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Chemokine patterning by glycosaminoglycans and interceptors

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1. ABSTRACT

Chemokines mediate leukocyte emigration from blood into tissues. This process is triggered by chemokines binding and signaling through their cognate G-protein-coupled receptors on leukocytes and requires the involvement of leukocyte and endothelial cell adhesion molecules. Additionally, *in vivo* chemokine activity depends on their interaction with "auxiliary" molecules expressed by the vascular endothelial cells. Secreted chemokines can be immobilized on the luminal and abluminal endothelial cell surfaces by glycosaminoglycans. In order to be targeted to their presentation sites on the luminal endothelial cell surface, the tissue-derived chemokines have to cross the endothelial cell barrier. For inflammatory chemokines this is accomplished by active transport involving Duffy antigen, an 'interceptor' expressed by venular endothelial cells. Other chemokine interceptors, D6 in particular, may act as scavenging decoys and are involved in clearance of chemokines. The interceptor-mediated transport or elimination of chemokines, together with their immobilization by glycosaminoglycans, lead to chemokine patterning at the blood-tissue interface and within tissues. The resulting chemokine gradients induce leukocyte emigration from blood and may also be necessary for directed leukocyte migration within tissues.

2. INTRODUCTION

The development of efficient innate and adaptive immunity depends on the ability of different subpopulations of leukocytes to leave the circulation and migrate within tissues into defined microanatomical sites where they can exert their diverse effector functions. Chemokines have been implicated in driving both leukocyte emigration from blood and directed migration in tissues (1). These chemokine effects, like *in vitro* leukocyte chemotaxis, are conveyed through specific heptahelical G-protein coupled receptors (GPCRs). The molecular diversity within this system is extensive as over 40 different human chemokine ligands may interact with one or more of the 20 known chemokine GPCRs (1). In fact, almost all chemoattractants, not just chemokines, and many other endogenous and exogenous mediators use GPCRs to transmit their signals. The interaction of cognate ligands with the extracellular portion of GPCRs induces allosteric conformational changes leading to spectrum of immediate signaling events through the intracellular portion of the receptor (2). The "texture" (profile, magnitude and quality) of the cellular response depends on the particular receptor and the cell type expressing it, along with the nature of the ligand, and where it is positioned on the antagonist-agonist scale. Ligand engagement also induces complex sequelae of receptor unresponsiveness which effectively terminate

signaling reactions initiated through the GPCR (3, 4). These features make GPCRs ideal for rapid transduction of signals from the cell membrane and, at the same time, provide fine regulation of the resultant cellular reactions. Arguably, immediate signal transduction and its swift termination by GPCRs constitute the basis for continuous orientation and persistent directional cell migration which take place in chemokine gradients. Because chemokines are small molecules, they can rapidly diffuse from the source of their production. The Brownian movement of chemokines has been traditionally used to explain how spatial tissue gradients of chemokines are generated and can persist in the tissues, albeit for finite periods of time. Yet, the direct application of the second law of thermodynamics to modelling the *in vivo* dissemination of chemokines has some limitations. First, natural barriers exist that are impermeable for chemokines, such as the vascular structures of the blood-brain barrier (5) or epithelial cells lining exocrine glands (6). Second, physiological fluid currents which persist *in vivo* influence the diffusion of chemokines. For example, the flow of interstitial fluid reverses the gradients of chemokines in the vicinity of the lymphatic vessels (7) whereas the stream of blood eliminates them altogether at the blood-tissue interface (8). Third, the free dissemination of chemokines in the tissues is halted by their interaction with molecules which can efficiently capture chemokines. While chemokines may interact with many different molecules, two major molecular classes of avid chemokine binders have been suggested to profoundly affect the tissue distribution of chemokines and, as a result, chemokine activity *in vivo*. On one hand, all secreted chemokines bind glycosaminoglycans (GAGs), linear sulfated sugars built of repeating disaccharide units. Extracellular GAGs decorate cell surfaces and matrix proteins and immobilize chemokines which, as a consequence, form functional solid phase gradients that can persist for long periods of time. On the other hand, chemokines can interact with a group of atypical heptahelical receptors. These are highly homologous to classical chemokine GPCRs but, because of the modified or missing canonical DRYLAIV motif in the second intracellular loop, do not couple to G-proteins or initiate downstream intracellular signaling.. The most prominent and unifying feature of these "mute" receptors is their ability to efficiently internalize chemokines, hence their suggested name, 'interceptors' (for *inter*nalizing re*ceptors*). The outcome of the chemokine interaction with their respective interceptors differs: chemokines may be either translocated intact across biological barriers to perform specific *in vivo* tasks or, alternatively, targeted to lysosomes for efficient degradation. Whether transporting or scavenging, the interactions of chemokines with interceptors lead to the establishment of tissue patterns which would not be able to form as a result of the free diffusion of chemokines.

This review outlines the interaction of chemokines with GAGs and interceptors and discusses how these contribute to the establishment of functional chemokine patterns in tissues and thus importantly impinge on the *in vivo* activities of chemokines.

3. CHEMOKINE-INDUCED LEUKOCYTE EMIGRATION

Chemokines can induce leukocyte egress from blood into tissues. This activity is responsible, at least in part, for the specificity of homeostatic migration of functional leukocyte subsets into various lymphoid and non-lymphoid microenvironments and also for the cellular diversity of leukocyte infiltrates characteristic for many inflammatory pathologies (1). Cell egress from blood is a tightly orchestrated process where chemokines act in concert with adhesion molecules of different classes expressed on leukocyte and endothelial cell membranes (9-11). The progressively occurring binding interactions between leukocyte and endothelial adhesion molecule pairs stepwise strengthen leukocyte attachment and are visible as distinct morphological phases of leukocyte-endothelial adhesion (9, 10). During this process, chemokines wield their effects initially on leukocyte adhesion to endothelium. Chemokines can slow down the rolling velocity of leukocytes and induce the conversion of leukocyte tethering and rolling into firm adhesion (12). This is achieved by rapidly increasing the affinity of leukocyte integrins (13, 14). Recent investigations using intravital microscopy and *in vitro* flow chamber models illuminated the events leading from chemokine triggering their cognate GPCR to integrin activation and firm leukocyte adhesion (reviewed in (11, 12, 14). Furthermore, in the process of leukocyte-endothelial cell interaction chemokines apparently also contribute to the (i) lateral leukocyte migration on the apical endothelial surface (15, 16) (ii) trans-endothelial migration, (iii) abluminal leukocyte locomotion (17) and (iv) penetration of the subendothelial, adventitial and perivascular structures (14). During transendothelial migration chemokines, acting together with growth factors, may also provide anti-apoptotic signals which significantly enhance the efficiency of the transmigration process (18). This may be particularly relevant for cells with a relatively short life span, such as neutrophils. The chemokines involved in leukocyte emigration may come from a number of cellular sources. They can be produced by the endothelial cells themselves and either directly secreted or stored for prolonged periods of time in Weibel-Palade bodies untill rapid exocytosis induced by type I endothelial activation e.g. by vasoactive amines (19). Alternatively, chemokines may be generated extravascularly by any of the tissue cells (20) and subsequently transcytosed across endothelial cells. In addition, chemokines derived in peripheral tissues may be channeled through afferent lymphatics to induce the recruitment of leukocytes into lymph nodes through the high endothelial venules (21, 22). However, irrespective of their origin, it is clear that to affect the recruitment of blood leukocytes, chemokines have to interact with endothelial cell surfaces (23).

4. CHEMOKINE PATTERNING ON BLOOD-TISSUE INTERFACE

By drawing parallels with chemotaxis (cell migration along soluble gradients of chemoattractants) it was initially believed that soluble chemokines were

responsible for inducing leukocyte-endothelial cell adhesion and emigration. However, soluble chemokines are instantly washed away by the blood flow, and may act as negative regulators of leukocyte emigration by desensitizing chemokine receptors before the leukocytes have a chance to engage with the endothelial cell surface (24-27). It is noteworthy, that plasma chemokines may also induce the release of their target leukocytes from bone marrow, be they neutrophils, eosinophils or monocytes, leading to increased circulating numbers and, at least in some cases, enhanced emigration of these cells into peripheral sites (28-30). It is not apparent which factors determine if the pro- or anti-emigratory actions of plasma chemokines dominate in various inflammatory and infectious diseases and their experimental models. However, it is clear that soluble chemokines are not able to direct circulating leukocytes to the appropriate vascular segments for their consequent emigration.

4.1. Chemokine immobilization on luminal endothelial surface

As an alternative to the "soluble chemotactic gradient" hypothesis, it was proposed that in order to facilitate leukocyte adhesion and emigration chemokines have to be immobilized on the luminal endothelial cell surface (8). Such solid phase chemokines "presented" on the luminal endothelial membrane would stimulate only the leukocytes which started rolling on the endothelial surface, but not those in circulation. Indeed, many chemokines bind *in situ* to the endothelial cells of postcapillary and small venules (31-34). Furthermore, immunoelectron microscopy showed chemokine immobilization on the luminal endothelial cell membrane of postcapillary and collective venules in skin. Chemokines were preferentially immobilized by the endothelial cell microvilli, the sites of direct contact with adherent leukocytes (34).

4.2. GAG binding of chemokines

Because all secreted chemokines, just like the majority of cytokines and growth factors, bind sulfated GAGs (35), it was suggested that chemokine immobilization on the luminal endothelial surface is accomplished by endothelial GAGs, heparan sulfate in particular (34, 36-38). Distinct patterns of chemokine recognition by different GAGs exist (39). Therefore GAGs typical for individual microenvironments in health and disease may lead to the retention of some but not other chemokines in diverse vascular beds, different organs and tissues as well as extravascular cells, structures and matrices (40-43). For some of the GAGs which bind chemokines their well-defined core proteins have been identified (44-46). However, it is not clear how much of the structural specificity of GAGs is actually determined by the sequences of their protein cores. Alternatively, structural patterns of GAGs may depend on the cell-specific make-up of the enzyme machinery responsible for GAG synthesis and sulfation. This would result in different cellular proteins bearing similar GAG structures that are characteristic for a particular cell type. Irrespective of the cause of GAG specificity in different cells, it is likely to result in distinct patterns of chemokine immobilization on cells and in matrices in different microanatomical sites. In turn, these specific fingerprints of chemokine binding contribute an additional level of selectivity to the process of leukocyte recruitment from blood and may determine direction, extent and composition of leukocyte infiltrates within the tissues.

4.2.1. Differential GAG binding of homologous chemokines

Several chemokine receptors have been shown to have multiple chemokine ligands; also, vice versa, individual chemokines may bind to several different receptors (1). These facts have led to the general assumption that chemokines, particularly pro-inflammatory chemokines, bear considerable redundancy. However, the highly specific fingerprints of their receptor specificities indicates that the assertion of redundancy is wrong for the majority of chemokines (1). Only few chemokines have similar affinities for the same cognate receptor resulting in a complete overlap of their *in vitro* activities. However, even those which have similar receptor-binding profiles, show differential interactions with GAGs as, for example, the two CCR7 ligands, CCL19 and CCL21. In comparison to CCL19, CCL21 carries an extended 36 amino acid C-terminus with 12 basic residues, allowing for its very efficient immobilization by GAGs. Thus, CCL19 is likely to stay in solution longer and diffuse away from the sites of its production, whilst CCL21 is likely to be readily immobilized and remain bound to the cell surfaces and matrices. The situation may be similar for the two known CCR4 agonists, CCL17 and CCL22. CCL17 avidly binds to GAGs, is readily immobilized and presented by the endothelium *in situ*, whereas CCL22, a more potent chemokine, is not (40, 47). The paradigm of dissimilar GAG binding abilities contributing to differential function of homologous chemokines is most dramatically illustrated by the isoforms of CXCL12. The six known human CXCL12 isoforms result from alternative gene splicing and differ only in the C terminal region encoded by the fourth exon (48). In comparison with alpha- and beta- isoforms, CXCL12gamma has a long C-terminus with 18 basic residues and three BBXB GAG-binding motifs providing an additional GAG-binding domain within this molecule (49). This C-terminal extension is responsible primarily for the very slow off-rate of GAG binding and the unprecedented affinity of CXCL12gamma to GAGs, which is the highest measured for any chemokine (49). This feature should result in instantaneous immobilization and retention of CXCL12gamma close to the sites of its *in vivo* production, whereas the alpha- and beta- isoforms of CXCL12 should be able to diffuse away and act as "hormones" remote from their sites of origin. These are only few known examples where homologous chemokines or chemokine isoforms acting on the same receptor, by virtue of their differential ability to be immobilized by GAGs, may accomplish different *in vivo* tasks. The systematic comparative investigation of differences in GAG binding characteristics of homologous ligands of other chemokine receptors, e.g. CCR1, CCR2, CCR3, CCR5, CXCR2 and CXCR3, is still outstanding. It is likely that such analyses will reveal other key differences in properties of chemokines sharing the same cognate GPCR.

4.2.2. Chemokine presentation by GAG

It has not been established experimentally if chemokines can trigger their cognate receptors while bound to GAGs in either cis- or trans- configuration. In fact, it is quite possible that tri-molecular complexes GAG-chemokine-GPCR cannot form due to chemical constraints. Thus, in order to associate with their cognate receptors, chemokines immobilized by GAGs may need to be released from their GAG binding. In this case, GAG "presentation" of chemokines may only ensure their tissue patterning, i.e. their concentration in functionally productive microenvironments and the establishment of tissue gradients. This scenario is very likely to happen with lymphotactin (XCL1) which was shown recently to exist in two interconvertible conformational states: one allowing its binding to heparin and another allowing its XCR1 agonism (50).

Despite recognized homologies in chemokine structures, there are considerable differences in how distinct chemokines interact with GAGs (51). Hence, for different chemokines the likelihood of simultaneous interaction with GAG and GPCR may differ. For some chemokines, e.g. CXCL8, such interaction is possible, at least theoretically, because the GAG- and receptor- binding domains within CXCL8 are distinct and located at the diametrically opposed sides of the molecule (36). Moreover, CXCL8 binding to heparan sulfate enhances its biological activity in neutrophil chemotaxis (36) possibly as a result of GAG-induced conformational changes in chemokine which increase its receptor affinity as well as its structural stability (52). It should be noted, that heparan sulfate may also enhance the *in vitro* chemotactic activity of CXCL8 by another mechanism. Formation of CXCL8 and heparan sulfate complexes in the chemotactic chamber may increase the concentration of chemokines immobilized on the polycarbonate membranes (36). Such surface- bound (haptotactic) and not soluble (chemotactic) CXCL8 gradients were shown to be responsible for directed leukocyte migration in Boyden-type *in vitro* cell migration assay (53). Unlike CXCL8, several other chemokines, for example CCL2 and CXCL12, show an overlap between their receptor and GAG binding domains (54, 55). Accordingly, the *in vitro* activity of these and several other chemokines was reduced by the addition of GAGs. (56-62). Irrespective of whether GAGs and GPCRs share the same functional binding domains on chemokines or not, soluble GAGs administered systemically can act as functional chemokine antagonists *in vivo* possibly by removing chemokines from their natural GAG anchoring (63-65).

4.2.3. Chemokine activity *in vivo* depends on their interaction with GAGs

The ability of chemokines to bind GAGs is a prerequisite of their *in vivo* effects on leukocyte recruitment. This was shown first using CXCL8 (1-63), a mutant with reduced GAG binding capacity (34) and later with several non-GAG binding mutants of CC chemokines (66) as well as lymphotactin (XCL1) (67) In some *in vitro* models of leukocyte migration, the GAG- binding domains of chemokines are also required for the optimal activity of chemokines (34, 68). A more direct proof of involvement of heparan sulfate in leukocyte emigration came from studies using mice deficient in N-acetyl glucosamine N-deacetylase-N-sulfotransferase-1, an enzyme responsible for sulfation of heparin. The conditional knockout of this enzyme in endothelial cells led to a decrease in leukocyte recruitment. Mechanistically, this was due to diminished leukocyte rolling, as well as reduced luminal presentation and transport of chemokines across the endothelium (69). However, endothelial cell transcytosis of chemokines may also be accomplished, as discussed below, by another molecule, DARC.

4.2.4. GAG binding of chemokines induces oligomerizatrion and protects them from proteolytic degradation

Chemokines can dimerize and oligomerize, and these reactions are actually supported by their binding to GAGs (70). Therefore, it is possible that one chemokine member of an oligomer interacts with GAG while another one triggers the cognate GPCR. Chemokine oligomerization is apparently required, at least for some chemokines, for leukocyte adhesion to endothelium (66, 71), but not for the transendothelial migration step (72, 73). The oligomerization of other chemokines, for example CCL11, is not induced by GAGs. Nevertheless, *in vivo*, but not *in vitro*, chemotactic activity of CCL11 is enhanced by addition of heparin which can protect CCL11 from proteolytic cleavage (74). Free, but not GAG-bound, chemokines are efficiently cleaved by multiple proteases resulting in some cases in truncated variants devoid of biological activity. Protecting chemokines from degradation in the tissues may be an important facet of the *in vivo* activity of immobilized GAGs as well as soluble ones (74-76). Mast cells are the only known source of the natural soluble GAG, heparin, and thus may contribute to protecting chemokines from proteases. Accordingly, the *in vivo* pro-emigratory activity of chemokines was shown to depend on the presence of mast cells (77). However, mast cells may also contribute to leukocyte emigration by release of their stored mediators TNFα and histamine which induce the endothelial surface expression of selectins (77-79). In general, enzymatic cleavage of chemokines contributes greatly to the control of their *in vivo* activity. In some cases chemokine truncation by proteases, especially CD26, results in derivatives with enhanced agonistic activity. Detailed discussion of this aspect of chemokine biology exceeds the scope of this text and the reader is referred to the authoritative reviews on this topic (80-82).

5. CHEMOKINE-INDUCED TRANSENDOTHELIAL LEUKOCYTE MIGRATION

During leukocyte emigration, in addition to inducing firm leukocyte-endothelial adhesion, chemokines also can stimulate the transendothelial leukocyte migration step. Chemokine-driven adhesion and subsequent transmigration can be uncoupled from each other and, in some cases, may be separated by the lateral movement of leukocytes on the luminal endothelial cell surface, a step which is also mediated by chemokines (16, 15). Leukocyte adhesion and transmigration may take place in response to two different chemokines acting successively on the same

leukocyte, or by the same chemokine inducing both steps through sequential engagement of different chemokine receptors (83). It is not entirely clear where on the endothelium chemokines have to be positioned in order to optimally induce leukocyte transmigration. Theoretically, chemokines immobilized on the luminal surface may be able to induce transmigration and also lateral intraluminal locomotion (84) by haptotactic, haptokinetic or haptorepulsive mechanisms (53, 85). To date, haptorepulsion has been demonstrated for CXCR3 ligands triggering their cognate receptor on plasmocytoid dendritic cells only (85). The immobilized gradients of chemokines form spontaneously under various *in vitro* experimental conditions. Therefore, they may contribute, unnoticed, to other *in vitro* migratory mechanisms, including chemotaxis (53) and possibly also chemorepulsion. Chemorepulsion (known also as chemofugetaxis) denotes the movement away from the source of a gradient. It has been shown to be a possible mechanism of migratory cell responses to CXCL8 and CXCL12 (86, 87). In the blood vessels, chemokines can also accumulate on the abluminal endothelial cell surface and in the basal membrane. GAGs may be involved in this immobilization process too (88). For example, subendothelial heparan sulfate proteoglycans were shown to become major binders of CCL2 upon renal ischemia/reperfusion (89). The transmigration mechanism apparently involves leukocytes that are adherent to the luminal endothelial surface sensing the abluminal chemokines by extending their foot-like projections, pseudopodia, through either the intercellular junctions or transcellular channels. The lateral shear of the blood flow aids this process by stimulating pseudopodia formation (84, 88, 90, 91). Two alternative routes of transendothelial leukocyte migration have been described: the transcellular path, when the migrating cells pierce their way through the endothelial cytoplasm (92) and the paracellular way through the junctions between the endothelial cells (92-94). Currently, it is not clear if the two alternative transendothelial migration routes involve different cell migratory mechanisms, diverse patterning of chemokine signals or differential involvement of other molecular switches within the endothelial cells (95, 96).

6. CHEMOKINE PATTERNING IN EXTRAVASCULAR TISSUES

Besides patterning chemokines on the luminal and abluminal endothelial surfaces, GAGs contribute to chemokine binding to the extracellular matrices (97) and thus may influence leukocyte migration in the tissues. In addition to endothelial cells, GAGs immobilize chemokines on surfaces of various extravascular cells in different sites. Diverse (patho)physiological processes may involve chemokines anchored on surfaces of motile and sessile cells positioned extravascularly. For example, recently it was shown that homeostatic chemokine CCL21 is immobilized on cell membranes of dendritic cell in the paracortical areas of lymph nodes (98) .Such immobilized CCL21 can influence the development and outcome of immunological synapse formation with potential consequences for the ensuing immune responses (98) and possibly their suppression by regulatory T cells (99). It is also likely that chemokines can bind to the surface of the mesenchymal scaffold cells within the lymphoid organs and in immobilized rather than soluble form drive and orchestrate the intricate motility steps and cellular encounters required for the development of the acquired immunity (100-102). Similarly to endothelial cells, epithelial structures may also immobilize chemokines. It was shown that transepithelial migration of eosinophils across the airway endothelium is mediated by eotaxins immobilized on the epithelial cell membrane in part by GAGs and in part by protein molecules (103). As chemokines are highly interactive molecules, not only GAGs but also different endogenous cell surface and matrix (glyco)proteins can bind them specifically (104-108) and pattern them *in vivo*. For example collagen type IV binds specifically CXCL12, CXCL13 and CCL21, but not several other chemokines (106) Podoplanin, a glycoprotein expressed by lymphatic endothelial and other cells binds specifically CCL21 (108) and thus contributes to the immobilization of this chemokine.

Curiously, chemokines themselves were also suggested to provide “presentation scaffolds” for some but not other of their family members (109). The formation of chemokine heteromers leads to the upregulation of the chemotactic responses to the components and may explain some of the synergistic effects observed with several defined combinations of chemokines (110, 111). However, in addition to several ways of passive chemokine immobilization in the tissues, active cellular mechanisms exist which modify the levels of chemokines and their positioning in the tissues.

7. CHEMOKINE PATTERNING BY INTERCEPTORS

Recently a new family of chemokine--binding serpentine membrane proteins homologous to classical chemokine GPCRs has been identified (112, 113). These molecules can efficiently internalize chemokines, hence their suggested name, “interceptors” for internalizing receptors. The interceptor-mediated endocytosis of chemokines takes place in the apparent absence of conventional G-protein mediated signaling. Independently of the outcome of chemokine engulfment by their interceptors (either transport across the biological barriers or scavenging), these molecules may contribute, albeit by different mechanisms, to the establishment of chemokine gradients in the tissues. Currently four *bona fide* chemokine interceptors have been characterized: (i) Duffy antigen receptor for chemokines (DARC), (ii) D6, (iii) CCX-CKR, also known as CCR11 and (iv) RDC1 (also called CXCR7). Additionally, an orphan receptor HCR (also known alternatively as CCRL2 or CRAM) as well as possibly several others currently still orphan receptors may function as chemokine interceptors. The concept of interceptors appears to extend beyond chemokines as other chemoattractants may have receptors which behave in a similar fashion. Anaphylatoxin C5a binds to “non-signaling” receptor C5L2 with the scope of potential outcomes ranging from silencing to enhancing C5a effects and, as a result, influencing the parameters of experimental

inflammatory pathologies (114, 115). The pioneering work on the biology of chemokine interceptors may pave the way for the discovery and understanding of interceptors in general. Here we illustrate how chemokine interceptors, despite being "silent", may contribute to the induction of leukocyte emigration by chemokines and impinge on their other activities *in vivo*.

7.1. Chemokine transcytosis by venular endothelial cells

In order to appear on the luminal endothelial cell membrane and to affect circulating leukocytes, tissue-derived chemokines have to cross the endothelial cell barrier. It was shown that extravascular chemokines are actively transported by endothelial cells of venules and small veins in the abluminal to luminal direction (34, 116) Naturally, chemokines being small proteins can also diffuse from tissue into the blood through the junctions between the endothelial cells. This, however, is more likely to take place in the capillaries, the most permeable vascular bed with the facility for bidirectional molecular exchange between tissues and blood. Yet the inflammatory and homeostatic leukocyte emigration from blood into the tissues seldomly takes place in the capillaries but rather in venules and small veins. Additionally, passive diffusion of chemokines across the endothelium is not likely to target chemokines to the luminal microvilli, sites where chemokines were shown to appear following their transcytosis by the endothelial cells (34).

7.2. DARC, an interceptor for inflammatory CC and CXC chemokines: role in chemokine transcytosis

When the process of chemokine transcytosis by the endothelial cells was described (34), it was suggested that in addition to GAGs, DARC an interceptor expressed by venular endothelial cells (117, 118) may be involved in chemokine binding and transcytosis. DARC is a promiscuous receptor: in human and mice it binds almost all inflammatory CC and CXC chemokines (119-121) and, characteristically for interceptors, internalizes them without transmitting G-protein mediated signals (122). *In vitro* studies of chemokine transport across the monolayers of DARC transfectants, as well as *in vivo* mechanistic and disease models in DARC deficient mice, cumulatively support the notion that in nucleated cells this interceptor functions as a transcytosis receptor for inflammatory chemokines (123-126). Accordingly, DARC expression is required for optimal *in vitro* and *in vivo* chemokine-induced leukocyte migration and emigration, respectively (123). The studies on the contribution of DARC to chemokine transcytosis have been hampered by the fact that primary or transformed endothelial cells propagated *in vitro* do not carry DARC spontaneously. For example, human umbilical vein endothelial cells show no DARC expression *in situ*, whereas microvascular endothelial cells become devoid of it before their first passage. In heterologous transfectants DARC appears in the cell membrane and often localizes to the cellular junctions. Following the binding its cognate chemokine ligands, DARC is rapidly internalized from the cell surface into a yet unknown intracellular vesicular compartment. This actually signifies a cell signaling mechanism induced by chemokine and mediated by DARC, though clearly different from that transmitted through the fully competent chemokine GPCRs. Following internalization, a part of the internalized chemokine cargo reappears again extracellularly. In polar cells DARC transports chemokines unidirectionally, only from the baso-lateral side to the apical surface, but not *vice versa* (own unpublished data). Thus, DARC can create functional patterns of tissue-derived inflammatory chemokines on the luminal endothelial cell surface. As discussed above, heparan sulfate on the endothelial cells was also suggested to facilitate the transport of chemokines across the endothelial cells (69). It is possible that two alternative DARC- and heparan sulfate- mediated chemokine transcytosis pathways exist in parallel in endothelial cells. Alternatively, these two molecules may be part of the same transport mechanism and divide between them the tasks in different steps of transcytosis: chemokine capture, internalization, transport and presentation.

Not all venules in all anatomical sites express DARC. Therefore one can imagine that DARC expression by the endothelial cells is a rate limiting step in the process of chemokine-induced leukocyte emigration. The upregulation of DARC expression by the venular endothelium and the appearance of DARC immunoreactivity in vascular segments normally devoid of it, occur in several inflammatory and autoimmune diseases. (127-132). However, it is not yet clear if the induction of DARC expression in the inflammatory lesions is required for their development, or occurs as a consequence of ongoing inflammation.

Chemokine transcytosis by DARC across venular endothelial cells may, at least hypothetically, provide a mechanism of chemokine elimination from the tissues. Clearance of chemokines, however, in the overwhelming majority of tissues is more likely to take place passively by diffusion through the junctions between the endothelial cells of blood capillaries and lymphatic vessels. However, for example in the brain where the molecular exchange across the blood brain barrier is limited, DARC expressed by the blood vessel endothelium may play a role in the clearance of the inflammatory chemokines from the brain parenchyma.

7.2.1. DARC on erythrocytes

In addition to venular endothelial cells, DARC is expressed by the erythrocytes of Duffy antigen positive individuals (122, 133, 134) . Duffy antigen "negative" individuals of African origin do not express DARC on erythrocytes. This trait has possibly been selected by malaria, two pathogenic strains of which use DARC to invade erythrocytes (134, 135). The Duffy-"negative" phenotype is due to a point mutation in the binding region of the erythroid GATA1 promoter (136). Therefore Duffy "negatives" still bear DARC on venular endothelial cells (118) and presumably other sites of its expression, e.g. Purkinje neurons of the cerebellum (137). DARC on erythrocytes acts as a sink for chemokines in circulation (133, 138, 139) preventing the activation of leukocytes in blood. In addition, erythrocyte DARC functions as a chemokine depot in blood. Here it maintains the levels of its cognate chemokines, not only on erythrocytes but also in

plasma, (140, 141) by preventing their rapid loss primarily into kidneys and urine. To date it is not clear which purpose is served by maintaining active chemokine reserves in blood.. Also, surprisingly little is known about the consequences of Duffy "negative" phenotype on the development of human diseases mediated by chemokines.

7.3. CCX-CKR, an interceptor for homeostatic chemokines CCL19, CCL21 and CCL25

DARC binds and was suggested to transport inflammatory chemokines only. Hence, it is not apparent how homeostatic chemokines produced in the tissues are transported to the luminal endothelial cell surface. Such transport across the high endothelial venules of the lymph nodes was shown for CCL19 (116) and presumed to take place for CCL21 (21). Chemokine interceptor CCX-CKR, which binds both of these chemokines and additionally CCL25, may accomplish the transcytosis of these homeostatic chemokines (142, 143). In addition to multiple epithelia (143), CCX-CKR is also expressed by the endothelial cells. In full agreement with a potential role in chemokine transport, it was shown using CCX-CKR deficient mice that this interceptor contributes to the migration of a dendritic cell subpopulation to lymph nodes (143). Conversely, it mitigates the homing of embryonic thymic precursors to the developing thymus (143). The investigation of CCX-CKR behavior *in vitro* in transfected cells demonstrated that primarily degradation of CCL19 takes place following its internalization by CCX-CKR (144). The outcome was similar although not as robust as that observed with interceptor D6 which leads to efficient degradation of its cognate inflammatory CC chemokines (145, 146). It is possible that the potential outcome of chemokine internalization by this, as well as other interceptors, either chemokine transport and presentation or degradation, may depend on the cell type and the relative preponderance of transcytotic or lysosomal pathways. There are indications that D6, chemokine interceptor most commonly associated with scavenging chemokines (113), may also play a role in chemokine transport and retention within microenvironments (own unpublished results).

7.4. D6, an interceptor for inflammatory CC chemokines

D6 binds many inflammatory CC chemokines (147). Unlike DARC, it is not expressed in blood vessels but in the endothelial cells of lymphatic vessels (148), in placental syncytiotrophoblast (149) and in different leukocyte populations (150). In contrast to DARC, D6 is internalized independently of its occupancy by cognate chemokines (146, 151). Therefore the majority of the D6 molecules expressed by a cell can be found intracellularly (146). Chemokines internalized by D6 in transfected cells were shown to be delivered through the endosomes into the lysosomes for their degradation (113, 145, 146). Tissue-derived chemokines are drained through the lymphatics into the lymph nodes where they are transposed onto the endothelial cells of high endothelial venules and can induce the homing into the draining lymph nodes of apposite leukocytes (22). D6 expressed by the lymphatics may serve as gatekeeper by restricting the diffusion of inflammatory chemokines from tissue into the draining lymph nodes and consequent leukocyte recruitment (22, 152). D6 may scavenge chemokines not only during their entry into the lymphatics but also all along their diffusion towards the lymph nodes. In clear accord with its role as a chemokine scavenger, D6 knockout mice have exhibited exaggerated skin inflammation following topical treatment with phorbol esters and Freund's adjuvant (152, 153). Also, in an inflammation-dependent two step chemical skin carcinogenesis model, D6 was shown to function as a tumor suppressor by scavenging chemokines and reducing the number infiltrating lymphocytes and mast cells required for the tumorigenesis (154). D6 expressed by the syncytiothrophoblasts scavenges maternal blood chemokines and can reduces the extent of fetal loss in pregnant mice treated systemically by inflammatory stimuli (149).

The use of D6 -/- mice in ovalbumin-induced "asthma" model uncovered a rather ambiguous parthomechanistic role of this interceptor. On one hand, D6 scavenged some but not all of its cognate chemokine ligands and reduced the leukocyte emigration into the lungs. On the other hand its presence resulted in increased airway hyperreactivity, by yet unknown mechanism (155). Even more surprisingly, D6 knockout mice were shown to be relatively protected from experimental autoimmune encephalitis which develops following immunization with MOG (156). Currently there is no unequivocal explanation for this observation; though, adoptive cell transfer experiments suggest the involvement of D6 in the effective generation of the adaptive immune response. Thus, D6 may play a yet unrecognized role in chemokine transport or retention within functional microenvironments which contribute to the initiation of immunity. Alternatively, it is possible that D6 scavenging of chemokines is required for the establishment of their functional gradients in the tissues. Such tissue gradients may drive the migratory steps, e.g. the entry of the antigen presenting cells into the lymphatic vessels. Indeed, diminished migration of CD11c+ dendritic cells from skin sites of antigen injections was seen in D6 deficient mice (156). Additionally, several subsets of leukocytes express D6 which in these cells appears to down-modulate their migratory responses to CC chemokines *in vitro* and *in vivo* (150). Because even immediate responses to CC chemokines are mitigated in the presence of D6, it is unlikely that chemokine degradation is the mechanism responsible for this effect. Alternatively, it is possible that the expression of D6 influences the signalling properties or the surface expression of classical chemokine GPCRs, e.g. through the consumption of the intracellular intermediates or heterodimerization with signalling receptors with consequent impediment of their function. Such heterodimerization was shown to take place between the two CXCL12 receptors, CXCR4, its classical GPCR, and CXCR7, its putative interceptor (157).

7.5. CXCR7, an interceptor for CXCL12 and CXCL11

In contrast to other interceptors CXCR7 has an intact canonical DRYL motif, but the adjacent amino acids are modified to: SIT instead of AIV. Also, there is considerable controversy as to the ability of this molecule to convey conventional chemokine signals, cell migration

and calcium mobilization in particular, in response to binding its two cognate ligands CXCR11 and CXCR12 (157-160). Notions supporting the lack of GPCR signaling start to prevail, nevertheless currently it is not possible to exclude that the ability of CXCR7 to either transmit GPCR signals or not may depend on the biochemical make-up of the cells which bear it. CXCR7 is expressed by subsets of leukocytes (161), activated endothelium including that in tumor vasculature as well as tumor cells themselves (160, 162). Three recent publications demonstrated the contribution of CXCR7 to the primordial germ cell migration in zebra fish (163-165) and suggested that this CXCL12-mediated process is streamlined in the presence CXCR7. Also in this process CXCR7 may act either on patterning of chemokine gradients by scavenging CXCL12 (165) or by heteromerizing with CXCR4 (155). Mice lacking CXCR7 die at birth due to deective cardiac development and valve malformations (155) mechanism of which still remains enigmatic.

7.6. Classical chemokine GPCRs as interceptors

There are indications that classical chemokine receptors may become uncoupled from G-proteins and subsequent signaling events and, as a consequence, act similarly to interceptors. Such unresponsive state of chemokine receptors was shown to be induced by pharmacological or cytokine treatment of the leukocytes (166), or to emerge as a result of leukocyte senescence (167). In some microenvironments, e.g. endothelial or epithelial cells, the "non-signaling state" of chemokine receptors occurs spontaneously in the absence of exogenous stimulation (168) and is possibly due to these cells lacking appropriate G proteins or downstream effectors. Such "disconnected" chemokine GPCRs may either passively absorb chemokines or induce chemokine internalization. Once internalized chemokines may be either transported or degraded as shown for CXCR4 on bone marrow endothelium and for CXCR3B expressed by salivary gland epithelium, respectively (168, 169). Curiously, ligand internalization by fully competent classical chemokine receptors which remain plugged into productive G-protein mediated signaling may be their important functional feature and contribute to chemokine clearance from the tissues, as shown for example for CCR2 (170). By this mechanism leukocytes which emigrate in the first wave bind tissue chemokines via classical GPCRs and eliminate them, thus limiting the extent of the subsequent migratory responses. Currently it is hard to judge how pervasive this mechanism is in limiting leukocyte migration to chemokines. However, leukocytes recruited by chemokines can not only mitigate but also augment the subsequent leukocyte migration as they produce secondary chemoattractants, including chemokines. For example, monocytes migrating in response to CCL5 secrete CCL2 which can attract additional monocytes (own unpublished data). The examples above illustrate that cognate chemokine receptors expressed by tissue and motile cells may contribute, akin to GAGs and interceptors, to patterning chemokines *in vivo*.

8. CONCLUSIONS

This review sketches some of the molecular pathways of chemokine action *in vivo*. These pathways are complex and involve, in addition to the interaction with classical chemokine receptors, several auxiliary molecules which dramatically influence the ability of chemokines to exert their action *in vivo*. Successful therapeutic strategies aimed at blocking the effects of chemokines can hardly be developed without the knowledge on the pathophysiological molecular partners of chemokines and the profound influence they have on chemokine behavior. Also, better understanding the mechanisms required for the *in vivo* actions of chemokines as well as those which naturally limit and terminate them, may yield a plethora of novel attractive molecular targets for future anti-inflammatory therapies

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Abbreviations: CCL: CC chemokine, CCR: CC chemokine receptor, CCX-CKR: CCX chemokine receptor, CXCL: CXC chemokine, CXCR: CXC chemokine receptor, XCL1: lymphotactin, XCR1: lymphotactin receptor; DARC: Duffy antigen/receptor for chemokines, GAG: glycosaminoglycans, GPCR: G-protein coupled receptor,

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