

The role of chemokines in neutrophil biology

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1. ABSTRACT

Neutrophils are the first to be recruited to a site of infection or a diseased site. Among various inflammatory mediators, CXC chemokines including IL-8 (CXCL8), MIP-2 (CXCL2), and KC (CXCL1) are the most critical for such recruitment. Neutrophils have been considered as effector cells that kill bacteria or destroy affected tissues mainly through the production of reactive oxygen species. Recent studies, however, revealed that neutrophils are involved in the production of chemokines in response to a variety of stimulants including LPS, TNF- α , and IFN- γ , thereby contributing to immunomodulation. These functions are also regulated by selectins during infiltration into various sites. In this review, I summarize the current knowledge on this area and propose that neutrophils are a fascinating target for basic as well as clinical scientists.

2. INTRODUCTION

Neutrophils are continuously produced in the bone marrow, and are promptly recruited to a site of inflammation or an injured tissue through the bloodstream in response to infection or injury. As compared with classic chemoattractants, such as complement protein C5a and leukotriene B₄, CXC chemokines, such as IL-8 (CXCL8), MIP-2 (CXCL2), and KC (CXCL1), are selective for neutrophils. Recent studies revealed that these CXC chemokines are the main chemotactic mediators involved in neutrophil recruitment in various *in vivo* models, as reviewed previously (1). Neutrophil recruitment can be categorized into several steps, namely mobilization from the bone marrow, rolling along and tight adhesion to endothelial cells, and transmigration. All the steps are regulated by CXC chemokines (2).

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At a site of inflammation or a diseased site, neutrophils exhibit various activities such as bacterial killing, tissue destruction, and angiogenesis through the oxidative burst, degranulation, and production of vascular endothelial growth factor (VEGF). These activities are also regulated by chemokines, as described in this review. In addition, recent studies revealed that neutrophils are directly or indirectly responsible for the production of chemokines, thereby regulating an immune response, as described in this review.

Consequently, in this review, I focus on the mechanisms by which chemokines regulate neutrophil recruitment and how chemokines induce various activities.

3. NEUTROPHIL RECRUITMENT

3.1. Mobilization of neutrophils from the bone marrow

A variety of agents including G-CSF mobilize neutrophils from the bone marrow where stem cells differentiate into neutrophils, whereas SDF1 (CXCL12) causes the retention of neutrophils in the bone marrow. It was found recently that G-CSF suppresses SDF1 (CXCL12) expression in the bone marrow, thereby inducing the release of neutrophils from the bone marrow (3). In addition to G-CSF, LPS also induces neutrophilia, which is caused by down-regulation of CXCR4 expression on neutrophils (4). Furthermore, KC (CXCL1) also induces neutrophil release from the bone marrow, and the release is augmented by low doses of CXCR4-blocking antibody that otherwise shows no mobilizing effect, suggesting that KC may act on the SDF-1 (CXCL12)/CXCR4 axis (5). CXCR4 is a sole receptor for SDF1 (CXCL12), and of note is that surface expression of CXCR4 decreases in peripheral neutrophils as compared with bone marrow neutrophils and further decreases in peritoneal exudates neutrophils (5). Indeed administration of the cyclized CXCR4 agonist peptide (CTCE-0021) with improved stability elevates blood neutrophils (6).

CXC chemokines, such as IL-8 (CXCL8), MIP-2 (CXCL2), and KC (CXCL1), contribute greatly to several disease models in which neutrophils are recruited to diseased sites, as reviewed previously (1). G-CSF also plays an important role in regulating neutrophil responsiveness to IL-8 (CXCL8) and MIP-2 (CXCL2) *in vivo*, because G-CSF receptor-deficient mice do not have the expected neutrophilia after administration of human IL-8 (CXCL8) (7). Of note is that neutrophils from G-CSF receptor-deficient mice also exhibit significant defects of chemotaxis and adhesion in response to IL-8 (CXCL8) and formyl-methionyl-leucyl-phenylalanine (fMLP) (7).

Neutrophils mobilized in response to rat MIP-2 (CXCL2) were found to express more CD11b and CD49d (8). Blockade of CD18 increased this mobilization, whereas that of CD49d decreased it dramatically (8). Therefore CD18 and CD49d appear to play contrasting roles in neutrophil retention and release within the bone marrow. Although CD11b/CD18 (Mac-1) is expressed on the cell membrane, a proportion of it is targeted to specific (also known as secondary) and tertiary granules. Upon cell

activation by chemotactic factors including IL-8 (CXCL8) and certain cytokines such as TNF α , the surface expression of Mac-1 increases several-fold within minutes due to the translocation of such granules and their fusion with the cell surface (9, 10). In addition to CD11b, it is also known that CD66b is up-regulated by activation with IL-8 (CXCL8) (11). CD66b is a member of CD66 family, and is phosphorylated following stimulation with fMLP, platelet-activating factor and phorbol myristate acetate (12).

3.2. Rolling along and tight adhesion to endothelial cells

During inflammation, neutrophils roll along the walls of postcapillary venules, CXC chemokines playing a critical role in this process. For this purpose, tissue-derived CXC chemokines have to traverse endothelial cells. An electron microscopic study showed that IL-8 (CXCL8) is internalized by venular endothelial cells abluminally and then transcytosed to the luminal surface, and that IL-8 (CXCL8) is presented to the adherent neutrophils on the endothelial cell membrane, predominantly in association with the endothelial cell projections. The C terminus of IL-8 (CXCL8) is required for endothelial cell binding, transcytosis, and the ability of IL-8 (CXCL8) to recruit neutrophils *in vivo*, suggesting that such transcytosis is essential for neutrophil recruitment (13).

Two molecules responsible for such transcytosis have been identified so far. One is Duffy antigen and the other is endothelial heparan sulfate. Duffy antigen is expressed on red blood cells, capillaries, and postcapillary venular endothelial cells, and binds certain CXC and CC chemokines. The Duffy antigen exogenously expressed in human endothelial cells facilitates the movement of CXC chemokines, in this case Gro- α (CXCL1), across an endothelial monolayer, and neutrophil migration towards CXC chemokines, such as Gro- α (CXCL1) and IL-8 (CXCL8), is enhanced across an endothelial monolayer expressing Duffy antigen. In agreement with this *in vitro* observation, IL-8 (CXCL8)-driven neutrophil recruitment in the lungs was markedly attenuated in mice deficient in Duffy antigen (14). On the other hand, mice deficient in the enzyme N-acetylglucosamine N-deacetylase-N-sulfotransferase-1 in endothelial cells and leukocytes, which is required for the addition of sulfate to heparin sulfate chains, showed impaired neutrophil infiltration. In these mice, chemokine transcytosis across endothelial cells and presentation on the cell surface were reduced, resulting in decreased neutrophil firm adhesion and migration (15).

Neutrophils scan the surface for IL-8 (CXCL8), which is transcytosed and immobilized through the mechanisms described above. The administration of MIP-2 (CXCL2) and KC (CXCL1) induced a dose- and time-dependent increase in neutrophil rolling, which was significantly inhibited by an antibody against P-selectin, suggesting that MIP-2 (CXCL2) and KC (CXCL1) induce P-selectin-dependent rolling (2).

Although IL-8 (CXCL8) arrests rolling neutrophils *in vitro* (16), no neutrophil arrest chemokine had been demonstrated *in vivo* until recently. In inflamed

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cremaster muscle venules in TNF- α -treated mice, neutrophil adhesion was almost completely abrogated in E-selectin (-/-) mice treated with pertussis toxin, an inhibitor of chemokine signal transduction, and significantly reduced in CXCR2 (-/-) mice treated with a monoclonal antibody blocking E-selectin. However, the adhesion was not abrogated in E-selectin (-/-) or CXCR2 (-/-) mice (17). Therefore, CXC chemokine and E-selectin appear to mediate tight adhesion cooperatively.

Phosphoinositide-3 kinase γ (PI-3K γ) can be activated directly by the $\beta\gamma$ dimer of heterotrimeric G proteins coupled to CXCR2. PI-3K γ null mice showed a significant decrease in KC (CXCL1)-induced neutrophil adhesion in venules of exteriorized cremaster muscle. In wild-type mice rolling neutrophils showed rapid and sustained adhesion, but in PI-3K γ (-/-) mice, adhesion was not triggered at all. Lethally irradiated wild-type mice reconstituted with the bone marrow cells of PI-3K γ null mice showed a 50% decrease in KC (CXCL1)-induced neutrophil adhesion, suggesting that PI-3K γ must function in the adhesion of neutrophils (18).

3.3. Transmigration

Neutrophils then move in an amoeboid manner across the endothelial cell barrier, followed by passage across the subendothelial basal lamina. The former process involves homophilic interaction of CD31 and JAM-A on neutrophils and endothelial cells where CD31 and JAM-A act sequentially to mediate neutrophil migration through venular walls (19). On the other hand, the latter process may involve proteolysis. Because the basal lamina comprises a dense meshwork of extracellular matrix proteins, including type IV collagen, laminin, fibronectin, and glycosaminoglycan, it is thought that the matrix proteins are degraded by matrix metalloproteases stored in specific (also known as secondary) and tertiary granules of neutrophils. Among MMPs, MMP-9 is thought to be a candidate that participates in such degradation. MMP-9 is indeed induced by not only ligation of L-selectin and Mac-1 (20) but also IL-8 (CXCL8) and TNF- α (21). In MMP-9 (-/-) mice, neutrophil infiltration was impaired at the peak during zymosan-induced experimental peritonitis (22). In such mice, however, neutrophils infiltrated into the peritoneal cavity at later stages, suggesting that other mechanisms compensate for the function of MMP-9.

3.4. Fate of transmigrated neutrophils

Peripheral blood neutrophils circulate through the vascular system with a lifespan of 6-12 h (23), after which they die due to apoptosis, but under inflammatory conditions neutrophil apoptosis is inhibited *in vitro* (24) as well as *in vivo* (25). Such prolongation of life span may allow neutrophils to perform effector functions such as killing bacteria. In one study, IL-8 (CXCL8) and Gro- α (CXCL1) greatly suppressed neutrophil apoptosis, whereas they augmented superoxide anion production and phagocytic activity towards *E. coli* (26). In another study, however, IL-8 (CXCL8) failed to suppress neutrophil apoptosis, whereas GM-CSF, IL-6, and IL-15 suppressed it through down-regulation of Bax (27). In a second study, even when neutrophils migrated through human umbilical

vein endothelial cells (HUVEC) in response to IL-8 (CXCL8), neutrophil apoptosis was only minimally inhibited, while neutrophils transmigrating through TNF- α or IL-1 β -treated HUVEC were strongly protected against apoptosis (28). Overall, although it is not known at present why such a discrepancy arose, IL-8 (CXCL8) appears to be ineffective in prolonging the neutrophil lifespan *in vivo*.

3.5. Genomic changes induced by transmigration

Microarray analysis revealed dramatic gene expression differences between neutrophils which accumulated in the air spaces in response to bronchoscopic instillation of LPS and circulating neutrophils in man (29). Approximately 15% of expressed genes, including inflammatory- and chemotaxis-related ones and ones for antiapoptotic and IKK-activating pathways, exhibit altered expression levels. Functional analysis also showed increased superoxide release, decreased apoptosis, decreased IL-8 (CXCL8)-induced chemotaxis, and a different pattern of IL-8 (CXCL8), MIP-1 β (CCL4), MCP-1 (CCL2), and TNF- α release in air space neutrophils as compared with those of circulating neutrophils. Of note was that many of these changes were not caused by treatment with LPS *in vitro*, suggesting that neutrophils sequestered in the lungs become fundamentally different from those resident in the circulation. In another *in vitro* study, IL-8 (CXCL8) and LPS induced partially overlapping transcriptional profiles, 50% of IL-8 (CXCL8)-responsive genes being concomitantly regulated by LPS. IL-8 (CXCL8) also modulated a significant number of genes unresponsive to LPS (30). Future studies should clarify whether or not neutrophils sequestered in tissues such as the lungs in response to IL-8 (CXCL8) or MIP-2 (CXCL2) also show such dramatic differences.

4. CHEMOKINES AS SECRETAGOGUES

Neutrophils secrete their granule contents in response to a variety of secretagogues, including chemokines, TNF- α , and C5a, and/or upon interaction with endothelial cells via integrins.

Recently, a member of the TNF superfamily of ligands, B-lymphocyte stimulator (BLyS), was found to be stored in G-CSF-activated neutrophils and secreted by them. Although IL-8 (CXCL8) and Gro- α (CXCL1) did not induce BLyS *de novo* synthesis in neutrophils, they acted as potent secretagogues for BLyS stored in such activated neutrophils (31). A cytokine, TNF-related apoptosis-inducing ligand (TRAIL), is also synthesized, stored, and released from interferon-activated neutrophils upon stimulation with IL-8 (CXCL8) (32). In such cells, TRAIL is mainly retained in secretory vesicles and light membrane fractions that are rapidly mobilizable to the cell surface upon exposure to IL-8 (CXCL8). TRAIL is subsequently secreted by the cells. BLyS is important in B cell maturation and survival, whereas TRAIL exerts selective, apoptotic activities towards tumor and virus-infected cells, as well as immunoregulatory functions on activated T cells, and therefore IL-8 (CXCL8)-induced release of BLyS and TRAIL from neutrophils may play physiological and pathological roles *in vivo*.

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Table 1. Chemokine receptors on neutrophils

Receptors	Stimulants	Up or down regulation	References
CXCR2	substance P	Up	34
CCR1	GM-CSF	Up	36
	substance P	Up	34
	unknown	Up	37
CCR2	unknown	Up	37
	none		33
CCR6	TNF- α	Up	38
CXCR4	LPS	Down	4
	none		5

Representative ligands: CXCR2: CXCL1 (KC, Gro- α), CXCL2 (MIP-2), CXCL8 (IL-8), CCR1: CCL4 (MIP-1 β), CCL5 (RANTES), CCR2: CCL2 (MCP-1), CCR6: CCL20 (LARC), CXCR4: CXCL12 (SDF-1)

Table 2. Chemokines produced by neutrophils

Chemokines	Stimulants	References
IL-8 (CXCL8)	LPS, adherence	39, 40
	LPS, GM-CSF+Zymosan	46
MIP-2 (CXCL2)	substance P	34
MIP-1 α (CCL3)	substance P	34
	LPS, LPS+GM-CSF	41, 42
	Zymosan	46
MIP-1 β (CCL4)	LPS	42
	Zymosan	46
Gro- α (CXCL1)	Zymosan	46
IP-10 (CXCL10)	IFN- γ , IFN- γ +LPS, IFN- γ +TNF- α	43
MIG (CXCL9)	IFN- γ +LPS, IFN- γ +TNF- α	44
MIP-3 α (CCL20)	LPS, TNF- α	45
MIP-3 β (CCL19)	LPS, TNF- α	45

5. CHEMOKINE RECEPTOR EXPRESSION ON NEUTROPHILS AND CHEMOKINE PRODUCTION BY NEUTROPHILS

Neutrophils not only express CXCR1 and 2 but also CXCR4 (5) and CCR2 (33). Neutrophils also express CCR1 and CCR6 under certain circumstances.

CXCR2 is up-regulated by neuropeptide substance P through neurokinin-1 receptor (34). Although human peripheral blood neutrophils do not express CCR1 (35), GM-CSF treated neutrophils express CCR1 and respond to MIP-1 α (CCL3), MCP-3 (CCL7), and RANTES (CCL5) (36). CCR1 is also expressed on substance P treated neutrophils (34) and neutrophils from adjuvant-immunized rats (37). CCR2 had been regarded as a receptor for monocyte chemoattractant MCP-1 (CCL2), but recently it was reported that CCR2 is expressed on neutrophils from adjuvant-immunized rats (37) and neutrophils from the bone marrow (33). CCR6, a receptor for LARC (MIP-3 α , CCL20), is also expressed on TNF- α treated neutrophils (38). These findings suggest that a variety of chemokines in addition to IL-8, MIP-2 and KC may participate in neutrophil infiltration and activation particularly under pathological conditions (Table 1).

Upon treatment with a variety of stimulants, such as LPS, TNF- α , IFN- γ and G-CSF, neutrophils produce chemokines, including IL-8 (CXCL8), Gro- α (CXCL1), MIP-1 α (CCL3), MIP-1 β (CCL4), IP-10 (CXCL10), and MIG (CXCL9) (39, 40, 41, 42, 43, 44, 45,

Table 2). Although there is a discrepancy regarding the production of IL-1 β , IL-6, and TNF- α by neutrophils (45, 46), the production of chemokines, such as IL-8 (CXCL8), Gro- α (CXCL1), MIP-1 α (CCL3), and MIP-1 β (CCL4), has been confirmed with highly purified neutrophils (46). Of note is that the production of each chemokine requires a relatively selective combination of stimulants. For instance, LPS and TNF- α induce the production of IL-8 (CXCL8), Gro- α (CXCL1), and MIP-1 α (CCL3), whereas IFN- γ plus LPS and IFN- γ plus TNF- α induce that of IL-8 (CXCL8), Gro- α (CXCL1), MIP-1 α (CCL3), IP-10 (CXCL10), and MIG (CXCL9) (45). In support of the pathophysiological relevance of such *in vitro* findings, there are several reports on detection of chemokines in neutrophils in human pathologies (47, 48, 49) and several studies using a variety of animal models (50, 51, 52). A recent study showed that macrophage recruitment to cutaneous polyacrylamide gel-induced granulomas is the result of a sequence of inflammatory processes initiated by mast cell-derived TNF- α followed by neutrophil influx and MIP-1 α / β release (50). Another study showed that *Toxoplasma gondii* triggered neutrophil synthesis of MIP-1 α (CCL3), MIP-1 β (CCL4), RANTES (CCL5), and MIP-3 α (CCL20), followed by DC activation (51). The other study showed that neutrophils produce IP-10 (CXCL10) and MIG (CXCL9) in response to IFN- γ to recruit T cells in a delayed-type hypersensitivity response to HSV-1 antigen (52). Neutrophils themselves, however, do not always produce significant amounts of cytokines and chemokines under various conditions. For instance, we recently reported that, although neutrophils are required for the production of MCP-1 (CCL2) that leads to the accumulation of killer T cells upon injection of late apoptotic tumor cells, neutrophils themselves do not produce MCP-1 (CCL2) (53). In another study, we also found that in Con A-induced hepatitis neutrophils play a role in IFN- γ production and that neutrophils are not IFN- γ producers but augment IFN- γ production by T cells (54). Future studies should elucidate the mechanism by which neutrophils regulate the production of cytokines and chemokines *in vivo*.

6. PRIMING OF THE OXIDATIVE BURST BY IL-8 (CXCL8)

Superoxide-producing phagocyte NADPH oxidase consists of a membrane-bound flavocytochrome b(558), cytosolic factors p47(phox), p67(phox), and p40(phox), and small GTPase Rac2, all of which are translocated to the membrane to assemble the active complex following neutrophil activation.

Although IL-8 (CXCL8) does not activate NADPH oxidase, it potentiates the oxidative burst induced by stimulants such as fMLP and P-selectin. During priming, sialyl Lewis X epitope, a ligand for P-selectin, was redistributed to one end of the neutrophils, thereby facilitating oxidative burst induced by soluble P-selectin (55). Later, the same group showed that, during priming, PSGL-1, a major ligand for P-selectin that contains sialyl Lewis X carbohydrate structures, was redistributed to form

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a more compactly accumulated cluster, PSGL-1-containing lipid microdomains (also referred to as lipid rafts) (56). Another study also showed that, during priming, IL-8 (CXCL8) enhanced the Btk- and ERK1/2-dependent phosphorylation of p47(phox), as well as the recruitment of flavocytochrome b(558), p47(phox), and Rac2 into lipid microdomains, thereby potentiating the oxidative burst in response to fMLP (57). Consequently, the mechanism of priming by IL-8 (CXCL8) involves recruitment of NADPH oxidase components, fMLP receptor, and PSGL-1 into lipid microdomains.

7. INFLAMMATION AND CARCINOGENESIS

Inflammation is associated with carcinogenesis. In the lungs, the influx of neutrophils into the airways may be an important process linking inflammation with carcinogenesis, and induction of oxidative DNA damage by reactive oxygen species and myeloperoxidase-related metabolic activation of chemical carcinogens appear to be crucial for the process (58). On the other hand, when patients with early gastric cancer underwent partial gastrectomy, *Helicobacter pylori* eradication completely normalized mucosal lesions, and resulted in complete absence of neutrophil infiltration and a significant decrease in the tissue IL-8 (CXCL8) level, thereby presumably contributing to a reduction in the risk of carcinogenesis (59). Indeed it was found that IL-8 (CXCL8) promoter polymorphism increases the risk of gastric cancer, which is associated with the IL-8 (CXCL8) level and neutrophil infiltration (60). In another study involving an experimental tumor model, co-implantation of a foreign body, a gelatin sponge, into a benign tumor-bearing mouse caused conversion of the tumor into a highly malignant one, which was due to neutrophil infiltration (61). All these findings may be explained by neutrophil-derived reactive oxygen species.

Neutrophils are also directly or indirectly involved in VEGF release, thereby contributing to carcinogenesis. Infiltrating neutrophils positive for MMP-9 were found to be involved in VEGF release and activation from pancreatic islets during carcinogenesis of the islets in transgenic mice in which SV40 large T antigen oncogenes were expressed in all islets under control of an insulin gene promoter. Neutrophil depletion and MMP inhibitors significantly reduced such angiogenic switching, as did genetic ablation of MMP-9 (62, 63). In this model, it is not known what signals trigger the recruitment of neutrophils positive for MMP-9. In this regard, it should be noted that MIP-2 (CXCL2)-induced angiogenesis is mainly mediated by neutrophil-derived VEGF-A, because neutrophils from mice deficient in the src family kinases, Hck and Fgr (hck(-/-)fgr(-/-)), normally migrate and release MMP-9 in response to MIP-2 (CXCL2) *in vitro*, whereas they are completely unable to release VEGF-A or cause an angiogenic response (64).

8. PERSPECTIVES

Chemokines act on neutrophils in collaboration with cytokines such as TNF- α and/or

selectins *in vivo*. On the other hand, the process of recruitment, in particular rolling and adhesion, causes much more dramatic changes in neutrophil functions than treatment with chemokines alone. Therefore, research has been and will be directed towards elucidation of the molecular mechanisms underlying such changes, and the physiological and pathological roles of the changes.

It is accepted that neutrophils play a critical role as producers of chemokines in immunological and pathological settings. The *in vivo* role of neutrophils as regulators of the production of chemokines, however, has not been fully explored. Research along these lines would provide us with a new perspective on neutrophil function.

9. ACKNOWLEDGEMENTS

This work was partly supported by a research grant from Toho University, Faculty of Science.

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Key Words: Chemokines, Neutrophils, Regulation, Recruitment

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