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Intravascular Leukocyte Chemotaxis: The Rules of Attraction

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1. Introduction

The security system of the body against pathogenic invaders includes leukocytes that efficiently scan the organism. Leukocytes within the vasculature utilize the comprehensive circulatory system to examine the blood vessels of the entire body for signs of *e.g.* bacteria displayed on vascular endothelial cells. Upon infection, a successful immune response is dependent on prompt recruitment of leukocytes from the bloodstream to the afflicted site where they exert their effector functions. A critical aspect of leukocyte recruitment out of vasculature is the chemotactic gradient that guides leukocytes over the blood vessel wall, and further through the extracellular matrix towards the affected site. Leukocyte recruitment is a strictly regulated cascade of events involving different molecular mechanisms. To rapidly and efficiently reach their target, specific interactions between circulating leukocytes and vascular endothelium orchestrates leukocyte activation and guides them already within blood vessels to optimal transmigration sites at endothelial *loci* close to the source of inflammation.

Despite the obvious need for effective leukocyte recruitment to eradicate bacteria and to maintain tissue homeostasis, amplified and dysregulated recruitment of leukocytes is a key factor in diverse disorders including autoimmune diseases and sepsis. For many of these conditions, therapeutic options are limited and unspecific. Understanding the triggering signals, involved molecules and underlying mechanisms by which the body enhances, controls and limits immune responses is therefore critical for the development of novel therapeutic interventions.

In this chapter we summarize leukocyte recruitment during inflammation, highlighting a recent finding, namely intravascular leukocyte chemotaxis.

2. Leukocyte recruitment and chemotaxis

Over the last years, research groups have been dedicating their efforts to delineate the cellular and molecular mechanisms behind leukocyte recruitment using a wide range of *in vivo* imaging techniques (*e.g.* fluorescence intravital microscopy, spinning disk confocal, as well as two-photon confocal microscopy). These techniques, together with genetically altered mice (transgenic or knockout) combined with fluorescently labeled proteins and antibodies, allow detailed examination of leukocyte-endothelial cell interactions, adhesion

molecule expression and chemokine distribution. Thereby, an expanded and more detailed version of the leukocyte recruitment cascade was established.

Leukocyte recruitment can be described as a sequential process having at least five distinct events, as depicted in **Figure 1**, induced by upregulation of endothelial adhesion molecules and molecular guidance signals (chemotactic stimuli).

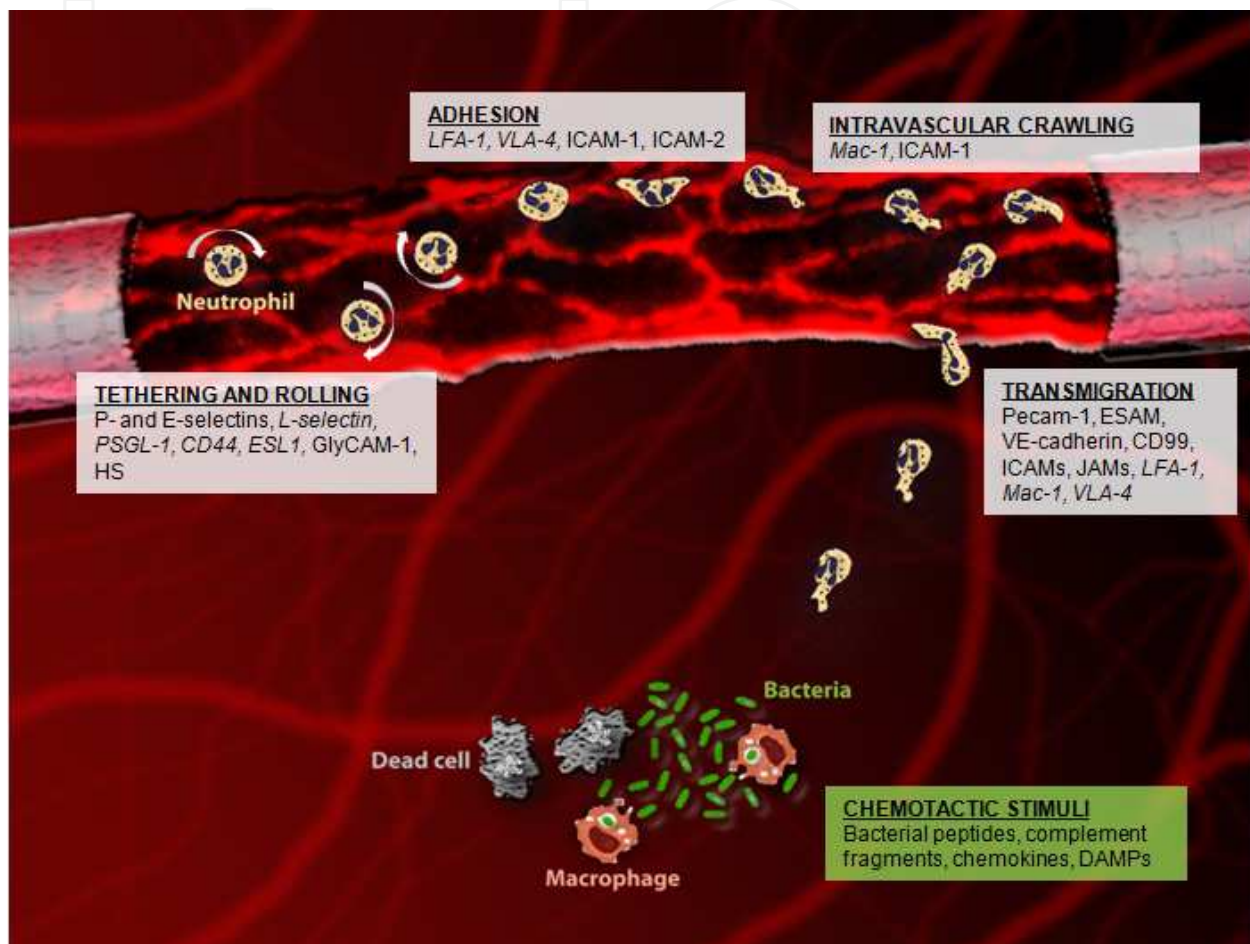


Fig. 1. The leukocyte recruitment cascade. The vessels is stained red by monoclonal antibodies to CD31 conjugated to Alexa Fluor 555, and cartoon neutrophils are added to the phtograph. The white boxes contain involved adhesion molecules, where the ones expressed by neutrophils are written in *italics*. Illustration adapted with permission from Phillipson M, Kubes P, *Nature Medicine*, 2011.

2.1 Leukocyte tethering and rolling

In order to leave the vasculature at the site of infection, leukocytes have to become margined, leave the center of the blood stream, and decelerate to come in contact with the vascular endothelium. However, due to the force of blood flow in postcapillary venules (shear rate ~ 150 to 1600 s^{-1} , depending on flow rate and vessel diameter) collisional contact duration between leukocytes and unstimulated endothelium is brief (*i.e.* $<25 \text{ ms}$) (Simon S. I., Goldsmith H. L., 2002). Specific interaction mechanisms between leukocytes and activated endothelium under shear flow are therefore required for leukocyte recruitment to inflammatory foci.

In fact, during inflammation, locally released stimuli (*e.g.* bacterial peptides, complement fragments, chemokines, histamine, and damage-associated molecular patterns) activate endothelial cells in the nearby venules to upregulate adhesion molecules on the plasma membrane which will aid leukocyte tethering, slow rolling and adhesion to the endothelium, leading ultimately to leukocyte transmigration into the tissue.

Selectins are a family of long adhesive molecules, extending from the plasma membrane, which facilitate attachment of circulating leukocytes to the endothelium (Patel K. D. *et al.*, 1995; Kansas G.S., 1996). Increased expression of P- and E-selectin (CD62P and CD62E, respectively) on activated venular endothelium induces leukocyte tethering (Kunkel E. J., Ley K., 1996; Petri B. *et al.*, 2008). While P-selectin is stored in Weibel-Palade bodies within the endothelial cells, E-selectin requires *de novo* synthesis. Once tethered, leukocytes can rapidly release and reengage selectin ligand bonds, resulting in a slow rotational movement along the vessel wall termed rolling (Norman M. U., Kubes P., 2005; Kelly M. *et al.*, 2007; Ley K. *et al.*, 2007). Rolling dynamics is optimized by force-regulated transitions from catch bonds to slip bonds¹, which explains the requirement for a shear threshold to support rolling (McEver R. P., Zhu C., 2010). L-selectin (CD62L), constitutively expressed on leukocytes, participates redundantly with P- and E-selecting, and supports both capture and rolling of leukocytes in blood vessels (Kunkel E. J., Ley K., 1996; Petri B. *et al.*, 2008).

Each of the three selectins binds with different affinity to sialylated and fucosylated oligosaccharides including sialyl Lewis^x (sLe^x) moieties, which are present on multiple glycolipids and glycoproteins on leukocytes and endothelium (McEver R. P., 2001; Simon S. I., Green C. E., 2005; Kelly M. *et al.*, 2007). The best characterized selectin ligand is PSGL-1 (P-selectin glycoprotein ligand-1), a heavily sialylated mucin present on leukocytes and endothelial cells, which can serve as a ligand to P-, E- and L-selectins although it binds P-selectin with the highest affinity (Kansas G. S., 1996). Besides PSGL-1, other ligands have been identified for E-selectin, *e.g.* sialophorin (leukosialin, CD43), hematopoietic cell E-selectin ligand (HCELL, CD44), and E-selectin ligand-1 (ESL1) (Kelly M. *et al.*, 2007; Ley K. *et al.*, 2007). L-selectin can also bind other ligands *e.g.* glycosylation-dependent cell adhesion molecule-1 (GlyCAM-1), mucosal addressin cell adhesion molecule-1 (MAdCAM-1), podocalyxin (CD34), heparan sulfate (HS) and sulphated glycoprotein-200 (Sgp200) (Wang L. *et al.*, 2005).

Rolling along the endothelium provides leukocytes a great opportunity to interact with and be further activated by chemokines or other inflammatory mediators presented on the luminal endothelium.

2.2 Intravascular chemokine presentation to rolling leukocytes

To initiate activation and recruitment of circulating leukocytes to tissue, tissue-derived chemokines need to be presented to rolling leukocytes at the apical endothelium. Within blood vessels, immobilization of chemokines on the endothelium is essential to avoid that they are washed away from the site of inflammation by the blood flow.

¹ *Catch bond*: a bond that prolongs its lifetime in response to tensile force; *Slip bond*: a bond that shortens its lifetime in response to tensile force (McEver R. P., Zhu C., 2010).

Heparan sulfate proteoglycans (HSPGs) are proteins bearing covalently attached complex polysaccharide chains that are negatively charged (heparan sulfate, HS), and are found on cell surfaces of most cell types as well as in the extracellular matrix (Bernfield M. *et al.*, 1999, Parish C., 2005). Chemokines and a variety of positively charged proteins bind to HS through specific and/or electrostatic interactions (Lindhal U., Kjellén L., 1991; Lindhal U., 2007). Indeed, interstitially released chemokines were shown to cross the endothelium and to be presented by luminal HSPGs to leukocytes both *in vitro* (Ihrcke N. S. *et al.*, 1993; Parish C., 2005; Wang L. *et al.*, 2005; Lindhal U., 2007) and *in vivo* (Massena S. *et al.*, 2010). Binding of chemokines to endothelial HSPG may promote molecular encounters between rolling leukocytes and chemokines, and thereby further inducing leukocyte activation. Moreover, endothelial HS acts as a ligand for L-selectin aiding neutrophil slow rolling (Wang L. *et al.*, 2005), which increase the propensity for leukocyte-chemokine encounters.

How chemokines originating from the afflicted site or released by tissue leukocytes reach the luminal side of the endothelium in order to be presented by HS to leukocytes is not completely established. However, electron microscopy studies suggested that chemokines bound to HS are transported through the endothelium by transcytosis (Middleton J. *et al.*, 1997) as endothelium exposed to interleukin-8 (IL-8, CXCL8) contained IL-8 within intracellular caveola, while no chemokines were found at endothelial cell junctions. Nevertheless, under the experimental conditions of this study, it is impossible to tell if the intracellular chemokines are being transported through endothelium towards the apical membrane or if they are on their way to lysosomes for degradation. In addition, soluble chemokines passing through junctions cannot be detected using electron microscopy, as they would be lost during tissue preparation prior to examination.

Edema formation is one of the cardinal signs of inflammation, and is caused by increased vascular permeability and consequent plasma leakage. The primary cause of increased vascular permeability is leakage of plasma through paracellular gaps (Curry F. E., Adamson R. H., 2010; Lindbom L., Kenne E., 2011), which is regulated by the interplay of adhesive forces between adjacent endothelial cells and counter adhesive forces generated by endothelial actomyosin contraction (Mehta D., Malik A. B., 2006). The physiological importance of this event for leukocyte recruitment is debated. It is well documented that increased vascular permeability in presence of inflammatory mediators is accompanied by increased leukocyte adhesion and diapedesis (Curry F.E., Adamson R. H., 1999; Michel C. C., Curry F. E., 1999). Nevertheless, temporal and spatial uncoupling between these two events has also been described. During inflammation, vascular permeability can increase at a faster rate than leukocyte transmigration (Kim M. H. *et al.*, 2009), suggesting that increased vascular permeability precedes leukocyte recruitment. Further, in aseptic wounds, vascular permeability and leukocyte extravasation were shown to be uncoupled (Curry F.E., Adamson R. H., 1999; Kim M. H. *et al.*, 2009). It is generally believed that increased permeability supports chemokine influx into the vessels and one hypothesis is that the increase in vascular permeability during inflammation grants the paracellular transport of chemokines for rapid presentation to intravascular leukocytes, guiding them out to the afflicted area in the tissue. A recent study using intravital spinning-disk confocal microscopy in anesthetized mice revealed that chemokines added extravascularly became accumulated intra-luminally at endothelial cell junctions (Massena S. *et al.*, 2010). High junctional sequestration of chemokines suggests that chemokines are transported either paracellularly into blood vessels or longitudinally on the apical endothelial cell membrane

towards junctions after being transcytosed. Further, this observation might simply reflect high concentrations of HS in junctional regions. However, these findings suggest that increased vascular permeability during inflammation does not necessarily account for amplified leukocyte extravasation *per se*, but instead might promote cytokine/chemokine transport and thereby induce leukocyte recruitment.

Endothelial cells display extraordinary phenotypic and functional heterogeneity. Endothelial cell structural features such as shape, thickness, molecular characteristics of apical membrane and junctions, as well as the thickness of the luminal glycocalyx are some of the features, which vary across the vascular tree (Van Den Berg B. M. *et al.*, 2003; Aird W. C., 2007). Heparan sulfate is known to display miscellaneous structural features in various tissues and on different cell types (Lindhall U., Li J. P., 2009), which accounts for binding of proteins in a selective fashion. Differences in proteoglycan composition (altered structure of HS epitopes or sequences, and/or expression pattern of different syndecans) might result in different chemokine binding properties, explaining the observed differences in leukocyte recruitment of different organs upon diverse inflammatory stimuli.

2.3 Leukocyte activation and adhesion to the endothelium

After being stimulated by chemokines sequestered on the endothelium, rolling leukocytes adhere to the endothelium by rapid formation of shear-resistant bindings mediated by specialized leukocyte integrins (Rose D. M. *et al.*, 2007). Integrins are noncovalently associated heterodimeric cell surface adhesion molecules consisting of combinations of α and β -molecules. Leukocytes express at least 10 members of the integrin family belonging to the β_1 -, β_2 - and β_7 -subfamilies (Luo B. H. *et al.*, 2007). Leukocyte adhesion molecules relevant for recruitment belong to the β_1 - and β_2 -integrin families (Ley K. *et al.*, 2007), of which LFA-1 (Lymphocyte function-associated antigen-1, ITGAL, CD11a/CD18, $\alpha_L\beta_2$), Mac-1 (Macrophage antigen-1, ITGAM, CD11b/CD18, $\alpha_M\beta_2$) and VLA-4 (very late antigen-4, CD49d/CD29, $\alpha_4\beta_1$) are the most studied.

Members of the β_1 -subfamily (also called VLA integrins) contain the β_1 -subunit associated to one of at least six different α subunits (Hemler M. E., 1990). VLA-4 integrin is amply expressed on peripheral blood B-lymphocytes, T-lymphocytes and monocytes (Hemler M. E., 1990). Peripheral blood neutrophils are believed to generally be devoid of cell surface β_1 -integrin structures (Hemler M. E., 1990), even though some reports claim that immature neutrophils expressing surface VLA-4 can also be found in circulation (Lund-Johansen F., Terstappen L. W., 1993; Pillay J. *et al.*, 2010).

Most circulating leukocytes express integrins in a low affinity state (Carman C. V., Springer T. A., 2003). Upon binding of chemokines to G-protein-coupled receptors (GPCRs) expressed on leukocytes, a complex intracellular signaling network is triggered within milliseconds (Shamri R. *et al.*, 2005; Ley K. *et al.*, 2007). This induces integrins to undergo an almost instantaneous change in avidity and ligand affinity (Von Andrian U. H. *et al.*, 1992; Shamri R. *et al.*, 2005; Hyduk S. J., Cybulsky M. I., 2009). Thus, inside-out signaling after chemokine binding to GPCRs shifts the integrins from a resting to an active conformation (Simon S.I., Goldsmith H. L., 2002; Simon S. I., Green C. E., 2005), which is necessary for binding to its ligands expressed on activated endothelial cells (Ley K. *et al.*, 2007).

Differential leukocyte expression levels of integrins and chemokine receptors as well as receptor affinity for chemokines might account for selective arrest and recruitment of leukocyte subtypes. Additionally, chemokine-triggered signaling networks can regulate distinct integrins in specific leukocyte subtypes, contributing for differential leukocyte recruitment.

2.3.1 Neutrophil adhesion

β_2 -integrin dependent neutrophil adhesion is fundamental for effective bacterial clearance. In fact, the genetic disorder leukocyte adhesion deficiency I (LAD I) is characterized by a profound defect in leukocyte recruitment and therefore severe immunodeficiency, due to neutrophils failing to adhere to the activated endothelium since the surface levels of β_2 -integrins are dramatically reduced or absent (Bunting M. *et al.*, 2002).

It was recently found that binding of the β_2 -integrin LFA-1 to intercellular adhesion molecule-1 (ICAM-1, CD54) expressed by endothelial cells, mediates neutrophil firm adhesion to the vascular endothelium under shear flow (Shamri R. *et al.*, 2005; Phillipson M. *et al.*, 2006; Ley K. *et al.*, 2007; Petri B. *et al.*, 2008). LFA-1 is also able to bind to other immunoglobulin superfamily members, ICAM-2 (CD102) and ICAM-3 (CD50), albeit with lower affinity relative to ICAM-1 (De Fougères A. R. *et al.*, 1994), in addition to JAM-A (junction adhesion molecule-A).

However, there is some evidence that neutrophils adhere via other adhesion molecules than LFA-1, or by non-adhesion processes such as physical trapping, described to occur in lung capillaries or liver sinusoids (Doerschuk C. M. *et al.*, 1990; Wong J. *et al.*, 1997; Norman M. U., Kubes P., 2005). Indeed, anti-CD18 treatment did not reduce leukocyte recruitment to the lung of rabbits (Doerschuk C. M. *et al.*, 1990), or in the rat liver (Jaeschke H. *et al.*, 1996). In the liver, neutrophils have been reported to adhere via CD44 interacting with sinusoidal hyaluronan (McDonald B. *et al.*, 2008). Furthermore, under systemic inflammatory conditions, such as sepsis, neutrophils have been suggested to adhere to the endothelium via VLA-4 (Ibbotson G. C. *et al.*, 2001). This integrin was also proposed to be involved in neutrophil adhesion in the lung microvasculature (Ibbotson G. C. *et al.*, 2001).

2.3.2 Monocyte adhesion

In contrast to neutrophil adhesion, β_2 -integrins seem to play a moderate role in monocyte arrest, since monocytes both adhere and polarize after blockade of β_2 -integrins as well as after blockade of ICAM-1 or ICAM-2 *in vitro* (Schenkel A. R. *et al.*, 2004). Instead, β_1 -integrins seem to play a more substantial role in monocyte adhesion to the endothelium. In fact, recent mouse models showed that monocytes firmly adhered to endothelium by VLA-4 binding to endothelial VCAM-1 (vascular cell adhesion molecule-1, CD106) (Luscinkas F. W. *et al.*, 1994; Meerschaert J., Furie M. B., 1995; Lee T. D. *et al.*, 2003; Ley K. *et al.*, 2007; Soehnlein O. *et al.*, 2009).

2.3.3 Lymphocyte adhesion

During lymphocyte recruitment to peripheral tissues, LFA-1 is the dominant integrin involved in firm adhesion (Dustin M., Springer T. A., 1989; Shamri R. *et al.*, 2005) by binding

to its ligand ICAM-1 (Shamri R. *et al.*, 2005). As described for neutrophils, LFA-1 prevails in a low affinity state on most circulating lymphocytes. Stimulation of lymphocyte-GPCRs rapidly shifts LFA-1 integrin to a high avidity state (Carman C. V., Springer T. A., 2003). This high avidity state of LFA-1 on T-lymphocytes is transient, peaking 5 to 10 minutes after receptor stimulation and returns to the low affinity state by 30 min to 2 hours (Dustin M., Springer T. A., 1989).

T-lymphocytes are not only recruited to tissue, they also home to lymph nodes. *In vitro* studies of T-lymphocyte adhesion to the specialized lymph node endothelium (high endothelial venules, HEV), demonstrated that besides LFA-1, VLA-4 is involved in T-cell adhesion (Faveuw C. *et al.*, 2000). Adhesion was reduced by 40-50% upon treatment with inhibiting antibodies to either integrin. Interestingly, the effects of VLA-4 and LFA-1 antibodies were additive, giving >90% inhibition of T-lymphocyte adhesion.

2.4 Intravascular chemotactic gradients and leukocyte crawling

Using time-lapse *in vivo* microscopy, adherent neutrophils and monocytes were recently observed to crawl significant distances within the vessels (Phillipson M. *et al.*, 2006; Auffray C. *et al.*, 2007; Phillipson M. *et al.*, 2009). The crawling neutrophils were searching the endothelium for optimal sites for transmigration, since inhibition of crawling significantly delayed neutrophil transmigration (Phillipson M. *et al.*, 2006; Sumagin R. *et al.*, 2010).

2.4.1 Neutrophil crawling

Intravascular crawling of neutrophils is dependent on the leukocyte β_2 -integrin Mac-1 and its ligand ICAM-1 on endothelial cells (Phillipson M. *et al.*, 2006; Sumagin R. *et al.*, 2010). Compared to LFA-1, Mac-1 binds a wider spectrum of ligands, including complement fragment iC3b, fibrinogen, fibronectin, laminin, collagen, myeloperoxidase, elastase, JAM-B and -C to name just a few (Simon S. I., Green C. E., 2005; Kelly M. *et al.*, 2007; Luo B. H. *et al.*, 2007) suggesting that this integrin might have other roles apart from intravascular crawling.

Neutrophil crawling on the stimulated endothelium occurs in two distinct stages. In the initial phase directly following neutrophil adhesion, a mechanotactic signal provided by shear stress induces neutrophil crawling perpendicular to blood flow until an endothelial cell junction is encountered (Phillipson M. *et al.*, 2009). This observation has also been made *in vitro* when adherent neutrophils crawled perpendicular to the direction of flow when shear was applied to the system (Phillipson M. *et al.*, 2009). However, as soon as the crawling neutrophils meet the junction, the shear stress signal is ignored and neutrophils instead begin to follow the junction. Considering that endothelial cells are elongated in the direction of flow, perpendicular crawling generates the greatest probability for a neutrophil to find an endothelial junction in the shortest period of time.

More recently, the existence of an intravascular gradient of chemokines (macrophage inflammatory protein-2, CXCL2 [MIP-2]; keratinocyte-derived chemokine, CXCL1 [KC]) originating from the infection or released by tissue leukocytes on endothelial cells has been described (Massena S. *et al.*, 2010). Indeed, this chemotactic gradient is sequestered on endothelial HS and provides directional cues to crawling neutrophils, which follow this gradient to optimal transmigration sites close to the origin of the infection (Massena S. *et al.*,

2010). However, whether haptotactic gradients can be established by all chemokines remains unclear, since different chemokines have diverse affinity to HS (Lindhal U., Kjellén L., 1991; Lindhal U., 2007).

Directional intravascular crawling along a chemotactic gradient expedites neutrophil recruitment, compared to when no chemokine gradient is formed due to homogenous extravascular chemokine concentrations (Massena S. *et al.*, 2010). Disruption of the chemokine gradient is translated into random crawling and inefficient recruitment of neutrophils which ultimately leads to a decreased ability to clear infections, as seen in *Staphylococcus aureus* infected mice with truncated HS chains (overexpressing heparanase, Massena S. *et al.*, 2010).

2.4.2 Monocyte crawling

Intravascular crawling monocytes have been reported *in vivo* (Auffray C. *et al.*, 2007; Sumagin R. *et al.*, 2010), and crawling on endothelium is critical to reach optimal transmigration sites, as demonstrated *in vitro* (Schenkel A. R. *et al.*, 2004).

Whereas the integrin Mac-1 alone is responsible for crawling of neutrophils, LFA-1 and Mac-1 integrins were in some studies reported to play a redundant role in monocyte crawling via binding to ICAM-1 and ICAM-2 (Schenkel A. R. *et al.*, 2004; Sumagin R. *et al.*, 2010). Blockade of each of these molecules led to a pirouette behavior at the adhesion site (Schenkel A. R. *et al.*, 2004). Nevertheless, monocytes were shown to be able to adhere and polarize.

Recently, a distinct role for each of these integrins on monocyte crawling under different endothelial activation states has been described. Monocytes were shown to crawl long distances on resting endothelium in a patrolling behavior (*i.e.* monitoring healthy tissue) in a LFA-1-dependent manner (Auffray C. *et al.*, 2007; Sumagin R. *et al.*, 2010). However, upon inflammatory stimulation, monocyte crawling became Mac-1-dependent and assumed a neutrophil-like crawling pattern, *i.e.* similar crawling distance and confinement ratio (Sumagin R. *et al.*, 2010). These results have been suggested to correspond to differences between two different monocyte populations rather than to a shift in integrin expression upon inflammatory stimulation.

Two monocyte subsets distinguished by their expression levels of selectins, integrins and chemokine receptors have already been characterized in various mammals (Geissman F. *et al.*, 2003; Gordon S., Taylor P. R., 2005). These phenotypic differences encompass distinct effector functions. A monocyte subset termed "resident" (CX₃CR1^{hi} CCR2⁻ Ly6C⁻ in mice; CD14^{lo} CD16⁺ in humans) is involved in tissue remodeling and wound repair (Gordon S., Taylor P. R., 2005; Auffray C. *et al.*, 2007; Soehnlein O. *et al.*, 2009). In contrast, another monocyte subset denominated "inflammatory" (CX₃CR1^{lo} CCR2⁺ Ly6C⁺ in mice; CD14^{hi} CD16⁻ in humans) is specialized in pro-inflammatory activities such as bacterial phagocytosis, secretion of inflammation-promoting cytokines and reactive species as well as proteolytic activity (Gordon S., Taylor P. R., 2005; Auffray C. *et al.*, 2007; Soehnlein O. *et al.*, 2009). Resident monocytes, express high amounts of LFA-1. In contrast, inflammatory monocytes do not express LFA-1, even though no differences were found for Mac-1 between the two monocyte subsets (Auffray C. *et al.*, 2007).

It is possible that the two subsets of circulating monocytes might use different integrins and display different crawling patterns to achieve the different effector functions.

2.4.3 Lymphocyte crawling

In vitro, adherent T-lymphocytes have been reported to crawl over the luminal surface of the endothelium in a LFA-1-dependent manner (Shulman *et al.*, 2009). LFA-1 is also responsible for T-lymphocyte adhesion, but the distribution of the membrane LFA-1 is altered correlating with changes in cell morphology as soon as the T-lymphocyte starts to migrate (Smith A. *et al.*, 2005). LFA-1 turnover at numerous focal points ensures rapid crawling and resistance to detachment by shear forces (Shulman *et al.*, 2009). Low expression levels of LFA-1 were detected at the leading edge of the cell and high expression level in the non-attached uropod at the rear (Smith A. *et al.*, 2005). Interestingly, LFA-1 in the leading edge was not in a high-affinity state, as detected by use of specific antibodies that recognize LFA-1 in different conformational states (Smith A. *et al.*, 2007). Instead LFA-1 in the leading edge was in an intermediate affinity conformation allowing crawling possibly by weaker interactions with ICAM-1.

Published studies have identified intravascular natural killer T-lymphocytes (NKT cells) with possible sentinel functions for the detection of bacteria in the blood (Geissmann F. *et al.*, 2005; Lee T. D. *et al.*, 2010; Thomas S. Y. *et al.*, 2011). These cells are distinguished by their restricted repertoire of T-cell receptor (TCR) variants that recognize lipids and glycolipids presented by CD1d (Kawano T. *et al.*, 1997; Brossay L. *et al.*, 1998). NKT cells primarily reside and wander within the vasculature of the liver and spleen (Geissmann F. *et al.*, 2005; Bendelac A. *et al.*, 2007) but have also been suggested to accumulate in smaller amounts in the vascular compartment of the lung (Thomas S. Y. *et al.*, 2011). The mechanisms underlying adhesion and crawling of NKT cells are still poorly understood. It has been reported that treatment of mice with blocking antibodies to LFA-1 and ICAM-1 induced rapid detachment of adherent NKT cells from sinusoidal endothelium (Thomas S. Y. *et al.*, 2011). In contrast, blocking VLA-4 or VCAM-1 had no effect. Integrin activation typically relies on inside-out signaling after chemokine binding to GPCRs (Ley K. *et al.*, 2007). However, genetic ablation of CXCR6 (the major chemokine receptor expressed on NKT cells) or treatment with an inhibitor of GPCRs, did not induce detachment of NKT cells from liver microvasculature (Geissmann F. *et al.*, 2005; Lee T. D. *et al.*, 2010). Furthermore, previous studies on NKT cells transferred into CD1d-deficient mice suggested that TCR activation was not a prerequisite for NKT cells sinusoidal adhesion (McNab F. W. *et al.*, 2005; Wei D. G. *et al.*, 2005). Crawling was also unimpeded in mice treated with anti-CD1d antibody (Lee T. D. *et al.*, 2010). Interestingly, upon infection with the blood-borne pathogen *Borrelia burgdorferi* (a spirochete injected intravenously through tick bite), NKT cells were reported to slow their crawling and to accumulate in clusters on Kupffer cells in a GPCRs-dependent way (CXCR3, and CD1d) (Lee T. D. *et al.*, 2010). Kupffer cells are specialized ramified macrophages, which line the walls of liver sinusoids and prevent the dissemination of pathogens via the blood by capturing and engulfing them. Kupffer cells can then present glycolipid antigens via CD1d (Lee T. D. *et al.*, 2010). In the absence of Kupffer or NKT cells, dissemination of *Borrelia burgdorferi* occurred, suggesting a role for NKT cells in vascular surveillance for blood-borne pathogens captured by Kupffer cells (Lee T. D. *et al.*, 2010).

2.5 Diapedesis: Trans- and paracellular routes

Leukocyte diapedesis out of vasculature into affected tissue can occur both between neighboring endothelial cells (paracellularly through junctions) and directly through the endothelium (transcellularly) (Feng D. *et al.*, 1998; Shaw S. K. *et al.*, 2001; Carman C. V., Springer T. A., 2004; Engelhardt B., Wolburg H., 2004; Yang L. *et al.*, 2005; Phillipson M. *et al.*, 2006). The route employed most likely depends on inflammatory stimuli, as well as the type of leukocyte and vascular bed.

Diapedesis has been reported to be mediated by numerous endothelial adhesion molecules expressed in high density at endothelial junctions, such as platelet-endothelial cell adhesion molecule 1 (PECAM-1, CD31), CD99, vascular endothelial-cadherins (VE-cadherins), endothelial cell-selective adhesion molecule (ESAM), ICAM-1 and -2 and JAMs (Luscinskas F. W. *et al.*, 2002; Engelhardt B., Wolburg H., 2004; Yang L. *et al.*, 2005; Ley K. *et al.*, 2007; Lou O. *et al.*, 2007; Petri B. *et al.*, 2008; Woodfin A. *et al.*, 2011). Other molecules involved in leukocyte transmigration are integrins expressed on leukocytes (*e.g.* LFA-1, Mac-1, VLA-4) (Ley K. *et al.*, 2007; Petri B. *et al.*, 2008; Woodfin A. *et al.*, 2011). The specific molecules involved in either of the transmigration pathways remains to be identified.

The different molecules appear to mediate leukocyte transmigration in either a stimulus-specific or leukocyte-specific manner. For example PECAM-1, ICAM-2 and JAM-A mediate leukocyte transmigration in response to interleukin-1 β (IL-1 β) but not to tumor necrosis factor-alpha (TNF- α) (Wang S. *et al.*, 2006; Ley K. *et al.*, 2007). Direct activation of leukocytes by TNF- α , fMLP (N-formyl-methionyl-leucyl-phenylalanine) or leukotriene-B₄ (LTB₄) appears to bypass the need for these molecules. Studies of activated mouse cremaster muscle and intravital microscopy in mice knocked down for ESAM gene (Ley K. *et al.*, 2007) have shown that ESAM does not show a stimulus-specific role but appears to mediate neutrophil rather than T-lymphocyte transmigration.

Neutrophils have been found to transmigrate predominantly through the paracellular route, *i.e.* between adjacent endothelial cells (Phillipson M. *et al.*, 2006; Woodfin A. *et al.*, 2011). Paracellular transmigration was found to be dependent on the ability for leukocytes to crawl to optimal transmigration sites at the endothelial cell junctions. In Mac-1 deficient mice, due to inhibition of intravascular crawling, transcellular transmigration predominated (Phillipson M. *et al.*, 2006).

Using *in vivo* spinning disk or multi-photon confocal microscopy, profound anatomical changes of the endothelium that facilitated leukocyte extravasation without compromising vascular barrier integrity were observed (Phillipson M. *et al.*, 2008; Petri B. *et al.*, 2011). Docking cup-like structures were formed by endothelial cells (endothelial projections) at the base of the transmigrating neutrophil, which has also been described for T-lymphocytes *in vitro* (Carman C. V., Springer T. A., 2004). The endothelial projections extended towards the top of the neutrophil and eventually formed a dome that surrounded the entire neutrophil, prior to basolateral opening and neutrophil migration further into tissue. If the dome formations were prevented, neutrophil transmigration was delayed (Petri B. *et al.*, 2011), further implicating an active role of endothelium during leukocyte diapedesis, while maintaining the barrier function and vascular permeability.

2.5.1 Migration through the subendothelial basement membrane and pericyte sheet

To overcome the barrier of the blood vessel and finally reach the inflamed tissue, leukocytes also have to transmigrate across the subendothelial basement membrane (BM) surrounding the venular endothelium. This has been shown to occur in areas low in collagen IV, laminin-10 and nidogen-2 (Wang S. *et al.*, 2006). These areas were seen to be closely associated to gaps between pericytes (Wang S. *et al.*, 2006). Interestingly, leukocytes have been observed to initiate transmigration through endothelium at sites superimposing these specific areas. How intravascular crawling leukocytes can detect these areas from the luminal side of the endothelium remains unknown.

2.6 Extravascular crawling

Following leukocyte diapedesis across the vessel wall, further movement in tissue is required in order for the leukocytes to reach the affected site to exert their effector functions. As within the vasculature, leukocyte movement in the tissue is guided by chemotactic gradients leading to the source (Foxman E. F. *et al.*, 1997; Lindbom L., Werr J., 2002). Upon binding to GPCRs on leukocytes, chemoattractants trigger downstream signaling, which translates to cytoskeletal reorganization, polarization and directional locomotion (Friedl P. *et al.*, 2001). Migrating leukocytes thereby adopt a polarized morphology consisting of a leading edge and a tail-like uropod (Friedl P. *et al.*, 2001).

In order to initiate movement, leukocytes have to establish adhesive contacts with the tissue stroma via interactions between the extracellular integrin domains and components of the extracellular matrix (ECM) (Friedl P. *et al.*, 2001; Lindbom L., Werr J., 2002). Stimulation by encountered chemoattractants activates surface integrins and recruits additional integrins from cytoplasmic stores (Diamond M. S., Springer T. A., 1994; Friedl P. *et al.*, 2001). Binding to ECM macromolecules triggers integrin-mediated signals, which regulate further integrin apposition, actin assembly, cell polarity, and migration (Friedl P. *et al.*, 2001).

Accumulating evidence suggests that leukocyte chemotaxis in the ECM is mostly associated with β_1 -integrins while a limited role for β_2 -integrins is described (Sixt M. *et al.*, 2001). Members of the β_1 -family shown to be involved in leukocyte locomotion (Gao J. X., Issekutz A. C., 1997; Werr J. *et al.*, 1998; Sixt M. *et al.*, 2001) show high affinity interactions with proteins of the ECM, including fibronectin, vitronectin, collagen and laminin (Hemler M. E., 1990). Circulating neutrophils are not believed to constitutively express β_1 -integrins. However, it has been suggested that upregulation of β_2 -integrins by chemotactic stimuli induced an outside-in signaling leading to mobilization of β_1 -integrins to the neutrophil surface, in order to prepare recruited neutrophils for subsequent interactions with ECM (Lindbom L., Werr J., 2002). There are also studies demonstrating upregulation of neutrophil surface expression of β_1 -integrins in association with emigration from the vasculature (Kubes P. *et al.*, 1995; Werr J. *et al.*, 1998).

Another adhesion molecule involved in extravascular crawling is L-selectin. Indeed, studies using L-selectin-deficient mice revealed no role of L-selectin on leukocyte rolling or adhesion, but transmigration was significantly impaired (Hickey M. J. *et al.*, 2000). Furthermore, leukocytes in L-selectin-deficient mice were unable to respond to directional cues (platelet activating factor [PAF]; KC) in the interstitium (Hickey M. J. *et al.*, 2000). These findings provided strong evidence of an important L-selectin function in leukocyte

emigration and extravascular locomotion. Intriguingly, L-selectin expression on emigrated leukocytes is dramatically reduced in comparison to levels on circulating leukocytes (Hickey M. J. *et al.*, 2000). Shedding of L-selectin upon transmigration has been reported both *in vitro* (Smith C. W. *et al.*, 1991; Allport J. R. *et al.*, 1997) and *in vivo* (Juttila M. A. *et al.*, 1989). These results raise the possibility that L-selectin early in the recruitment cascade triggers downstream signals that modulate consecutive transmigration and migration in the interstitium (Hickey M. J. *et al.*, 2000).

Further, integrins bind different components of the tissue stroma and with diverse affinities (Lindbom L., Werr J., 2002). Leukocyte chemotaxis in the tissue is therefore influenced not only by the stimuli encountered but also by the matrix proteins in the tissue.

2.6.1 Prioritizing chemotactic cues

During bacterial infections, the chemotaxing leukocytes are exposed to a cacophony of different chemotactic gradients of diverse origins. Chemoattractants originate from the bacteria themselves (*e.g.* fMLP; lipopolysaccharide [LPS]) or complement fragments bound to bacteria (*e.g.* complement fragment C5a), but also from nearby activated leukocytes and endothelial cells (*e.g.* LTB₄; IL-8) (Foxman E. F. *et al.*, 1997; Heit B. *et al.*, 2008; Muller W. A., 2011). A microenvironment where numerous chemoattractants are encountered requires tightly regulated intracellular mechanisms for leukocytes to readily prioritize between the cues, in order for them to effectively reach the target. Thus, to fulfill their missions, leukocytes need to find the bacteria without being distracted by opposing gradients. A hierarchical relationship between chemotactic factors has developed, and “end-target” chemotactic factors like bacterial products *versus* the “intermediate” chemokines released by activated endothelium or tissue leukocytes have been shown to activate separate signaling pathways in neutrophils (p38-mitogen-activated protein kinase [p38MAPK] and phosphoinositide 3-kinase [PI3K], respectively [Campbell J. J. *et al.*, 1997; Heit B. *et al.*, 2002]). In this way, neutrophils are able to sort signals in the noisy environment of inflammation, and respond to chemotactic cues in a hierarchical manner, preferring “end-target” chemoattractant factors like fMLP and C5a over “intermediate” chemokines like IL-8 (Foxman E. F. *et al.*, 1997). This has been suggested to occur through the p38MAPK pathway inhibiting PI3K through relocalization of phosphatase and tensin homologue (PTEN) on the basolateral cell membrane of the polarized moving cell (Heit B. *et al.*, 2008). However, additional parallel scenarios of how chemotaxing leukocytes are directed to their goal have been described (PLA2, cGMP and DOCK2/phosphatic acid). Despite the mentioned advances in understanding how leukocytes find their way in tissue, many issues remain to be deciphered considering the very complex nature of the extravascular environment during infection. Leukocytes will not only encounter bacterial chemoattractants and chemokines, but also cytokines, lipids and complement fragments. Cytokines (IL-1 β , TNF- α) are released simultaneously during infection and induce chemokine production. Chemokines and cytokines have been shown to act in concert to direct leukocyte delivery and activation, and chemokines of different families have been demonstrated *in vitro* to synergistically enhance influx of both neutrophils and monocytes (Gouwy M. *et al.*, 2008; Kuscher K. *et al.*, 2009). Further, interplay between lipid chemoattractant LTB₄, the cytokine IL-1 β and the chemokine ligands to first CCR1 and later CXCR2 was shown in a model of arthritogenesis (Chou R. C. *et al.*, 2010). This vividly demonstrates that leukocyte recruitment *in vivo*, in

contrast to during *in vitro* settings hardly is the result of release of a single chemokine, and that synergistic as well as opposing effects of involved signaling molecules are to be expected to enhance as well as steer the inflammatory response. This interplay needs to be further clarified and the importance of these observations has to be confirmed *in vivo*, to determine therapeutic targets during different inflammatory conditions.

3. Additional stimuli for leukocyte recruitment

Inflammation is closely linked to hypoxia, and numerous leukocytes are detected at sites of tissue ischemia (Eltzschig H. K., Carmeliet P., 2011). Severe hypoxia causes both apoptosis and necrosis of somatic cells, which results in release of various damage-associated danger signals, like DAMPs (danger associated molecular pattern molecules). DAMPs include molecules originating from the cytosol or the nucleus, such as adenosine triphosphate (ATP), formylated peptides from mitochondria, heat shock proteins, chromatin and galectins, that often undergo denaturation when leaving the intracellular milieu, where after they become pro-inflammatory (Kono H., Rock K. L., 2008). Even though most of them are not considered directly chemotactic, they induce leukocyte recruitment by activating tissue macrophages and nearby endothelial cells to secrete pro-inflammatory cytokines (*e.g.* IL-1 β) and chemokines (*e.g.* IL-8), in addition to upregulating expression of adhesion molecules on endothelial cells (Muller W. A., 2011). However, formylated peptides originating from mitochondria were recently found to recruit neutrophils via activation of the fMLP receptor formyl-peptide receptor-1 (McDonald B. *et al.*, 2010; Zhang Q. *et al.*, 2010).

During hypoxia and cell injury, recruited neutrophils are believed to contribute to wound healing processes by clearing the area of debris through phagocytosis. In addition to phagocytosis, leukocytes have recently been acknowledged for their role in angiogenesis and tissue remodeling both during health and disease. Neutrophils produce and store within their granules pro-angiogenic molecules such as VEGF-A (vascular endothelial growth factor-A, [Gaudry M. *et al.*, 1997]) and MMP-9 (matrix metalloproteinase-9, [Ardi V. C. *et al.*, 2007]). VEGF is a key player in blood vessel formation and has a direct chemotactic effect on endothelial cells, while the pro-angiogenic function of MMP-9 is attributed to its ability to digest extra cellular matrix (ECM), which pave way for new vessels as well as release and thereby activate ECM-bound VEGF and other growth factors. The neutrophils are in fact the only cells in the body that release MMP-9 free of its endogenous inhibitor TIMP (tissue inhibitor of metalloproteinase), and are therefore capable of deliver highly active MMP-9 to sites of angiogenesis (Ardi V. C. *et al.*, 2007). The pro-angiogenic capacity of neutrophils has been demonstrated in a corneal injury model, where the number of infiltrated neutrophils positively correlated to angiogenesis and VEGF levels (Gong Y., Koh D. R., 2010). Neutrophil depletion significantly impaired tissue healing in this model, as well as the release of VEGF. Further, neutrophils recruited to islets of Langerhans transplanted to striated muscle were recently shown to be crucial for revascularization to occur, as transplantation of islets to neutropenic mice resulted in complete inhibition of islets revascularization (Christoffersson G. *et al.*, 2010). Neutrophils were shown to accumulate at sites for islet engraftment and were specifically localized at the newly formed vessels, as demonstrated in **Figure 2**.

Monocytes have also been shown to have pro-angiogenic properties. For instance, tissue healing following myocardial infarction requires sequential mobilization of the two

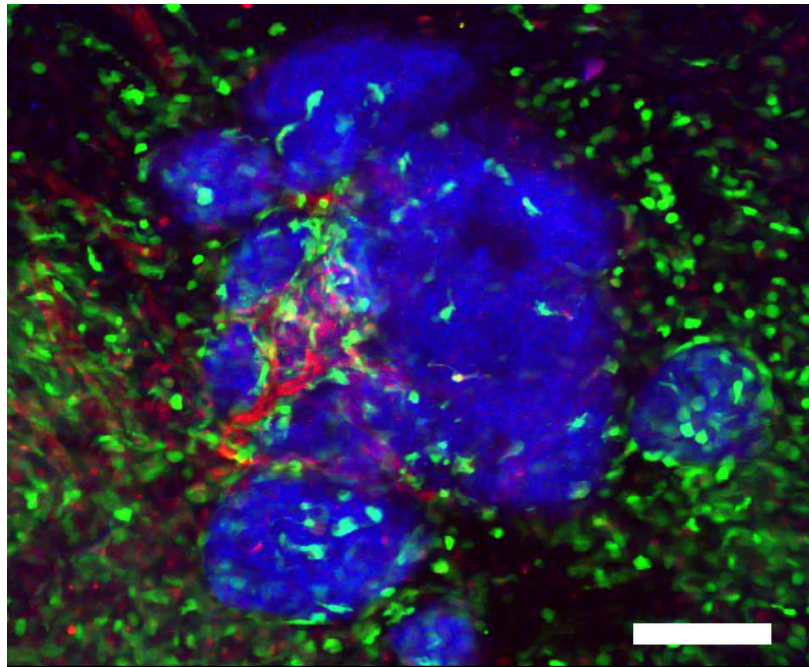


Fig. 2. Isolated pancreatic islets (blue) transplanted to muscle causes Gr1⁺ cell (green) accumulation (courtesy of G. Christoffersson). Blood vessels are stained with anti-CD31 (red). Scale bar equals 100 μ m.

described monocyte subsets which exhibit opposing phenotypes (Nahrendorf M. *et al.*, 2007). Recruited “inflammatory” monocytes exhibit proteolytic activity and inflammatory functions, whereas monocytes of the “resident” subtype contribute to angiogenesis and have attenuated inflammatory properties (Nahrendorf M. *et al.*, 2007).

In the field of tumor biology, it is known that the ability of tumors to create an immunomodulating microenvironment to escape cytotoxic immune cells and to allow for angiogenesis is central for tumor growth. Different factors have been shown to skew tumor-associated leukocytes including neutrophils and macrophages from a pro-inflammatory, anti-tumorigenic to a pro-tumorigenic, pro-angiogenic phenotype (Fridlender Z. G. *et al.*, 2009; Hanahan D., Weinberg R. A., 2011; Rolny C. *et al.*, 2011). Indeed, the angiogenic switch in islet dysplasia and tumors in the RIP1-Tag2 transgenic mouse model of pancreatic cancer was mediated by tumor-infiltrated MMP-9 expressing neutrophils (Nozawa H. *et al.*, 2006).

The identity of these pro-angiogenic leukocytes is currently being investigated, and whether circulating subpopulations with pro-angiogenic and anti-inflammatory properties exist, or if they attain their phenotype giving the stimuli, are under debate. Myeloid-derived suppressor cells (MDSCs) are a heterogenic population that increases in numbers in the spleen and bone marrow of tumor bearing mice, and consists of monocyte and neutrophil subsets with potent ability of suppressing immune functions such as T-lymphocyte activation (Bronte V. *et al.*, 2000). In mice, they express CD11b and Gr-1, where the Gr-1^{hi} CD11b⁺ corresponds to immature and mature neutrophil subpopulations while the Gr-1^{int} CD11b⁺ to the monocyte subset (Peranzoni E. *et al.*, 2010; Youn J. I., Gabrilovich D. I., 2010). How other surface markers differ from the classical pro-inflammatory neutrophils or monocytes are not completely established, but the roles of neutrophils during restitution and angiogenesis described above might indeed involve the neutrophil subset of MDSC.

Further characterization of the identities of leukocytes involved in tissue restitution and angiogenesis, and even more importantly their functions during these situations, is of great relevance.

4. Therapeutic interventions

A variety of disorders are associated with leukocyte activation and infiltration. Asthma (Fanta C. H., 2009; Broide D. H. *et al.*, 2011; Minnicozzi M. *et al.*, 2011); emphysema (Martinez F. J. *et al.*, 2011); atherosclerosis (Ross R., 1999; Hansson G., 2005); inflammatory bowel disease (Khor B. *et al.*, 2011); rheumatoid arthritis (Olsen N. J., Stein C. M., 2004; O'Dell J. R., 2004; Scott D. L., Kingsley G. H., 2006); multiple sclerosis (Frohman E. M. *et al.*, 2006); sepsis (Hotchkiss R. S., Karl I. E., 2003); and allograft rejection after transplantation (Savasta M., Lentini S., 2011; Arias M. *et al.*, 2011), are just some examples of this broad spectrum. Although many details remain to be delineated, the consensus is that the over-exuberant, maladaptive and/or uncontrolled inflammation is in the pathogenesis of these conditions and leads to tissue injury.

Over the past few years, pharmacotherapeutic advances have been made, with most therapeutic options focusing on means to prevent leukocyte activation and recruitment. Anti-adhesion therapies directed against different adhesion molecules have been evaluated. However, despite positive data from animal studies, many of the integrin antagonists have failed in clinical trials or are associated with severe side effects (Rutgeerts P. *et al.*, 2009; Fontoura P., 2010; Del Zoppo G. J., 2010).

A new approach for reducing leukocyte recruitment to tissue was recently described in mice (Maiguel D. *et al.*, 2011). This study was based upon a nearly 20-year-old observation for eosinophils, which, in response to activating antibodies trapping VLA-4-integrin in a high-avidity state, were able to adhere but not migrate (Kuijpers T. W. *et al.*, 1993). Accordingly, the recent study screened for selective small-molecule Mac-1 agonists, named leukadherins. These agonists caused increased intravascular adhesion, but not transmigration of neutrophils, resulting in reduced leukocyte recruitment in experimental models of acute peritonitis and nephritis. Integrin clustering or outside-in signaling were not induced by binding of the agonist, which might account for the lack of detectable vascular injury, even though the effects on tissue blood flow by the increased number of intravascular adherent neutrophils remains to be studied. These observations are all very intriguing, even though further experimental evaluation of this course of action is required.

5. Conclusion

Leukocyte recruitment is a hallmark event in acute and chronic inflammation. Tightly regulated activation of circulating leukocytes and intravascular leukocyte guidance by the establishment of chemokine gradients within blood vessels is fundamental for leukocytes to efficiently transmigrate to the inflamed tissue, where they finally exert their effector functions. Specific interactions between transmigrating leukocytes and the activated vascular endothelium orchestrate profound anatomical changes of the endothelium which facilitate leukocyte extravasation while maintaining the barrier function and vascular permeability. Targeting intravascular leukocyte chemotaxis and the gating property of the endothelium would limit leukocyte transmigration and/ or vascular permeability during

detrimental inflammation. Understanding the underlying mechanisms behind these processes might therefore contribute for the development of novel therapeutic interventions.

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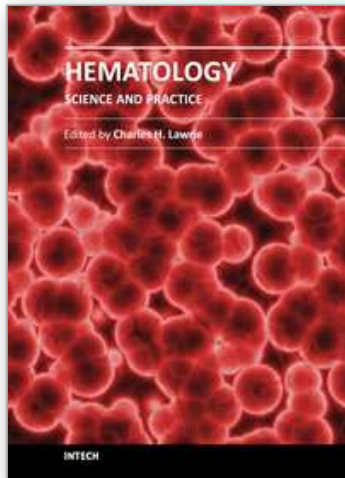
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Hematology encompasses the physiology and pathology of blood and of the blood-forming organs. In common with other areas of medicine, the pace of change in hematology has been breathtaking over recent years. There are now many treatment options available to the modern hematologist and, happily, a greatly improved outlook for the vast majority of patients with blood disorders and malignancies. Improvements in the clinic reflect, and in many respects are driven by, advances in our scientific understanding of hematological processes under both normal and disease conditions. Hematology - Science and Practice consists of a selection of essays which aim to inform both specialist and non-specialist readers about some of the latest advances in hematology, in both laboratory and clinic.

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