CXCL1 provides a sensitive arrest assay, as neutrophils rapidly (within 15 s) transition from rolling to arrest on the endothelium. This assay provides the ability to distinguish the arrest step from adhesion stabilization, as neutrophils lacking signaling molecules involved in the latter process, such as PI3Kγ (Smith et al., 2006) and PKCθ (Bertram et al., 2012), quickly detach and return to the bulk flow. Since chemokine signaling also contributes to the stabilization and strengthening of leukocyte adhesion following arrest by enhancing LFA-1 mobility and allowing clustering to occur (Constantin et al., 2000; Giagulli et al., 2004, 2006), the analysis of rapid arrest *in vivo* is critical for assessing the functional role of molecules in inside-out activation of LFA-1. CXCL1 (also known as keratinocyte-derived chemokine) is the primary chemokine involved in murine neutrophil arrest through CXCR2 and activation of the $G\alpha_{i2}$ signaling cascade (Ley, 2003; Smith et al., 2004; Zarbock et al., 2007a). In the context of inflammation, both the PSGL-1 and CXCR2 signaling pathways that regulate LFA-1 activation contribute to neutrophil adhesion and recruitment (Smith et al., 2004; Zarbock et al., 2007b). PSGL-1 signaling seems to induce only the extended conformation of LFA-1, and even pulling on LFA-1 as it transiently engages with ICAM-1 in slow rolling is not sufficient to induce rapid neutrophil arrest mediated by the high affinity conformation. By contrast, in the presence of chemokines, neutrophil LFA-1 reaches its high affinity state and mediates arrest. Another potential difference between these two signaling pathways is that chemokine receptors trigger rapid and locally restricted LFA-1 activation (Shamri et al., 2005), whereas PSGL-1 signals to LFA-1 may not be as spatially confined (Alon and Ley, 2008; Kuwano et al., 2010). It has been proposed that the downstream signaling proteins involved in integrin activation may be preassembled into complexes, thus allowing for leukocyte arrest to occur on the timescales observed experimentally (Alon and Ley, 2008). Consistent with this idea, a signaling module containing SKAP55 (Src kinase-associated phosphoprotein of 55 kDa), ADAP (adhesion and degranulation promoting adaptor protein), RIAM (Rap1-interacting adaptor molecule), and Kindlin-3 exists constitutively in unstimulated human T lymphocytes, and inducibly associates with the common integrin activator Rap1 GTPase (Kliche et al., 2012).

NEW MECHANISTIC INSIGHTS INTO INSIDE-OUT LFA-1 ACTIVATION

LFA-1 has been a model for studying integrin activation for more than 20 years. The role of inside-out signaling in regulating LFA-1 structure and affinity is a topic of great interest not only in the fields of immunology and leukocyte adhesion, but also among integrin biologists. Until recently, the roles of two families of common integrin co-activators, talins and Kindlins, in regulating LFA-1 conformational activation had not been studied. Using mixed chimeric mice with genetic deletion of either *Fermt3* encoding Kindlin-3 or *Tln1* encoding talin-1, we tested the individual roles of these two proteins in neutrophil slow rolling and arrest mediated by LFA-1 (Lefort et al., 2012). We reasoned that since different conformations of LFA-1 are induced by stimulating either the PSGL-1 or CXCR2 signaling cascades, it would be possible to separately test whether talin-1 and Kindlin-3 were involved in LFA-1 extension versus headpiece opening. We observed that LFA-1-dependent neutrophil slow rolling on E-selectin/ICAM-1 substrates in flow chambers and on inflamed endothelium *in vivo* was impaired in talin-1-deficient neutrophils, but was unaffected by knockout of Kindlin-3. In contrast, both talin-1 and Kindlin-3 were required for CXCL1-stimulated rapid neutrophil arrest and soluble ICAM-1 binding to LFA-1, indicating an important role for these co-activators in reaching the high affinity state of LFA-1. Our findings in neutrophils were corroborated by studies measuring LFA-1 affinity states using the reporter antibodies KIM127 (Robinson et al., 1992), NKI-L16 (van Kooyk et al., 1991), mAb 24 (Dransfield et al., 1992), and 2E8 (Carreno et al., 2010) in human HL-60 cells stimulated with an active Rap1a peptide. Together, these results show that talin-1 is needed for LFA-1 extension, while Kindlin-3 is involved in transition of the LFA-1 headpiece to its high affinity state. These data are the first to demonstrate distinct functions of talin-1 and Kindlin-3 in inducing specific conformers of LFA-1. It remains to be shown whether talin-1 plays an additional role in LFA-1 headpiece opening to reach the high affinity state, or whether LFA-1 extension is a prerequisite for headpiece opening in the context of chemokine-induced neutrophil arrest. Furthermore, previous studies of cultured effector T lymphocytes derived from LAD-III patients suggest that Kindlin-3 is needed for LFA-1 extension in the context of chemokine stimulation (Manevich-Mendelson et al., 2009). It is apparent that there are diverse mechanisms among the various modalities of integrin activation in leukocytes.

What do these new insights into talin-1 and Kindlin-3 regulation of LFA-1 structure tell us about the mechanisms of inside-out activation? It was not surprising to find that talin-1 is needed for LFA-1 extension. As shown for $\alpha_{IIb}\beta_3$ in a reconstituted system, the talin head domain (but not intact, autoinhibited talin) is sufficient to induce extension of the integrin extracellular domain (Ye et al., 2010). Several studies have shown that talin directly disrupts the basal association of integrin TMDs by contacting the salt bridge between the α and β chains close to the inner membrane leaflet (Wegener et al., 2007; Anthis et al., 2009). Talin is likely involved in further rearrangement of the TMDs, as mutations that disrupt the α/β salt bridge do not fully overcome the requirement for talin in integrin activation (Tadokoro et al., 2003; Wegener et al., 2007). That Kindlin-3 was not required for neutrophil slow rolling mediated by the extended/closed conformer of LFA-1 (Lefort et al., 2012) suggests that it may not be involved in initial disruption of the LFA-1 TMDs.

However, structural studies of LFA-1 suggest that the TMDs and lower legs are separated even further in high affinity than in intermediate affinity LFA-1 (Nishida et al., 2006). Thus, Kindlin-3 may be involved in inducing swingout of the hybrid domain by causing a further separation of the TMDs. It is also possible that Kindlin-3 mediates the indirect linkage of the β_2 integrin tail to the actin cytoskeleton through a scaffolding function, thus providing an anchor for force-induced conversion of ligated integrins to the high affinity state. It was recently found that LFA-1-mediated neutrophil slow rolling is not dependent on the linkage of LFA-1 to the actin cytoskeleton (Shao et al., 2012), but chemokine-stimulated arrest of lymphocytes (Shamri et al., 2005) and neutrophils (Shao et al., 2012) is impaired by reagents that disrupt actin microfilaments. These studies support the idea that LFA-1 extension and headpiece opening are distinctly regulated processes, and that Kindlin-3 may be involved in the transition to a high affinity state through an indirect interaction with the actin cytoskeleton. Kindlins have been shown to bind to several actin-binding proteins and complexes, including integrin-linked kinase (Mackinnon et al., 2002), migfilin (Tu et al., 2003), focal adhesion kinase, and α-actinin (Has et al., 2009). The expression and role of these adaptor molecules in LFA-1 activation will need to be tested in leukocyte arrest under flow conditions.

The distinct roles of talin-1 and Kindlin-3 in neutrophil slow rolling and arrest raise several questions about the two signaling pathways that stimulate these separate behaviors. Clearly, LFA-1 engages ICAM-1 during slow rolling interactions induced by PSGL-1 signaling. If chemokines directly stimulate only LFA-1 extension and not conversion to the high affinity state, why does CXCR2 engagement lead to neutrophil behavior qualitatively distinct from the PSGL-1 pathway that has also been shown to induce LFA-1 extension? Likewise, if force on the LFA-1/ICAM-1 bond results in affinity maturation to the long-lived state, how are neutrophils able to use LFA-1 for rolling interactions that require a transient bond with a fast off-rate? Structural communication between the βI and αI domains through the α7 helix is needed for headpiece opening (Shimaoka et al., 2003a). Perhaps Kindlin-3 provides a permissive signal or induces a structural rearrangement that allows coupling of the βI and αI domains, such as swingout of the hybrid domain. We speculate that Kindlin-3 may be actively excluded from binding to the β_2 cytoplasmic tail after inside-out activation of LFA-1 by the PSGL-1 signaling pathway and this prevents the transition to the open headpiece and neutrophil arrest. This could occur by promoting the binding of a competing molecule to the Kindlin-3 binding NPKF site on the β_2 tail, or by regulating phosphorylation of threonine residues that also contribute to Kindlin-3 binding (Ma et al., 2008). Indeed, T cell receptor signaling affects the phosphorylation of β_2T758 and promotes binding of 14-3-3 proteins and LFA-1 activation (Fagerholm et al., 2005; Gronholm et al., 2011). Whether this is also true for chemokine signaling is unknown.

A potential alternative mechanism for LFA-1 extension resulting in distinct slow rolling and arrest behaviors after PSGL-1 and CXCR2 signaling, respectively, may be that chemokines also stimulate an increase in LFA-1 mobility in the membrane (Constantin et al., 2000) that could lead to ligand-driven LFA-1 clustering and subsequent firm adhesion. This mechanism would require that LFA-1 ligands like ICAM-1 are pre-clustered on endothelial cells (Barreiro et al., 2008), because integrin clustering is most likely a post-ligand binding event (Kim et al., 2004). In the case of slow rolling, if LFA-1 is immobile following PSGL-1 engagement then clustering may be disfavored and the force on individual LFA-1/ICAM-1 bonds could be sufficiently high so that LFA-1 resides in the slip bond regime with short enough lifetimes (high k_{off}) to support rolling behavior. Clearly, much remains to be tested to uncover the mechanisms regulating LFA-1 structure and affinity on the surface of leukocytes.

CONCLUDING REMARKS

LFA-1 plays important roles in the trafficking of multiple leukocyte subsets during the immune response. At least two distinct signaling pathways impact the adhesive function of LFA-1 by altering its structure, and thus affinity for extracellular ligands. Post-ligand binding LFA-1 clustering alters its distribution on the plasma membrane. Our understanding of how cellular factors regulate LFA-1 conformation by binding to its short cytoplasmic tails has evolved as Kindlin-3 and talin-1 have been identified as major and distinct players. The concepts explaining how structural rearrangements are propagated through the TMDs and integrin legs to the ligand-binding headpiece are being refined. It has recently become clear that force plays an integral role in these processes, and future work will be aimed at determining the molecular requirements and mechanisms of LFA-1 affinity regulation by force in rolling and arresting leukocytes.

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Aspects of VLA-4 and LFA-1 regulation that may contribute to rolling and firm adhesion

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Very Late Antigen-4 (CD49d/CD29, alpha4 beta1) and Lymphocyte Function-associated Antigen-1 (CD11a/CD18, alphaL beta2) integrins are representatives of a large family of adhesion receptors widely expressed on immune cells. They participate in cell recruitment to sites of inflammation, as well as multiple immune cell interactions. A unique feature of integrins is that integrin-dependent cell adhesion can be rapidly and reversibly modulated in response to cell signaling, because of a series of conformational changes within the molecule, which include changes in the affinity of the ligand binding pocket, molecular extension (unbending) and others. Here, we provide a concise comparative analysis of the conformational regulation of the two integrins with specific attention to the physiological differences between these molecules. We focus on recent data obtained using a novel technology, based on small fluorescent ligand-mimicking probes for the detection of integrin conformation in real-time on live cells at natural receptor abundance.

Keywords: integrins, VLA-4, LFA-1, conformation, affinity, cell adhesion, rolling, tethering

INTRODUCTION

Integrins are a large family of adhesion receptors widely expressed on different cell types that participate in cell-matrix, or cell-cell interactions. These receptors can transmit signals in two directions. From the outside of a cell, ligation of the integrins results in the activation of a number of signaling pathways. Integrins can also serve as mechanosensors probing mechanical properties of the extracellular environment (Hogg et al., 2011). Ligation of other receptors, including different G-protein coupled receptors, cytokine, and chemokine receptors, Fc-receptors and others, can lead to the propagation of an inside-out signal toward the integrin (Hogg et al., 2011). This can result in a series of conformational changes within integrin molecules leading to a rapid increase or decrease of the integrin ligand binding affinity, molecular extension (unbending), movement of integrin domains (such as hybrid domain swing-out), and changes in integrin lateral mobility. These events directly modulate cell adhesion behavior (Askari et al., 2009).

In the peripheral blood, the majority of leukocytes exhibit a non-adhesive phenotype in which cells move freely with flowing blood. On these cells, integrins usually exist in a resting (inactive) non-adhesive state. On encountering soluble or immobilized ligands cellular behavior can be rapidly altered. Cells may roll on endothelial cells, arrest and firmly adhere, and transmigrate, leaving the blood vessel and crossing the endothelial barrier. Surprisingly, a number of steps in the cell adhesion cascade can be mediated by the same integrin molecule, existing in different conformational states that can be rapid and reversibly regulated through cellular signaling. Here we discuss recent insights into integrin conformational regulation. We will focus on two major leukocyte integrins (CD49d/CD29, Very Late Antigen-4, alpha4 beta1 integrin), and (CD11a/CD18, Lymphocyte Function-associated Antigen-1, alphaL beta2 integrin).

STRUCTURAL AND FUNCTIONAL DIFFERENCES BETWEEN THE TWO INTEGRINS

In the membranes of cells, integrins exist as heterodimers composed of one alpha and one beta subunit. In humans, 18 alpha and 8 beta subunits have been identified that combine to form at least 24 different heterodimers (Huhtala et al., 2005). An important feature of integrins is the presence of the so-called "inserted domain, or I-domain," homologous to the von Willebrand factor A domain (vWFA). It can be found in every beta- (I-like domain), but only several alpha-subunits. This domain directly participates in the binding of the integrin ligands. Because of its homology to the vWFA alpha I-domains several groups prefer the term A-domain.

The alpha subunit with an inserted I-domain represents a late evolutionary acquisition. Even though teleost fish and several tunicata genomes contain integrin alpha subunits that have the inserted alpha I-domain, the leukocyte-specific integrin subunit orthologs, which include alpha D, alpha M, alpha X, and

alpha L, are absent. Moreover, the beta2 integrin subunit that is known to form a dimer with each of these alpha subunits was also not found (Huhtala et al., 2005). Thus, it appears that the development of leukocytes, bearing diverse immune functions (as found in vertebrates), requires a set of leukocyte-specific integrin subunits. What would be the major physiological advantage to have these integrins? To answer this question we have to compare what is known about the physiological differences between alpha I-domain containing integrins (such as LFA-1) and leukocyte integrins lacking alpha I-domain, such as VLA-4.

According to the UniGene EST profile the overall expression patterns of integrin alpha 4 subunit (ITGA4) and integrin alpha L subunit (ITGAL) are very similar. These integrins are expressed in tissues associated with blood and lymphatic tissues. Blood, bone marrow, lymph, lymph nodes, spleen, and thymus are primary sites of expression. However, one major difference is that while LFA-1 expression is usually attributed to mature leukocytes, VLA-4 integrin is strongly expressed on CD34+ early hematopoietic stem progenitor cells (HSPCs). VLA-4 expression is critical for homing and retention of HSPCs, since blocking VLA-4-specific interactions using mAbs or small molecule antagonists is sufficient to induce cell mobilization into peripheral blood (Coulombel et al., 1997; Oostendorp and Dormer, 1997; Gazitt, 2004; Chigaev et al., 2011d). This observation is also confirmed by the fact that the expression of VLA-4 is more pronounced in "germ cell tumors." (Compare the VLA-4 and LFA-1 UniGene EST profiles at http://www.ncbi.nlm.nih.gov/unigene/).

Two major integrin functions, related to cell adhesion, are usually assigned to VLA-4 and LFA-1. First, these integrins directly participate in cell arrest under flow, where firm adhesion is mediated by activated (high-affinity, unbent) integrins. Second, VLA-4 and LFA-1 contribute to cell-cell interactions that are critical for immune system responses. For example, both integrins play a part in the formation of immunological synapse, and participate in cell co-stimulation. There are also a number of differences between the two integrins related to both of these two functions. We postulate that these differences can be related to distinct structural and functional characteristics of these molecules, and thus, can provide a clue to the mystery of leukocyte-specific alpha I-domain-containing integrins.

Integrins are thought to be firm adhesion receptors. Historically, LFA-1 was one of the first integrins for which firm cell adhesion on activated cells was described. LFA-1 was unable to sustain cell rolling, and therefore, a selectin-mediated rolling step was envisioned to be necessary. However, under specific conditions that include LFA-1 mutagenesis, truncation, or treatment with allosteric antagonist rolling on LFA-1 can be observed (**Table 1**). This led to a multi-step recruitment paradigm, where cells will first roll on selectins, and after encountering activating

Table 1 | Examples of different functional roles of LFA-1 and VLA-4-dependent adhesive interaction.

Function	LFA-1/ICAM-1 interaction	VLA-4/VCAM-1 interaction
Tethering or rolling under shear flow	No tethering or rolling under shear flow; requires selectin-mediated rolling (Lawrence and Springer, 1991; von Andrian et al., 1991). No contribution to lymphocyte rolling on high endothelial venules (Warnock et al., 1998). Rolling can be artificially induced when mutated I domains or isolated wild type I domains are used (Salas et al., 2002). Small molecule allosteric antagonist XVA143 stimulates rolling on ICAM-1 (Salas et al., 2004).	Shown to mediate tethering or rolling (Alon et al., 1995; Berlin et al., 1995). Integrin activation is not required for tethering or rolling (Alon et al., 1995).
Engraftment of non-obese/severe combined immunodeficiency mice by human stem cells	Treatment with anti-LFA-1 antibodies caused partial inhibition of engraftment (by ∼20%) (Peled et al., 2000).	Treatment with anti-VLA-4 antibodies prevented engraftment (Peled et al., 2000).
NK cell and NKT cell recruitment to bone marrow	LFA-1 does not participate (Franitza et al., 2004).	VLA-4 is critical for recruitment (Franitza et al., 2004).
Recruitment of cells to lungs during *Streptococus pneumoniae* infection	No LFA-1 contribution found (Kadioglu et al., 2011).	T cell recruitment solely dependent on VLA-4; neutrophil recruitment depends also on Mac-1 (Kadioglu et al., 2011).
Phagosome maturation in macrophages	Search for "LFA-1 AND phagosome maturation" in PubMed database returned no items.	VLA-4 (and VLA-5) are critical for phagosome maturation. Integrin-deficient macrophages have impaired bactericidal activity (Wang et al., 2008).
T-B-cell interactions *in vivo*	LFA-1/ICAM-1 interactions are critical for polyclonal B-cell activation in host-versus-graft model (Lopez-Hoyos et al., 1999).	Blocking of VLA-4 had no effect (Lopez-Hoyos et al., 1999).
Immunological synapse	LFA-1-dependent interaction represent an important part of immunological synapse, playing a role in adaptive immune responses (Dustin, 2008; Springer and Dustin, 2012).	Only a few papers describe involvement of VLA-4 in immunological synapse formation and signaling (Burkhardt, 2008; Carrasco and Batista, 2006).

Publications where similar integrin functions were reported are not included as the authors intentionally focused on functional differences between the two integrins.

stimuli they will arrest, and transmigrate (von Andrian et al., 1991; Springer, 1994). The discovery that in addition to firm adhesion VLA-4 can mediate cell tethering and rolling (Alon et al., 1995) represents the first indication of a functional difference between VLA-4 and LFA-1. More detailed analysis revealed that VLA-4 supports a number of adhesive interactions that are directly related but not limited to the maintenance of immune cells through hematopoiesis (Imai et al., 2010), as well as intrinsic immune responses. Thus, VLA-4 participates in chemokine-dependent cell arrest on endothelium, NK, and MKT cell recruitment to bone marrow, cell recruitment in response to bacterial infections, bacterial killing, etc. In contrast, LFA-1-dependent cell adhesion is critical for modulating adaptive immune responses that include T-B-cell interaction, Antigen Presenting Cell (APC)-T-cell interaction, regulation of TCR signaling, host-versus-graft reaction, binding, etc. (**Table 1**).

Thus, another functional difference between the two integrins is related to their role in the immune system. It appears that VLA-4, representing an ancient integrin family expressed on leukocytes, is predominantly related to certain types of innate antigen-independent non-specific immune responses, where no significant role for LFA-1 is shown. LFA-1 is predominantly related to the signaling pathways, where antigen-dependent adaptive immunity plays a critical role (**Table 1**).

Furthermore, the appearance of leukocyte-specific alpha I-domain-containing integrins during evolution coincides with the emergence of the BCR-TCR-MHC-based adaptive immune system. The whole genome duplication that occurred at the dawn of jawed vertebrate evolution provides a mechanism for the emergence of novel genes that included a set of leukocyte-specific alpha and beta subunits. The "big bang" that created vertebrate adaptive immune system could be responsible for leukocyte integrin evolution as well (Flajnik and Kasahara, 2010). Thus, it is not surprising that leukocyte-specific alpha I-domain-containing integrins, such as LFA-1, are functionally linked to the adaptive immune system and BCR/TCR/MHC-related signaling pathways.

FLUORESCENT PROBES AS TOOLS FOR INTEGRIN STUDIES

We postulated that the physiological difference in the integrin function could be directly related to a primary integrin function: binding of the integrin ligand under different signaling conditions. To study the real-time regulation of integrin affinity and conformation, we developed a set of small fluorescent probes. [For VLA-4 see (Chigaev et al., 2001, 2003b, 2004) and for LFA-1 see (Chigaev et al., 2011b)]. These molecules were designed using small molecule integrin antagonists, developed by pharmaceutical companies, and have been shown to bind to the natural ligand binding sites. Therefore, these molecules mimic the binding of a natural ligand (Chigaev et al., 2003b; Zwartz et al., 2004), and because of an intrinsically higher affinity and commercial availability, these probes can be used in homogeneous assays to study rapid integrin conformational changes on live cell and in real-time (Chigaev et al., 2003a,b; Chigaev and Sklar, 2012). For example, for the detection of a real-time affinity change, the experimental concentration of the probe is required to be below the dissociation constant (K_d) for its binding to the resting integrin, and above the K_d for the physiologically activated integrin. Therefore, the transition from the low affinity to the high affinity state after "inside-out" activation through a G-protein coupled receptor, leads to an increase in the binding of the probe that can be detected using a conventional flow cytometer. Physiological signaling pathways involving cAMP and cGMP that lead to integrin deactivation result in rapid probe dissociation (Chigaev et al., 2001, 2008, 2011a,b). Moreover, different integrin affinities can be detected through analysis of ligand dissociation rates. Slower rates correspond to states of higher affinity (Chigaev et al., 2003b).

Vertical extension of the VLA-4 integrin molecule can be detected using a FRET-based approach, where a fluorescent probe bound to the integrin headgroup serves as the donor, and octadecyl rhodamine B incorporated into the cell membrane, serves as the acceptor (Chigaev et al., 2003a, 2007, 2008). Using these and other approaches (Chigaev et al., 2009) which depend upon the ability of the flow cytometer to discriminate fluorescent signals from a cell and the volume around it, a complex picture of conformational regulation of integrin has emerged.

INTEGRIN CONFORMATION IN THE REGULATION OF INTEGRIN DEPENDENT CELL ADHESION

Integrins can exist in multiple conformational states. For LFA-1, at least three states that differ in ligand binding affinity (low, intermediate, and high affinity) have been reported. Moreover, application of an external force can lead to the stabilization of ligand binding [or "catch bond" (Kong et al., 2009)], while lateral shear force can significantly modify the adhesive properties of LFA-1 (Hogg et al., 2011). For VLA-4, the discovery of several distinct signaling mechanisms that can independently regulate the affinity of the ligand-binding pocket and molecular unbending (or extension, detected using FRET-based approaches), was an early indication of the conformational complexity of this non I-domain-containing integrin (Chigaev et al., 2007). Next, it was found that after inside-out activation through wild type Gαi-coupled GPCRs, ligand binding affinity and molecular extension exhibited distinctly different time courses (Chigaev et al., 2007). In contrast, the fact that VLA-4 deactivation through Gαs-coupled GPCRs only affected the affinity of the integrin ligand binding pocket (Chigaev et al., 2008), provided a plausible explanation for the differences in cell adhesion at rest and after cAMP-dependent integrin deactivation [see Figure 7A in Chigaev et al. (2008)]. The use of conformationally sensitive antibodies in real-time on living cells allowed us to answer several questions regarding the role of the hybrid domain movement during inside-out activation and ligand engagement as described below (Chigaev et al., 2009; Njus et al., 2009). Using this information we can reconstruct a model of integrin conformational states for a non I-domain containing integrin (VLA-4).

HYBRID DOMAIN

On resting cells, in the absence of ligand, VLA-4 exhibits a low affinity, bent conformation with a hidden hybrid domain epitope (based on the lack of HUTS mAb binding). Although, the approach for assessing VLA-4 extension is based on FRET between the fluorescent ligand bound to the integrin headgroup and a membrane bound fluorescent acceptor, the observation

that inside-out activation rapidly induces FRET signal unquenching suggests that at rest the VLA-4 headgroup is closer to the membrane. The inside-out activation through a Gαi-coupled GPCR in the absence of a ligand has only a small effect on hybrid domain movement. In this case, the short-term exposure of the HUTS epitope was maximal during the first 30 s after GPCR signal, and it was undetectable 4 min after activation based on the rate of HUTS Mab binding (Chigaev et al., 2009). Under similar conditions, the high affinity state of the VLA-4 ligand binding pocket was sustained for more than 15 min, in the presence of a non-desensitizing mutant of the GPCR (Prossnitz, 1997; Chigaev et al., 2007, 2011a). Thus, at least for VLA-4, no direct connection between exposure of the hybrid domain epitope [and an outward swing of a beta-1 subunit hybrid domain (Mould et al., 2003; Mould and Humphries, 2004)] with the high affinity activated state can be established.

Multiple VLA-4-specific compounds, with binding affinities spanning more than three orders of magnitude, were all capable of inducing exposure of the hybrid domain epitope (Njus et al., 2009). Moreover, a quantitative analysis of the fractional occupancy of the VLA-4 ligand binding pocket revealed that EC_{50}s for the ligand-induced epitope exposure were virtually identical to the K_is determined in the competition assay with the fluorescent VLA-4 specific ligand. This suggests that occupancy of the VLA-4 ligand binding pocket by a direct (competitive) VLA-4 ligand is directly translated into HUTS epitope exposure, which can be also detected in real-time (Chigaev et al., 2009; Njus et al., 2009). This approach was recently adapted for the discovery of VLA-4 allosteric antagonists (see below) (Chigaev et al., 2011c,d).

EXTENSION AND AFFINITY

The inside-out activation through Gαi-coupled GPCRs induced rapid VLA-4 extension that can be detected using a FRET-based approach. In the presence of the ligand this created an extended conformation with an exposed hybrid domain epitope. However, in the case of wild type GPCRs the high affinity state existed only for a few minutes. After that, the binding affinity rapidly returned to a resting low affinity state. On the contrary, VLA-4 molecular extension detected using FRET persisted for several tens of minutes (Chigaev et al., 2007). This created a sustained low affinity extended state that was similar to the state induced by Gαs-GPCR induced deactivation (Chigaev et al., 2008) or the nitric oxide (NO) and cGMP-dependent signaling (Chigaev et al., 2011a). We envision that this state could sustain cell rolling. Significant similarity between cAMP and cGMP-dependent signaling mechanisms, together with a specific role of cAMP-dependent guanine-nucleotide-exchange factors for small GTPases (Rap1 and Rap2), which are implicated in integrin-mediated cell adhesion (Bos, 2006), suggest that cyclic nucleotides may represent a universal, and previously underestimated mechanism of integrin regulation.

Another VLA-4 state was observed after cell treatment with phorbol ester. Phorbol 12-myristate 13-acetate rapidly elevated VLA-4 affinity in a dose dependent manner. However, it failed to stimulate any extension of the molecule as detected using FRET. Moreover, the addition of calcium ionophore fully restored VLA-4 extension (Chigaev et al., 2007). This led us to hypothesize that cytoplasmic Ca^{2+} elevation is obligatory for molecular unbending, in contrast to the diacylglycerol-dependent step, which regulates the affinity of the ligand binding pocket. The recent finding that two adaptor proteins (talin-1 and kindlin-3) can independently regulate LFA-1 integrin extension and ligand binding affinity (Lefort et al., 2012) provides additional support for our current model (Chigaev et al., 2007).

INTEGRIN CONFORMATION AND CELL ADHESION

How are multiple VLA-4 conformations translated into cell adhesive properties? To address this question, see the data summarized in **Table 2**. Two different approaches to study cellular behavior for differing VLA-4 conformations were used: (1) real-time cellular aggregation in solution in a VLA-4/VCAM-1 dependent cell adhesion model system (Zwartz et al., 2004), and (2) cell rolling in a parallel plate on low density recombinant human VCAM-1 (DiVietro et al., 2007).

The real-time analysis of cell aggregation in solution showed a strong correlation between the initial rate of aggregation and

Table 2 | Behavior of VLA-4 conformational states using several approaches.

Cell treatment, activation (pathway)	Small fluorescent ligand binding (LDV-FITC)[a]			Extension of the molecule (FRET-based assay)[b]	Real-time cell aggregation in solution[c]		Rolling on low density rhVCAM-1[d]	
	Association rate, k_{on}	Dissociation rate, k_{off}	Overall affinity		Initial rate of cell aggregation	Number of aggregates at steady state	Capture frequency	Tether duration
Resting state	Fast	Fast	Low	Bent	Slow	Low	Low	Short
fMLFF, FPR activation (Gαi signal)	Fast	Slow	High	Extended	Rapid	High	High	Long
Phorbol ester (PKC)	Fast	Slow	High	Bent	Slow	High	Low	Long
fMLFF + Forskolin (Gαi and Gαs)	Fast	Fast	Low	Extended	Rapid	Medium	Low	Short

[a] *Based on data from Chigaev et al. (2001, 2003b, 2007, 2008).*

[b] *Based on data from Chigaev et al. (2003a, 2007, 2008).*

[c] *Based upon Chigaev et al. (2007, 2008).*

[d] *Unpublished data.*

the extension of VLA-4 detected using FRET. On the other hand, the overall number of aggregates at steady state was related to the overall ligand-binding affinity that was largely determined by the dissociation rate of soluble ligand (LDV-FITC, k_{off}, **Table 2**). It is worth noting that such an unambiguous result was possible since under the chosen experimental conditions only a small number of VLA-4/VCAM-1 bonds were needed to form and sustain cellular aggregates. According to an experimental estimate, in most cases this number was less than three bonds per aggregate (Zwartz et al., 2004). Thus, molecular extension seemed to facilitate the initial VLA-4-VCAM-1 ligand interaction and therefore, promote initial receptor engagement. Slow ligand dissociation, in the high affinity state stabilizes cellular interactions, and therefore, results in a larger number of cell aggregates.

In the parallel plate rolling assay at very low density of immobilized ligands, the formation of multiple consecutive "bonds" between receptor and its counter-structure is relatively unlikely. Therefore, under these experimental conditions the kinetics of transient tether formation and its dissociation provides insight into the functional consequences of nascent adhesive contacts (Grabovsky et al., 2000). We hypothesized that integrin extension, because of the exposure of the ligand binding site, directly affects the efficiency of tether formation. On the other hand, the affinity of the binding pocket determines the life-time of the integrin-ligand interaction and thus, regulates the duration of the adhesive event. To test this idea we studied tether frequency and duration for the four affinity states described previously (**Table 2**). The high affinity extended state of VLA-4 induced by stimulation through a Gαi-coupled receptor produced rapid accumulation of cells and long tether duration, when compared to the low affinity bent resting state. Phorbol ester treated cells showed low cell recruitment and long tether duration. This state was previously described as a high affinity bent conformation of VLA-4. Treatment with fMLFF/forskolin (intended to reproduce cAMP elevation through Gαs signaling), which generates a low affinity unbent (extended) state, showed tether duration similar to the resting cells. However, the tether frequency was unexpectedly low for an unbent conformation. This unanticipated result might result from our inability to accurately estimate the number of very short tethers and merits further investigation. Thus, the parallel plate data were generally consistent with the predicted behavior except for the low affinity extended state to promote efficient cell recruitment and adhesion (Chigaev et al., 2007).

Taken together, the overall scheme of the VLA-4 conformation regulation can be generalized as follows (**Figure 1**). At rest, the low affinity bent conformation prevents cell tethering and rolling because of the positioning of the ligand binding site. If ligand engagement occurs, it would have a very short life time. However, it is also possible that a series of engagements of integrins or other receptors [selectins for example (Kuwano et al., 2010)] could provide a signal resulting in molecular extension. This could lead to rolling on an extended low affinity integrin. Rapid activation by Gαi-coupled GPCR induces a short-lived high affinity extended state (seconds to minutes), followed by a sustained extended low affinity state. If during the short period that VLA-4 engages its counter-structure, a long-lived tether will form. Under shear and external force this interaction can potentially be sustained for a longer period of time because of mechanical (catch bond) or signaling/cytoskeletal events. If no engagement of the integrin occurred, a low affinity extended state could be ideally suited for rolling under shear.

LFA-1 CONFORMATION

A similar approach employing a small fluorescent ligand mimicking probe was used to study LFA-1 conformational regulation. Because several small molecules, direct and allosteric antagonists, are known to specifically bind to LFA-1, multiple fluorescent probes based, for example, on BIRT0377 (Larson et al., 2005), Genentech compounds (Gadek et al., 2002; Chigaev et al., 2011b), and others can be used.

Employing this approach, recent studies of LFA-1 conformational regulation revealed notable similarities and differences in the regulation of VLA-4 and LFA-1. In a manner analogous to VLA-4, LFA-1 can be rapidly activated by Gαi-coupled GPCRs, with the overall activation time-frame dependent on the rate of GPCR desensitization. Similar to VLA-4, LFA-1 can be rapidly deactivated by Gαs-coupled GPCRs. Also similar to VLA-4, modulation of the ligand dissociation rate can be observed for different LFA-1 affinity states (Chigaev et al., 2011b). However, unlike VLA-4, without inside-out activation (at rest), the binding of the fluorescent ligand to LFA-1 was extremely slow, at least 10 times slower than was expected for diffusion-limited binding. This suggests that an additional structural mechanism prevents rapid binding of the ligand to resting LFA-1. In the case of VLA-4 the binding of the ligand is unobstructed, and ligand binding rates are close to the rates expected for the diffusion-limited binding regardless of activation state, the k_{on} ranged from $\sim$3–5 $\times$ 10^6 $M^{-1}s^{-1}$ (Chigaev et al., 2001). We postulate that such a blocking mechanism explains the inability of native LFA-1 to support cell rolling, where the absence of its rapid engagement by the ligand in the inactive state leads to the requirement for the selectin-mediated rolling step (**Table 1**) (Chigaev et al., 2011b). A recent funding that rolling on E- or P-selectin induces the extended but not high-affinity conformation of LFA-1 through a signaling mechanism triggered by PSGL-1 engagement adds more complexity to the overall scheme of LFA-1 conformational regulation (Kuwano et al., 2010).

LIGAND BINDING RATES, I-DOMAIN, AND INTEGRIN PHYSIOLOGY. IS THERE A RELATIONSHIP?

For adhesion receptors, the kinetics of ligand-receptor interaction, which includes "bond" formation and its dissociation, is a critical factor that determines the type of adhesive interaction. The rapid forward rate (on-rate) is specifically important for cell rolling, because of the requirement for rapid molecule engagement under flow (Lawrence and Springer, 1991). As discussed above, the major difference between the two integrins is that the on-rates for the binding of small ligands to VLA-4 and LFA-1 are dramatically different. The on-rate for ligand binding to VLA-4 approaches the diffusion-limited binding rate for a ligand of similar size (Chigaev et al., 2003b). For the LFA-1-specific ligand, this rate is at least an order of magnitude slower. We believe that this LFA-1-specific kinetic property is directly

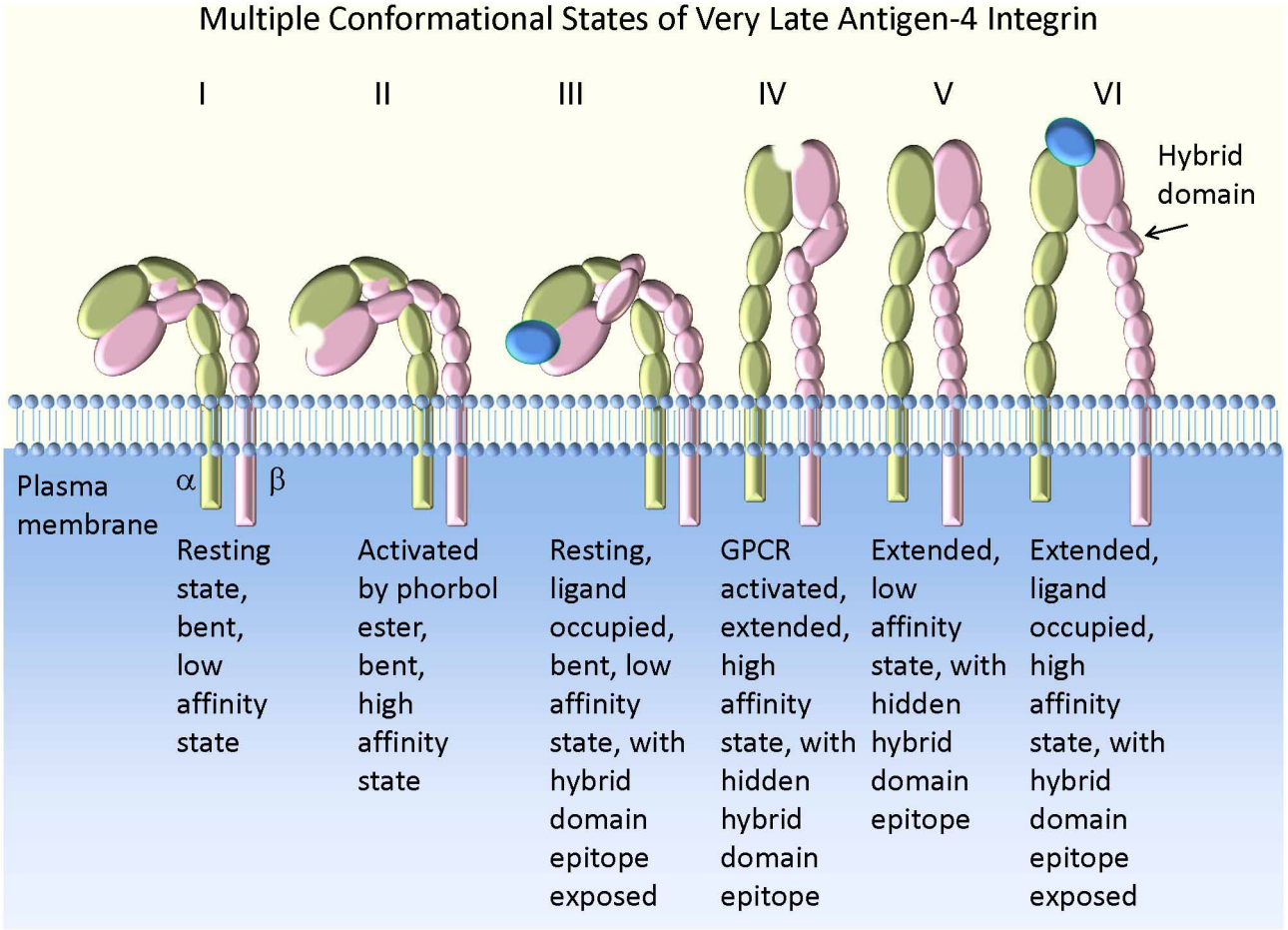

FIGURE 1 | Model of VLA-4 integrin conformation and affinity. The bent low affinity state is observed on resting cells (I). Activation by phorbol ester creates a high affinity state lacking molecular extension as detected by a FRET-based approach (II). This conformation results in the slow accumulation of cell aggregates in suspension (Chigaev et al., 2007), with a low tether capture frequency but a long tether duration in the rolling assay (**Table 2**). The addition of VLA-4 specific ligand to resting cells leads to exposure of a hybrid domain (LIBS) epitope (III). However, the ligand binding affinity remains low [see Figures 2, 4 in Chigaev et al. (2009)]. This state is bent (or at least not fully extended) because a further molecular extension can be detected with a FRET-based approach (Chigaev et al., 2003a, 2004, 2007). Activation through a wild type Gαi-coupled GPCR induces a high affinity extended conformation (IV). This conformation results in the rapid accumulation of cell aggregates in suspension (Chigaev et al., 2007), with high tether capture frequency and long tether duration (**Table 2**). The low affinity extended (or at least partially extended) conformation (V) can be detected for several minutes after signaling from wild type Gαi-coupled GPCR, because of relatively faster desensitization of the ligand binding affinity than relaxation of the conformation (Chigaev et al., 2007). Conformation V may also result from consecutive stimulation through Gαi-coupled and Gαs-coupled receptors (Chigaev et al., 2008). In suspension this translates into rapid cell aggregation that reaches a steady-state intermediate between resting (I) and Gαi-coupled GPCR activated states (IV) [see Figure 7A in Chigaev et al. (2008)]. A low tether capture frequency and short tether duration was detected in the rolling assay (**Table 2**) (see text for details). The ligand occupied and extended high affinity state (VI) was detected after Gαi-coupled GPCR activation in the presence of ligand. The molecule affinity and extension were preserved by the use of a non-desensitizing GPCR mutant (Prossnitz, 1997; Chigaev et al., 2003a, 2007). This conformation results in the rapid formation of a large number of aggregates in cell suspension (Chigaev et al., 2008), with high tether capture frequency and long tether duration in the rolling assay (**Table 2**). The exposure of the hybrid domain (LIBS) epitope can be also used to determine VLA-4 ligand binding affinity for unlabeled ligands (Chigaev et al., 2009; Njus et al., 2009).

translated into the well documented inability of LFA-1 to support tethering and rolling under natural conditions (Lawrence and Springer, 1991; von Andrian et al., 1991). These conditions do not include the cases where LFA-1 conformation was changed by mutations, I-domain isolation (Salas et al., 2002), or a small molecule XVA-143 (Salas et al., 2004). We suggest that these manipulations lead to a conformational change that facilitates LFA-1 ligand binding site exposure, and therefore, promotes rapid ligand-receptor engagement.

It worth noting that the possibility of a dramatic difference in the ligand kinetics between VLA-4 and LFA-1 (and Mac-1) was first suggested by Alon et al. (1995). These authors proposed that for VLA-4, which can mediate tethering and rolling without cell activation, rapid ligand association and dissociation rates would be observed. This conclusion was based on the analogy with other rolling receptors, i.e., selectins. These authors also suggested that for the LFA-1 interaction with ICAM-1, the ligand binding kinetics would be different (Alon et al., 1995). Now, experimental

data directly supporting this concept are available (Chigaev et al., 2011b).

Because a major structural difference between VLA-4 and LFA-1 is the presence of an additional "inserted" I-domain, which was acquired by the I-domain containing leukocyte integrins at the time their emergence, it is tempting to attribute the difference in ligand binding kinetics to the presence of the domain. Without inside-out signaling, binding of ligand to LFA-1 is virtually absent, leading to the hypothesis that, at rest, the LFA-1 ligand binding site is "shielded" by some part of the molecule (Chigaev et al., 2011b). A rapid conformational rearrangement of LFA-1 upon activation (Shamri et al., 2005) could release this putative ligand binding site "protection," and as a result mediate rapid receptor engagement. The presence of such intra- or intermolecular "protection" is supported by the fact that an isolated alpha L I-domain expressed on the cell surface was very effective in supporting cell rolling (Salas et al., 2002). We propose that a downward bending of the molecule that simply changes I-domain orientation would be insufficient to prevent binding of a small fluorescent ligand to LFA-1. In contrast, for VLA-4, the binding of the small fluorescent probe was not obstructed in its bent conformation. The VLA-4-specific small fluorescent ligand binding rate was close to its diffusion limit, where a FRET-based extension assay can be successfully performed (Chigaev et al., 2003a, 2004, 2007). We envision that a competitive protection mechanism that can be similar to an "endogenous ligand" (Alonso et al., 2002) could serve as a "shield" for the ICAM-1 binding site.

The physiological difference between the two integrins seems to be related to the function of the immune system. VLA-4 appears to be more important for innate antigen-independent immune responses, and LFA-1 for adaptive immunity. The presence of an additional protective mechanism for the binding of a ligand to the LFA-1 binding site suggests that LFA-1/ICAM-1-mediated interactions will be more difficult to establish. This is not surprising to researchers who performed side-by-side comparative studies of the two integrins. However, from a biological perspective, this seems to provide an additional "check" for adaptive immune responses, where intercellular immune cell interaction can directly lead to unwanted, or excessive immune activation and result in cell and tissue damage. This notion is additionally supported by the idea that the appearance of the leukocyte-specific alpha I domain-containing integrins (such as LFA-1) during vertebrate evolution coincides with the emergence of the BCR-TCR-MHC-based adaptive immune system.

One apparent exception from this observation is the crucial role of LFA-1 in chemokine-dependent arrest and trafficking of neutrophils, which is traditionally envisioned as a part of innate immunity. However, without questioning the well-established role of neutrophils in the rapid destruction of infectious agents, we would like to point toward emerging roles of neutrophils in immune regulation. As recent reports suggest, neutrophils can capture antigen and migrate to lymph nodes. They can also produce a repertoire of cytokines, chemokines, and angiogenic factors, provide signals for maturation of APCs, participate in the immune cells crosstalk that includes B and T cells, regulate adaptive immunity, and participate in the resolution of inflammation [for review see (Chakravarti et al., 2007; Kumar and Sharma, 2010; Mantovani et al., 2011)]. Thus, neutrophils should not be only envisioned as innate "weapons of mass destruction," but also as emerging regulators of immune responses. Will some of these functions require LFA-1 integrin for the mediation of immune cell-cell interactions? We think that it is possible.

INTEGRIN PHYSIOLOGY AND ITS IMPLICATION FOR DRUG DISCOVERY

Another remarkable difference between LFA-1 and VLA-4 integrins is the type of integrin antagonists identified in the attempt to regulate integrin dependent adhesion for therapeutic purposes. A majority of compounds specific for VLA-4 and several other integrins, including αIIbβ3 and αvβ3, are competitive (direct) inhibitors (Shimaoka and Springer, 2003) (formally agonists that promote LIBS). Until recently, no VLA-4-specific allosteric antagonists had been described (Chigaev et al., 2011d). For a competitive drug, the ligand binding site location is very close (or overlaps) with its natural ligand binding site. Therefore, direct competition with the integrin natural ligand can be observed. On the other hand, a large number of LFA-1 specific compounds are allosteric antagonists for two different allosteric sites on LFA-1 (Shimaoka and Springer, 2003). Is there a plausible explanation that can account for such distinction? Can different ligand binding properties provide an insight into such a peculiar difference?

Direct competitive inhibitors are expected to be ineffective in blocking LFA-1-dependent cell adhesion, if on resting cells, the LFA-1 binding domain is "hidden" and only exposed after inside-out activation. The binding of these compounds to LFA-1 would only be possible after an inside-out signal. Because LFA-1 activation and engagement can occur locally, right on the spot, where activating receptors, LFA-1, and ICAM-1 are juxtaposed at the site of contact (Shamri et al., 2005; Laudanna and Alon, 2006), competitive inhibitors would be highly inefficient in competition with natural ligands. For integrins possessing a ligand binding site that is exposed at rest (such as VLA-4), binding of competitive inhibitors would occur at any time, and binding site occupancy would simply depend on binding affinity and drug concentration.

On the other hand, binding of allosteric antagonists to their binding site, which is spatially separated from the ligand binding pocket, should be independent of the natural binding site exposure. Therefore, these compounds can occupy LFA-1 prior to its activation, and thus, should be more efficient in blocking LFA-1-dependent cell adhesion. We postulate that because of this property, in screening assays aimed at identifying LFA-1-specific antagonists, the number of allosteric "hits" was artificially enriched. This resulted in the predominance of LFA-1-specific allosteric antagonists (Shimaoka and Springer, 2003).

Is it possible to identify allosteric antagonists for integrins with an exposed ligand binding site? Using an approach that relies upon the exposure of the Ligand Induced Binding Site epitope (LIBS) to distinguish VLA-4 competitive antagonists (Njus et al., 2009), several VLA-4-specific allosteric antagonists were identified (Chigaev et al., 2011d). These molecules, although not competing directly with VLA-4-specific ligands, blocked VLA-4-dependent cell adhesion. Moreover, they mobilized early hematological progenitors into the peripheral blood, which is a

well-documented ability of anti-VLA-4 blocking antibodies or competitive inhibitors (Bonig et al., 2008, 2009; Zohren et al., 2008; Ramirez et al., 2009). Moreover, because several of the identified molecules are FDA approved drugs that have been used over the past 30 years for treatment of non-hematological diseases, it appears that these anti-VLA-4 allosteric properties account for the previously reported hematological side effects (Chigaev et al., 2011c).

CONCLUSIONS

An evolutionary divergence among ancient and more modern leukocyte integrins, containing an inserted alpha I-domain, provides a plausible mechanism to account for structural and functional differences between VLA-4 and LFA-1. A new set of fluorescent approaches has made it possible to study the affinity and conformation of these integrins in real-time on live cells at natural receptor abundance using several homogeneous assays. The ability of VLA-4 to bind ligand in the low affinity resting state as well as the high affinity activated state allows it to serve as an adhesion receptor for rolling as well as firm attachment. The inability of LFA-1 to bind ligand in its natural resting state suggests that its normal function is as a firm attachment receptor in conjunction with selectins as rolling receptors. These ligand binding differences provide an explanation to account for the fact that VLA-4 inhibitors are typically competitive, while inhibitors for LFA-1 are typically allosteric. Moreover, the ability of integrins to independently regulate molecular extension as well as affinity through known physiological pathways suggests a means for independent regulation of the adhesive capture efficiency as compared to adhesive duration.

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Touch of chemokines

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Chemoattractant cytokines or chemokines constitute a family of structurally related proteins found in vertebrates, bacteria, or viruses. So far, 48 chemokine genes have been identified in humans, which bind to around 20 chemokine receptors. These receptors belong to the seven transmembrane G-protein-coupled receptor family. Chemokines and their receptors were originally studied for their role in cellular trafficking of leukocytes during inflammation and immune surveillance. It is now known that they exert different functions under physiological conditions such as homeostasis, development, tissue repair, and angiogenesis but also under pathological disorders including tumorigenesis, cancer metastasis, inflammatory, and autoimmune diseases. Physicochemical properties of chemokines and chemokine receptors confer the ability to homo- and hetero-oligomerize. Many efforts are currently performed in establishing new therapeutically compounds able to target the chemokine/chemokine receptor system. In this review, we are interested in the role of chemokines in inflammatory disease and leukocyte trafficking with a focus on vascular inflammatory diseases, the operating synergism, and the emerging therapeutic approaches of chemokines.

Keywords: chemokine, chemokine receptor, arrest, oligomerization, vascular inflammatory diseases, leukocyte trafficking, therapeutics

INTRODUCTION

With the exceptions of CX3CL1/fractalkine and CXCL16/SR-PSOX, chemoattractant cytokines or chemokines constitute a family of small soluble signaling molecules of approximately 70 amino acid residues with a molecular weight of 7–12 kDa. In addition to their monomeric form, these proteins are able to associate, forming dimers, tetramers, or multimers (i.e., to oligomerize). Chemokines have crucial roles in both homeostasis and disease. Their homeostatic roles include leukocyte maturation and trafficking, development, tissue repair, and angiogenesis (Ransohoff, 2009). As disease modulators, chemokines have roles in a wide variety of inflammatory and immune responses through the chemoattraction of innate and adaptive immune cells. To date, around 50 chemokines have been identified in humans, which have been grouped into one of four families, CXC, CC, CX3C, and XC, based on the arrangement of cysteine residues involved in the formation of disulfide bonds (**Table 1**). In the CXC and CX3C chemokine family, one or three amino acid residues are inserted between the first two of four cysteine residues, respectively. The first and third cysteine residues are absent in the XC subfamily that possesses only one disulfide bond. In the CC subfamily, the first two cysteines are juxtaposed. Another family has been recently described in the zebrafish genome, namely the CX family, which lacks one of the four cysteine residues highly conserved amongst chemokines (Nomiyama et al., 2008). All chemokines arose from a single ancestral gene, originating approximately 650 million years ago (Nomiyama et al., 2010). Amongst vertebrates, the zebrafish genome has the highest number of chemokine genes with more than 100 genes while both pufferfish Tetraodon and Fugu genomes contain less than 20 chemokine genes each. The human genome encompasses more than 50 different chemokine genes and pseudogenes. These genes have undergone a rapid evolution in both their sequences and their family gene size. The conventional name is still often used, which may lead to some confusion while the International Union of Immunological Societies/World Health Organization Subcommittee on Chemokine Nomenclature has assigned a name to each chemokine and chemokine receptor (Bacon et al., 2001). A large number of human chemokine genes are known to be clustered on specific chromosomal regions. There are two major gene clusters comprising exclusively either CXC or CC genes on chromosome 4q13.3-q21.1 and 17q12, respectively (**Table 1**). These major clusters can be subdivided into two regions. For the CXC gene cluster, the regions are named GRO and IP10 while the regions of the CC gene cluster are called MCP and MIP (Nomiyama et al., 2010). The GRO region contains the CXCL1–CXCL8 genes and the IP10 region the CXCL9–CXCL13 genes, respectively. In the CC major cluster, the MCP and MIP regions comprise 6 and 12 genes, respectively (CCL2, CCL7, CCL11, CCL8, CCL13, CCL1 versus CCL5, CCL16, CCL14, CCL15, CCL23, CCL18, CCL3, CCL4, CCL3L3, CCL4L1, CCL3L1, CCL4L2). In addition to the two major clusters, a CC "mini"-cluster is found on chromosome 7 (comprising the CCL26 and CCL24 genes), on chromosome 9 (CCL27, CCL19, CCL21), and on chromosome 16 (CCL22, CX3CL1, and CCL17), respectively. Both XCL1 and XCL2 are also found in a "mini"-cluster on chromosome 1.

Table 1 | Human chemokine genes.

Name	Official symbol	Conventional name	Chromosome	Gene size (kb)	Number of exons	Number of amino acids (mature form)	Cluster name	Receptor[1]
CCL								
CCL1	CCL1	TCA3; I-309	17q11.2	2.85	3	73	MCP	CCR8
CCL2	CCL2	MCP-1; MCAF; JE	17q11.2-q21.1	1.93	3	76	MCP	CCR2, CCR3, DARC, CCBP2
CCL3	CCL3	MIP-1α; LD78α	17q12	1.90	3	69	MIP	CCR1, CCR5, CCBP2
CCL3L1	CCL3L1	LD78β	17q12	1.89	3	70	MIP	CCR1, CCR5
CCL3P1	CCL3L2	–	17q21.1	–			MIP	
CCL3L3	CCL3L3	LD78β	17q12	1.88	3	70	MIP	CCR1, CCR5
CCL4	CCL4	MIP-1β	17q21-q23	1.79	3	69	MIP	CCR5, CCBP2
CCL4L1	CCL4L1	LAG-1	17q12	1.80	3	69	MIP	CCR5, CCBP2
CCL4L2	CCL4L2	LAG-1	17q12	1.80	3	68	MIP	CCR5, CCBP2
CCL5	CCL5	RANTES	17q11.2-q12	8.88	3	68	MIP	CCR1, CCR3, CCR5, DARC, CCBP2, CCRL2
CCL7	CCL7	MCP-3; MARC	17q11.2-q12	2.01	3	76	MCP	CCR1, CCR2, CCR3, CCR5, DARC, CCBP2
CCL8	CCL8	MCP-2	17q11.2	2.35	3	76	MCP	CCR2,CCR3
CCL11	CCL11	Eotaxin,	17q21.1-q21.2	2.51	3	74	MCP	CCR3,CCR5, DARC, D6
CCL13	CCL13	MCP-4	17q11.2	2.21	3	82	MCP	CCR2, CCR3, CCR5, DARC, CCBP2
CCL14	CCL14	HCC-1	17q11.2	3.07	4	74	MIP	CCR1
CCL15	CCL15	HCC-2	17q11.2	4.46	4	92	MIP	CCR1, CCR3, DARC
CCL16	CCL16	HCC-4; LEC	17q11.2	4.98	3	97	MIP	CCR1
CCL17	CCL17	TARC; ABCD-2	16q13	11.29	3	71	"Mini"-CC 16	CCR4, DARC, CCBP2
CCL18	CCL18	DC-CK1; PARC; AMAC-1	17q11.2	7.2	3	69	MIP	DARC
CCL19	CCL19	MIP-3β; ELC; Exodus-3	9p13	1.71	4	77	"Mini"-CC 9	CCR7, CCRL1, CCRL2
CCL20	CCL20	MIP-3α; LARC; Exodus-1	2q33-q37	3.72	4	70, 69		CCR6
CCL21	CCL21	6Ckine; SLC; Exodus-2	9p13	1.14	4	111	"Mini"-CC 9	CCR7,CCRL1
CCL22	CCL22	MDC; STCP-1; AMCD-1	16q13	7.41	3	69	"Mini"-CC 16	CCR4, DARC, CCBP2
CCL23	CCL23	CKβ8; MPIF-1	17q11.2	4.91	4	99, 116	MIP	CCR1, FPR2
CCL24	CCL24	Eotaxin-2; MPIF-2	7q11.23	1.92	3	93	"Mini"-CC 7	CCR3
CCL25	CCL25	TECK	19p13.2	34.81	5	127, 61		CCR9, CCRL1
CCL26	CCL26	Eotaxin-3, MIP-4α, IMAC	7q11.2	20.22	3	71	"Mini"-CC 7	CCR3
CCL27	CCL27	CTACK; ILC; ESKINE	9p13	0.80	3	88	"Mini"-CC 9	CCR10
CCL28	CCL28	MEC	5p12	30.88	3	108		CCR3, CCR10

CXC								
CXCL1	CXCL1	GRO-α; MGSA-α; MIP-2; KC	4q13.3	1.85	4	73	GRO	CXCR2, DARC
p-CXCL1	CXCL1P	–	4q13.3	–	4		GRO	
CXCL2	CXCL2	GRO-β; MGSA-β; MIP-2α	4q13.3	2.24	4	73	GRO	CXCR2, DARC
CXCL3	CXCL3	GRO-γ, MGSA-γ; MIP-2β	4q13.3	2.18	3	73	GRO	CXCR2, DARC
CXCL4	PF4	PF4	4q13.3	0.92	3	70	GRO	CXCR3
CXCL4L1	PF4V1	PF4-ALT; CXCL4V1	4q13.3	1.18	4	70	GRO	
CXCL5	CXCL5	ENA-78	4q13.3	3.05	4	78	GRO	CXCR2, DARC
CXCL6	CXCL6	GCP-2	4q13.3	2.20	3	77	GRO	CXCR1, CXCR2, DARC
CXCL7	PPBP	NAP-2; beta-TG; CTAP-III	4q13.3	1.75	3	81, 85, 94	GRO	CXCR2, DARC
p-CXCL7	PPBPL1	–	4q13.3	–			GRO	
CXCL8	IL8	IL8	4q13.3	3.21	4	79, 82	GRO	CXCR1, CXCR2, DARC
CXCL9	CXCL9	MIG	4q21.1	6.02	4	103	IP10	CXCR3
CXCL10	CXCL10	IP10; CRG-2	4q21.1	2.42	4	77	IP10	CXCR3
CXCL11	CXCL11	I-TAC,	4q21.1	2.51	4	73	IP10	CXCR3, CXCR7, DARC
CXCL12	CXCL12	SDF-1α	10q11.1	14.94	4	68		CXCR4, CXCR7
CXCL12	CXCL12	SDF-1β	–	–	–	72		
CXCL12	CXCL12	SDF-1γ	–	–	–	98		
CXCL13	CXCL13	BCA-1; BLC	4q21.1	100	4	87	IP10	CXCR5
CXCL14	CXCL14	BRAK	5q31	8.60	4	77		?
CXCL16	CXCL16	SR-PSOX	17p13.2	6.39	5	225[2]		CXCR6
CXCL17	CXCL17	DMC	19q13.2	14.44	4	98		?
XC								
XCL1	XCL1	Lymphotactin; SCM-1α; ATAC	1q23	5.60	3	93	"Mini"-CC 1	XCR1
XCL2	XCL2	SCM-1β	1q24.2	3.23	3	93	"Mini"-CC 1	XCR1
CX3C								
CX3CL1	CX3CL1	Fractalkine; Neurotactin; ABCD-3	16q13	12.54	3	355[2]	"Mini"-CC 16	CX3CR1
NOT ASSIGNED								
MIF	MIF	Macrophage migration inhibitory factor, glycosylation-inhibiting factor	22q11.23	0.84	3	114		CXCR2, CXCR4; CXCR7, CD74

Human gene and protein data were collected from the web sitesEntrezGene: http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene and http://www.uniprot.org/, respectively.

[1]*Only agonist receptors are indicated (adapted from Schall and Proudfoot, 2011).*

[2]*Each chemokine domain of CXCL16 and CX3CL1 is constituted by 76 amino acids.*

Besides their structural classification, another organization of chemokines has been proposed based on their expression and their functional activity. This classification groups chemokines into three "families": pro-inflammatory, homeostatic, and mixed function (Mantovani et al., 2006). Pro-inflammatory chemokines are up-regulated under inflammatory conditions and are involved in the leukocyte recruitment to inflamed sites. Homeostatic chemokines are expressed constitutively at non-inflamed sites and are involved in homeostatic migration and homing of cells in physiological conditions such as lymphocyte homing. Some chemokines have both properties, and are thus called mixed-function chemokines.

Chemokines act by binding specialized receptors on the target cell surface. These chemokine receptors are also grouped into four families, CXCR, CCR, XCR, and CX3R, based on the chemokine family they bind (Nomiyama et al., 2011). The entire group of chemokine receptors belongs to the seven transmembrane domain G-protein-coupled receptors that usually combine the receptor to the Gαi subunit of heterotrimeric G proteins. So far, around 25 human chemokine receptor genes have been identified (**Table 2**). Interestingly, 12 of these receptors are found on human chromosome 13 and stretched around 13.5 megabases. In addition, several decoy receptors have been reported to bind chemokine ligands without eliciting signal transduction. These comprise CXCR7, CCBP2, Duffy antigen receptor for chemokines (DARC), CCRL1, and CCRL2. The first chemokine receptor genes appeared in the most primitive vertebrate, agnathan lamprey (hagfish), around 480 million years ago (Nomiyama et al., 2011).

The chemokine/chemokine receptor system can be considered as a "puzzle" since many receptors have different chemokines as ligands and vice versa. However, thanks to the multiple combinations allowed, this system offers robustness. Indeed, even if one chemokine or receptor does not function, another one can replace it.

CHEMOKINES IN LEUKOCYTE TRAFFICKING AND INFLAMMATORY DISEASES

Leukocyte recruitment represents a fundamental episode during infection, in inflammatory disorders, such as atherosclerosis, as well as in autoimmune diseases, such as in psoriasis, rheumatoid arthritis, and chronic lung disease (Luster et al., 2005). Initially, leukocyte extravasation was described as a three-step process namely rolling, activation, and arrest. Recently, new insights have allowed defining a more complex process by adding several steps to the three original, including tethering (or capture), slow rolling, adhesion strengthening, spreading, intravascular crawling, and finally paracellular and transcellular transmigration.

Whereas capture and slow rolling are mediated by reversible and transient interactions between E-, L-, or P-selectin and ligands such as P-selectin glycoprotein-1 (PSGL-1), the adhesion of leukocytes to endothelial cells is mediated by the interaction of VCAM1 and ICAM-1, receptor for advanced glycation end-products (RAGE), or mucosal vascular cell-adhesion molecule 1 (MADCAM1) with leukocyte integrins. The common structure of integrins is a non-covalently associated α and β subunit. So far, 16 α subunits and 8 β subunits have been identified, and various combinations form at least 22 heterodimers. The principal neutrophil β2-integrins are CD11a/CD18 (LFA-1) and CD11b/CD18 (Mac-1, CR3; Luo et al., 2007) although neutrophils can express p150,95/αxβ2 (CD11c/CD18) and at low level very late antigen (VLA) 4/α4β1 (CD49d/CD18; Zarbock et al., 2012). LFA-1 and Mac-1 have been shown to mediate neutrophil adhesion by interacting with ICAM-1 while αxβ2 is able to bind the N-terminal part of the alpha chain of fibrinogen (Loike et al., 1991; Diamond and Springer, 1993; Lum et al., 2002). During neutrophil adhesion, LFA-1 and Mac-1 appear to have sequential roles binding ICAM-1 under shear conditions (Neelamegham et al., 1998; Hentzen et al., 2000). In a two-step process neutrophils adhere first to ICAM-1 by interacting with LFA-1 and then Mac-1 acts as a stabilizer of the LFA-1/ICAM-1 bond.

The transition of rolling to leukocyte arrest and activation is triggered by chemokines such as CXCL1/GRO-α while others like CCL2/MCP-1 *per se* are rather promoting transmigration. Arrest of rolling leukocytes is triggered by an increase in the affinity of integrins by chemokines (Ley et al., 2007; Chavakis et al., 2009).

Different cell types, such as mesenchymal stem cells, endothelial cells, and circulating blood cells including leukocytes or platelets produce and release a broad range of chemokines and other chemoattractants that facilitate and enhance the recruitment of leukocytes. Some of these pro-inflammatory mediators circulate in the plasma, others are only found in the inflamed tissue, and yet others are presented on endothelial cells. Furthermore, additional to direct endothelial deposition from the luminal side, chemokines are transported via caveolae through the endothelium and presented to the apical side of the cell instead of diffusing through endothelial cell junctions (Pruenster et al., 2009). This transcytosis requires the DARC (Middleton et al., 1997). Recently, a new mechanism has been highlighted introducing the concept of lymphocyte transendothelial migration by intraendothelial vesicle-stored chemokines beneath the apical membrane (Shulman et al., 2011).

Chemokines bind chemokine receptors expressed on leukocytes to induce activation. In addition, most chemokines are also able to bind extracellular matrix components, including glycosaminoglycans (GAGs), to get immobilized and be presented to leukocytes. This is essential in order to avoid to be swept away under flow conditions from the cell surface. This coimmobilization with adhesion molecules will promote leukocyte activation, adhesion, and migration.

The following section will give several examples of chemokine contribution in leukocyte trafficking and in inflammatory diseases with a particular focus on vascular inflammatory diseases.

CHEMOKINES IN PLATELETS

As outlined above, activated platelets are able to release chemokines as well as a battery of different mediators to modulate inflammation. Thus, platelets have been found to be involved in different diseases with an inflammatory component such as obesity, acute lung injury, or coronary artery disease where they interact with both endothelial cells and leukocytes leading to a diversity of effects (van Gils et al., 2009; von Hundelshausen et al., 2009). Platelets release chemotactic cytokines stored in α-granules upon activation. *Inter alia*, CXCL4/PF4, CCL5/RANTES, CXCL7/NAP-2, CXCL12/SDF-1, CXCL1/GRO-α, or CXCL5/ENA-78 are able

Table 2 | Human chemokine receptor genes.

Name	Conventional name	Chromosome	Gene size (kb)	Number of exons	Number of amino acids	Ligands
CXCR						
CXCR1	IL8R1; IL8RA; CMKAR1	2q35	4.15	2	350	CXCL6, CXCL8,
CXCR2	IL8R2; IL8RB; CMKAR2	2q35	11.96	4	360	CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8, MIF
CXCR2P1	CXCR2P; IL8RBP	–		1		
CXCR3A	IP10-R; MigR; CMKAR3;	Xq13.1	2.60	2	368	CXCL4, CXCL4L1, CXCL9, CXCL10, CXCL11,
CXCR3B	IP10-R; MigR; CMKAR3;	–	–	2	415	
CXCR3-alt		–	–	2	267	
CXCR4	LAP3; LCR1	2q21	2.60	2	352, 356	CXCL12, MIF
CXCR5	BLR1; MDR15	11q23.3	12.43	2	372, 327	CXCL13
CXCR6	BONZO; CD186	3p21.26	4.87	2	342	CXCL16
CCR						
CCR1	CKR1; CMKBR1; MIP1aR	3p21	6.63	2	355	CCL3, CCL3L1, CCL3L3, CCL5, CCL7, CCL14, CCL15, CCL16; CCL23,
CCR2A	CMKAR2; CD182; CKR2B	3p21.31	7.18	2	374	CCL2, CCL7, CCL8, CCL13,
CCR2B	CKR2B	–	–	3	360	
CCR3	CKR3; CMKBR3	3p21.3	24.32	3	355	CCL2, CCL5, CCL7, CCL8, CCL11, CCL13, CCL15, CCL24, CCL26, CCL28,
CCR4	CKR4; CMKBR4; ChemR13	3p24	3.33	2	360	CCL17; CCL22
CCR5	CMKBR5; CKR5	3p21.23	6.06	3	352	CCL3, CCL3L1, CCL3L1, CCL4, CCL4L1, CCL4L2, CCL5, CCL7, CCL11, CCL13
CCR6	BN-1; DCR2; CKR-L3	6q27	27.33	3	374	CCL20
CCR7	BLR2; CMKBR7; EBI1	17q12-q21.2	11.71	3	378	CCL19, CCL21
CCR8	CKRL1; CMKBR8; CMKBTER1	3p22	3.97	2	355	CCL1
CCR9A	GPR-9-6; GPR28	3p21.3	16.67	4	359	CCL25
CCR9B	GPR-9-6; GPR28	–	–	–	357	CCL25
CCR10	GPR2	17q21.1-q21.3	2.42	2	362	CCL27, CCL28
XCR						
XCR1	CCXCR1; GPR5	3p21.3	7.68	2	333	XCL1, XCL2
CX3CR						
CX3CR1	CMKDR1; GPR13; CCRL1	3p21.3	18.24	4	355, 387, 362	CX3CL1
DECOYRECEPTORS						
CXCR7	RDC1; GPR159	2q37.3	12.61	2	362	CXCL11, CXCL12, MIF
CCRL1	CCR11; CCBP2; VSHK1; CCX-CKR; PPR1	3q22	5.29	2	350	CCL19, CCL21, CCL25
CCRL1P1	dJ509I19.4	6q23.3		1		
CCRL2	CKRX; CRAM-A; CRAM-B; HCR	3p21	2.29	3	344, 256	CCL5, CCL19
CCBP2	D6	3p21.3	57.81	3	384	CCL2, CCL3, CCL4, CCL4L1, CCL4L2, CCL5, CCL7, CCL11, CCL13; CCL17, CCL22,
DARC	Duffy; FY	1q21-q22	2.48	2	336, 338	CCL2, CCL5, CCL7, CCL11, CCL13, CCL15, CCL17, CCL18, CCL22, CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8, CXCL11
FORMYL PEPTIDE RECEPTOR						
FPR2	FPRL1; LXA4R; FMLP-R-II; FMLPX; FPR2A; FPRH1	19q13.3-q13.4	9.33	3	351	CCL23, Lipoxin A4, serum amyloid A, β amyloid peptide Aβ42, SAA, MMK, Hp-(2-20)

Human gene and protein data were taken from the web sitesEntrezGene: http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene and http://www.uniprot.org/, respectively.

to mediate the endothelial adhesion of different cells including monocytes, neutrophils, and progenitor cells (Lievens and von Hundelshausen, 2011).

Platelets secrete CXCL4 which is the first and most abundant chemokine identified in releasates from activated platelets and which is involved in a wide range of physiological processes

such as proliferation and angiogenesis. This chemokine is also involved in numerous pathological processes. High levels of CXCL4 are positively correlated with Crohn's disease activity index (Simi et al., 1987). In Heparin-induced thrombocytopenia (HIT), autoantibodies developed against high molecular complexes of CXCL4/heparin or CXCL4/GAG side chains. The presence of HIT antibodies can lead to platelet activation and depletion through platelet consumption in venous thrombosis (Greinacher, 2009). CXCL4 exerts chemotactic activities on different cells including neutrophils, monocytes (Deuel et al., 1981), and activated T-lymphocytes in a pertussis toxin-sensitive manner (Mueller et al., 2008). Recently, CXCL4 has also been shown to be able to induce a specific macrophage type with specific phenotypic and functional characteristics (Gleissner, 2012). Moreover, it promotes adhesion of neutrophils on endothelial cells (Petersen et al., 1999). Although CXCL4 has been reported to bind to and stimulate CXCR3 and a splice variant thereof (CXCR3B), the functional importance of these two receptors for the biological activity of CXCL4 is not clear. For instance, a recent report found CXCL4 to be involved in ligand driven monocyte down-regulation of chemokine receptors CCR1, CCR2, and CCR5 by releasing the respective ligands (CCL2-4) from CXCL4-activated monocytes in absence of CXCR3 highlighting the connection between platelets and monocytes (Schwartzkopff et al., 2012). CXCL4 can induce exocytosis and firm neutrophil adhesion to endothelium when incubated with the appropriate co-stimuli. A contribution of CXCL4 in cardiovascular diseases has been described in both human and mouse where CXCL4 has been found in the endothelium, neovasculature, macrophages, and calcified regions of atherosclerotic carotid arteries (Pitsilos et al., 2003). Moreover, a strong positive correlation between both luminal and neovascular CXCL4 staining and coronary artery disease and between CXCL4 in macrophages and the presence of symptomatic atherosclerotic disease has been found. In a murine model of atherosclerosis, the knock-out of CXCL4 has been shown to exert an atheroprotective effect reducing atherosclerotic lesion formation (Sachais et al., 2007). Activation of platelets results in a release of stored-P-selectin -CXCL4 and CCL5 from granules. CCL5 has been detected on the luminal surface of atherosclerotic murine and human carotid arteries or neointimal lesions after arterial injury and can be deposited on inflamed or atherosclerotic endothelium by activated platelets, thereby triggering monocyte recruitment under flow (von Hundelshausen et al., 2001; Schober et al., 2002). The deposition of platelet chemokines can be facilitated by platelet-derived microparticles (Mause et al., 2005). Injection of activated platelets into the tail vein of atherosclerosis prone mice results in exacerbated atherosclerotic lesions and increased endothelial deposition of CXCL4 and CCL5 dependent on the presence of P-selectin (Huo et al., 2003). Therefore, platelet adhesion molecules such as P-selectin are mediating transient interactions with endothelial cells enabling a local delivery of soluble chemokines. We have discovered that heterophilic interactions between CXCL4 and CCL5 (see below) are responsible for enhanced monocyte recruitment into the arterial wall which explains to a certain extent why activated platelets are strong promoters of atherosclerosis. Peptides inhibiting the association of CXCL4 and CCL5 decrease atherosclerosis and macrophage content of lesions (von Hundelshausen et al., 2005; Koenen et al., 2009).

A non-allelic variant form of CXCL4, called CXCL4L1 or PF4ALT which differs only in three amino acids in the C-terminal α-helix of the protein has been identified in different kind of cells including leukocytes, endothelial, or smooth muscle cells (Lasagni et al., 2007). CXCL4L1 is capable of inducing endothelial cell chemokinesis and has been characterized as a potent anti-angiogenic regulator similar to CXCL4. Important differences of CXCL4L1 and CXCL4 are a lower affinity for CCL5 (Sarabi et al., 2011) and GAGs, e.g., heparin (Dubrac et al., 2010). Substantiating the role of CCL5–CXCL4 heterodimers CXCL4L1 failed to increase CCL5-triggered monocyte adhesion (Sarabi et al., 2011). The decreased affinity of CXCL4L1 compared to CXCL4 may have critical implications for cell adhesion since CXCL4L1 will not be retained at its site of expression. The existence of heparan sulfate in the subendothelial extracellular matrix has been found to regulate the arrest function of CCL5 and CCL4/MIP-1β (Gilat et al., 1994). On the endothelial surface under flow conditions, both platelet-derived and recombinant CCL5 are able to bind to activated endothelium and to trigger the firm arrest and transmigration of monocytes (von Hundelshausen et al., 2001).

Moreover, the oligomerization of CCL5 is crucial for CCR1-mediated leukocyte arrest on inflamed endothelium but not for their transmigration via CCR5 (Baltus et al., 2003). In bronchial mucosa of patients with chronic obstructive pulmonary disease (COPD), CCL5, and to a lesser extent CXCL7, have been found to be the most abundant chemokine expressed in the bronchial epithelium and are associated with an increase of neutrophil activation (Di Stefano et al., 2009).

CXCL12 or SDF-1α, which is the ligand for CXCR4 and CXCR7, has both proatherogenic and antiatherosclerotic properties (Weber et al., 2011). Blocking the CXCR4–CXCL12 axis leads to a release of different leukocyte subsets into the circulation. In this context, monocytosis and neutrophilia are conditions positively correlated with the development and severity of atherosclerosis. On the other hand has CXCL12 been demonstrated to be crucial for the healing of arterial lesions by the regenerative capacity of progenitor cells which are attracted to adhere be CXCL12 (Massberg et al., 2006). In addition to its production by platelets, CXCL12 is expressed in the bone marrow and in cells directly relevant to atherogenesis, including endothelial cells, smooth muscle cells, and leukocytes, which enables it to regulate the trafficking and localization of immature and maturing leukocytes, including bone marrow stem cells, neutrophils, T cells, and monocytic cells (Abi-Younes et al., 2000; Zeiffer et al., 2004; Stellos et al., 2009). Furthermore, CXCL12 has been thought to play a pro-inflammatory role in various autoimmune diseases, especially in rheumatoid arthritis and nephritis, in murine lupus erythematosus as well as in ongoing experimental autoimmune encephalomyelitis (Meiron et al., 2008; Karin, 2010). Recently, changes in CXCL12 signaling patterns have been found to be necessary for bone marrow neutrophil mobilization and are involved in polymicrobial sepsis, where its inhibition resulted in peritoneal cavity neutropenia (Delano et al., 2011). While both CCL5 and CCL7/MCP-3 are able to activate and to induce the chemotaxis of eosinophil and basophil granulocytes in allergy (Baggiolini and Dahinden, 1994), CCL11/Eotaxin has been found

to be a powerful attractant for eosinophils and has also been identified in atherosclerotic lesions (Baggiolini et al., 1997; Haley et al., 2000).

The expression of CXCL7 is restricted to the platelet-lineage. Proteolytic cleavage of the carboxy-terminal part of pro-platelet basic protein (PPBP) and the proteolytic removal of the N-terminal part of PPBP produces two other chemokines namely connective tissue-activating peptide III (CTAP-III) and beta-thromboglobulin (beta-TG; Walz and Baggiolini, 1990; von Hundelshausen et al., 2007). Dependent on CXCR2, CTAP-III, and CXCL7 promote neutrophil and monocyte adhesion to human endothelial cells under flow conditions, respectively (Schenk et al., 2002; Baltus et al., 2005). The chemotactic potential of CXCL7 is also enhanced in COPD patients (Traves et al., 2004).

CXCL5/ENA-78 has been shown to act as a potent chemoattractant and activator of neutrophil function via CXCR2 (Ahuja and Murphy, 1996). CXCL5 has also been found to be strongly correlated with the number of neutrophils in patients with acute respiratory distress syndrome (Goodman et al., 1996).

During early atherosclerosis, CXCL1/GRO-α immobilized on the surface of endothelial cells via heparin proteoglycans induces the firm adhesion of rolling monocytes expressing CXCR2 (Schwartz et al., 1994; Huo et al., 2001; Boisvert et al., 2006). Moreover, a recent study has shown that *in vivo*, lysophosphatidic acid increased the progression of atherosclerosis and recruited leukocytes to the vessel wall during early atherogenesis via lysophosphatidic acid receptor-mediated release of endothelial CXCL1 (Zhou et al., 2011). A study conducted on elderly COPD patients has also indicated that CXCL1 might be a relevant candidate biomarker for this disease (Tsai et al., 2010).

CHEMOKINES IN MAST CELLS

In addition to their role as sentinels in the recognition of pathogens, mast cells (like platelets) are able to communicate with immune cells facilitating the recruitment of leukocytes to sites of infection. Indeed, mast cells are able to produce different chemokines including CCL4, CXCL8, or CCL11 assisting in the recruitment of $CD8^+$ T cells, eosinophils, and natural killer cells, respectively (Abraham and St John, 2010).

CCL3/MIP-1α and CCL4/MIP-1β can initiate diverse cellular responses that regulate both acute and chronic inflammation via their interaction with CCR1 and CCR5. In addition, proteoglycan-bound CCL4 is used to effectively activate and induce the adhesion of circulating lymphocytes for their extravasation through lymph node endothelium (Tanaka et al., 1993). The quaternary structures of CCL3 and CCL4 are decisive for their biological activity. Aggregation of CCL3 and CCL4 can be considered as polymerization processes of MIP-1 dimers, which constitute the basic unit of MIP-1 proteins. MIP-1 monomers form dimers of the CC-type by creating an anti-parallel β-sheet of the N-termini (Lodi et al., 1994; Czaplewski et al., 1999; Ren et al., 2010). MIP-1 dimers associate to polymers consisting up to 50 units forming a double helixed rod like structure. Polymerization of MIP-1 protects MIP-1 from proteolytic degradation while the positively charged region of MIP-1, which is crucial for the receptor binding, is buried. The continuous and slow release of monomers from the polymer leads to a shallow gradient with a long gradient and effective range for leukocyte recruitment.

CXCL8/interleukin-8/IL8 has been found in intracellular granules from skin mast cells and mast cell lines (Möller et al., 1993). Recently, Kim et al. (2010) have shown that CXCL8 synthesis is induced via the leukotriene B4/leukotriene B4 Receptor 2 pathway in response to IL-1β in human primary mast cells and mast cell line HMC-1. CXCL8 released by mast cells is implicated in the selective chemotaxis of CXCR1-expressing natural killer cells (Burke et al., 2008). CXCL8 also induces neutrophil migration and activation by binding to G-protein-coupled receptors on their surface, namely human CXCR1 and CXCR2 (Wuyts et al., 1998). During inflammation, CXCL8 is produced and presented to the endothelial surface in association with GAGs. In a recent study, using obligate monomeric and dimeric IL8 mutants, the oligomerization state of CXCL8 was shown to have an influence on the kinetics of the neutrophil extravasation. The dimeric form initiated a fast robust but short lived vascular efflux whereas the monomeric form resulted in a weaker but longer-lasting response (Das et al., 2010). Also, this chemokine is among the most important in the recruitment of inflammatory cells, mostly neutrophils, in COPD (Barnes, 2004).

CHEMOKINES IN DENDRITIC CELLS: CCL17 AS EXAMPLE

CCL17/TARC (thymus and activation regulated chemokine) together with CCL22/MDC (macrophage-derived chemokine) are expressed in relevant amounts by mature dendritic cells but occur as well in other cell types such as fibroblasts. CCL17 is constitutively expressed in the thymus (Saeki and Tamaki, 2006). CCL17 is a ligand for CCR4, which is predominantly expressed on Th2 lymphocytes, basophils, and natural killer cells. Recently, dendritic cell-derived CCL17 has been found to be critical in atherosclerosis (Weber et al., 2011). Indeed, deficiency of CCL17 in $Apoe^{-/-}$ mice results in a reduction of the plaque formation in aortic root since CCL17 inhibits the expansion of atheroprotective Tregs and attracts $CD4^+$ and $CD3^+$ T cells.

MEMBRANE-BOUND CHEMOKINES

In addition to different types of cells such as T cells, macrophages, cytokine-induced smooth muscle cells, and endothelial cells, CXCL16/SR-PSOX has been recently identified for the first time in platelets (Seizer et al., 2011). This protein constitutes an atypical chemokine because it is expressed as a cell surface bound molecule but is also found in a soluble form after shedding. CXCL16 has also been involved in different diseases. Thus, a low plasma concentration of CXCL16 has been associated with coronary artery disease and has been found in atherosclerotic lesions in human and mice (Wuttge et al., 2004; Sheikine and Hansson, 2006). *In vivo* and *in vitro*, homocysteine, a homolog of cysteine that can promote atherosclerosis (Harker et al., 1976), has been found to stimulate CXCL16 production and deposition on the surface of endothelial cells via both production of ROS and a PPARγ-dependant pathway, thereby increasing adhesion of lymphocytes to endothelial cells (Postea et al., 2008).

Like CXCL16, CX3CL1 is an atypical multimodular chemokine that exists both in a membrane-tethered or soluble form. The immobilized form consists of a chemokine domain anchored to

the plasma membrane through an extended mucin-like stalk, a transmembrane helix, and an intracellular domain. Besides, CX3CL1 has an anti-apoptotic and a proliferative effect on smooth muscle cells (White et al., 2010). Previous data have shown that CX3CL1 could serve as an adhesion molecule (Fong et al., 1998; Goda et al., 2000). However, more recent data indicated that, although CX3CL1 might mediate leukocyte adhesion, this phenomenon occurred only under low shear force and not under physiological conditions (Kerfoot et al., 2003). Regarding endothelial cells, CX3CL1 is expressed on the surface of IFN-γ/TNF-α-activated HUVEC and promotes leukocyte adhesion to atherosclerotic mouse arteries *in vivo* and under arterial flow *in vitro*. More precisely, CX3CL1 expressed by inflamed endothelial cells is recognized by CX3CR1 on activated platelets. Ligation of platelet CX3CR1 results in platelet activation and subsequent exposure of P-selectin on the surface of adherent platelets (Schulz et al., 2007). The inflamed CX3CL1-expressing endothelial cells can also recruit the non-classical subset of monocytes which highly express CXC3CR1 (Geissmann et al., 2010). Under homeostatic conditions, the disruption of the CX3CL1–CX3CR1 axis leads to a specific reduction of circulating non-classical monocytes in mice (Landsman et al., 2009). Addition of full-length recombinant soluble CX3CL1 to human monocytes has also shown to decrease apoptosis triggered by serum deprivation or treatment with 7-β-hydroxycholesterol. This reduction of apoptosis occurred in both CX3CR1-expressing $CD14^{++}CD16^{-}$ and $CD14^{+}CD16^{+}$ monocyte subsets. However, the precise mechanism is still unclear. A recent study shows that platelets over-expressing CX3CR1 on their surface are recruited alone or in association with monocytes to the site of inflammation. This phenomenon might contribute to an acceleration of atherosclerotic lesions (Postea et al., 2012).

CHEMOREPULSION

Another aspect to take into consideration in the involvement of chemokines in leukocyte trafficking is the fact that chemokines could favor a "flight" of leukocytes from a tissue to reach the blood circulation or another tissue. In this case, leukocytes might run away from a chemokine gradient. This reverse migration from a peak concentration of chemokine is named chemorepulsion or fugetaxis. However, chemorepulsion refers more to a mediator that, depending on its concentration, can either repel or recruit cells using the same receptor. This phenomenon has been comprehensively studied in the context of T-cell trafficking during the process of thymic emigration and for which an extensive review has been recently published (Bunting et al., 2011). It has been suggested that chemorepulsion could participate in the thymic egress of human thymocytes. Thus, high concentration of CXCL12 has been shown to repulse human single positive thymocytes *in vitro* and this "run away" could be abolished using a neutralizing CXCL12 antibody (Poznansky et al., 2000). Moreover, this chemokine has also been shown to be a chemorepulsive agent of firm adhesion to activated pancreatic islet microvascular endothelium for both diabetogenic CD4 and CD8 T cells from NOD/LtJ mice. This repulsion results in a decrease of T-cell integrin activation in a CXCR4-independent manner (Sharp et al., 2008). Using a modified flow chamber containing a transwell insert on which HUVECs are cultured, Lee et al. (2009) have shown that T cells that have extravasated in response to subendothelial CCL5 may intravasate after exposure to subendothelial CXCL12 under flow conditions. High concentration of a chemokine, as already observable in the typically bell shaped response upon increasing chemokine concentration, is an important factor. However the exact molecular mechanisms of chemokine-induced chemorepulsion are still ill defined. Using a CXCL12 model, Zlatopolskiy and Laurence (2001) postulated that chemokine-mediated repulsion would be triggered by an excess of free ligand in the vicinity of the cell that would lead to a dimerization of the receptor, followed by an internalization of the ligand/receptor complexes. Internalization, digestion of the ligand, and recycling of the receptors would be realized under the same way than during the chemoattraction process. The difference would take place through the localization of the recycled receptors. The reappearance of the internalized receptors may occur not on the apical side of the cell but on the basal side resulting in a reverse movement. Summarizing, the gradient dependent direction of a chemokine triggered movement is concentration dependent. Thus, at least two different signaling pathways have to exist at the beginning, converging later again to reorganize the cytoskeleton for cell polarization and movement. Possible explanations for the chemorepulsion at high concentrations and chemoattraction at low concentrations are the chemokine dimerization at high concentrations, high- and low-affinity binding sites for chemokines on their cognate receptor, rapid recycling of GPCRs, apical rearrangements of recycled GPCRs, the oligomerization or homodimerization of GPCR with receptor and non-receptor proteins, and allosteric mechanisms.

GENETIC VARIATIONS IN CHEMOKINE GENES

Different studies have been carried out in order to evaluate the relationship between chemokine/chemokine receptor genes and inflammatory diseases including cardiovascular diseases. **Table 3** provides several examples illustrating the association of chemokine/chemokine receptor polymorphisms with cardiovascular diseases.

In order to identify genes involved in cardiovascular diseases and before the emergence of genome-wide association studies (GWAS), many efforts have been undertaken with gene candidate studies. In these studies several chemokine or chemokine receptor gene candidates have been found to be associated with cardiovascular diseases. For instance, a polymorphism in the promoter region of the *CCL5* gene called rs2107538 has been found associated with coronary artery disease (Simeoni et al., 2004). However, an extensive analysis based on the MONICA/KORA Augsburg Case-Cohort, Athero-Express, and CARDIoGRAM Studies has been recently carried out. Though an association between high CCL5 levels and an unstable plaque phenotype has been found, no associations of either CCL5 serum levels or its content in carotid plaques or its different genotypes with CAD or other coronary events has been established (Herder et al., 2011). The result of this study suggests that CCL5 protein levels and its gene variants might not be considered as biomarkers for the risk of coronary events in humans. As discussed by Altshuler et al. (2008), studies of candidate genes are performed on specific variants that have a small *a priori* probability of being disease-causing. Those studies are also able to generate false positives due to the lack of knowledge of the

Table 3 | Examples of chemokine/chemokine receptor single nucleotide polymorphisms (SNP) associated with cardiovascular diseases.

Gene	SNP	Associated with	*p*-Value	References
CCL2	rs1024611	Myocardial infarction	0.005 and 0.009[a]	McDermott et al. (2005)
			<0.001 and 0.001[b]	
		Coronary artery disease	<0.005	Szalai et al. (2001)
CCL5	rs2107538	Acute coronary syndrome	0.0073	Simeoni et al. (2004)
		Coronary artery disease	0.0038	
CCL11	rs1129844	Myocardial infarction	0.012 and 0.008[c]	Zee et al. (2004)
CXCL5	rs352046	Acute coronary syndrome	0.005	Zineh et al. (2008)
CXCL8	rs4073	Acute coronary syndrome	0.004	Zhang et al. (2011)
CXCL12	rs1746048	Myocardial infarction (early onset)	1×10^{-8}	Kathiresan et al. (2009)
		Atherosclerosis severity and progression	0.009	Kiechl et al. (2010)
		Coronary artery disease	3×10^{-10}	Schunkert et al. (2011)
	rs1801157	Myocardial infarction	0.007	Luan et al. (2010)
	rs501120	Coronary heart disease	1.4×10^{-6}	Franceschini et al. (2011)
		Myocardial infarction	0.002	Qi et al. (2011)
		Coronary artery disease	9.46×10^{-8}	Samani et al. (2007)
CCR2	rs1799864	Heart failure	0.015	Ortlepp et al. (2003)
		Myocardial infarction	0.007	Ortlepp et al. (2003)
			0.054[d]	Petrkova et al. (2003)
	rs34948438	Myocardial infarction	0.0013[e]	Karaali et al. (2010)
			0.0016[f]	
CCR5	rs333	Myocardial infarction	0.001	Kallel et al. (2012)
			0.0013	Karaali et al. (2010)
		Severe calcific aortic stenosis	0.037[g]	Ortlepp et al. (2004)
		Myocardial infarction	0.003[h]	Singh et al. (2012)
CX3CR1	rs3732379	Coronary artery disease	0.03	McDermott et al. (2001)
		Acute coronary syndrome	0.001	Moatti et al. (2001)
		Single in-stent restenosis	0.006	Niessner et al. (2005)
		Recurrent in-stent restenosis	0.011	
		Myocardial infarction	0.006[i]	Singh et al. (2012)

[a] *In multivariable adjustment and multivariable adjustment of pooled-sex cohort, respectively.*

[b] *In multivariable adjustment and multivariable adjustment of male cohort, respectively.*

[c] *In an age and smoking and body mass index, hypertension, diabetes, and randomized treatment assignment adjusted recessive model of inherence, respectively.*

[d] *In female cohort.*

[e] *In patients carrying CCR5 rs34948438 wildtype (wt)/deletion (Δ) genotype.*

[f] *Individuals carrying the CCR5 rs34948438 heterozygote or homozygous variant genotype (Δ/Δ + wt/Δ).*

[g] *In patients carrying the CCR5 rs333 SNP or CTGF -447C allele.*

[h] *In individuals carrying both CCR5 rs1799987 and rs333 SNPs.*

[i] *In individuals carrying both CX3CR1 rs3732378 and rs3732379SNPs.*

genetic background of cases and controls. This could explain the low reproducibility in candidate gene studies and lack of recovery between GWAS and candidate gene studies.

Amongst the different GWAS for cardiovascular disease performed during the last years, chemokine *CXCL12* gene polymorphisms have been associated with CAD (e.g., Samani et al., 2007; Kathiresan et al., 2009; Franceschini et al., 2011; Schunkert et al., 2011). In addition, the study conducted by Mehta et al. (2011) found the CAD risk locus 10q11 to regulate the level of CXCL12 transcripts.

CHEMOKINE SYNERGISM BY HETEROMERIZATION

The regulation of chemokine activity during initiation and development of inflammatory diseases is crucial to reach a fast and directed response. There is evidence that the activity of chemokines can be modulated by posttranslational processing (reviewed by Proost et al., 2003) and synergistic cytokines, e.g., IFN-γ (Mortier et al., 2011). Especially at the early phase of inflammation the concentration of a specific chemokine might not be high enough for a sufficient cell response. Hence synergism would aid to speed up the chemokine-induced response of leukocyte migration and to increase combinatorial specificity (Gouwy et al., 2005; Paoletti et al., 2005). A mixture of low concentrated individual synergizing chemokines behaves like the receptor agonist at an adequate concentration. Although the synergism of some single chemokines has been explored, so far a complete overview how many chemokines are involved is still elusive. It was previously shown that chemokine receptor induced chemotaxis may be

enhanced by addition of chemokines which have *per se* no effect are not cognate ligands of the respective receptor and are called synergy-induced chemokines (Paoletti et al., 2005). Currently it is still unclear how this effect may be mediated in detail. Several underlying mechanisms are conceivable and can depend mainly on the respective chemokine partners and their receptors. It is possible that homeostatic and inflammatory chemokines that exhibit a different functional activity can form heteromers and act together in a synergistic way. Furthermore the signaling of GPCR-agonists can be enhanced by non-ligand CXC- and/or CC-type chemokines. Additionally, the GPCR-agonist mono and dimer equilibrium may regulate the signaling of the specific GPCR, which results in a different recruitment pattern of the target cells (Drury et al., 2011). It is of strong interest how the chemokine–chemokine interactions occur *in vivo* but it is difficult to find feasible approaches for a direct observation of the processes in living organisms. Some examples for a chemokine–chemokine synergism are given in the next parts.

CHEMOKINE HETEROMERIZATION

Interaction between receptor agonist and non-ligand chemokines influences the activity of the chemokine receptor. All chemokines exhibit a typical tertiary structure homology which consists of a disordered N-terminus followed by three anti-parallel β-strands and the C-terminal α-helix. The quaternary structures of CC and CXC chemokines are different. Whereas the CXC-type forms dimers with a central β-sheet, the CC-type dimerizes through the interaction of both N-termini. In case of CC- and- CXC-type heteromers it is difficult to predict the proper structure. Our workgroup previously showed the synergistic interaction of CXCL4 and CCL5 to accelerate atherosclerosis by triggering monocyte arrest on endothelium (von Hundelshausen et al., 2005; Koenen et al., 2009). The synergistic effect is based on the heteromerization of these two chemokines, since peptides disrupting the heteromers abolish this synergism. Interestingly, the quaternary structure of the CCL5–CXCL4 complex features a CC-type heteromer, which exhibits paired N-termini, yet results in better receptor activation.

The response to CCR4 in skin-homing T-lymphocytes is enhanced by co-expressed chemokines in the inflamed skin. For example the CXCR3-agonist CXCL10 enhances the chemotaxis of CCR4-transfected preB-cells and T cells due to interaction with the CCR4-agonist CCL22 (Sebastiani et al., 2005). Further enhancement of the CCR4 activity evolves from the direct interaction of CCL22 with the CCR7-agonist CCL19. In addition, CCL22 was also shown to interact with the CCR3-agonist CCL7. In this last case, it was shown that a sequence of five amino acids of the first β-strand from CCL7, which contains two positively charged arginine residues, is needed to synergize with CCL22 and hence increases the CCR4 activation. In the same study a CCL4–CCL7 chimera lost the synergetic activity, being generated by substituting the first β-strand of CCL7 with that of the non-synergizing CCL4, lacking the positively charged amino acids. Thus the first β-strand of a chemokine, containing positive and hydrophilic amino acids, seems to have a crucial role in synergism and heteromer formation.

Furthermore monocyte recruitment is enhanced by the homeostatic chemokines CCL19 and CCL21 which are both CCR7-agonists. They synergize with CCL7 and CCL2 that result in an augmented CCR2 response to recruit monocytes (Kuscher et al., 2009). Interestingly the induced monocyte recruitment by CCL7 is enhanced 100 times by CCL19 and CCL21 whereas CCL2 showed less synergistic activity. By comparing a specific motif, comprising five amino acids in the first β-strand of all four chemokines, it has further been shown that CCL7 and CCL21 exhibit more positively charged amino acids which correlates with a higher synergistic effect confirming the importance of the first β-strand. Synergism of chemokines by heteromerization was also shown for other chemokines (Paoletti et al., 2005; Allen et al., 2007; Koenen et al., 2009). The authors suggest that heteromers of synergistically acting chemokines lead to a high affinity conformation of the respective receptor. Another study (Venetz et al., 2010) showed that heteromerization of CXCL12 with the inflammatory CXCR3-agonist CXCL9 results in a higher response of CXCR4-expressing T cells and malignant B cells on tumor vasculature.

ANTAGONISM BY CHEMOKINE DIMERS

Even if the neutrophil migration toward CXCL8 is enhanced by different CXC- and also CC-chemokines, i.e., CCL2 and CXCL12, the dimerization of CXCL8 decreases its binding to CXCR1 (Fernando et al., 2004; Weber and Koenen, 2006). This effect might not be due to structural change but rather to a loss of conformational flexibility which leads to a low-affinity configuration. Thus the dimer is not competent enough to bind the receptor N-domain. Moreover, heteromerization of CXCL8 with CXCL4 reduces the chemotactic propensity of CXCL8 (Dudek et al., 2003). These heterodimers enhance the anti-proliferative effect of CXCL4 on endothelial cells in culture, and the CXCL8-induced migration of CXCR2 transfected Baf3 cells as well (Nesmelova et al., 2005; Weber and Koenen, 2006). Inhibition of CXCL8-induced monocyte arrest is evoked by CXCL4. This effect might also be due to a less flexible CXCL8 molecule that has a lower affinity for its receptor. However, the availability of the monomer-dimer equilibrium of CXCL8 is crucial to regulate tissue-specific neutrophil recruitment given that the recruitment profile differs due to altered GAG-binding interaction (Gangavarapu et al., 2012).

Recently, it could be shown that a monomeric or dimeric state of CXCL12 plays a crucial role for the CXCR4 activation and its mode of signaling (Ray et al., 2012). The dimeric CXCL12 activates recruitment of β-arrestin 2 to CXCR4 and chemotaxis of CXCR4-expressing breast cancer cells, whereas the monomeric CXCL12 promotes the CXCR4 signaling through Gαi and Akt. Furthermore, another recent study (Drury et al., 2011) demonstrated that monomeric CXCL12 compared with the dimeric variant exhibits more contact sites for CXCR4 and thus results in different receptor signaling. To our knowledge it has not been tested, but supposedly a different or even inverse activity, e.g., the chemorepellent activity of higher concentrated CXCL12, may well be dependent on the preponderance of CXCL12 dimers.

Not only chemokine heteromerization may influence receptor driven signal transduction but as well the homooligomeric state changes the biological activity by either buried receptor binding sites, e.g., in polymeric MIP-1 or the different kinetics of monomeric versus dimeric CXCL8. These insights will be helpful to develop specific drugs interfering with oligomerization motifs thereby suppressing or enhancing desired chemokine effects.

BINDING TO GPCRs

In order that chemokine–chemokine partners unfold synergism it is suggested that first chemokine heteromers form and subsequently receptor binding follows. Besides the formation of a heteromeric chemokine complex, the binding to a receptor is required to mediate the synergistic effects. GAGs, as co-receptors of GPCRs, can also induce heteromerization of chemokines (Crown et al., 2006). In addition, it is speculated that instead of heteromerization, as mentioned above, different receptor binding sites for CCL2 and CCL7 are responsible for the synergistic activity as it was previously shown for CXCR3-agonists (Colvin et al., 2004).

Homeostatic chemokines like CCL21, CCL19, CXCL12, and CXCL13 are synergizing to promote a regulated lymphocyte trafficking across the lumen or basal lamina of high endothelial venules (HEVs) in lymph nodes. For example, CXCL12 augments through its receptor CXCR4 the CCR7-induced chemotaxis of T cells and therefore helps to transfer them across the HEVs without direct interactions with the CCR7-ligands CCL19 and CCL21 (Bai et al., 2009). Here the signaling through CXCR4 has a major impact because in T cells, deficient in CXCR4, no cooperative effect was observed. The synergistic effect is merely evident at suboptimal concentrations of the CCR7-ligands CCL19 and CCL21. In summary, CXCL12–CXCR4 signaling has the ability to cause a maximal T-cell response by a suboptimal CCR7-ligand concentration. A similar observation was also previously shown for CXCL13 (Paoletti et al., 2005; Bai et al., 2009). However, it is remarkable that the heteromerization of CXCL13 with CCR7-ligands is thought to be the responsible mechanistic reason for synergism, whereas synergy of CXCL12 with CCR7-ligands is independent of direct chemokine–chemokine interaction. In fact, it is assumed that CXCL12 increases the CCR7-signaling by ERK phosphorylation and actin polymerization in T cells (Bai et al., 2009). A similar conclusion was provided by van Damme's group who showed that the synergism of CXCL8 or CXCL12 with CCL2 is mediated through CXCR1/2 (CXCL8) and CXCR4 (CXCL12; Gouwy et al., 2008, 2009). When the concentration of CCL2 is low CXCL8 helps to chemoattract monocytes. This requires binding of CXCL8 to CXCR1 and CXCR2. A further example is the synergism of CXCL12 with CCL2 where correct binding and signaling to CXCR4 and CCR2 is essential for synergistic interactions. Additionally a recent study has shown that CCR1-agonists like CCL5 and CCL3 are enhancing CXCR4-induced ERK phosphorylation and chemotaxis of mononuclear cells and it was further observed that this cooperative effect is inhibited by blocking CCR1 with specific antibodies and AMD3100 (Gouwy et al., 2011).

In summary, synergism of chemokines crucially depends on increased activation of the GPCR by heteromerization of ligand and non-ligand chemokines or cooperative interactions after chemokine activation of distinct GPCRs. Heteromerization of receptors has been observed. However the mechanistic role of these complexes in respect of ligand binding is still unclear, it might be that chemokine heteromers can stabilize and change the functional activity of receptor heteromers (Thelen et al., 2010; Kramp et al., 2011).

THERAPEUTICS

GPCRs as therapeutic targets have been reviewed extensively (Rek et al., 2009; Koenen and Weber, 2010b, 2011; Bennett et al., 2011; Schall and Proudfoot, 2011; Kanzler et al., 2012). A lot has already been done and it is still in progress to find appropriate therapeutic drugs, particularly for the prevention and treatment of HIV. Since chemokines and the subsequent receptor signaling are involved in many diseases, there is hope that good antagonists will increase the means to treat them.

The different interactions between chemokines, which result in a changed biological activity, can be used to find new targets against inflammatory diseases. There are several possibilities for therapeutically targeting chemokines involved in inflammation. In the next part, several examples are illustrating how to alter inflammatory properties by blocking heterophilic interactions, multiple chemokine axes, direct blocking of chemokine receptors as well as blocking of GAG-binding sites.

MODIFIED CHEMOKINES

Modifying the target chemokine is one option to create antagonists since changing the molecular structure leads to a different binding pattern and receptor response. Especially the N-terminal part is crucial for receptor signaling and thus a change in this domain can lead to alteration or loss of receptor activation. Variants of chemokines with an extended or modified N-terminal part are for example N-methylated CCL5 (Met-RANTES) or aminooxypentane-RANTES which block the CCL5 receptors CCR1, CCR3, and CCR5 (Proudfoot et al., 1996; Elsner et al., 1997; Proudfoot et al., 1999; Veillard et al., 2004). In liver fibrosis and atherosclerosis it was shown that inhibiting CCL5 receptors through Met-RANTES was sufficient to reduce inflammation in mice (Veillard et al., 2004; Berres et al., 2010). This phenomenon was observed *in vivo* and *in vitro*. Another example is R6H-CXCL8, a variant of CXCL8 with substitutions on the conserved ELR-triad and CXC-motif which exclusively activates CXCR1 without effecting CXCR2. This is based on a distinct CXCL8 binding mechanism: the CXCR2 activation is mediated by the N-terminal ELR- and CXC-motif whereas the N-loop of CXCL8 is essential for CXCR1 activation (Sarmiento et al., 2011). Furthermore, the above mentioned CXCL8 variant displays anti-inflammatory properties since it activates CXCR1 by desensitization of the CXCR2 response in human neutrophils. In fact, this agonist could help to clarify the biological and physiological function, especially of CXCR1, in inflammatory diseases. Thus R6H-CXCL8 is a potential candidate as a therapeutic molecule.

GLYCOSAMINOGLYCAN BINDING AFFINITY

Most chemokines have the ability to bind GAGs located on the cell surface. The enhancement or reduction of this property can diminish the GPCR signaling by an indirect blockade of chemokine binding to its receptor, since GAGs are co-factors for GPCR activation. It is assumed that chemokines first bind the GAG co-receptor followed by GPCR activation.

A variety of chemokines was previously designed with altered GAG-binding affinities resulting in a loss of GPCR activation (Proudfoot et al., 2008; Shahrara et al., 2008; Rek et al., 2009). The activity of the pro-inflammatory chemokine CCL5 depends

on the binding to GAGs. The substitution of positively charged residues into alanine in the 40s loop ([44AANA47]-CCL5mutant) results in defective heparin binding and loss of the ability to recruit monocytes. The heteromerization of both CCL5 variants leads to non-functional heteromers with a lack of GAG-binding efficiency (Johnson et al., 2004; Koenen and Weber, 2010b). Another study (Braunersreuther et al., 2008) additionally confirms [44AANA47]-CCL5 as a potential therapeutic agent against atherosclerosis. But in contrast to Met-RANTES, [44AANA47]-CCL5 does not directly abolish GPCR activation. Thus variations of CCL5 mutants acting in different ways can lead to anti-inflammatory properties by a direct blockade of the GPCR or by indirect inhibition through prevention of chemokine binding to GAGs on the cell surface. Another way to block chemokine activity using the affinity for GAGs is to design dominant-negative mutants with a higher GAG-binding affinity compared to the wild type chemokine (Brandner et al., 2009). H23K-RANTES showed attenuation of autoimmune uveitis in rats based on displacement of wild type CCL5 from its proteoglycan-co-receptor. Mutants with increased GAG-binding potential were designed for CCL2, as well. The PA508 mutant of CCL2 exhibits no ability for CCR2 activation but a fourfold higher GAG affinity compared to the wild type CCL2 (Piccinini et al., 2010). In a recent study in mice, PA508-CCL2 showed prevention of neointima formation and reduction of tissue damage after myocardial infarction without notable side effects. Therefore, it could be a candidate as a therapeutic agent in reducing restenosis in stents (Liehn et al., 2010). Additionally, a mutant of CXCL12 with a deficiency in heparan sulfate binding can still transduce signals through CXCR4 but is not able to promote transendothelial migration *in vitro*. *In vivo* experiments could further show that this mutant efficiently down-regulates the CXCR4 expression and desensitizes the chemotactic response toward CXCL12. Hence, this modified chemokine might work in anti-inflammatory therapies (O'Boyle et al., 2009).

SMALL MOLECULES AND ANTIBODIES

The development of small molecules blocking GPCR activation is a powerful tool for the treatment of inflammatory diseases. Major efforts have been done to find drugs for blocking HIV infection. Maraviroc (Celsentri/Selzentry; Pfizer) was established as a functional anti-HIV drug by blocking CCR5 as important entry receptor. In inflammatory diseases, like atherosclerosis, TAK779 and nbI-74330 antagonists for CCR5 and CXCR9, respectively, represent suitable therapeutic agents (Koenen and Weber, 2010b). Antagonizing CXCR4 by TAK779 additionally blocks leukocyte trafficking induced by CXCL12 (Sohy et al., 2009). Recently, DF 2156A was introduced as a novel dual inhibitor of CXCL8 receptors CXCR1 and CXCR2 (Bertini et al., 2012). This dual function is based on a non-competitive inhibition resulting in a stabilized binding between DF 2156A and the two CXCL8 receptors due to formation of specific ionic bonds in the allosteric binding site (Bertini et al., 2012). Some CXCR2- and CCR2-specific antagonists (i.e., reparixin and MLN1202) have already been tested as therapeutic drugs in clinical trials, like MLN1202, which is a CCR2-blocking monoclonal antibody shown to reduce high-sensitivity CRP as surrogate parameter for atherosclerosis (Allegretti et al., 2008; Gilbert et al., 2011).

CHEMOKINE HETEROMERIZATION

As mentioned before some chemokines inherently exhibit synergistic function toward other chemokines which mostly depends on heteromerization. Disruption or changing these critical heterophilic interactions might entail a decrease in the physiological response which has an impact on the degree of inflammation. A prominent example is the heterophilic interaction between CXCL4 and CCL5 which results in a synergistic enhancement of CCL5 induced signaling accompanied by increased monocyte recruitment to the inflamed endothelium (Koenen et al., 2009; Koenen and Weber, 2010a). Interruption of the chemokine heteromerization by cyclic peptides was shown to eliminate synergistic effect *in vitro* and *in vivo*. Recently it was shown (Grommes et al., 2012)that small peptide antagonists, disrupting CXCL4–CCL5 heteromer formation in mouse models of acute lung injury, result in improved lung edema, less neutrophil infiltration, and reduced tissue damage. Thus targeting heterophilic chemokine interactions can act as therapeutic approach by attenuating inflammatory disease in a mild way.

CONCLUSION

It is still elusive which consequences the blockade of one chemokine has when entering clinical trials. For example, the dendritic cell-derived CCL17 could be identified as a catalyzer for atherosclerosis due to interference of Treg homeostasis in mice (Weber et al., 2011). Blocking CCL17 with an antibody abolished this pro-inflammatory effect. Nevertheless the blocking mechanism is unclear and which consequences the blocking has for other physiological signal cascades. For example the CXCL12–CXCR4 axis is crucial for the CXCL12-dependent recruitment of progenitor cells. Consequently a reduction of the CXCR4 level diminishes this important process in regeneration but inversely a decreased expression of CXCR4 was efficient to limit myocardial infarct size in mice (Liehn et al., 2011). Detailed knowledge and clarity of how a specific chemokine oligomerizes, binds to GAG and its GPCR as well as its interaction with other chemokines, with regard of the resulting signal cascade and immune response, are required.

Targeting GAG-binding sites of specific chemokines is a promising approach for developing drugs against chemokine driven diseases, given that the GPCR binding is not directly affected. Also the disruption of chemokine–chemokine interactions seems to become attractive, since synergistic effects can be prevented without reducing the function of the respective chemokine *per se*.

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Arrest functions of the MIF ligand/receptor axes in atherogenesis

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Macrophage migration inhibitory factor (MIF) has been defined as an important chemokine-like function (CLF) chemokine with an essential role in monocyte recruitment and arrest. Adhesion of monocytes to the vessel wall and their transendothelial migration are critical in atherogenesis and many other inflammatory diseases. Chemokines carefully control all steps of the monocyte recruitment process. Those chemokines specialized in controlling arrest are typically immobilized on the endothelial surface, mediating the arrest of rolling monocytes by chemokine receptor-triggered pathways. The chemokine receptor CXCR2 functions as an important arrest receptor on monocytes. An arrest function has been revealed for the *bona fide* CXCR2 ligands CXCL1 and CXCL8, but genetic studies also suggested that additional arrest chemokines are likely to be involved in atherogenic leukocyte recruitment. While CXCR2 is known to interact with numerous CXC chemokine ligands, the CLF chemokine MIF, which structurally does not belong to the CXC chemokine sub-family, was surprisingly identified as a non-cognate ligand of CXCR2, responsible for critical arrest functions during the atherogenic process. MIF was originally identified as macrophage migration inhibitory factor (this function being eponymous), but is now known as a potent inflammatory cytokine with CLFs including chemotaxis and leukocyte arrest. This review will cover the mechanisms underlying these functions, including MIF's effects on LFA1 integrin activity and signal transduction, and will discuss the structural similarities between MIF and the *bona fide* CXCR2 ligand CXCL8 while emphasizing the structural differences. As MIF also interacts with CXCR4, a chemokine receptor implicated in CXCL12-elicited lymphocyte arrest, the arrest potential of the MIF/CXCR4 axis will also be scrutinized as well as the recently identified role of pericyte MIF in attracting leukocytes exiting through venules as part of the pericyte "motility instruction program."

Keywords: chemokine, leukocyte recruitment, arrest, signal transduction, atherosclerosis, inflammation

INTRODUCTION

Leukocyte recruitment and arrest are central steps in inflammatory reactions and associated diseases, including atherosclerosis (**Box 1**). Identifying the main players mediating chemotaxis and arrest is therefore crucial. Boisvert et al. (1998) revealed an important role for the chemokine receptor CXCR2 in mediating monocyte recruitment into atherosclerotic lesions by showing a reduced lesion size and macrophage content in atherosclerosis-prone *Ldlr*$^{-/-}$ mice transplanted with *Cxcr2*-deficient bone marrow. Although mice do not express an ortholog of the CXCL8/IL8 ligand of human CXCR2, the Cxcr2 ligand Cxcl1 (also known as KC/Gro-α) was detected in advanced lesions in mice (Boisvert et al., 1998)[1]. However, a subsequent study in 2006 showed that the reduction in lesion size in *Cxcl1*-deficient *Ldlr*$^{-/-}$ mice didn't exceed half of what was observed in the bone marrow-specific *Cxcr2* knock-out (Boisvert et al., 2006), suggesting the presence of other relevant Cxcr2 ligands with an important role in monocyte recruitment during atherogenesis. In fact, one such factor was uncovered a year later and was found to be the inflammatory cytokine macrophage migration inhibitory factor (MIF). MIF was originally discovered half a century ago as a T-cell-derived factor inhibiting the random migration of macrophages out of capillary tubes and thus was termed macrophage migration inhibitory factor. However, following its cloning and the biochemical characterization and preparation of MIF protein, MIF was later on redefined to be a pleiotropic inflammatory cytokine with critical roles in physiological immunity but also inflammatory diseases and cancer (Bernhagen et al., 1993; Calandra and Roger, 2003). Although the migration inhibitory activity of MIF was not studied and characterized much further, the eponymous name "MIF" was kept up over the years. It was thus largely unexpected, when MIF was identified as a ligand of CXCR2, exhibiting chemokine-like properties, and shown to be a crucial pro-atherogenic factor (Bernhagen et al., 2007). Intriguingly, MIF/CXCR2 interaction

[1]Throughout the manuscript, the letter format of all gene and protein notations was chosen to conform with internationally agreed gene/protein nomenclature guidelines: all letters of human genes/proteins are in uppercase, whereas for mouse genes/proteins, only the first letter is in uppercase and the remaining letters are in lowercase. Gene names are in italics.

Box 1 | The leukocyte adhesion cascade.

Leukocyte arrest on inflamed endothelium can be divided into three main steps: rolling, adhesion, and transmigration. Leukocyte rolling is mediated by the binding of leukocyte-derived PSGL1 to the selectins P-selectin and E-selectin on inflamed endothelial cells (ECs). Next, chemokines triggering their respective G protein-coupled receptor (GPCR) on the leukocyte cell surface promote leukocyte integrin activation, resulting in leukocyte arrest. Finally, leukocytes transmigrate across the endothelium into the vessel wall, which can occur by paracellular (through endothelial junctions) or transcellular route (through the EC body). This three-step model has been refined over the last years, to include tethering (capture), rolling, slow rolling, arrest, adhesion strengthening, intraluminal crawling, and transmigration (Ley et al., 2007).

was found to trigger the recruitment and arrest of monocytes, whereas MIF-mediated T-cell recruitment could be traced to an interaction of MIF and yet another chemokine receptor. This was CXCR4, a CXC chemokine receptor thought to be much more specific regarding its ligand spectrum than CXCR2 (Bernhagen et al., 2007). Interestingly, MIF-mediated monocyte recruitment had previously been described in other inflammatory diseases, such as arthritis and glomerulonephritis (Lan et al., 1997; Morand et al., 2006), but was thought to represent an indirect event at the time. And although the third MIF receptor CD74 was already identified as a MIF-interacting membrane protein in 2003, a direct role of CD74 in MIF-mediated monocyte chemotaxis and arrest was not revealed until the discovery of CXCR2/CD74 complexes in 2007. Interestingly, it is now also clear that CD74 has a role in atherogenesis (Sun et al., 2010).

This review discusses MIF's role as a chemokine-like mediator, addressing its structure, receptor binding capacity and importance in leukocyte recruitment, particularly arrest, in the context of atherosclerosis.

MIF AS AN IMPORTANT CHEMOKINE-LIKE FUNCTION – CHEMOKINE

Chemokines are 8–12 kDa cytokines with chemotactic properties, playing a fundamental role in leukocyte trafficking (Bajetto et al., 2002; Weber et al., 2004; Charo and Ransohoff, 2006). Typically, a chemokine consists of a disordered N-terminus containing a characteristic cysteine motif, an N-loop region, three antiparallel β-strands linked by turns designated 30s-, 40s-, and 50s-loop, and a C-terminal α-helix, which together form the typical chemokine fold (Clark-Lewis et al., 1995). Chemokines share 20–50% gene and amino acid homology, and are classified into four groups depending on the presence and spacing of their N-terminal cysteine residues. These groups comprise the C, CC, CXC, and CXXXC chemokines, with the CXC and CC groups being most prominent (Murphy et al., 2000). The N-terminal cysteine motif stabilizes the chemokine structure by forming two disulfide bonds, one between the first cysteine with a cysteine in the 30s-loop, and the other one between the second cysteine and a cysteine in the 50s-loop (Fernandez and Lolis, 2002). Chemokines exert their specific function by binding to rhodopsin-like G protein-coupled receptors (GPCRs), which contain a seven-transmembrane domain and signal through heterotrimeric G proteins (Thelen and Didichenko, 1997; Murphy et al., 2000; Thelen, 2001; Bajetto et al., 2002; Charo and Ransohoff, 2006). The receptors are classified according to the chemokines they bind (Murphy et al., 2000). It has been suggested that for all chemokine sub-groups, the binding mechanism follows a so-called two-site-binding mechanism. First, there is an interaction between the N-loop of the chemokine with the N-terminus of the receptor (site I interaction). This results in a conformational change of the receptor and allows a second interaction between the N-terminus of the chemokine and the extracellular loops of the receptor (site II interaction) (Clark-Lewis et al., 1995; Rajagopalan and Rajarathnam, 2006).

In the last decade, there was a raising need to establish an additional chemokine category, to accomodate proteins that exhibit similar functions as the prototypical, "classical" chemokines, but that lack the typical chemokine structure. Characteristics of this group of "chemokine-like function (CLF) – chemokines" were defined as follows: (i) CLF chemokines are released during infection, inflammation, or cell stress by non-classical export or due to cell death; (ii) they do not usually share the typical chemokine fold and the N-terminal residues with the classical chemokines; (iii) they exhibit chemokine-like activities in particular promoting chemotaxis; and (iv) they typically interact with a GPCR, preferentially functioning as non-cognate ligand of a classical chemokine receptor (Degryse and de Virgilio, 2003; Yang et al., 2004; Oppenheim and Yang, 2005; Noels et al., 2009). Some representatives of this sub-group and their characteristic features are listed in **Table 1**.

MIF is a typical CLF chemokine, as missing cysteines in its N-terminus do not allow for a classification of MIF into one of the four prototypical chemokine classes, although MIF shares several features with chemokines (**Box 2**). As such, MIF mediates the recruitment of monocytes, T-cells, neutrophils, endothelial progenitor cells, and tumor cells (Ren et al., 2003; Gregory et al., 2006; Bernhagen et al., 2007; Takahashi et al., 2009; Brandau et al., 2010; Dessein et al., 2010; Simons et al., 2011). Furthermore, MIF is immobilized on the endothelial cell (EC) surface (Schober et al., 2004; Bernhagen et al., 2007), where it induces leukocyte arrest (Schober et al., 2004; Amin et al., 2006). Interestingly, MIF was shown to directly mediate these chemokine-like functions by triggering the activation of leukocytic integrins (**Box 3**) through the CXC chemokine receptors CXCR2 and CXCR4 on monocytes/neutrophils and T-cells, respectively (Bernhagen et al., 2007; Zernecke et al., 2008; Kraemer et al., 2012), as discussed in more detail below.

In addition, MIF indirectly enhances leukocyte arrest by inducing the expression of adhesion molecules or other chemokines. This has been observed for both endogenous and exogenous MIF on ECs and leukocytes, either by MIF stimulation alone, or by MIF in combination with other pro-inflammatory stimuli. For example, *Mif*-deficient mice show a reduced adhesion of leukocytes to the endothelium of the cremaster microvasculature upon injection of inflammatory agents such as Tnfα and lipopolysaccharide (LPS), or chemokines such as Cxcl1 or Ccl2

Table 1 | Chemokine-like function (CLF) chemokines.

Name	Secretion mechanism	Chemotaxis	Additional CLF feature	Interacting chemokine receptor	Other receptor	Reference
Aminoacyl-tRNA synthetases (AaRS), mini-tyrosyl-tRNA synthetase (mini-TyrRS)	Apoptosis/cell death	Monocytes, neutrophils, T-cells, immature DCs	ELR motif	CCR5, CCR3, CXCR1	–	Wakasugi and Schimmel (1999), Wakasugi et al. (2002), Yang et al. (2002)
Complement factor 5a (C5a)	–	DCs, monocytes, macrophages, neutrophils, eosinophils	Modulation of cytokine release	–	C5aR, C5L2	Wennogle et al. (1994), Sozzani et al. (1995), Riedemann et al. (2004), Gao et al. (2005)
Cyclophilin	Secretory pathway unknown (possibly non-classical)	Murine bone marrow cells, eosinophils, neutrophils, T-cells	Integrin-mediated adhesion of T-cells	–	CD147	Colley et al. (1991), Xu et al. (1992), Price et al. (1994), Allain et al. (2002), Yurchenko et al. (2002), Suzuki et al. (2006), Khromykh et al. (2007)
α-Defensins	Cell death	Immature DCs, memory, and CD8 T-cells	–	Chemokine receptor of unknown identity	–	Yang et al. (2001)
β-Defensins	Cell death	Immature DCs, memory and CD8 T-cells, monocytes	Augment cytokine production	CCR6	TLR4	Yang et al. (1999), Biragyn et al. (2002), Hoover et al. (2002), Oppenheim et al. (2003)
Cathelicidins (LL37, Cramp-1)	Cell death and possibly specific secretion	Monocytes, neutrophils, mast cells, T-cells	various	–	–	Yang et al. (2001), Soehnlein et al. (2011), Wantha et al. (2013)
High-mobility group binding protein-1 (HMGB-1)	Non-classical export/cell death	DCs, immature DCs, neutrophils, macrophages	Cytokine expression, modulation of VCAM1/ICAM1 expression	CXCR4 (in complex with CXCL12)	RAGE, TLR2/4	Andersson et al. (2000), Fiuza et al. (2003), Pullerits et al. (2003), Yang et al. (2007), van Zoelen et al. (2009), Schiraldi et al. (2012)
Macrophage migration inhibitory factor (MIF)	Non-classical export/(cell death?)	Monocytes, T-cells, neutrophils, EPCs, tumor cells	Pseudo-ELR motif	CXCR4, CXCR2 (CXCR7?)	CD74	Bernhagen et al. (2007), Noels et al. (2009), Cho et al. (2010), Dessein et al. (2010), Tarnowski et al. (2010)
Thioredoxin (TRX)	Non-classical export/apoptosis	Monocytes, neutrophils, T-cells	Cytokine expression	Unknown	TNF-R-super-family member 8 (TNFRSF8/CD30)	Bertini et al. (1999), Schwertassek et al. (2007)
Urokinase (uPa)	Non-classical export/secretory vesicles	Monocytes, keratinocytes, fibroblasts	–	–	FPRL1, uPAR	Quax et al. (1994), Takahashi et al. (1998), Resnati et al. (2002), Roychoudhury et al. (2006)
Y-box protein-1 (YB-1)	Non-classical export/apoptosis	Mesangial cells	–	–	Notch-3	Frye et al. (2009), Rauen et al. (2009)

DC, dendritic cell; EPC, endothelial progenitor cell; FPRL1, formyl peptide receptor-like 1.

Box 2 | Macrophage migration inhibitory factor (MIF).

Macrophage migration inhibitory factor (MIF) is a pleiotropic inflammatory cytokine with chemokine-like functions, thus placing it into the CLF chemokine class. The sequence of MIF differs by only one residue from a protein mediator called glycosylation-inhibiting factor (GIF). The name "macrophage migration inhibitory factor" goes back to the initial discovery of MIF in 1966, when MIF-containing T-cell supernatants were found to inhibit the random migration of guinea pig macrophages out of capillary tubes (David, 1966). As a CLF chemokine, MIF acts as a chemoattractant for leukocytes, endothelial progenitor cells, and certain tumor cells, and mediates many pro-inflammatory processes through the induction of cytokines, chemokines, and adhesion molecules. Also, MIF counteracts the anti-inflammatory activity of glucocorticoids. MIF has been implicated in a variety of acute and chronic inflammatory diseases like sepsis, atherosclerosis, rheumatoid arthritis, inflammatory lung disease, or systemic lupus erythematosus. MIF also is a tumor promoter in most models (Calandra and Roger, 2003). MIF signals through its high affinity receptor CD74, a surface form of the MHC class II invariant chain Ii, and through the chemokine receptors CXCR2 and CXCR4. Apart from CLF functions, MIF exhibits evolutionarily conserved catalytic activities as an oxidoreductase and tautomerase activity. The physiological and pathophysiological relevance of these catalytic functions, which can readily be detected *in vitro*, is still unclear (Kraemer et al., 2012). The tautomerase activity of MIF is shared by D-dopachrome tautomerase (D-DT), a protein with 34% amino acid homology to MIF in humans, and 27% in mice. D-DT also binds to CD74 and has a broad overlapping spectrum of functions and therefore was recently designated as MIF-2 (Merk et al., 2012). Unfortunately, MIF is frequently mixed up with another chemokine with a similar name, i.e., macrophage inflammatory protein-1α (MIP-1α or CCL3). This chemokine also acts in a pro-inflammatory manner and is produced and secreted by macrophages, but unlike MIF, MIP-1α is classified into the CC chemokine class and is formally not related to MIF by structure.

Box 3 | Integrins.

Integrins are αβ heterodimeric transmembrane proteins mediating the arrest of cells to the extracellular matrix (ECM) or other cells through interaction with ECM proteins (e.g., fibronectin, laminin, collagen, and vitronectin) or integrin ligands (e.g., VCAM1, ICAM1), respectively. Currently, 19α and 8β subunits have been identified in vertebrates, which can be assembled into 24 different integrins. Well-studied are the β_1-integrin VLA4 ($\alpha_4\beta_1$) and the β_2-integrins LFA1 ($\alpha_L\beta_2$, CD11a/CD18) and MAC1 ($\alpha_M\beta_2$, CD11b/CD18), which bind to VCAM1 and ICAM1, respectively (Zhang and Wang, 2012). The association strength between a specific integrin heterodimer and a ligand is called "integrin affinity." It is dependent on the composition and conformation of the integrin, the latter being modulated by intracellular signaling events triggered by, e.g., GPCR stimulation. For LFA1, at least 3 different conformations have been demonstrated. In its "closed," inactive state, the extracellular domain of LFA1 is bent towards the plasma membrane, with the ligand-binding headpiece situated close to the membrane, preventing ligand binding. An intermediate affinity of LFA1 has been linked to an "intermediate extended" state with a closed ligand-binding headpiece extending above the plasma membrane. The high affinity, "open" conformation of LFA1 is coupled to the "opening" of the ligand-binding headpiece through conformational rearrangements. This "open headpiece" of LFA1 has been shown to be necessary and sufficient for cell arrest under flow (Lefort and Ley, 2012).
Besides integrin affinity, cellular adhesion strength is affected by the integrin density, or valency, on the cell surface. This is regulated by integrin expression and clustering, and contributes to the joined, synergistic strength of all integrin-ligand interactions, called "integrin avidity" or "functional affinity." In addition to intracellular signaling cascades regulating integrin affinity (called "inside-out signaling"), ligand binding by integrins also mediates "outside-in signaling" affecting cellular processes, as for example gene expression, cell proliferation and survival.

(Gregory et al., 2004; Fan et al., 2011; Santos et al., 2011). For TNFα, this could be linked to a reduced basal and TNFα-triggered expression of the integrin ligands VCAM1 and ICAM1, and of the cytokines IL6, CXCL8, and CCL2 in *MIF*-deficient ECs, possibly through a reduced TNFα-induced p38 activation in the absence of MIF (Cheng et al., 2010). Similarly, the cremaster muscle microvasculature of *Mif*-deficient mice showed a diminished LPS-induced Vcam1 expression pattern (Gregory et al., 2009). Also, Cxcl1-induced chemotaxis was significantly reduced in *Mif*-deficient neutrophils and was associated with a reduced mitogen-activated protein kinase (Mapk) activation (Santos et al., 2011). Similarly, Ccl2-triggered monocyte chemotaxis was severely decreased in the absence of endogenous Mif. This was associated with a reduced Rho GTPase and Mapk activation, and a reduced expression of the α4 integrin and of the Mapk-regulating protein Mkp1 in *Mif*-deficient macrophages (Fan et al., 2011). Likewise, exogenous MIF has been linked to an enhanced expression of chemokines and adhesion molecules. For example, injection of mice with recombinant MIF increased monocyte adhesion and endothelial transmigration in the microvasculature (Gregory et al., 2006; Fan et al., 2011). This was mostly dependent on Cd74 (Fan et al., 2011) and associated with enhanced Ccl2 secretion from microvascular ECs *in vitro*, without affecting endothelial Vcam1 expression *in vivo* (Gregory et al., 2006). In contrast, exogenous MIF upregulated ICAM1 on a human EC line *in vitro* (Lin et al., 2000) and reduced Icam1 expression was seen in the atherosclerotic aorta of mice treated with a Mif blocking antibody (Burger-Kentischer et al., 2006). These observations seem to be dependent on the vascular bed from which the ECs derive, as in contrast to the MIF-induced arrest responses on microvascular ECs, exogenous MIF could not induce leukocyte rolling or arrest on HUVECs. However, TNFα-induced leukocyte rolling and adhesion on HUVECs were enhanced by exogenous MIF, which could be linked with an increase in endothelial P-selectin expression, while the TNFα-induced expression of E-selectin, VCAM1, and ICAM1, or of different chemokines were unaltered in the presence of exogenous

MIF (Cheng et al., 2010). Furthermore, MIF mediates neutrophil accumulation in MIF-triggered lung inflammation by inducing the chemokines Cxcl1 and Cxcl2/Mip2 in alveolar macrophages through Cd74/extracellular signal regulated kinase (Erk) signaling (Takahashi et al., 2009) and can promote neutrophil chemotaxis in particular in the presence of actively expressed surface CD74 (Bernhagen et al., 2007). In addition, MIF has been shown to increase the surface expression of ICAM1 and VCAM1 on human monocytes via PI3K/AKT, p38, and NF-κB (Amin et al., 2006), which can be shed to soluble adhesion molecules, capable of mediating leukocyte chemotaxis (Kitani et al., 1998; Tokuhira et al., 2000). In conclusion, MIF is a pivotal mediator of leukocyte chemotaxis and arrest, by both direct mechanism or through the induction of other chemokines or adhesion molecules.

In addition to its chemotactic and arrest properties, MIF exerts pro-inflammatory and anti-apoptotic functions, either through receptor activation by extracellular MIF (as described in more detail below) or through intracellular interactions, e.g., with JAB1/CSN5 or with the pro-apoptotic proteins BIM and p53 (Kleemann et al., 2000; Jung et al., 2008; Liu et al., 2008; Noels et al., 2009). Furthermore, comparable to other CLF chemokines, MIF is secreted upon diverse inflammatory or stress factors (**Table 2**). This secretion occurs through a non-classical pathway, a so-called export pathway, as MIF lacks a typical N-terminal consensus secretion sequence required for classical endoplasmic reticulum/Golgi-mediated protein secretion (Flieger et al., 2003). Of note, MIF secretion is not only observed in immune cells, but other cell types with a prominent role in atherogenesis including ECs and SMCs can also be triggered to secrete MIF (Bernhagen et al., 1994; Noels et al., 2012).

STRUCTURE – FUNCTION RELATIONSHIPS OF MIF AS A CXCR2 LIGAND

MIF is a conserved protein, ubiquitously expressed in mammals. Furthermore, MIF homologs have been identified in avians, fish, plants (*Arabidopsis thaliana*), the nematode *Caenorhabditis elegans*, cyanobacteria, ticks, and parasites, amongst others (Calandra and Roger, 2003; Kim et al., 2010). The human and mouse MIF protein consist of 114 amino acids (excluding an N-terminal methionine, which is processed after ribosomal synthesis), with a total molecular weight of 12.3 kDa. The human and mouse orthologs share a 90% sequence homology and lack a conventional leader sequence targeting proteins for classical secretion (Bernhagen et al., 1994). In addition to cytokine and chemokine-like activities, MIF is unusual in featuring two evolutionarily conserved catalytic activities, being an oxidoreductase and a tautomerase activity. Despite its consensus Cys-Xaa-Xaa-Cys motif, structural similarities between MIF and oxidoreductases such as thioredoxin or glutaredoxin are of remote nature only. In contrast, MIF possesses three-dimensional structural homology with the bacterial isomerases 4-oxalocrotonate-tautomerase, 5-carboxymethyl-2-hydroxymuconate isomerase, and chorismate mutase (Rosengren et al., 1997; Kleemann et al., 1998; Calandra and Roger, 2003). Furthermore, MIF's overall architecture resembles that of human D-dopachrome tautomerase (D-DT). Also, MIF and D-DT share overlapping biological functions, both bind the CD74 receptor, and activate similar signaling pathways (Sugimoto et al., 1999; Merk et al., 2012). Therefore, "MIF-2" was recently suggested as an alternative name for D-DT (Merk et al., 2012). Remarkably, a MIF ortholog can even be found in the hookworm *Ancylostoma ceylanicum* (aceMIF), sharing 28–35% sequence homology with human MIF. Like the human ortholog, aceMIF shows tautomerase activity, binds to CD74 and has chemoattractant properties (Cho et al., 2010). Although the global topology is similar to human MIF, protein surface and electrostatic potential are distinct (Cho et al., 2007).

The MIF monomer consists of two antiparallel α-helices and a four-stranded β-sheet (excluding two really short β-strands), as displayed in **Figure 1**. Although MIF has no sequence homology to other chemokines, the 3D structure of the MIF monomer resembles the dimeric form of CXCL8 and other CXC chemokines (Weber et al., 2008) (**Figure 1**). But, the monomer is not the only existing form of the MIF protein. Nuclear magnetic resonance (NMR) analysis suggested that MIF dimerizes, whereas

Table 2 | MIF expression and secretion in cell types relevant in atherogenesis.

Cell type	Basal expression	MIF expression (secretion) upregulated by	Reference
Monocytes/macrophages	Yes	LPS, TNFα, IFNγ, CD40L, ATII, oxLDL, bacterial exotoxins, hypoxia, glucocorticoids	Calandra et al. (1994), Schmeisser et al. (2005), Burger-Kentischer et al. (2002), Calandra et al. (1998), Schmeisser et al. (2005), Calandra et al. (1995)
T-cells	Low	T-cell activation (αCD3, PMA/ionomycin) glucocorticoids	Bloom and Bennett (1966), Bacher et al. (1996), Bacher et al. (1996)
B-cells	Yes	Tumor stress signals	Wymann et al. (1999), Reinart et al. (2013)
ECs	Low	LPS, oxLDL, hypoxia, thrombin	Nishihira et al. (1998), Burger-Kentischer et al. (2002), Schober et al. (2004), Schmeisser et al. (2005), Simons et al. (2011), Zhang et al. (2012), Shimizu et al. (2004)
SMCs	Low	oxLDL, hypoxia	Chen et al. (2009), Zhang et al. (2012)

LPS, lipopolysaccharide; TNF, tumor necrosis factor; IFN, interferon; ATII, angiotensin II; oxLDL, oxidized low density lipoprotein; ECs, endothelial cells; SMCs, smooth muscle cells.

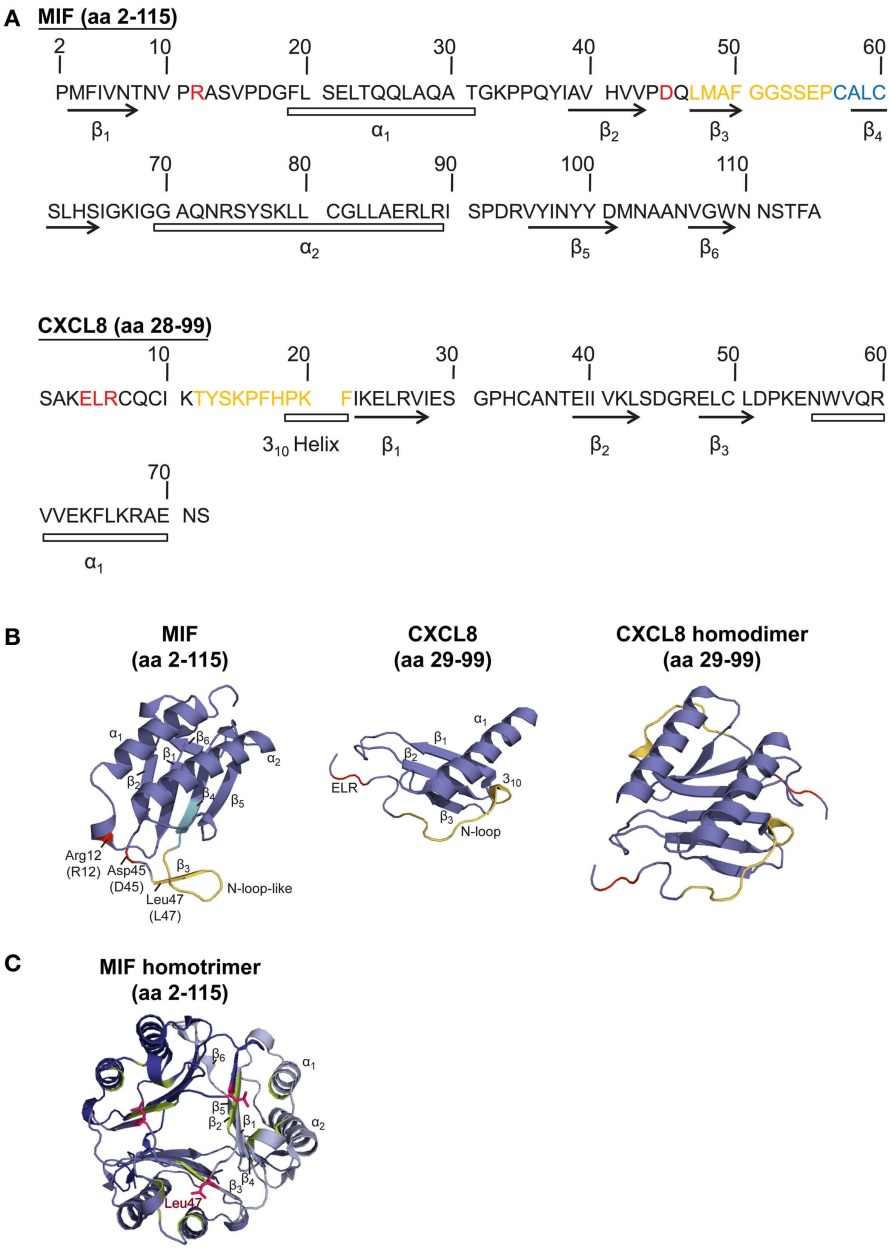

FIGURE 1 | Sequence and structure comparison of human MIF and the cognate CXCR2 ligand CXCL8. **(A)** Amino acid (aa) sequence comparison of MIF and CXCL8. **(B,C)** Comparison of the crystal structure of the MIF monomer, CXCL8 monomer, CXCL8 dimer, and MIF trimer. To **(A,B)**: CXCL8 (aa 28–99) is the predominant form of CXCL8. α-Helices and β-sheets are indicated. Important amino acids and motifs are highlighted: ELR or pseudo-(E)LR (red); N-loop (for CXCL8) or N-like loop (for MIF; orange); CALC motif, forming the catalytic center of MIF's oxidoreductase activity (blue). The MIF structural information is according to Orita et al. (2001), the crystal structure for CXCL8 was based on data from Clore et al. (1990). **(C)** Crystal structure of the MIF homotrimer (Orita et al., 2001), showing the barrel-shaped homotrimeric structure and the inter-subunit interactions between two β-strands of one subunit with β-sheets of adjacent subunits. Further stabilization is provided by the hydrophobic interaction of Leu47 (pink) of the β3-strand of one subunit with an adjacent hydrophobic pocket (green) on a second subunit, comprising amino acids mainly positioned on the β2-strand. For details, see text.

X-ray crystallography revealed human MIF as a trimer. The barrel-shaped homotrimeric structure is stabilized by conserved inter-subunit interactions between two β-strands of one subunit with β-sheets of adjacent subunits (Sugimoto et al., 1996; Sun et al., 1996a). Further stabilization is provided by the hydrophobic interaction of Leu47 on the β3-strand of one subunit with an adjacent hydrophobic pocket on a second subunit, comprising amino acids mainly positioned on the β2-strand (El-Turk et al., 2012) (**Figure 1**). However, X-ray and NMR structural analyses are performed at mg/ml concentrations of the analyte, which

are far off the physiological concentrations found in the cell or in extracellular fluids. Overall, the physiological oligomerization state of MIF remains elusive. Crosslinking studies revealed the coexistence of MIF monomers, dimers, and trimers (Sugimoto et al., 1996; Sun et al., 1996a,b), probably influenced by local MIF concentrations. At physiological conditions, an equilibrium of monomers and dimers has been described, whereas at concentrations >10 μg/ml, trimeric or higher-ordered oligomers seem to be preferred (Mischke et al., 1998; Calandra and Roger, 2003; Philo et al., 2004; El-Turk et al., 2008). Interestingly, homotrimeric MIF seems to drive inflammatory responses in the corneal epithelium, as recently reported by Reidy et al. (2013). Nevertheless, it is unlikely that the homotrimer is the only active form, as disruption of the homotrimeric structure with the MIF inhibitor ebselen leads to an increased chemotactic response (Ouertatani-Sakouhi et al., 2010). In addition, concentration extrapolations into the physiological ng/ml range would likely favor a predominant population of the monomeric state. Yet, monomeric MIF is thought to be intrinsically unstable, necessitating yet unknown mechanisms for its stabilization (Bernhagen and Lue, unpublished observations).

To study the structure-activity relationship of MIF in the context of its interaction with CXCR2, it appeared obvious to look for homologous structural features with the cognate CXCR2 ligand CXCL8, which, in its dimeric form, shares structural homology with the MIF monomer (**Figure 1**). Bioinformatic prediction analysis in conjunction with mutational studies revealed an important receptor interacting motif in MIF that resembles the N-terminal Glu-Leu-Arg (ELR) motif carried by a sub-group of CXC chemokines, as shown for CXCL8. This MIF motif, consisting of Asp45-X-Arg12, was termed "pseudo-(E)LR" motif, as the glutamate (Glu/E) was substituted with an aspartic acid (Asp/D) (Hebert et al., 1991; Weber et al., 2008). The non-adjacent residues of this pseudo-(E)LR motif are located in neighboring loops of the MIF protein with similar spacing as in the true ELR motif (**Figure 1**). Site-directed mutagenesis studies showed an almost complete inhibition of CXCR2 binding when the R12A or D45A mutation was introduced into MIF. The MIF-R12A mutant also exhibited a complete loss of chemotactic and arrest function. Also, the MIF-D45A mutant showed a reduced chemotactic and arrest activity in *in vitro* assays, whereas its hyperactivity towards neutrophil recruitment in a peritonitis model was shown to be CXCR4-mediated (Weber et al., 2008). Furthermore, evidence became available for an interaction of the pseudo-(E)LR motif with the extracellular loops EL-2 and EL-3 of CXCR2 (Kraemer et al., 2011). Still, it should be noted that Arg12 and Asp45 of the pseudo-(E)LR motif are located close to the critical Leu47 residue, which is involved in inter-subunit hydrophobic interactions modulating the conformation and stability, but not the oligomerization state, of homotrimeric MIF (El-Turk et al., 2012) (**Figure 1**). The reduced leukocyte adhesion activity, which was observed for the pseudo-(E)LR mutants (Weber et al., 2008), might thus not solely result from changes in the direct MIF receptor interaction locus, but could also result in part from modifications of the conformational stability of MIF by disturbance of the Leu47 region.

Interestingly, Kraemer et al. (2011) revealed the involvement of an N-like loop in MIF in binding to CXCR2. This loop spans 10 amino acids from position 47 to 56 but is structurally different from the N-loop of CXC chemokines (**Figure 1**; **Table 3**). Whereas the classical N-loop found in CXC chemokines contains 1–3 basic residues and interacts with the N-terminus of the receptor, the N-like loop of MIF has an acidic isoelectric point (pI) and interacts with EL-1 and parts of EL-2 as well as with the N-terminus of CXCR2, according to peptide spot array analysis. Importantly, short MIF N-like loop-derived peptides blocked monocyte arrest and inhibited MIF/CXCR2 interaction in a receptor competition assay, verifying the importance of the N-like loop of MIF for CXCR2 binding (Kraemer et al., 2011). Furthermore, amino acids in the region between residues 50 and 68 are critical for obtaining potent MIF neutralizing antibodies, which block important biological activities such as cell proliferation and glucocorticoid overriding *in vitro* and MIF-driven septic responses *in vivo* (Kerschbaumer et al., 2012). This confirmed that the N-like loop region of MIF is critical for MIF-driven receptor-mediated processes. Moreover, site-specific mutations of the cysteines at positions 57 and 60 and the use of peptides covering the region 50–65 further underscored that the sequence region of the N-like loop, i.e., region 47–68, is critical for a variety of MIF activities (Kleemann et al., 1998, 1999; Nguyen et al., 2003).

Table 3 | The N-like loop of MIF shows only limited similarity with the N-loop of CXC chemokines.

Chemokine	N-loop sequence
CXCL1	LQTLQ GIHP
CXCL2	LQTLQ GIHL
CXCL3	LQTLQ GIHL
CXCL5	LQTTQ GVHP
CXCL6	LRVTL RVNP
CXCL7	IKTTS GIHP
CXCL8	IKTYSKPFHP
MIF	LMAFGGSSEP

Adapted from Kraemer et al. (2011).

Taken together, as suggested by Kraemer et al. (2011), the binding of MIF to CXCR2 seems to follow a two-site-binding mechanism which is similar but not identical to that between CXCL8 and CXCR2. In contrast, no structure-activity relationship data are available yet for the interaction between MIF and its receptors CD74 and CXCR4. Interesting questions are therefore, whether the uncovered motifs mediating the MIF/CXCR2 interaction are also important for the interaction between MIF and CXCR4, whether for CD74 binding fully different regions are required, and whether CXCR7, for which an interaction with MIF has recently been implied, binds directly to MIF and utilizes the N-like loop and pseudo-(E)LR motif as well.

Interestingly, the anti-inflammatory drug AV411 (Ibudilast) and its analog AV1013, both allosteric inhibitors of MIF's tautomerase activity, were found to inhibit MIF-mediated CXCR2-dependent chemotaxis of monocytes (Cho et al., 2010). AV1013 binds into a pocket formed by several C-terminal residues of MIF. AV1013 binding apparently induces conformational changes leading to both an inactivation of the tautomerase site and changes at the MIF/CXCR2 interface, i.e., likely affecting the N-like loop

or pseudo-(E)LR motif. Alternatively, conformational changes in the tautomerase site could subsequently lead to conformational changes in the receptor interaction interface of MIF (Cho et al., 2010). Similarly, the hormonally inert isomer DT(4) of the thyroid hormone thyroxine [T(4)], inhibited MIF's tautomerase activity by binding to a hydrophobic pocket harboring this enzymatic function and reduced leukocyte accumulation in a carrageenan-induced airpouch model in wildtype but not *Mif*$^{-/-}$ mice, providing further evidence for a potential participation of the tautomerase site in MIF receptor-mediated chemotaxis (Al-Abed et al., 2011). Yet, disruption of the MIF trimer, and therefore of the active tautomerase site[2], increased MIF-mediated chemotaxis (Ouertatani-Sakouhi et al., 2010). In addition, Fingerle-Rowson et al. (2009) showed that the tautomerase-inactive mutant P2G-MIF, which contains a mutation of the crucial catalytic N-terminal Proline (Pro2)[3], could still bind CD74 and mediate growth regulation in a skin tumorigenesis model, although to a somewhat reduced level. This indicates that the enzyme activity *per se* is not essential for CD74 receptor binding. Also, in comparison with wildtype MIF, the capacity of P2G-MIF to compete with CXCL8 for binding to CXCR2-expressing cells was more reduced than ligand competition in the CD74 binding assay (Fingerle-Rowson et al., 2009). This suggests that the Pro2 residue and/or conformational changes in the tautomerase site affect MIF binding to CXCR2 more than the MIF-CD74 interaction.

MIF'S ARREST FUNCTION THROUGH ITS RECEPTOR CXCR2

MIF, immobilized on the endothelial surface, triggers the arrest of monocytes/neutrophils and T-cells through CXCR2 and CXCR4, respectively, by a rapid and transient activation of the leukocyte integrins LFA1 and VLA4 (**Box 3**) (Bernhagen et al., 2007). While the precise mechanism of MIF deposition on ECs has not yet been explored, the basic pI could be a likely explanation. Alternatively, ECs express CD74 which has been found to be modified by chondroitin sulfate, thus providing a possible anchoring site for MIF as well.

For immobilized classical chemokines, the GPCR/Gαi-mediated intracellular signaling cascade in leukocytes triggering integrin activation and leukocytic arrest, has been shown to be very complex, with currently 65 proteins identified to be possibly involved (Ley et al., 2007; Montresor et al., 2012). Three main stages of integrin activation are distinguished. These involve: (1) phospholipase C (PLC)-mediated calcium influx, (2) small GTPases, and (3) actin-binding proteins as talin-1 and kindlin-3, as described in more detail elsewhere (Ley et al., 2007; Lefort and Ley, 2012; Montresor et al., 2012) (**Figure 2**). However, this model of GPCR-mediated integrin activation cannot be universally applied to all conditions, and it is expected to be dependent on the GPCR, GPCR ligand, the integrin activated, and the biological context (Ley et al., 2007; Montresor et al., 2012). For MIF/CXCR2, few details are known about the exact molecular delineation of proteins involved in MIF receptor-mediated integrin activation. Initial inhibitor studies revealed MIF to mediate monocyte and T-cell adhesion by Gαi proteins and PI3K (Bernhagen et al., 2007), a kinase which is directly activated by the Gβγ dimer and which mediates adhesion stabilization of neutrophils to CXCL1 through CXCR2 (Smith et al., 2006). Also, MIF triggered CXCR2-dependent calcium transients. In mouse fibroblasts, recombinant MIF induces the activation of RhoA GTPase and Rho kinase, and *Mif*-deficient fibroblasts showed a reduction in RhoA GTPase activation and stress fiber formation (Swant et al., 2005). Of note, the latter has been linked with integrin clustering (Roovers and Assoian, 2003). Also VASP, LASP-1, IQGAP1, and NHERF1 were recently identified to interact with CXCR2 and to be involved in CXCL8-triggered chemotaxis (Neel et al., 2009, 2011; Wu et al., 2012), but it remains unknown whether this is directly linked to integrin activation, or whether these proteins are also involved in MIF/CXCR2-mediated arrest.

In addition to this direct link of MIF-CXCR2 signaling towards integrin activation, MIF has been shown to induce the migration of human chondrosarcoma cells by upregulating the transcription of the $\alpha_v\beta_3$ integrin through PI3K/AKT/NF-κB signaling in a CXCR2- and CXCR4-mediated way (Lee et al., 2012). This again indicates MIF's potential to mediate chemokine-like functions through indirect effects, by regulating the expression of proteins involved in leukocyte adhesion, as discussed previously.

CD74 AND CXCR4 AS ALTERNATIVE MIF RECEPTORS

CD74

In 2003, the CD74 protein was discovered as a high affinity receptor for MIF (Leng et al., 2003). CD74 is well-known as an MHC class II chaperone, as it is the membrane-expressed portion of the invariant chain (II), which typically regulates antigenic peptide loading to MHC class II proteins through its CLIP domain. However, CD74 can also be expressed in the absence of the MHC class II protein, thus executing functions as membrane receptor (Borghese and Clanchy, 2011). The CD74 receptor is a type II membrane-spanning protein with a short cytoplasmic N-terminus. As a result, accessory signaling molecules like Src, CD44, c-Met, or other co-receptors are necessary to mediate CD74 signaling by MIF, i.e., by forming a functional receptor-tyrosine-kinase-(RTK)-like complex (Bernhagen et al., 2007; Gordin et al., 2010). Signaling of MIF through CD74 has been linked with MIF's pro-inflammatory and anti-apoptotic functions. For instance, interaction of MIF with CD74 leads to the activation of MAPKs and other protein kinases. One example is the sustained and transient activation of the MAPK ERK1 and ERK2 (Lue et al., 2006; Shi et al., 2006). Sustained ERK activation is mediated by CD74/CD44 and protein kinase A and has been linked to cell proliferation and enhanced pro-inflammatory phospholipase A2 activity (Mitchell et al., 1999; Lue et al., 2006; Shi et al., 2006). Recently, also β-arrestin-1 was shown to be involved in MIF-triggered sustained ERK activation, mediating MIF internalization in a CD74- and clathrin-dependent manner (Xie et al., 2011). Another example is the effect of MIF and CD74 on the MAPK Jun N-terminal kinase (JNK). MIF either impedes JNK signaling and JNK-mediated apoptosis, or rapidly initiates JNK activation through CXCR4/CD74, activating the

[2]MIF tautomerase activity requires trimerization.

[3]In the paper by Fingerle-Rowson et al. (2009), MIF sequence numbering refers to the processed MIF protein after Met1 removal. Thus, the protein sequence of MIF in that paper starts with Pro1 rather than Pro2, the latter referring to the cDNA sequence. In the current article, we prefer to refer to this proline residue as Pro2, i.e., applying the numbering according to the cDNA sequence to be consistent with the sequence numbering of the mutants above.

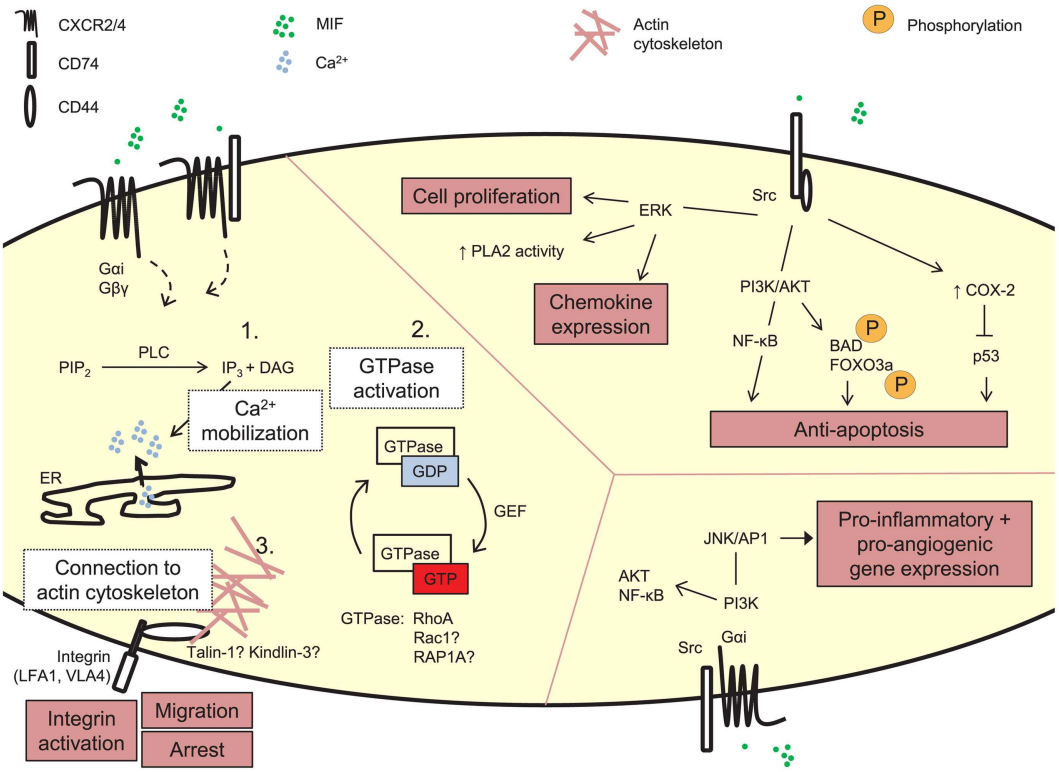

FIGURE 2 | Signaling by exogenous MIF. MIF can induce signaling cascades through its receptors CD74, CXCR2, and CXCR4. These pathways underlie MIF's biological functions, e.g., leukocytic integrin activation, cell proliferation, and anti-apoptosis, induction of pro-inflammatory gene expression. The detailed molecular mechanism underlying MIF's arrest function through its receptors CXCR2 and CXCR4 is still unexplored. Three main steps in GPCR-mediated integrin activation can be distinguished, i.e., PLC-mediated calcium mobilization, activation of small GTPases and recruitment of actin-binding proteins linking the integrin to the actin cytoskeleton. PIP2, phosphatidylinosytol 4,5-biphosphate; PLC, phospholipase C; IP3, inosytol 1,4,5-triphosphate; DAG, diacylglycerol; Ca^{2+}, calcium; ER, endoplasmic reticulum; GDP, guanosine diphosphate; GTP, guanosine triphosphate; guanine nucleotide exchange factor; PLA2, phospholipase A2; ERK, extracellular signal-related kinase; PI3K, phosphatidylinositol 3-kinase; NF-κB, nuclear factor-κB; BAD, BCL2-associated agonist of cell death; FOXO3A, forkhead box O3a; COX-2, cytochrome C oxidase subunit 2; JNK, c-Jun N-terminal kinase; AP1 (c-Jun), activator protein-1.

Src/PI3K/JNK/AP1 pathway, which results in the expression of the pro-inflammatory protein CXCL8 (Kleemann et al., 2000; Qi et al., 2009; Lue et al., 2011). Also, MIF-CD74 signaling has been shown to promote B-cell survival through CD44/PI3K/AKT-mediated NF-κB activation and NF-κB-induced CXCL8 secretion (Binsky et al., 2007; Gore et al., 2008). Furthermore, a CD74/Src/PI3K/AKT pathway links MIF to the phosphorylation of the pro-apoptotic proteins BAD and FOXO3a, providing a survival signal (Lue et al., 2007). Also, cyclooxygenase (COX)-2, which prevents the accumulation of p53, can be upregulated through this pathway, likewise contributing to survival (Mitchell et al., 2002).

In addition, CD74 has been shown to be involved in MIF-mediated leukocyte chemotaxis and arrest, although not in all circumstances. The administration of a CD74 blocking antibody reduced the MIF-dependent monocyte arrest on *ex vivo* carotid arteries from atherosclerotic mice (Bernhagen et al., 2007), and activation of macrophage CD74 by MIF leads to an ERK1/2-dependent release of Cxcl1 and Cxcl2, mediating the MIF-induced accumulation of neutrophils in the alveolar space (Takahashi et al., 2009). Furthermore, Ccl2-triggered monocyte arrest and endothelial transmigration were severely decreased in *Mif*- and *Cd74*-deficient mice. *Mif*- and *Cd74*-deficient macrophages also showed a reduced Ccl2-triggered chemotaxis *in vitro*, which was associated with a reduced Rho GTPase and Mapk activation. However, *Mif*-deficient macrophages showed a lower expression of the α_4 integrin and of the Mapk-regulating protein Mkp1, which was not the case for *Cd74*-deficient macrophages, suggesting that the involvement of endogenous Mif and Cd74 in Ccl2-induced monocyte recruitment or adhesion could at least partially be mediated through distinct mechanisms. Furthermore, exogenous MIF could restore deficient Ccl2-triggered leukocyte adhesion, but not endothelial transmigration, in $Mif^{-/-}$ and $Cd74^{-/-}$ mice to the same extent, indicating that exogenous MIF enhances CCL2-mediated leukocyte adhesion mostly independently of CD74. In contrast, exogenous MIF-induced leukocyte adhesion and transmigration were severely impaired in $Cd74^{-/-}$ mice (Fan et al., 2011), suggesting an alternative mechanism underlying MIF- versus CCL2/MIF-induced leukocyte adhesion *in vivo*. Alternatively, these different observations could result from differentially targeting vascular versus

leukocytic cells to a different degree, when examining MIF-induced versus CCL2/MIF-induced leukocyte adhesion, respectively. Of note, MIF also desensitized CCL2-mediated chemotaxis (Hermanowski-Vosatka et al., 1999), implying a MIF/CXCR-mediated cross-signaling mechanism as known for classical chemokines.

CXCR4

Another chemokine receptor known to interact with MIF is CXCR4, as shown by Bernhagen et al. (2007). Prior to this finding, the CXCL12/CXCR4 interaction was thought to be highly specific, from the receptor as well as from the ligand site (Murphy et al., 2000). The MIF/CXCR4 interaction together with the identification of the CXCL11 receptor CXCR7 as an additional receptor for CXCL12 (Balabanian et al., 2005; Burns et al., 2006; Thelen and Thelen, 2008), disproved the existence of a non-promiscuous interaction. In fact, CXCR4 is also known to interact with the HIV gp120 protein and a recent report suggests that serum ubiquitin also interacts with CXCR4 (Saini et al., 2010). CXCR4 is widely expressed, particularly on many cell types of the immune system (Murphy et al., 2000) (**Table 4**). The knockout of *Cxcr4* in mice is embryonically lethal, due to defects in hematopoiesis, vasculo-, cardio-, and neurogenesis (Ma et al., 1998; Zou et al., 1998). CXCR4 has prominently been implicated in cell recruitment processes, with CXCL12 mediating the recruitment of hematopoietic and vascular progenitor cells from the bone marrow (Mohle et al., 1998; Sainz and Sata, 2007). On the other hand, MIF has been shown to induce T-cell recruitment and arrest through CXCR4-induced, rapid $\alpha_4\beta_1$ (VLA4) integrin activation (Bernhagen et al., 2007). MIF was also identified as the critical autocrine CXCR4 ligand driving cell invasion by drug-resistant colon carcinoma HT-29 cells (Vera et al., 2008; Dessein et al., 2010). Little is known about the molecular details of MIF/CXCR4 signaling. In T-cells, MIF stimulation increases CXCL8 expression through both CXCR4 and CD74, depending on Src, PI3K, and JNK phosphorylation (Lue et al., 2011). Earlier on, CXCR4/CD74 heterodimers were found in monocytes, T-cells and fibroblasts, and MIF-induced AKT signaling was shown to be reduced both by blocking CD74 and CXCR4, indicating a functional CXCR4/CD74 MIF receptor complex (Schwartz et al., 2009).

CXCR7

Tarnowski et al. (2010) implicated a recently identified chemokine decoy receptor in MIF internalization and MIF-dependent adhesion of rhabdomyosarcoma cells. This seven-transmembrane-receptor, encoded by the *RDC-1* gene, was named CXCR7 and characterized as a receptor for CXCL11 and CXCL12 (Balabanian et al., 2005; Burns et al., 2006). CXCR7 is expressed on a variety of cells, including leukocytes, activated ECs, mature neurons, $CD34^+$ progenitor cells, and several tumor cell lines (Balabanian et al., 2005; Burns et al., 2006; Infantino et al., 2006; Zabel et al., 2009; Hattermann et al., 2010; Tarnowski et al., 2010; Shimizu et al., 2011). Unlike the prototypical chemokine receptors, CXCR7 carries two amino acid substitutions in the DRYLAIV motif (A/S and V/T) on the second intracellular loop, resulting in a change in the adaptor motif for G proteins and thus to a loss of G protein signaling (Zabel et al., 2009). Even though typical chemokine receptor signaling pathways, like calcium mobilization, are absent

Table 4 | MIF receptor expression in cell types relevant in atherogenesis.

Cell type	Receptor	Remark	Reference
Monocytes/macrophages	CXCR2		Murphy et al. (2000)
	CXCR4		Murphy et al. (2000), Sunderkotter et al. (2004), Ingersoll et al. (2010)
	CD74		Martin-Ventura et al. (2009)
Neutrophils	CXCR2		Murphy et al. (2000)
	CXCR4	Upon stimulation	Bruhl et al. (2003)
	No CD74		
T-cells	CXCR4		Murphy et al. (2000)
	CXCR2	On some $CD8^+$ T-cells, not on $CD4^+$ T-cells	Chuntharapai et al. (1994)
	CD74	On a subset of activated T-cells	Stein et al. (2007)
B-cells	No CXCR2		Chuntharapai et al. (1994)
	CXCR4		Nie et al. (2004)
	CD74		Gore et al. (2008)
ECs	CXCR2		Murdoch et al. (1999)
	CXCR4		Gupta et al. (1998)
	CD74	Only upregulated under inflammatory stimulation	Stein et al. (2007)
SMCs	CXCR2		Govindaraju et al. (2006)
	CXCR4		Schecter et al. (2001)
	CD74	In atherosclerotic plaques	Martin-Ventura et al. (2009)

ECs, endothelial cells; SMCs, smooth muscle cells.

for CXCR7, other signaling cascades have been described. For example, the β-arrestin-dependent internalization of chemokines by CXCR7 results in the activation of MAPK signaling and the CXCL12/CXCR7 interaction promotes Gαi-independent ERK and AKT phosphorylation, mediating T-cell chemotaxis and survival (Balabanian et al., 2005; Rajagopal et al., 2010; Kumar et al., 2012), although these findings have been controversial in part. On the other hand, CXCL11 binding to CXCR7 inhibits the CXCL12/CXCR4-mediated transendothelial migration of breast cancer cells (Dambly-Chaudiere et al., 2007; Boldajipour et al., 2008; Zabel et al., 2009). Thus, CXCR7 might play a role in MIF-mediated chemotaxis by generating MIF gradients leading to differential signaling, act as a co-receptor or influence chemokine crosstalk. Whether CXCR7 is implicated in a direct interaction with MIF or whether the observed effects by Tarnowski et al. (2010) are based on an indirect interaction, for instance by forming a functional complex with CXCR4 as identified by Luker et al. (2009), will have to be elucidated in the future. Nevertheless, the interaction of MIF with CXCR7 might be a further fine-tuning mechanism in the complex chemokine/chemokine receptor system.

RECEPTOR OLIGOMERIZATION

Receptor oligomerization is a further possibility to modulate ligand affinity, ligand internalization and signal transduction with an impact on cellular processes like cell arrest or cell activation. It is well established that chemokine receptors form dimers or even higher-order oligomers (Milligan, 2007; Thelen et al., 2010; Kraemer et al., 2013). Also, the MIF receptors CXCR2 and CXCR4 have been shown to homodimerize and even multimerize in a ligand-independent manner (Trettel et al., 2003; Hamatake et al., 2009; Wu et al., 2010). Moreover, CXCR2 and CXCR4 heterodimerize with various CXC receptors. For example, the MIF receptors CXCR4 and CXCR7 form a complex, thus modulating CXCL12-mediated CXCR4-dependent chemotaxis (Thelen and Thelen, 2008; Levoye et al., 2009). Also, CXCR2 heterodimerizes with CXCR1 (Wilson et al., 2005). Heterocomplex formation is not solely restricted to receptors of the same sub-family, but also exists between different chemokine receptor subtypes (Kraemer et al., 2013). The cooperation of the CXCR4 and CCR5 receptor, for example, is required for chemokine-induced T-cell stimulation at the immunological synapse (Contento et al., 2008). Furthermore, complexes between chemokine receptors and other receptor types were observed. As such, CXCR4 engages with the dopamine receptor and for CXCR2, a complex with the δ-opiod receptor (DOP) was demonstrated (Parenty et al., 2008; Kraemer et al., 2013). Interestingly, the CD74 receptor interacts with both CXCR2 and CXCR4 (Bernhagen et al., 2007; Schwartz et al., 2009). CXCR2/CD74 heterodimers are implicated in leukocyte recruitment. In this context, it was suggested that CD74 amplifies MIF/CXCR2-mediated signaling, as neutrophils, which lack CD74, only show a weak migratory response to MIF, whereas HL-60 cells, which do not express detectable levels of CD74, increasingly migrate to MIF after ectopic CD74 expression (Bernhagen et al., 2007). CXCR4/CD74 heterodimers were found in monocytes, T-cells, and fibroblasts. Both CXCR4 and CD74 mediate MIF-induced AKT signaling and a fast and transient activation of the JNK/AP1 pathway, suggesting the existence of a functional heterocomplex (Schwartz et al., 2009; Lue et al., 2011). Taken together, MIF/CXCR interactions play a role in inflammation and inflammatory leukocyte recruitment and arrest. The possibility that MIF interacts not only with a single receptor, but with a complex of receptors could further add to a highly controlled cell-, site- and disease-stage specific inflammatory cell adhesion process.

MIF IN ATHEROSCLEROSIS

Atherosclerosis is caused and sustained by inflammatory processes in the vessel wall. The deposition and oxidation of low density lipoprotein (LDL) in the intima drives EC and SMC activation, and the recruitment and infiltration of leukocytes (Weber and Noels, 2011). Whereas MIF is only detectable at low levels in healthy vessels, hyperlipidemia strongly enhances MIF expression in ECs, SMCs, monocytes, and T-cells in atherosclerotic lesions (Lin et al., 2000; Burger-Kentischer et al., 2002, 2006), and an even further upregulation during atheroprogression suggested a role for MIF in plaque destabilization (Burger-Kentischer et al., 2002) (**Table 5**). *In vitro*, leukocytes and vascular cells have been shown to express MIF upon several inflammatory triggers (**Table 2**). Typically, an initial secretion pulse of preformed MIF protein precedes *MIF* transcription (Simons et al., 2011). Also, all cell types involved in atherogenesis, including monocytes/macrophages, neutrophils, B- and T-lymphocytes, ECs, and SMCs, express at least one of the MIF receptors CD74, CXCR2, or CXCR4 (**Table 4**), suggesting that they do not solely act as MIF storage pools, but also respond to secreted MIF.

Of note, functional animal studies confirmed an atheroprogressive role of MIF, showing a reduced lesion size and inflammatory profile in *Mif*-deficient mice, or after treatment with a Mif blocking antibody (**Table 5**) (Pan et al., 2004; Burger-Kentischer et al., 2006; Bernhagen et al., 2007; Verschuren et al., 2009). Remarkably, Mif blockade even induced a regression of established atherosclerotic lesions (Bernhagen et al., 2007). Similarly, Mif neutralization reduced injury-induced restenosis, in which Mif expression is initially upregulated in SMCs, and in ECs and foam cells in a later stage (Chen et al., 2004; Schober et al., 2004).

These atheroprogressive effects of MIF can be linked with MIF's potential to trigger the expression of inflammatory mediators and mediate leukocyte recruitment and arrest directly or through the induction of adhesion molecules and chemokines in ECs and monocytes/macrophages (**Figure 3**) (Bernhagen et al., 1994; Calandra et al., 1994, 1995; Lan et al., 1997; Lin et al., 2000; Amin et al., 2006; Gregory et al., 2006, 2009; Takahashi et al., 2009; Cheng et al., 2010). Furthermore, the ability of MIF to stimulate oxidized LDL (oxLDL) uptake by macrophages (Atsumi et al., 2000), and its association with protease expression and a reduced PDGF-BB-induced SMC migration (Pan et al., 2004; Schrans-Stassen et al., 2005; Verschuren et al., 2005) may further contribute to MIF's plaque destabilizing properties in hyperlipidemia-induced atherogenesis. In the context of injury-induced neointima formation, interference with MIF's anti-apoptotic effect could underlie the enhanced apoptosis in conditions of Mif antibody treatment (Mitchell et al., 2002; Chen et al., 2004), whereas a report on MIF driving SMC proliferation could be linked to a decreased medial cell proliferation under conditions of MIF blockade (Chen et al., 2004).

Table 5 | MIF in atherosclerosis.

			Reference
MIF EXPRESSION IN ATHEROSCLEROSIS AND RESTENOSIS			
Native or diet-induced atherosclerosis			
Rabbit		Upregulated in macrohages, ECs, and SMCs from early atherosclerotic lesions	Lin et al. (2000)
Apoe$^{-/-}$ mouse		Enhanced in all cell types (monocytes, T-cells, ECs, SMCs), but mostly in monocytes	Burger-Kentischer et al. (2006)
Human		Enhanced in all cell types (monocytes, T-cells, ECs, SMCs) Further upregulated upon progression	Burger-Kentischer et al. (2002)
Injury-induced restenosis			
Apoe$^{-/-}$; *Ldlr*$^{-/-}$		Upregulated in medial SMCs (early) and ECs and foam cells (late)	Chen et al. (2004), Schober et al. (2004)
EFFECTS OF MIF BLOCKADE ON ATHEROSCLEROSIS IN MICE			
Native or diet-induced atherosclerosis			
Mif$^{-/-}$; *Ldlr*$^{-/-}$	High-fat diet	Smaller and less progressed lesions Reduced cell proliferation Reduced cathepsin expression	Pan et al. (2004)
Mif$^{-/-}$; *Ldlr*$^{-/-}$	Chow diet	Reduced lesion size Reduced macrophage content	Verschuren et al. (2009)
Apoe$^{-/-}$ +Mif blocking Ab	Chow diet	Only non-significant reduction in aortic lesion size Reduced macrophage content Reduced aortic expression of pro-inflammatory markers (CD40L, TNFα, IL12, ICAM1), the transcription regulators C-EBPβ and phospho-cJun, and of MMP2	Burger-Kentischer et al. (2006)
Atherosclerotic *Apoe*$^{-/-}$ +Mif blocking Ab	High-fat diet	Regression in established lesions Reduced macrophage and T-cell content	Bernhagen et al. (2007)
Injury-induced restenosis			
Ldlr$^{-/-}$ +Mif blocking Ab	Experimental angioplasty	Reduced neointimal size Reduced leukocyte recruitment Reduced cell proliferation in media and neointima Increased apoptosis in media and neointima	Chen et al. (2004)
Apoe$^{-/-}$ +Mif blocking Ab	Wire injury	No significant effect on neointimal size Reduced macrophage content Increased SMC and collagen content	Schober et al. (2004)
HUMAN EPIDEMIOLOGICAL STUDIES			
MIF SNP rs755622 (-173 CC genotype) risk factor for CHD and diverse inflammatory diseases *Although not confirmed by all studies*			Donn et al. (2001), Herder et al. (2008) Palomino-Morales et al. (2010)
MIF-173 CC genotype more frequent in Turkish children with cardiomyopathy			Col-Araz et al. (2012)
MIF SNP rs1007888 (GG genotype) associated with enhanced MI risk in female Czech patients			Tereshchenko et al. (2009)
Enhanced MIF plasma levels predictive for enhanced heart failure in CHD patients with impaired glucose tolerance or type 2 diabetes mellitus			Makino et al. (2010)
Increased MIF plasma levels in patients with ACS, associated with inflammatory marker expression (CRP, IL6)			Muller et al. (2012)
INFLAMMATORY/CARDIOVASCULAR EFFECTS OF MIF			
Monocytes/macrophages			
Enhances direct monocyte recruitment and arrest through CXCR2			Bernhagen et al. (2007)
Enhances CCL2-induced monocyte recruitment			Fan et al. (2011)
Enhances oxLDL uptake and degradation			Atsumi et al. (2000)

(Continued)

Table 5 | Continued

		Reference
Induces inflammatory mediators	TNFα, IL1β, IL6, IL8	Bernhagen et al. (1994), Calandra et al. (1994, 1995), Lan et al. (1997)
	NO, iNOS	Bernhagen et al. (1994), Lan et al. (1997)
Enhances expression of chemokines and adhesion molecules		Amin et al. (2006), Takahashi et al. (2009)
Interferes with p53-mediated apoptosis		Mitchell et al. (2002)
T-cells		
Enhances direct T-cell recruitment and arrest through CXCR2		Bernhagen et al. (2007)
SMCs		
Mif$^{-/-}$ SMCs: reduced cathepsin expression, reduced elastin/collagen degradation capacity		Pan et al. (2004)
Inhibits long-term PDGF-BB-induced SMC migration, despite short-term stimulatory effect		Schrans-Stassen et al. (2005)
Drives SMC proliferation in some studies (but not all)		Chen et al. (2004), Schrans-Stassen et al. (2005)
ECs		
Enhances the (cytokine-induced) expression of chemokines and adhesion molecules		Lin et al. (2000), Gregory et al. (2006, 2009), Cheng et al. (2010)
Other		
Colocalizes with MMP1/9 in human vulnerable plaques		Kong et al. (2005a,b)
Pro-angiogenic		Chesney et al. (1999), Ogawa et al. (2000); Amin et al. (2003)

Apoe, apolipoprotein E; Ldlr, low density lipoprotein receptor; ECs, endothelial cells; SMCs, smooth muscle cells; Ab, antibody; TNF, tumor necrosis factor; IL, interleukin; ICAM, intercellular adhesion molecule; MMP, matrix metalloproteinase; CEBP, CCAAT/enhancer binding protein; SNP, single nucleotide polymorphism; CHD, coronary heart disease; MI, myocardial infarction; ACS, acute coronary syndrome; CRP, C-reactive protein; oxLDL, oxidized low density lipoprotein; NO, nitrix oxide; iNOS, inducible nitric oxide synthase; PDGF, platelet-derived growth factor.

Importantly, human epidemiological studies support a pro-atherogenic role of MIF (**Table 5**). These studies showed a single nucleotide polymorphism (SNP) in the *MIF* promotor (*MIF*-173 CC genotype) to be a risk factor for coronary heart disease (CHD) (Herder et al., 2008) and to be more frequent in Turkish children with cardiomyopathy (Col-Araz et al., 2012). SNP rs1007888 (GG genotype) was associated with enhanced risk of myocardial infarction in female Czech patients (Tereshchenko et al., 2009). Furthermore, increased plasma levels of MIF were identified as a risk factor for increased heart failure in CHD patients with impaired glucose tolerance or type 2 diabetes mellitus (Makino et al., 2010), and were associated with inflammatory marker expression in patients with acute coronary syndrome (ACS) (Muller et al., 2012).

In conclusion, multiple animal and human studies support MIF's pro-atherosclerotic and pro-inflammatory role, and reveal MIF as an interesting target for drug development. The list of MIF inhibitors is steadily growing and includes small molecular weight or peptide drugs targeting mostly MIF's catalytic pocket or MIF trimerization (Garai and Lorand, 2009; Ouertatani-Sakouhi et al., 2010). An interesting therapeutic strategy would be to interfere with the CLF functions of MIF by blocking the interaction of MIF with its receptors. CXCR2 and CXCR4 inhibitors have recently been discussed in more detail (Liang, 2008; Stadtmann and Zarbock, 2012). However, an attractive alternative is a direct targeting of MIF instead of its receptors or devising strategies that would specifically target the MIF/CXCR interface but not other CXCR2- or CXCR4-mediated signaling effects, i.e., as stimulated by the cognate ligands CXCL8 or CXCL12, respectively. In the context of atherosclerosis, interference with MIF binding to both CXCR2 and CXCR4 by using a MIF blocking antibody interfered with MIF's pro-atherosclerotic functions (Bernhagen et al., 2007), while it would leave the protective homeostatic functions of the CXCL12-CXCR4 axis preserved (Koenen and Weber, 2010). Specific targeting of binding motifs in MIF, e.g., the pseudo-(E)LR and the N-like loop motifs critical for the MIF-CXCR2 interaction, provides an interesting strategy, but MIF motifs crucial for MIF-CXCR4 binding still remain to be identified. Finally, it is important to keep in mind that MIF exerts pleiotropic functions, and also behaves protective in different settings. For example, the MIF-CD74 axis is cardioprotective after myocardial ischemia/reperfusion injury (Miller et al., 2008; Qi et al., 2009; Luedike et al., 2012), and also exerts an important antifibrotic effect in experimental liver fibrosis (Heinrichs et al., 2011). Also, MIF polymorphisms associated with higher MIF expression were found to have a beneficial effect in community-acquired pneumonia (Yende et al., 2009). Therefore, possible negative side effects should always be carefully monitored for each new MIF inhibitor.

PERICYTES COORDINATE INTERSTITIAL LEUKOCYTE MIGRATION THROUGH MIF

MIF plays a pivotal role in leukocyte chemotaxis in the blood and other body fluids, in the arrest of leukocytes on the endothelium and their transmigration into the sub-endothelial space (Gregory et al., 2006; Bernhagen et al., 2007; Cheng et al., 2010; Santos et al.,

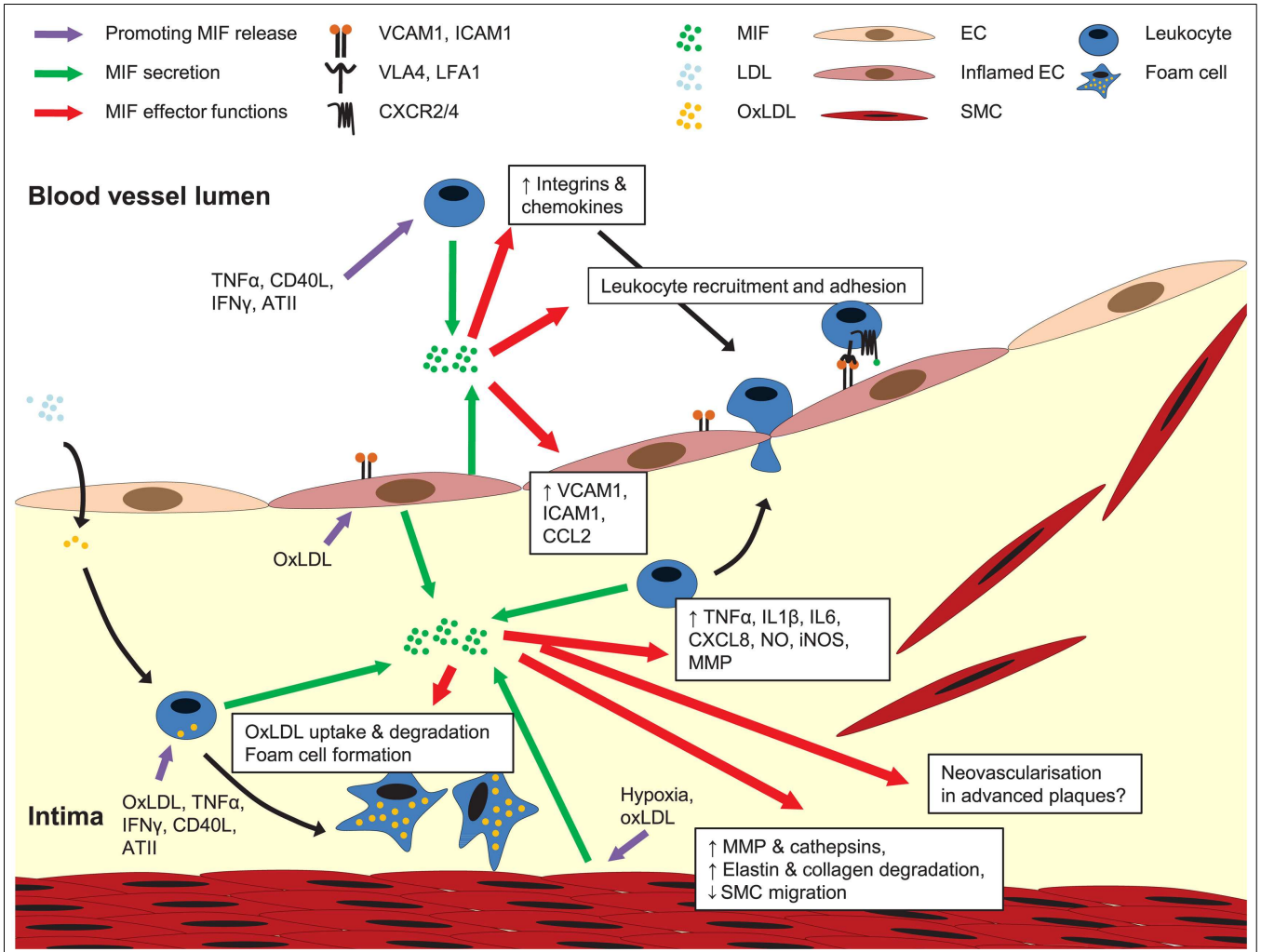

FIGURE 3 | Role of MIF in atherogenesis. MIF is secreted upon atherogenic stimulation of ECs, SMCs, and leukocytes. Once released, MIF activates leukocyte integrins, upregulates the expression of adhesion molecules and other chemokines, together mediating leukocyte recruitment and arrest on the endothelium. MIF is also implicated in pro-inflammatory cytokine expression, transdifferentiation of macrophages to foam cells, MMP and cathepsin induction in SMCs, and regulation of SMC proliferation and migration. A potential role for MIF in neovascularization in advanced plaques remains to be investigated. For more details, see text.

2011). Intriguingly, it was recently shown that MIF also plays a role in directing extravasated leukocytes in the peri-endothelial compartment to $NG2^+$ pericyte-rich regions along arterioles and capillaries (Stark et al., 2013) (**Box 4**). Stark et al. (2013) identified $NG2^+$ pericytes as the main source of MIF in the perivascular compartment of microvessels and demonstrated that stimulation of these cells with pro-inflammatory stimuli such as TNF, LPS, or damage-associated molecular patterns (DAMPs) released MIF and immobilized it on the cell surface of pericytes, probably through binding to CD74 and/or CXCR4. Furthermore, the release of MIF, CCL2, and CXCL8, together with the expression of ICAM1 on the pericyte surface mediated monocyte and neutrophil chemotaxis in an LFA1- and MAC1-dependent manner. As leukocyte extravasation occurs only in the postcapillary venules, which are covered by $NG2^-$ pericytes, but not in arterioles and capillaries containing $NG2^+$ pericytes, neutrophil and macrophage interaction with $NG2^+$ pericytes can only occur after successful interstitial migration from their entry point in postcapillary venules toward capillary and arteriolar pericytes, providing interstitial migration routes for the extravasated leukocytes (Murfee et al., 2005; Stark et al., 2013). The importance of MIF in this leukocyte migration track in the pericyte sheath was stressed by the observation that subcutaneous injection of the MIF inhibitor ISO-1 in mice reduced the number of accumulated neutrophils around $NG2^+$ pericytes in the skin microvasculature without affecting their extravasation (Stark et al., 2013). This finding, together with the capacity of MIF to induce CCL2-dependent leukocyte extravasation in postcapillary venules (Gregory et al., 2006), supports the concept, that the gradual interplay of MIF with different cell types, other chemokines, or inflammatory mediators is important for successful MIF-mediated leukocyte direction from the blood vessel lumen to the site of inflammation.

Box 4 | Pericytes.

Pericytes are also known as mural or rouget cells. They are essential components of microvessels, in which they are closely associated with the microvessel ECs, enveloped in a common basement membrane. Pericytes adhere to matrix proteins like fibrinogen, laminin, and collagen of the basement membrane through integrins. Due to their morphological and phenotypical heterogeneity, it is hard to distinguish them from other peri-endothelial cells, causing them to be often mixed up with vascular SMCs or mesenchymal cells. Also, no specific marker has been found yet. For example, pericytes covering postcapillary venules are $NG2^{-}$ αSMA^{+}, whereas pericytes on arterioles and capillaries are $NG2^{+}$. Some commonly used markers are α-smooth muscle actin (αSMA), alanyl(membrane)aminopeptidase (CD13), chondroitin sulfate proteoglycan 4 (NG2), melanoma cell adhesion molecule (CD146), platelet-derived growth factor receptor (PDGFR)-β, and desmin.

CONCLUDING REMARKS

MIF has been recognized as an important CLF chemokine mediating leukocyte recruitment and arrest in the context of many inflammatory diseases, in particular atherosclerosis. The receptors CD74, CXCR2, and CXCR4 have been identified to bind MIF and to mediate MIF-triggered arrest functions, but the molecular mechanisms underlying MIF-mediated receptor signaling toward different cellular functions still need further refinement. For example, the molecular sequelae of MIF-triggered events leading to integrin activation on leukocytes is still largely unexplored. In addition, the composition, balance, and differential functionality of different MIF receptor complexes needs further investigation. Identification and refinement of the critical receptor binding sites of MIF could stimulate the search for drugs that specifically interfere with MIF binding to only one or a certain selection of MIF receptors, with the aim to selectively interfere with only a subset of MIF's pleiotropic functions.

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CXCR2: from bench to bedside

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Leukocyte recruitment to sites of infection or tissue damage plays a crucial role for the innate immune response. Chemokine-dependent signaling in immune cells is a very important mechanism leading to integrin activation and leukocyte recruitment. CXC chemokine receptor 2 (CXCR2) is a prominent chemokine receptor on neutrophils. During the last years, several studies were performed investigating the role of CXCR2 in different diseases. Until now, many CXCR2 inhibitors are tested in animal models and clinical trials and promising results were obtained. This review gives an overview of the structure of CXCR2 and the signaling pathways that are activated following CXCR2 stimulation. We discuss in detail the role of this chemokine receptor in different disease models including acute lung injury, COPD, sepsis, and ischemia-reperfusion-injury. Furthermore, this review summarizes the results of clinical trials which used CXCR2 inhibitors.

Keywords: CXCR2, chemokine receptor, Gαi-signaling

INTRODUCTION

Inflammation is a defense reaction caused by infection or tissue damage. The aim of this process is to eliminate the inflammatory stimulus and protect the surrounding tissue from further damage making it necessary for the survival of the host. A defect in this system can severely affect the integrity of the organism and may be fatal. This is demonstrated in patients with hereditary or acquired immune deficiency. Neutrophil depletion can have detrimental effects in some disease models, but is beneficial in others (Henson and Johnston Jr., 1987; Weiss, 1989). The reduction of neutrophil recruitment in disease models elicited by bacteria resulted in decreased bacterial clearance and reduced survival (Craig et al., 2009). The same observation can be made in patients suffering from leukocyte adhesion deficiency (LAD). This disease is characterized by a defect in leukocyte extravasation, resulting in an inappropriate inflammatory response to injury or infection (Etzioni, 2010). Patients with this disease suffer from recurrent bacterial infections and have a reduced life expectancy (Etzioni, 2010).

Neutrophil granulocytes represent an important cellular component of the innate immune system and are recruited following an inflammatory stimulus in a coordinated sequence of events into inflamed tissue (Ley et al., 2007). The leukocyte recruitment cascade consists of different steps including capturing, rolling, slow rolling, adhesion, crawling, and other activation events prior transmigration (Ley et al., 2007). Leukocyte capturing and rolling is mediated by selectins (Ley et al., 2007), whereas slow leukocyte rolling and adhesion is predominantly mediated by integrins interacting with their ligands expressed on endothelial cells. Integrins are members of a large family of conserved adhesion receptors, which occur in a low affinity conformational state on circulating leukocytes. Selectin and immunoreceptor engagement and chemokine binding to their receptors activate signaling pathways leading to the activation of integrins (inside-out signaling). During adhesion, engaged integrins can signal into leukocytes (outside-in signaling), which stabilizes adhesion and initiates transmigration. The activation of neutrophils during the recruitment process is mediated by different mediators including selectins, chemokines, and integrin-mediated outside-in signaling.

Selectin engagement activates different signaling pathways leading to tyrosine phosphorylation, cytoskeletal rearrangement, β_2-integrin activation, cytokine secretion, and transcriptional activation. It has been shown that P- and E-selectin engagement induces β_2-integrin activation and reduces the rolling velocity on P-selectin/E-selectin and ICAM-1 (Zarbock et al., 2007b; Kuwano et al., 2010). Selectin engagement induces LFA-1 activation in a Syk (spleen tyrosine kinase)-dependent manner (Zarbock et al., 2007b). E-selectin engagement induces the phosphorylation of the Src kinase Fgr and the ITAM (immunoreceptor tyrosine-based activation motif)-containing adaptor proteins DAP12 and FcRγ (Zarbock et al., 2008a; Yago et al., 2010). DAP12 and FcRγ subsequently recruit and activate the tyrosine kinase Syk (Zarbock et al., 2008a). In neutrophils from $Fgr^{-/-}$ mice and $Lyn^{-/-}/Hck^{-/-}$ mice, DAP12, and Syk phosphorylation does not occur following E-selectin engagement (Zarbock et al., 2008a). In this signaling pathway, SLP-76 and the Tec family kinase Bruton's tyrosine kinase (Btk) are located downstream of Syk, whereas the signaling pathway downstream of Btk divides into a phosphoinositide 3-kinase (PI3K)γ- and PLCγ2-dependent pathway (Mueller et al., 2010; Block et al., 2012). Following E-selectin engagement the small GTPase Rap1 is activated downstream of PLCγ2 (Stadtmann et al., 2011). CalDAG-GEFI (Rasgrp2) and p38 MAPK are crucial signaling molecules between PLCγ2 and Rap1a (Stadtmann et al., 2011).

During rolling, leukocytes are exposed to different chemokines and chemoattractants presented on inflamed endothelial cells. Binding of chemokines to their receptors on leukocytes activates complex intracellular signaling networks which modulate integrin

activation and eventually lead to leukocyte adhesion mediated by binding of leukocyte integrins to their counter-receptors expressed on the endothelial cell surface.

Chemokine receptors are specific G protein-coupled receptors (GPCRs) on the cell surface and form specific subgroups depending on the binding capacities for members of distinct chemokine families. Chemokines are subdivided into different families depending on their structure characterized by the relative position of the first two cysteine residues of the chemokine representing the determining factor for the chemokine family classification (Baggiolini et al., 1994). For chemokines of the CC-chemokine family, the first two cysteines are adjacent to each other, whereas the first two cysteines in CXC chemokines are separated by one amino acid. Two chemokines are described so far showing a different positioning of their cysteines. Lymphotactin is characterized by the occurrence of only two cysteines and in fractalkine, the first two cysteines are separated by three amino acids (CX_3C; Kelner et al., 1994; Bazan et al., 1997). Until now, 10 receptors for CC-chemokines (CC-chemokine receptors, CCRs), seven for CXC chemokines (CXC chemokine receptors, CXCRs), and one CX_3C chemokine receptor (CX_3CR) are described (Murphy, 2002; Burns et al., 2006). Chemokine receptors on the cell surface of neutrophils are exposed to different chemokines during rolling on the inflamed endothelium. Following binding of the chemokine to its receptor, intracellular signaling cascades are activated resulting in integrin activation (Zarbock et al., 2012). Neutrophils express different chemokine receptors on their surface, like CXCR1, CXCR4, CCR2, and CX_3CR1, but for CXCR2 many different important functions are described. CXCR2 was cloned for the first time in 1991 from the human cell line HL-60 (Murphy and Tiffany, 1991). High affinity ligands for CXCR2, which is also expressed on other immune cells like mast cells, monocytes, and macrophages, are CXCL1, 2, 3, 5, 6, 7, and 8 (Olson and Ley, 2002). The most potent ligand of CXCR2 is CXCL8 as well as cleavage products of this chemokine (Van Damme et al., 1989). CXCL8 was first described and characterized as a product with chemotactic characteristics in the supernatant of LPS-stimulated human mononuclear cells in 1988 (Matsushima et al., 1988).

There is an important difference between human and murine neutrophils concerning chemokine receptor expression on the surface of neutrophils. Human neutrophils express CXCR1 and CXCR2, whereas murine neutrophils only express CXCR2, even if there are some recent reports about murine CXCR1 homologs (Fu et al., 2005; Moepps et al., 2006). High affinity ligands of CXCR1 are CXCL6 and 8 (Wolf et al., 1998). CXCL8 is the major CXCR2 ligand in humans, but in some cases, CXCL8 also binds to and mediates some functions via CXCR1. Rodents do not express CXCL8 (Reutershan, 2006).

Following adhesion, integrins may activate different signaling pathways that regulate several cellular functions including cell motility, polarization, respiratory burst, phagocytosis, proliferation, and apoptosis (Abram and Lowell, 2007). Integrin clustering and ligand-induced allosteric conformational changes likely initiate outside-in signaling and signalosome formation. The efficient protein tyrosine kinase (PTK) recruitment and activation of various signaling pathways require the formation of signalosomes (Ley et al., 2007). The two Src family kinase members Hck and Fgr are required for transducing LFA-1- and Mac-1-mediated outside-in signaling (Giagulli et al., 2006). However, these two Src family kinases are not required for chemoattractant-triggered upregulation of LFA-1 affinity and leukocyte arrest (Giagulli et al., 2006). Leukocyte adhesion strengthening can be abolished by blocking β_2-integrin-mediated outside-in signaling (Giagulli et al., 2006). Additionally, by eliminating WASP (Sato et al., 2012), the GEFs VAV1 and VAV3 (Gakidis et al., 2004), or PI3Kγ (Smith et al., 2006) representing important signaling molecules of leukocytes, adhesion strengthening can be blocked.

CHEMOKINE RECEPTORS ARE CHARACTERIZED BY DISTINCT STRUCTURAL PROPERTIES

Chemokine receptors are normally composed of 340–370 amino acids and show a 25–80% amino acid homology (Olson and Ley, 2002). GPCRs are seven-transmembrane proteins and consist of an α-subunit and a $\beta\gamma$-complex whereas the classification of these receptors depends on their α-subunit. Neutrophils express G_s-, G_i-, and G_q-family members with G_i-proteins representing the most important G proteins on neutrophils. This subclass mediates almost all pro-inflammatory effects of chemoattractants and can be subdivided into different subunits ($G\alpha_z$, $G\alpha_o$, $G\alpha_{i1}$, $G\alpha_{i2}$, and $G\alpha_{i3}$; Wilkie et al., 1992). With pertussis toxin (PTx), $G\alpha_i$-signaling with the exception of $G\alpha_z$-signaling can be blocked. Leukocytes abundantly express $G\alpha_{i2}$ and $G\alpha_{i3}$ (Jiang et al., 2002). A study by Zarbock et al. (2007a) demonstrated an important role for $G\alpha_{i2}$ for chemokine-induced neutrophil arrest in *in vitro* and *in vivo* models. A study using $G\alpha_{i2}$ deficient mice showed that $G\alpha_{i2}$ in non-hematopoietic cells is involved in leukocyte migration into the lung in an allergy model and after LPS application, whereas $G\alpha_{i2}$ in leukocytes is involved in regulating chemotaxis in response to chemokines (Pero et al., 2007). $G\alpha_{i3}$ regulates neutrophil migration via GIV ($G\alpha$ — interacting vesicle-associated protein; Ghosh et al., 2008), redistributes and localizes at the leading edge of the cell during the cell migration process (Ghosh et al., 2008). A number of $G\beta\gamma$-complexes can be formed, due to the fact that leukocytes express five different β-subunits and 12 γ-subunits (Wettschureck and Offermanns, 2005).

Chemokine receptors are special GPCRs and share some structural characteristics like an NH_2-terminal domain which is part of the chemokine binding site (Olson and Ley, 2002). They also have a conserved sequence in the second intracellular loop consisting of 10 amino acids (Olson and Ley, 2002) and share a characteristic cysteine within each extracellular domain and a short basic third intracellular loop (Murphy et al., 2000). The N-terminus of chemokine receptors which is characterized by a tyrosine sulfation motif (Murphy et al., 2000) is not important for the ligand binding affinity, however this domain is important for receptor triggering (Murphy et al., 2000). Recent studies made remarkable progress in the reconstruction of secondary and tertiary structures of GPCRs. Specialized methods like X-ray crystallography and electron microscopy revealed new insights into GPCR structures (Unger and Schertler, 1995; Pebay-Peyroula et al., 1997).

CXCR1 and CXCR2 are both $G\alpha_i$-coupled proteins and 78% of their amino acid sequences are identical (Reutershan, 2006). Divergent regions carrying the differences between the two receptors are the N-terminus, the C-terminus, the second extracellular

loop, and the fourth transmembrane domain (Nasser et al., 2007). These regions are probably responsible for functional differences between CXCR1 and CXCR2 which will be mentioned later on. CXCR2 is a member of the rhodopsin-like family of GPCRs, however the crystal structure of CXCR2 is not revealed, yet (Murphy et al., 2000).

GPCR-INDUCED SIGNALING

Binding of a chemokine to its receptor may induce many different cellular responses like adhesion, migration, and chemotaxis including cellular shape changes, reorganization of the actin cytoskeleton, upregulation of integrin expression, and integrin activation (Baggiolini, 1998).

The activation of a GPCR by the engagement of a chemoattractant results in an activation of the associated G protein, which dissociates into the GTP-bound Gα-subunit and the Gβγ-complex (Zarbock and Ley, 2008). Both, the α-subunit and the βγ-complex, are able to activate different signaling molecules (**Figure 1**).

Following dissociation, the α-subunit inhibits some adenylyl cyclase isoforms leading to a decrease of intracellular cAMP-levels and cAMP-dependent protein kinase activity (Sunahara et al., 1996). $G\alpha_i$-subunits are also involved in the activation of small GTPases following GPCR activation. $G\alpha_i$-dependent Ras activation induces the activation of phosphatidylinositol-3-kinase (PI3K) by directly binding to the catalytic subunit of PI3K (Rodriguez-Viciana et al., 1994).

The βγ-complex is able to activate PI3Kγ and the two phospholipase C (PLC) isoforms β_2 and β_3 (Camps et al., 1992; Hirsch et al., 2000). PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate to generate inositol trisphosphate (IP_3), which mobilizes calcium from non-mitochondrial stores, and diacylglycerol (DAG), which activates Ca^{2+}-independent and Ca^{2+}-dependent protein kinase C (PKC; Berridge and Irvine, 1984). Different PKC isoenzymes are required for activating cytotoxic effector functions of neutrophils (Mayer et al., 1996). PLC β_2-deficient neutrophils show enhanced chemotaxis and increased leukocyte recruitment in

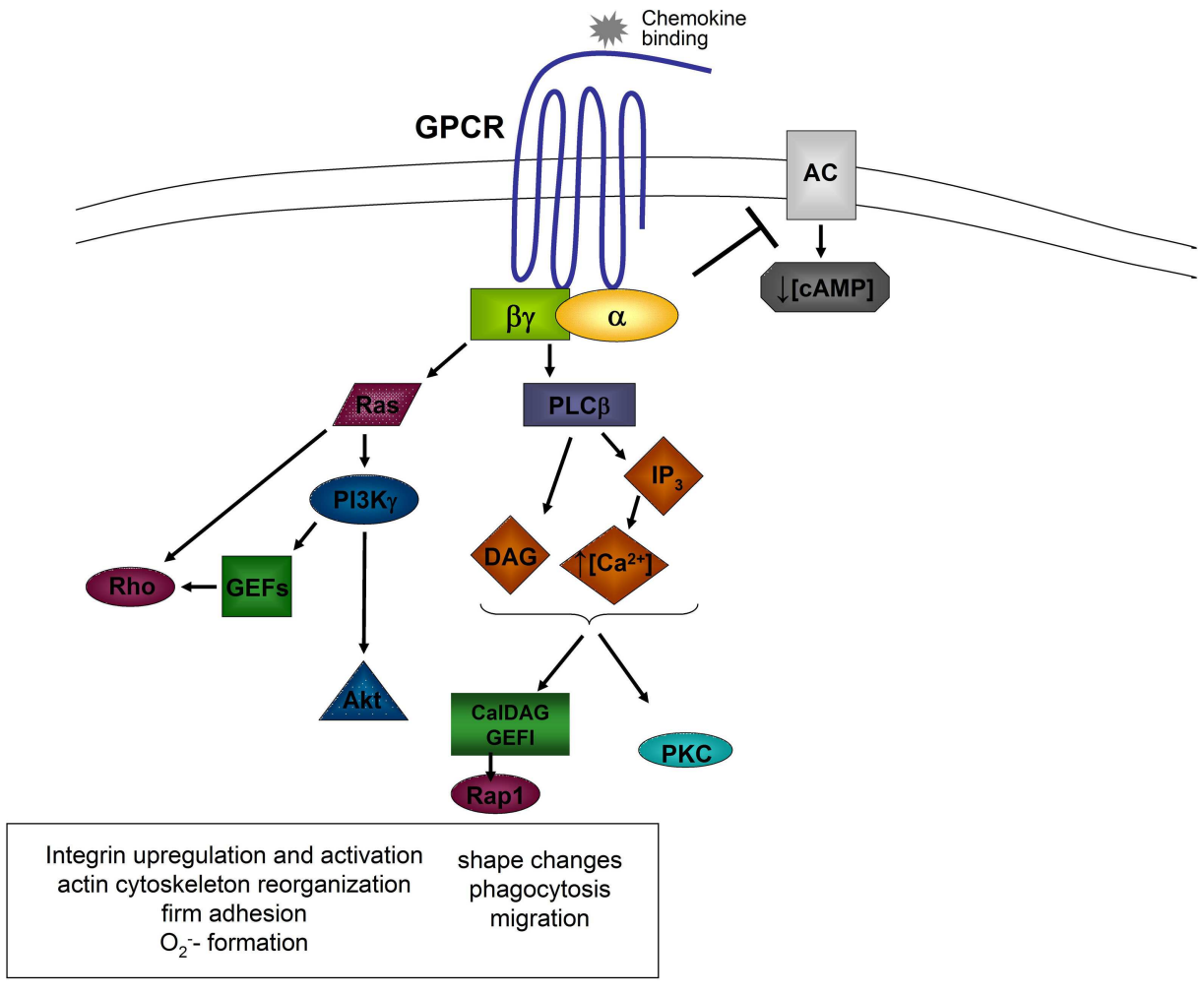

FIGURE 1 | General model of chemokine-induced signaling. Downstream of the α and βγ subunits, different signaling molecules are activated leading to different cellular functions. AC, adenylyl cyclase; Akt, proteine kinase B; Ca^{2+}, calcium; cAMP, cyclic adenine monophosphate; DAG, diacylglycerol; GEFs, guanine exchange factors; GPCR, G protein coupled receptor; IP_3, inositol triphosphate; PI3K, phosphatidylinositol-3-kinase; PKC, protein kinase C; PLCβ, phospholipase C beta; Rho, Ras, Rap1, small G proteins.

response to fMet-Leu-Phe (fMLP), but reduced chemoattractant-induced Ca^{2+} release and macrophage-1 antigen (Mac-1) upregulation (Jiang et al., 1997). Murine neutrophils deficient in both, PLC β_2 and PLC β_3, show impaired chemokine-stimulated O_2^- formation (Li et al., 2000). It has also been shown that PLC is involved in chemokine-induced $\alpha_4\beta_1$-integrin activation and monocyte adhesion (Hyduk et al., 2007). A recent study by Stadtmann et al. (2011) demonstrated that the GEF CalDAG-GEFI, which requires Ca^{2+} and DAG for activation, is necessary for chemokine-induced neutrophil arrest *in vivo*.

PI3Kγ, which can be activated by different βγ-subunits, catalyzes the phosphorylation of phosphatidylinositol-3,4-bisphosphate (PIP_2) to phosphatidylinositol-3,4,5-trisphosphate (PIP_3) which is able to bind to proteins containing pleckstrin homology domains, leading to downstream signaling (Varnai et al., 1999). Such an example for PI(3)Kγ-dependent downstream signaling is the activation of Akt, which clusters at the leading edge of migrating neutrophils (**Figure 1**; Servant et al., 2000).

The small GTPases of the Rho-family are also activated downstream of PI3Kγ (Servant et al., 2000). Neutrophils express Rac1 and Rac2 and both molecules regulate the migration of neutrophils, whereas Rac2 is also involved in the regulation of the respiratory burst (Glogauer et al., 2003; Gu et al., 2003). The activation of Rac is mediated by guanine nucleotide exchange factors (GEFs) by exchanging GDP for GTP. Different GEFs are expressed in neutrophils and these molecules are involved in Rac activation like the GEFs of the P-Rex family which are directly activated by the Gβγ subunit and PIP_3. In contrast, Vav family GEFs in neutrophils are activated in a Syk and Src kinases dependent pathway (**Figure 1**; Welch et al., 2002; Fumagalli et al., 2007). A recent study by Lawson et al. (2011) demonstrated that P-Rex-1- and Vav-1-deficiency mediates severe defects in GPCR dependent neutrophil activation. Another important molecule called DOCK2 (dedicator of cytokinesis 2), can also regulate the activity of Rac1 and Rac2 (Kunisaki et al., 2006) and influences cell polarity changes and translocation speed (Kunisaki et al., 2006). Activated Rac activates p21-activated kinase (PAK), which can subsequently induce the phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2), p38 mitogen-activated protein kinase, and c-Jun N-terminal kinase (Kim and Dinauer, 2001; Fumagalli et al., 2007).

CXCR2-triggered signaling uses the same molecules described above, but a recent study by Wu et al. (2012) revealed a new mechanism for the coupling of CXCR2 to its downstream signaling molecules. They showed that the PDZ scaffold protein Na^+/H^+ exchanger regulatory factor-1 (NHERF1) couples CXCR2 to its downstream effector PLC-β2, forming a macromolecular complex, through a PDZ-based interaction (Wu et al., 2012). Disruption of this complex led to a decrease of intracellular calcium concentrations on the molecular level, and suppressed neutrophil chemotaxis and migration on the cellular level following chemokine stimulation (Wu et al., 2012).

There are differences described for the signaling cascades downstream of either CXCR1 or CXCR2 activation. In contrast to CXCR2 dependent signaling, phospholipase D gets activated and neutrophils are primed to perform respiratory burst following CXCR1 activation (L'Heureux et al., 1995; Jones et al., 1996). Due to these data, it is likely that the activation of the two receptors play different roles under inflammatory conditions.

CXCR2 IS INVOLVED IN PHYSIOLOGICAL AND PATHOLOGICAL CONDITIONS

Neutrophils are essential for maintaining innate immune surveillance under normal conditions, but also represent a major contributor to tissue damage during autoimmune processes. Therefore, neutrophil homeostasis and recruitment are tightly regulated. CXCR2 plays a critical role in the regulation of neutrophil homeostasis (Eash et al., 2010; von Vietinghoff et al., 2010; Mei et al., 2012), because CXCR2-deficient mice demonstrate mild neutrophilia and severe neutrophil hyperplasia in the bone marrow (Shuster et al., 1995). A recent study demonstrated that CXCR2-signaling is a second chemokine axis that interacts antagonistically with CXCR4 to regulate neutrophil release from the bone marrow (Eash et al., 2010).

During inflammation, leukocyte extravasation from the blood vessel into inflamed tissue is one of the hallmarks of the immune system. However, leukocyte recruitment has to be tightly regulated, as excessive leukocyte extravasation may lead to the deterioration of the integrity of the organism and may worsen acute and chronic inflammatory diseases. Different chemokines, which are released during inflammation, direct leukocytes to the site of inflammation. The chemokine receptor CXCR2 and its ligands have been implicated in a variety of inflammatory disorders making it an interesting target for therapeutical approaches (Seitz et al., 1991; Boyle Jr. et al., 1998; Kurdowska et al., 2001; Bizzarri et al., 2006; Reutershan, 2006; Chapman et al., 2009). In several inflammatory disease models, blocking, or eliminating CXCR2 substantially reduces leukocyte recruitment, tissue damage, and mortality. Based on the physiological importance of CXCR2, selective CXCR2 inhibitors have been developed that are now being tested in clinical trials. Due to the structural similarities between CXCR1 and CXCR2 or an influence by the activity of one of these receptors on the activity of the other, CXCR2 manipulation may affect CXCR1 dependent functions. This possibility has to be tightly controlled, but experiments in the murine system do not include these cross-reactivities because murine neutrophils do not express CXCR1. The following part summarizes current knowledge about CXCR2 in inflammatory diseases and discusses its potential as a pharmaceutical target.

LUNG DISEASES

CXCR2 is found on many cells including leukocytes, endothelial, and epithelial cells. On endothelial cells, CXCR2 expression was demonstrated for the human and murine system (Murdoch et al., 1999; Addison et al., 2000). Additionally it was demonstrated that CXCR2 expression is important for angiogenesis and supports tumor growth (Addison et al., 2000; Keane et al., 2004). Lung epithelium of COPD patients expresses elevated levels of CXCR2, but not CXCR1, indicating different roles for these two receptors under COPD disease conditions (de Boer, 2002; Qiu et al., 2003).

Due to its expression on lung endothelial and epithelial cells, it is not surprising that CXCR2 has been implicated in different lung diseases. Several studies have identified an important role

for CXCR2 in acute lung injury (ALI), asthma, COPD, and cystic fibrosis (CF).

ACUTE LUNG INJURY

Acute lung injury is characterized by a damage of the alveolar-capillary barrier resulting in infiltration of neutrophils into the lungs, pulmonary edema, remodeling of the alveolar and small airway epithelium, and collagen deposition in the pulmonary interstitium. Lung fibrosis can result from ALI (Ley and Zarbock, 2008; Chapman et al., 2009). ALI may completely resolve or proceed to fibrosing alveolitis accompanied by persistent low oxygen in the blood (hypoxemia) and a reduced ability of the lung to expand with every breath (reduced pulmonary compliance; Rubenfeld et al., 2005). In both pulmonary diseases, a functional role for CXCR2 has been implicated.

Acute lung injury is a common disease with an incidence of 79 per 100,000 person-years in the United States (Rubenfeld et al., 2005). Despite the use of state-of-the-art treatment, this disease is associated with high mortality of up to 38% (Rubenfeld et al., 2005). Pneumonia and acid aspiration are intrapulmonary causes of ALI whereas trauma, massive transfusion, and sepsis are typical causes of extrapulmonary ALI. During ALI, neutrophils are recruited in the alveolar compartment and increased CXCL8 levels in the BAL fluid of patients with ALI have been positively correlated with the presence of activated neutrophils (Aggarwal et al., 2000; Kurdowska et al., 2001; Keane et al., 2002; Puneet et al., 2005). High levels of CXCL8 complexed with anti-CXCL8 autoantibodies were found in the alveolar fluid of patients suffering from ALI (Fudala et al., 2007). As these complexes can inhibit neutrophil apoptosis (Fudala et al., 2007), it is possible that this condition may prolong neutrophil survival and exacerbate the deleterious effects of neutrophil activation. These data identify CXCL8 as an important chemokine in the pathogenesis of ALI (Puneet et al., 2005). In addition to this, CXCL8 has also been associated with other pathophysiological aspects of ALI. Due to an increase in vascular permeability, higher levels of α_2 macroglobulin, and CXCL2 are found in the BAL of of patients with ALI (Kurdowska et al., 1997). CXCL8 and α_2 macroglobulin form complexes which maintain chemoattractant activity (Kurdowska et al., 2001). Based on this fact, it is likely that the complex perpetuates the inflammatory response in the lungs of ALI patients. Patients with CXCL8 gene polymorphisms accompanied with higher levels of CXCL8 have more prolonged and extensive lung injury which requires an extended time on ventilatory support (Hildebrand et al., 2007).

In animal models of ALI, a very important role for CXCL8 and CXCR2 has been clearly identified. Exposure to hyperoxic gas (Sue et al., 2004) and ventilator-induced lung injury (Strieter et al., 2005b) caused neutrophil recruitment into the lung, increased airway microvascular leakage, and induced lung edema. Each of these pathological changes was diminished in WT animals treated with neutralizing antibodies to CXCR2 or in CXCR2-deficient mice. Similar findings were observed following blocking CXCR2 in animal models of lung injury induced by lipopolysaccharide (LPS; Chapman et al., 2007), after lung infection with viral or bacterial challenge (Tsai et al., 2000; Del et al., 2001; Londhe et al., 2005b; Strieter et al., 2005a) and after induction of hemorrhagic shock (Lomas-Neira et al., 2004).

Angiogenesis is considered to be an important component of collagen deposition and fibrosis formation in the lungs (Keane et al., 2004). A variety of CXC chemokines possessing angiogenic properties are expressed in pulmonary edema fluid of patients with ALI (Keane et al., 2002) with CXCR2 as an important receptor mediating this effect (Addison et al., 2000). These data suggest that inhibition of CXCR2 may also be beneficial in reducing the development of pulmonary fibrosis in ALI patients.

CHRONIC OBSTRUCTIVE PULMONARY DISEASE

Chronic obstructive pulmonary disease (COPD) is characterized by a limitation of the airflow that is not fully reversible, and is usually progressive with an abnormal inflammatory response (Rabe et al., 2007). This disease is primarily caused by cigarette smoking where recurrent lung infections may induce a progressive decline in lung function. Small airway disease (obstructive bronchiolitis) and parenchymal destruction (emphysema) are the causes of the limited airflow. Bacterial and viral infections are frequent causes of COPD exacerbations. No medication exists that prevents the long-term decline in lung function, however at the present time, inhaled anticholinergics, β-adrenergic bronchodilators, and corticosteroids are used to treat the symptoms and exacerbations of COPD.

Chronic bronchitis is associated with excess mucus secretions in the large airways and a large number of recruited leukocytes, especially neutrophils (de Boer et al., 2000; Rabe et al., 2007). As chronic bronchitis is associated with mucus hypersecretion and neutrophil recruitment, it is not surprising that CXC chemokines and CXCR2 expression in the bronchial biopsies and sputum of COPD patients are increased (Traves et al., 2002; Qiu et al., 2003). Animal studies using lung infection models demonstrated that CXCR2 has an important role in this response (Tsai et al., 2000; Del et al., 2001; Miller et al., 2003; Londhe et al., 2005a). Similar observations can be made in humans suffering from COPD. Increased CXCR2 mRNA expression is present in bronchial biopsy specimens from COPD patients which goes along with the presence of tissue neutrophils during severe exacerbations of COPD (Qiu et al., 2003). These data suggest that CXCR2 inhibition may be a viable therapeutic approach against the inflammatory events occurring in the distal lungs of human COPD patients.

In contrast to the mucus hypersecretion in the large conducting airways, the presence of inflammatory mucus exudates in the peripheral airways contributes significantly to the airflow obstruction in COPD (Hogg et al., 2004). In animal studies using different COPD models, blocking CXCR2 inhibited mucus hypersecretion, and goblet cell hyperplasia (Miller et al., 2003; Stevenson et al., 2005; Chapman et al., 2007). However, these animal models investigated the mucus production in the large conducting airways, where the mucus rarely leads to airflow obstruction. Therefore, it will be important to assess the effect of CXCR2 blockade on mucus inflammatory exudates and obstruction in the peripheral airways.

ASTHMA

Asthma is a common chronic inflammatory disease of the airways characterized by variable and recurring symptoms, reversible airflow obstruction, bronchial hyperresponsiveness, chronic eosinophilic lung inflammation, and bronchospasm

(Bousquet et al., 2000). Symptoms include coughing, wheezing, chest tightness, and shortness of breath (Bousquet et al., 2000). Mild to moderate asthma is treated with a combination of inhaled beta-adrenergic bronchodilators and corticosteroids. However, severe asthmatics only respond poorly to inhaled beta-adrenergic agonists and corticosteroids. Severe asthma is characterized by a predominantly neutrophilic inflammation of the lung with airway remodeling.

During severe asthma, a positive correlation between increased expression of ELR$^+$ CXC chemokines, which carry the Glu-Leu-Arg (ELR) tripeptide motif at the NH2-terminus, and the presence of neutrophils in the lung exists (Kurashima et al., 1996; Lamblin et al., 1998; Norzila et al., 2000; Pease and Sabroe, 2002; Mukaida, 2003). In addition, it has been shown that increases in sputum CXCL8 precede the exacerbations of acute asthma (Mukaida, 2003). Viral and bacterial lung infections contribute significantly to the frequency of asthma exacerbations and studies in animals have shown an important role for CXCR2 in this response.

Eosinophils are present in sputum and bronchial biopsies of patients with mild to moderate asthma. Under physiological conditions, the expression and role of CXCR2 on eosinophils is uncertain. However, it is possible that this receptor plays a role on these cells under conditions of chronic lung inflammation. Given the fact that CXCR2 is highly expressed on the vascular endothelium and that animal studies have demonstrated a role for endothelial CXCR2 on mast cell migration into tissue following sensitization to allergen (Hillyer et al., 2003; Abonia et al., 2005; Hallgren et al., 2007), this may be important in inducing acute phase responses to allergen challenge.

The increased formation of blood vessels in airway mucosa with associated changes in the vascularity is a characteristic of human asthma (McDonald, 2001; Hashimoto et al., 2005). Th_2-dependent cytokines induce the synthesis of angiogenic chemokines (Matsuda et al., 2008), and different studies demonstrated that CXCR2 and its ligands are involved in the formation of new blood vessels in the lungs (Addison et al., 2000; Belperio et al., 2000; Babu et al., 2007; Mohsenin et al., 2007). CXCR2 is also expressed on airway smooth muscles and it could be speculated that the receptor is involved in the contractile and migratory responses of airway smooth muscle in chronic asthma (Govindaraju et al., 2006). This also raises the possibility that CXCR2 on airway smooth muscle cells is involved in the development of bronchial hyperresponsiveness in asthmatics. Indeed, inhalation of CXCL8 causes bronchoconstriction in pigs (Fujimura et al., 1999).

These data identify an important role for the CXC chemokines and CXCR2 in lung inflammation, lung histopathology, and abnormal physiology that is seen in asthma.

CYSTIC FIBROSIS

Cystic fibrosis is an autosomal genetic disease affecting most critically the lungs, and also the pancreas, intestine, and liver. CF is caused by a mutation in the gene for the protein CF transmembrane conductance regulator (CFTR) and the disease is characterized by abnormal transport of chloride and sodium across epithelium, leading to thick, viscous secretions (Clunes and Boucher, 2007). The absence or lack of functional CFTR in the airway epithelium leads to dysfunctional lung mucociliary clearance, recurrent lung infections, hypertrophy and hyperplasia of mucus secreting cells and glands, and small airway obstruction. The airways of patients suffering from CF are frequently infected with bacterial pathogens, which determine morbidity and mortality in these patients (Chapman et al., 2009). As a consequence of the airway infection, pro-inflammatory cytokines, and chemokines, including CXCL8, are produced that attract large numbers of neutrophils into the lung (Dean et al., 1993; Elizur et al., 2008). However, it is very likely that a variety of chemokines are involved in the pathology of CF as several non-ELR$^+$ CXC chemokines are also known to play a role in the inflammatory process of CF (Mackerness et al., 2008).

In CF patients, high levels of neutrophil elastase are found in airway secretions (Goldstein and Doring, 1986) which probably participates in the elevated mucus secretion in these patients (Voynow et al., 2004; Tirouvanziam et al., 2008). Studies of different animal models indicated that CXCR2 is involved in mucus hypersecretion and proliferation of mucus secreting cells in the airway. Neutrophils are also important for the antimicrobial response of the lungs (Tsai et al., 2000; Hartl et al., 2007). However, recently published studies suggest that CXCR1 rather than CXCR2 is the functionally important receptor involved in neutrophil degranulation (Geiser et al., 1993; Jones et al., 1996; Patel et al., 2001; Feniger-Barish et al., 2003). Therefore, blocking CXCR2 should not affect neutrophil phagocytosis and mediator release. As there is evidence showing the cleavage of CXCR1 on neutrophils, which disables the bacterial-killing capacity of neutrophils from CF patients (Hartl et al., 2007), the incidence of infection should be closely monitored.

SEPSIS

Sepsis is a major healthcare problem, affecting millions of individuals around the world each year, killing one in four, and increasing in incidence (Dombrovskiy et al., 2007; Andaluz-Ojeda et al., 2011). The immune system combats microbial infections but, in severe sepsis, its untoward activity seems to contribute to organ dysfunction. The inappropriate activation and positioning of neutrophils within the microvasculature contributes to the pathological manifestations of multiple organ failure.

Cummings et al. (1999) showed that the expression levels of CXCR2 on circulating neutrophils of septic patients are decreased by approximately 50% in comparison to control donors. This was associated with a reduced migratory activity of neutrophils toward ligands specifically binding to CXCR2 (CXCL1-3 and CXCL5), while migration toward IL-8 was unaffected (Cummings et al., 1999). The expression level of CXCR1 did not show any significant alterations, suggesting an important role of CXCR1 in septic patients (Cummings et al., 1999). CXCR2 downregulation can be explained by the high levels of soluble chemokines circulating within the plasma of septic patients (Phillipson and Kubes, 2011).

While CXCR2 is important for leukocyte extravasation into inflamed tissue and might be highly relevant for bacterial clearance and survival in bacteria-induced pulmonary inflammation, it has deleterious effects in sepsis. Blocking or eliminating CXCR2 decreased liver injury and mortality in a murine model of cecal ligation and puncture (CLP)-induced sepsis (Ness et al.,

2003). Although neutrophil recruitment into the peritoneum was delayed, bacterial clearance was not affected by eliminating CXCR2. Application of cell-penetrating lipopeptides, which block CXCR1- and CXCR2-signaling, reversed the lethal sequelae of sepsis, including multi-organ failure and disseminated intravascular coagulation in mice, indicating that CXCR2 is very important in sepsis (Kaneider et al., 2005).

REPERFUSION-INJURY

Ischemia-reperfusion-injury contributes to morbidity and mortality in a wide range of pathologies, including circulatory arrest, ischemic stroke, myocardial infarction, acute kidney injury, and trauma. Additionally, it is a common challenge during cardiothoracic and vascular surgery and organ transplantation. Due to the reduced metabolic supply in the ischemic organ, tissue hypoxia, and microvascular dysfunction occur. The subsequent reperfusion activates an innate and adaptive immune response (Eltzschig and Eckle, 2011) with a characteristic strong accumulation of inflammatory cells, predominantly neutrophils, into the injured organs leading to tissue injury (Eltzschig and Eckle, 2011).

Since neutrophil infiltration is a major cause of tissue injury, mechanisms involved in neutrophil recruitment following an inflammatory stimulus are interesting targets for therapeutic approaches. Several recent studies demonstrated a positive effect of inhibiting neutrophil recruitment into the region of ischemia-reperfusion (Kempf et al., 2011; Block et al., 2012).

Neutrophil depletion reduces tissue injury after myocardial ischemia-reperfusion in patients (Palatianos et al., 2004) and animals (Litt et al., 1989). In a model of myocardial infarction, Tarzami et al. (2003) demonstrated that the infarct size in CXCR2-deficient mice is significantly reduced in comparison to wildtype mice, predominantly mediated by CXCR2 on hematopoietic cells. More precise, investigation of the infarcted zone revealed a decreased number of infiltrated immune cells in $CXCR2^{-/-}$ mice (Tarzami et al., 2003). This finding is consistent with the important role of CXCR2 in leukocyte recruitment into inflamed tissue.

During organ transplantation, reperfusion-injury mediated by neutrophils is a major challenge, because it is associated with increased morbidity and mortality (King et al., 2000). Neutrophil accumulation in the kidney occurs rapidly after reperfusion, is associated with an increased CXCR2 and CXCL1 expression in the graft and is an important predictor of delayed graft function after kidney transplantation (Turunen et al., 2004). Blocking CXCR2 by an inhibitor reduces neutrophil accumulation in the kidney and maintains kidney function (Cugini et al., 2005). Inhibition of CXCR2 also reduced neutrophil recruitment and organ dysfunction in other models of ischemia-reperfusion-injury (Bertini et al., 2004; Souza et al., 2004; Belperio et al., 2005).

POSSIBLE THERAPEUTIC STRATEGIES BY BLOCKING CXCR2

As described above, CXCR2 and CXCR2 ligands are involved in many processes which can influence different disease conditions. Therefore, controlling CXCR2 and therewith CXCR2 dependent processes can be powerful therapeutic mechanisms. Affecting CXCR2 dependent pathways is possible in different ways. Distinct strategies including N-terminally modified chemokines, antibodies, and small-molecule antagonists were tested.

Several low molecular weight CXCR2 antagonists have been developed and tested in different *in vitro* and *in vivo* models. The first low molecular weight CXCR2 antagonist was described in 1998 in a study by White et al. (1998). The described antagonist was a selective non-peptide antagonist of CXCR2 and inhibited CXCL8 and GROα dependent neutrophil chemotaxis *in vitro*. Therefore, it was suggested as a potential tool for therapeutic application (White et al., 1998). Following the identification of this compound, a class of diarylureas was tested in different disease models for possible therapeutic usage.

Another type of CXCR2 inhibitors are allosteric inhibitors, which block CXCR2 function by blocking receptor signaling instead of chemokine binding. In the beginning of allosteric inhibitor investigation, a concentration-dependent inhibitory effect on CXCL8 function, such as neutrophil chemotaxis was reported (Souza et al., 2004). Non-steroidal anti-inflammatory drugs known as ketoprofen and ibuprofen were reported to be potent inhibitors of CXCL8 dependent neutrophil chemotaxis (Bizzarri et al., 2001). Subsequently, a series of potent allosteric CXCR2 inhibitors were described. Another non-competitive allosteric inhibitor is Reparixin (or Repertaxin), which specifically blocks CXCR1 or CXCR2-mediated neutrophil migration *in vitro* without affecting other receptors (Bertini et al., 2004). It was previously demonstrated that Reparixin inhibits CXCL8 induced neutrophil activation and blocks the increase of intracellular free calcium, elastase release, and production of reactive oxygen intermediates (Bertini et al., 2004). The application of this inhibitor in different disease models demonstrated that Reparixin is able to mediate beneficial effects in a bacteria-induced peritonitis, a venom-induced lung injury, and different models of ischemia-reperfusion-injury (Bertini et al., 2004; Souza et al., 2004; Cugini et al., 2005; Garau et al., 2005; Coelho et al., 2007). A recently published study by Zarbock et al. (2008b) demonstrated that Reparixin attenuates ALI by reducing neutrophil recruitment and vascular permeability. Because of suboptimal pharmacokinetic characteristics of Reparixin, related compounds were tested and DF 2162 was reported to have similar effects as Reparixin, but better pharmacokinetic characteristics (Coelho et al., 2007; Cunha et al., 2008; Chapman et al., 2009).

The pyrimidine series-based CXCR2 antagonist AZD-8309 has been clinically tested in different disease models. A recent report by Virtala et al. (2011) reported that AZD-8309 application reduced LPS-induced neutrophil recruitment and elastase activity about 50% in comparison to placebo application in a lower airway LPS model.

Different CXCR2 antagonists developed by GlaxoSmithKline were also reported to be able to inhibit chemokine binding to CXCR2, mediating decreased migration in response to CXCL1, 5, and 7 (Traves et al., 2004). Another antagonist was demonstrated to inhibit CD11b upregulation and shape changes as characteristics of neutrophil activation following stimulation with CXCL1 of neutrophils from COPD patients (Chapman et al., 2009). One of the antagonists of this group, SB-656933, was already tested in clinical trials of CF and ozone-induced tissue injury. In this study, oral administration of SB-656933 inhibited CXCL1-induced CD11b upregulation on neutrophils and reduced ozone-induced airway inflammation in a dose-dependent manner. This was quantified

by neutrophil counts in the sputum following ozone challenge (Lazaar et al., 2011).

Another recent study reported that the CXCR2 antagonist SCH527123, which is a potent inhibitor of neutrophil activation and trafficking in animal models, is a potent inhibitor of ozone-induced neutrophil recruitment in a human clinical trial (Holz et al., 2010). SCH527123 treatment of healthy humans resulted in significantly lower sputum total cell and neutrophil counts as well as CXCL8 levels following ozone treatment in comparison to prednisolon or placebo treated study participants (Holz et al., 2010).

GSK1325756, a specifically designed CXCR2 antagonist, was already tested in a phase I clinical trial, determining the effects of GSK1325756 in healthy adult volunteers (www.clinicaltrials.gov, study number NCT01267006). In this study, blood parameters were checked in participants treated with either a placebo or GSK1325756. In another group the effect of food to the levels and outcome of GSK1325756 was investigated.

As CXCR2 is involved in the pathogenesis of many diseases, this receptor and its ligands are interesting targets for clinical trials. However, it has to be kept in mind that blocking CXCR2 can have beneficial but also harmful effects. Blocking CXCR2 inhibits the inflammatory response. Following bacterial or viral infections, the inhibition of the immune response can be very dangerous. Pathogens cannot be eliminated accurately, leading to dissemination of the pathogen and systemic infection. On the other hand, under sterile inflammatory conditions, for example following ischemia-reperfusion-injury, decreasing the neutrophil recruitment and the immune response can be beneficial. Further studies and clinical trials are necessary to further elucidate the exact effects of blocking CXCR2 and/or its ligands under different disease conditions.

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Duffy antigen receptor for chemokines and its involvement in patterning and control of inflammatory chemokines

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Leukocyte functions are linked to their migratory responses, which, in turn, are largely determined by the expression profile of classical chemokine receptors. Upon binding their cognate chemokines, these G-protein-coupled receptors (GPCRs) initiate signaling cascades and downstream molecular and cellular responses, including integrin activation and cell locomotion. Chemokines also bind to an alternative subset of chemokine receptors, which have serpentine structure characteristic for GPCRs but lack DRYLAIV consensus motive required for coupling to G-proteins. Duffy antigen receptor for chemokines (DARC) is a member of this atypical receptor subfamily. DARC binds a broad range of inflammatory CXC and CC chemokines and is expressed by erythrocytes, venular endothelial cells, and cerebellar neurons. Erythrocyte DARC serves as blood reservoir of cognate chemokines but also as a chemokine sink, buffering potential surges in plasma chemokine levels. Endothelial cell DARC internalizes chemokines on the basolateral cell surface resulting in subsequent transcytosis of chemokines and their immobilization on the tips of apical microvilli. These DARC-mediated endothelial cell interactions allow chemokines produced in the extravascular tissues to optimally function as arrest chemokines on the luminal endothelial cell surface.

Keywords: atypical chemokine receptors, chemokines, DARC, duffy antigen, endothelial cells, erythrocytes, inflammation, transcytosis

DUFFY BLOOD GROUP ANTIGEN

The Duffy antigen receptor for chemokines (DARC) has recently become the focus of studies investigating interactions of inflammatory chemokines with erythrocytes during systemic inflammatory responses as well as with venular endothelial cells during chemokine-induced leukocyte adhesion and emigration. These studies uncovered new functional facets of this rather "old" molecule. DARC was first described in 1950 as the Duffy blood group antigen (Cutbush and Mollison, 1950; Cutbush et al., 1950). An antibody termed anti-Fy^a present in the plasma of a polytransfused hemophiliac, Mr. Duffy, was found to cause a delayed hemolytic transfusion reaction. In the following year, an antibody to the antithetic antigen, Fy^b, was found in a multigravida exposed to fetal Fy^b erythrocytes (Ikin et al., 1951). Subsequently, three "Duffy-positive" phenotypes were described: Fy(a+b−), Fy(a−b+), and Fy(a+b+), arising from combinations of the antithetical co-dominant *FYA* and *FYB* genes (Klein and Anstee, 2005). However, some individuals, designated "Duffy-negative," express neither Fy^a nor Fy^b antigens, Fy(a−b−). This phenotype results from a polymorphic form of the *FYB* gene, *FYB(ES)* "erythroid silent", present in up to 99% of West Africans and the majority of African Americans (68%; Mourant et al., 1976). The DARC (Fy) gene is located on chromosome 1, position 1922 and segregates with *Un* locus, having been the first to be assigned to an autosome in humans (Iwamoto et al., 1996). The two main alleles *FYA* and *FYB* differ in a single base substitution (125 G to A) in codon 42 in the NH_2 extracellular domain, encoding glycine in Fy^a and aspartic acid in Fy^b (Chaudhuri et al., 1995; Iwamoto et al., 1995). The *FYB(ES)* allele has a single T to C substitution at nucleotide −*67* within the erythroid GATA-1 promoter region, 33 bp upstream from the erythroid transcription starting point and 46 bp upstream from the start of the major transcript translation codon, thus preventing DARC transcription in erythroid cells only (Tournamille et al., 1995).

Hence *FYB(ES)* Fy(a−b−) individuals still express DARC at non-erythroid sites, e.g., on endothelial cells and possibly other cells (Peiper et al., 1995; Chaudhuri et al., 1997; Horuk et al., 1997). The Duffy-negative phenotype was first linked with resistance to malaria when Fy(a−b−) volunteers exposed to the bites of *Plasmodium vivax*-infected mosquitoes, in contrast to Duffy-positives, did not develop malaria (Miller et al., 1976). This confirmed the long standing clinical observation that African populations appeared resistant to this form of malaria, noted also during the treatment of neurosyphilis by therapeutic *P. vivax* inoculation (O'Leary, 1927; Boyd and Stratman-Thomas, 1933). Further work showed that this parasite requires DARC binding for entry into the erythrocytes (Miller et al., 1975; Horuk et al., 1993a), leading to the hypothesis that the Fy(a−b−) phenotype evolved as a result of selective pressure to protect its carriers from *vivax* but not *falciparum* malaria. Geostatistical maps show that in West Africa the areas of prevalence of the Fy(a−b−) phenotype of almost 100% (Howes et al., 2011), overlap with areas of expected but absent

p. vivax infection (Guerra et al., 2010). However, this resistance is not complete and some Fy(a−b−) populations, for example in Madagascar, both carry parasites asymptomatically and experience symptomatic *vivax* malaria (Ménard et al., 2010).

Other rare DARC polymorphisms include a C*265*T mutation in *FYB* leading to *FYX* allele and 90% reduction of DARC expression, the so called "Fy^b weak" phenotype, and the G*298*A polymorphism resulting in the Ala100Thr substitution (Olsson et al., 1998; Tournamille et al., 1998).

STRUCTURAL CHARACTERISTICS OF DARC

Human DARC contains 336 amino acids (molecular weight 35733) and was first predicted to have nine trans-membrane domains (Chaudhuri et al., 1993), but later shown to have seven, akin to other chemokine receptors (Neote et al., 1994). The extracellular amino-terminal domain of 65 amino acids harbors three potential *N*-glycosylation sites at residues 16, 27, and 33 (Czerwinski et al., 2007), and epitopes Fy^a, Fy^b, and Fy6 (Tournamille et al., 2003). The Fy6 epitope is between Q19 and W26 residues, the binding site of the reticulocyte binding protein of *P. vivax*. Accordingly, monoclonal anti-Fy6 antibody inhibits the invasion of human erythrocytes by *P. vivax* (Tournamille et al., 2005).

DUFFY ANTIGEN/RECEPTOR FOR CHEMOKINES

Duffy blood group antigen was designated DARC after it was shown to mediate the promiscuous binding of inflammatory CC and CXC chemokines to erythrocytes (Horuk et al., 1993b; Tournamille et al., 1997; Lee et al., 2003a; Pruenster and Rot, 2006; Ulvmar et al., 2011). DARC's extracellular N-terminal domain, which bears the blood group determinants, is linked with the fourth extracellular domain via a disulphide bond. These domains together create an active chemokine-binding pocket (Tournamille et al., 1997, 2003). Given the absence of a DRYLAIV motif, which is required G-protein coupling, no detectable chemokine-induced cell signaling has been recorded in either the form of calcium flux (Neote et al., 1994), GTPase activity (Horuk et al., 1993b), or gene transcription (Lee et al., 2003a). Thus, DARC is classified as an atypical chemokine receptor (Nibbs et al., 2003; Pruenster and Rot, 2006; Ulvmar et al., 2011; Graham et al., 2012). However, some intracellular responses take place following DARC ligation by cognate chemokines. It was demonstrated in heterologous transfectants that chemokine binding induces redistribution of DARC from the basolateral cell membrane, via an intracellular vesicular compartment onto the apical membrane and that chemokine cargo is translocated together with DARC (Pruenster et al., 2009). Such chemokine *in situ* binding mirroring exactly the ligand specificity of DARC (Hub and Rot, 1998) as well as chemokine transcytosis and luminal surface presentation (Middleton et al., 1997) have been shown to place in venular endothelial cells *in vivo* and *ex vivo* in intact viable tissues. Unlike other atypical chemokine receptors, D6 in particular, no degradation of chemokines occurs after their internalization by DARC. Accordingly, neutrophil and monocyte migration toward cognate chemokines was enhanced across cellular monolayers expressing DARC (Lee et al., 2003a; Pruenster et al., 2009). Also i*n vivo*, chemokine injections into transgenic mice, which over-express DARC on the endothelium, induced significantly greater leukocyte recruitment (Pruenster et al., 2009). Thus endothelial DARC mediates abluminal internalization and transcellular transport of chemokines. This activity of DARC prevents the escape of soluble tissue-derived chemokine molecules into circulation and allows them to associate with the tips of luminal microvilli and stimulate firm adhesion of leukocytes. Inflammation can further up-regulate DARC expression in postcapillary venules and veins, and induce DARC to appear in vascular segments usually devoid of it (Liu et al., 1999; Segerer et al., 2000; Patterson et al., 2002; Lee et al., 2003b; Bruhl et al., 2005; Gardner et al., 2006; Geleff et al., 2010). It is not clear whether DARC over-expression is the consequence of the development of the inflammatory lesions or their pre-requisite. Primary lymphatic vessels do not express DARC although a small segment, the podoplanin-dull pre-collectors, do express DARC, suggesting that chemokines mediated cell migration may occur at this site (Wick et al., 2008).

Despite the fact that chemokine internalization by DARC does not lead to their degradation, DARC may physically remove chemokines from extracellular environments and thus, e.g., negatively influence angiogenesis induced by extravascular pro-inflammatory chemokines. This was shown in mice over-expressing endothelial DARC, which have reduced angiogenic responses to CXCL2 (Du et al., 2002) and in the context of tumor angiogenesis (Horton et al., 2007). Also, DARC-deficient mice used in a transgenic model of prostate cancer developed tumors with increased vessel density, greater intratumor angiogenic chemokine levels, and augmented growth (Shen et al., 2006). CD82, a tetraspanin which was identified as a prostate cancer metastasis suppressor gene, apparently directly interacts with DARC which thus can inhibit tumor cell proliferation and induce senescence (Bandyopadhyay et al., 2006). It appears therefore that DARC may negatively affect tumor development and metastatic spread either directly by binding to CD82 or by removing angiogenic chemokines from perivascular spaces. Additionally, DARC has been shown to heterodimerize with a classical chemokine receptor CCR5, and through this interaction down-modulate CCR5 mediated signaling responses (Chakera et al., 2008).

THE ROLE OF DARC IN CHEMOKINE HOMEOSTASIS

Erythrocyte DARC was originally described as a chemokine "sink" (Darbonne et al., 1991) and this function was further supported when DARC was shown to reduce the levels of circulating inflammatory chemokines, thus dampening systemic leukocyte activation (Dawson et al., 2000). Chemokines in circulation can induce the desensitization of their cognate receptors. By protecting circulating leukocytes from chemokine excess, DARC may preserve subsequent leukocyte responsiveness to chemokine signals present on the endothelial surface or in the tissues. Conversely, systemic pre-exposure to chemokines may prime leukocytes for enhanced chemokine-induced migration (Brandt et al., 1998) or other effector functions (Green et al., 1996; Hauser et al., 1999). These two opposing potential outcomes may explain the following apparently conflicting observations in DARC-deficient mice exposed to various inflammatory stimuli (Dawson et al., 2000; Reutershan et al., 2009; Vielhauer et al., 2009; Mei et al., 2010; Zarbock et al., 2010). In an initial study DARC knockout (KO) animals received systemic LPS and responded by a marked increase

in neutrophil infiltrate in the lungs and livers as compared to the wild type controls (Dawson et al., 2000). Another study showed that DARC KO mice have significantly less leukocyte infiltrate in the bronchoalveolar lavage in response to chemokine instilled into pulmonary airspace (Lee et al., 2003a). These experiments used DARC KOs lacking this receptor on all cells. Subsequently, bone marrow chimeras were constructed allowing the examination of respective roles of DARC on erythrocytes and endothelium (Lee et al., 2006). Here, mice lacking erythrocyte DARC had significantly fewer airspace neutrophils following intratracheal LPS instillation, suggesting that erythrocyte DARC supports leukocyte emigration. The lack of DARC in the lungs was associated with higher chemokine concentrations in the airspaces compared with mice lacking DARC on erythrocytes. In a model of LPS-inhalation-induced acute lung injury neutrophil migration into the alveolar spaces was increased in DARC KO animals, along with elevated levels of CXC chemokines (Reutershan et al., 2009). In chimeric animals, the absence of erythrocyte DARC was the most significant factor determining leukocyte trafficking. Difference between the outcomes in these two studies may be due to the divergent dose of LPS administered. With higher LPS concentrations the role for erythroid DARC as a sink may become more significant (Reutershan et al., 2009). Of note is that during severe systemic inflammation erythrocyte-bound chemokines amounted to 30% of plasma chemokine concentrations, suggesting only a limited sink effect of erythrocyte DARC during severe inflammation (Reutershan et al., 2009). Conversely, in humans, following endotoxin challenge several hundred fold increases in chemokine levels in erythrocyte lysates were noted (Mayr et al., 2008). Further investigation into the role of DARC in acute lung inflammation revealed that a lack of DARC in mice results in down-regulation of CXCR2 on neutrophils because of high levels of circulating chemokines during severe inflammation (Zarbock et al., 2010). It this study DARC was essential for chemokine-mediated leukocyte recruitment, whereby DARC KO animals were protected from acid-induced lung injury and experienced preserved oxygenation. This occurred as a result of impaired leukocyte arrest on endothelial cells and consequently reduced pulmonary neutrophil recruitment. Adoptive transfer of neutrophils showed that the latter effect is dependent on neutrophils and independent of endothelial cells and erythrocytes, suggesting that the contribution of DARC is in the maintenance of receptor expression in the environments with excess ligands. Because neutrophils, which are activated by chemokines in the systemic circulation (Colditz et al., 2007), may be passively trapped in the lung circulation and contribute to the lung damage (Rot, 1991), inflammatory models in other organs may be more revealing in dissecting local vs. systemic effects of DARC on chemokine-driven leukocyte emigration. Renal models of inflammation have shown that DARC-deficient mice are protected from ischemic and LPS-induced acute renal injury (Zarbock et al., 2007). Furthermore, chemokine presentation on renal endothelial cells was absent, and renal neutrophil recruitment was impaired, in the context of lower inflammatory chemokine levels during systemic inflammation (Zarbock et al., 2007). In contrast, Vielhauer et al. (2009) studied tubule-interstitial inflammation and glomerulonephritis in DARC-deficient mice and demonstrated that in these models macrophage and T lymphocytes were recruited equally well in DARC KO and wild type mice.

Both human and murine studies suggest that DARC can sustain inflammatory chemokines levels on erythrocytes and in plasma (Jilma-Stohlawetz et al., 2001; Fukuma et al., 2003), but the biological purpose of this reservoir function is not clear. Basal plasma CCL2 levels are one-third lower in DARC KO mice than in control wild type animals (Fukuma et al., 2003). When CCL11 or hCXCL1 were administered intravenously, these chemokines disappeared more rapidly from the plasma of DARC KOs (Fukuma et al., 2003). Duffy-negative humans were noted to have significantly lower basal CCL2 levels than Duffy-positives (Jilma-Stohlawetz et al., 2001) and following endotoxin administration, higher levels of plasma CCL2 were observed in Duffy-positive individuals as compared to the Duffy-negative ones (Mayr et al., 2008). Also CCL2 and CXCL1 levels, but not CXCL8 or CCL4 levels were higher in erythrocyte lysates of Duffy-positive individuals at baseline, whereas following endotoxin administration CCL2 and CXCL8, but not CCL4, levels increased significantly in erythrocyte lysates of Duffy-positive subjects. Given that chemokine plasma levels, including of CXCL8 (Wong et al., 2008) and CCL2 (Bozza et al., 2007) have been shown to be predictive of survival and correlate with sepsis severity, it is tempting to speculate that the loss of DARC expression may affect the outcome in sepsis. It has been recently suggested that chemokines with different binding affinities for DARC can modify the levels of other erythrocyte-bound and free plasma chemokines, affecting resultant leukocyte responses (Mei et al., 2010). In addition, heparin and activated coagulation factors can elute chemokines off erythrocyte DARC (Schnabel et al., 2010). Thus chemokines with range of affinities for DARC and other factors may significantly interfere with the ability of DARC to bind any particular chemokine introducing further complexity into mechanistic understanding of erythrocyte DARC function.

Recently, differences in plasma and serum chemokine levels were reported in persons with DARC Fy^a and Fy^b (Schnabel et al., 2010), although the mechanism for this is not apparent. Further work revealed that Fy^b erythrocytes have reduced surface DARC expression as compared to Fy^a erythrocytes; however, the binding affinity of DARC for chemokines was not appreciably different between these two phenotypes (Xiong et al., 2011). As discussed above, endothelial cells of post-capillary and collective venules and small veins express DARC, which functions here as a transcytosis receptor transporting chemokines from the basolateral to the apical side (Pruenster et al., 2009) where they are immobilized on the luminal surface. It is attractive to speculate that that individuals of alternative Fy^a vs. Fy^b DARC phenotypes may also show differences in chemokine-binding specificity and patterning by the endothelium, though to date there is no data to support this notion.

CONCLUSION

Since the discovery of its chemokine-binding properties, DARC has been mainly considered as a "decoy" receptor scavenging its ligands. Recent research shed new light on much more multifaceted activities of DARC. On erythrocytes, DARC acts on the one hand as a blood chemokine sink and, on the other, as a reservoir of

cognate chemokines buffering the bursts in their blood levels, and maintaining these, respectively. Both of these functions are absent in individuals with FYB(ES) DARC "negative" polymorphism. Future work should uncover molecular and cellular mechanisms explaining how the lack of erythrocyte chemokine sink and depot functions in these DARC-negative individuals affects pathomechanisms in various inflammatory diseases and cancer. In endothelial cells DARC functions as a transcytosis receptor leading to correct patterning of chemokines on the tissue–blood interface in venules and veins, thus supporting optimal chemokine-induced leukocyte endothelial cell adhesion and subsequent leukocyte emigration.

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