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REVIEW

Chemotactically Active Proteins of Neutrophils

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Abstract—Polymorphonuclear leukocytes (neutrophils) are the first cells that arrive at sites of infection or injury. There, besides their microorganism-targeted effector functions, activated neutrophils secrete numerous chemoattractants that recruit other leukocyte subtypes into the inflamed tissue. First, neutrophil activation leads to the upregulation of the gene expression of several classical chemokines of the CXC and CC families. Second, neutrophil granules contain preformed intracellular storage pools of chemotactically active proteins that are rapidly released upon neutrophil degranulation. The third pathway of generation of chemotactically active proteins by activated neutrophils—shedding and concomitant proteolytic processing of a membrane protein—has recently been demonstrated in our laboratory. In this review we summarize the essential features of chemoattractant production by neutrophils and their contribution to orchestrating the recruitment of leukocyte subtypes during inflammatory response.

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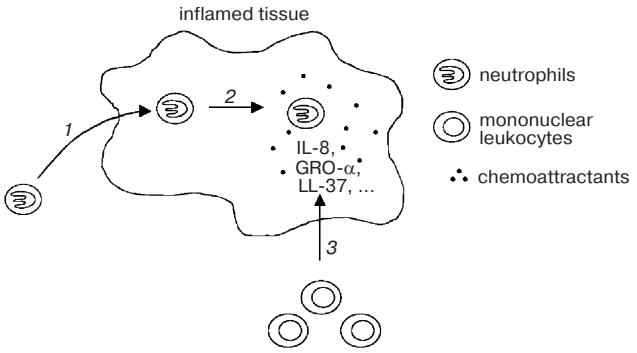
Neutrophils are the major effector cells of innate immunity; they represent the first line of defense against pathogenic microorganisms. These cells recognize and kill microorganisms by phagocytosis followed by subsequent secretion of reactive oxygen species and cytotoxic components of granules into phagosomes. Detailed analysis of these important effector functions of neutrophils is not considered in this review; we just recommend a recent review on this problem [1]. The contribution of neutrophils to host defense and innate immunity extends well beyond their role as professional phagocytes. In recent years it has become obvious that these cells play a key role in the recruitment of other types of leukocytes into inflamed tissue and provide links between innate and adaptive immune responses (Scheme 1). Early studies demonstrated biphasic innate immune responses: neutrophil infiltration into sites of inflammation usually preceded the appearance of mononuclear leukocytes (monocytes and lymphocytes) there [2-5]. Under conditions of experimental neutropenia, recruitment of mononuclear leukocytes into inflamed tissue was significantly suppressed and occurred with longer delay than in control. These data suggest that neutrophils accumulated in inflamed tissue secrete chemoattractants attracting monocytes and lymphocytes to the site of inflammation.

Indeed, subsequent studies employing the methods of reverse transcription polymerase chain reaction, immunohistochemistry, and *in situ* hybridization demonstrated the neutrophil accumulation at the sites of infection penetration and in damaged tissues is accompanied by induction of expression of numerous chemokines [6, 7]. Using Hu95A microarray (Affymetrix, USA), which can analyze the expression of 12,500 genes and detect differentially expressed genes, gene expression profiles of mature bone marrow neutrophils, circulating neutrophils, and neutrophils from inflamed tissues have been analyzed [8]. This analysis has shown that the release of mature neutrophils from the bone marrow to the blood circulation is not accompanied by major changes in gene expression. However, neutrophil migration from blood circulation into an inflamed tissue is accompanied by major changes in gene expression profile. Essentially, this is accompanied by induction of expression of genes

Abbreviations: CAP37) cationic antimicrobial protein of 37 kD; DTH) delayed type hypersensitivity; fMLP) N-formyl-methionyl-leucyl-phenylalanine; GROα) growth-regulated oncogene-α; HNP1-4) human neutrophil peptides 1-4; IFN-γ) interferon-γ; IL-8) interleukin-8; IP-10) interferon-γ-inducible protein of 10 kD; LPS) lipopolysaccharide; MCP-1) monocyte chemotactic protein-1; MIG) monokine induced by interferon-γ; MIP-1α, -1β, -3α, -3β) macrophage inflammatory protein-1α, -1β, -3α, -3β; suPAR) soluble form of the urokinase receptor; TNF-α) tumor necrosis factor-α; uPAR) urokinase receptor.



Biphasic innate immune response. Neutrophils are the first cells that infiltrate the site of inflammation (*1*). They are activated in the inflamed tissue and secrete numerous chemoattractants (*2*), which recruit other types of leukocytes (particularly monocytes and lymphocytes) to the site of inflammation (*3*)

Scheme 1

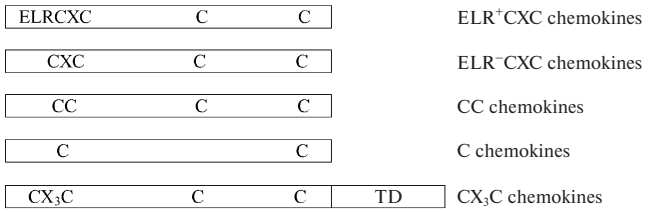
encoding numerous chemokines, which act on neutrophils, monocytes, lymphocytes, and other types of leukocytes. Similar results were obtained by another group, who studied alveolar transmigration of neutrophils using an experimental model of lung inflammation [9]. Comparison of neutrophils from peripheral blood and bronchoalveolar lavage fluid revealed that 15% of 14,131 genes analyzed by the microarray HG-U133A (Affymetrix) exhibited differential expression. Alveolar transmigration of neutrophils was accompanied by induction of a significant number of genes encoding chemokines.

Besides classical chemokines encoded by genes that are induced during neutrophil activation, activated neutrophils also secrete other proteins that can exhibit a chemoattractant effect on certain types of leukocytes. These proteins are components of neutrophil granules and they are readily secreted during degranulation of the activated cells. We have recently found another mechanism of generation of chemotactically active proteins during neutrophil activation; it includes shedding and concomitant proteolytic processing of a membrane protein followed by the release of a chemotactically active soluble form. In this review, we consider chemotactically active proteins secreted by activated neutrophils and their role in leukocyte recruitment in the inflammatory response.

CLASSICAL CHEMOKINES SECRETED BY NEUTROPHILS

Chemokines are a group of cytokines that act as the main mediators and regulators of leukocyte migration. They are small single-chain polypeptides (of 67-127 amino acid residues in length) exhibiting various identity degree (20-95%) in their primary structure [10]. About 50 chemokines are known to date. Based on location of cysteine residues in the polypeptide chain, all chemokines have been subdivided into four families. The largest families are CXC (or α-) and CC (or β-) chemokines. In the CXC chemokines, the cysteine residues closely located to the N-terminus are separated by one amino acid residue, whereas in the CC chemokines these cysteines are characterized by tandem localization (Scheme 2). The CXC chemokines are further subdivided into two groups, one of which contains the ELR motif (glutamate–leucine–arginine) immediately before the cysteines at the N-terminus (ELR^+CXC chemokines), whereas the other one lacks such motif (ELR^-CXC chemokines). The family of CX_3C chemokines has just one member, a membrane-bound glycoprotein CX_3CL1 also known as fractalkine. In the fractalkine molecule, the N-terminal cysteines are separated by three amino acid residues. C chemokines (lymphotoxin-α/XCL1 and lymphotoxin-β/XCL2) lack two of four conservative cysteine residues (Scheme 2). Chemokines are now designated by traditional abbreviations (e.g. IL-8 for interleukin-8) and also systematic nomenclature. According to this nomenclature, the chemokine name is formed from the name of the particular family to which this chemokine belongs (CXC, CC, C, or CX_3C), the letter L (ligand), and the number of the corresponding gene (e.g. the systematic name of IL-8 is CXCL8).

Except for CX_3CL1 and CXCL16 containing a transmembrane domain, all chemokines are secretory proteins [11]. Chemokine molecules are positively charged (they contain many basic amino acids) and they bind to negatively charged glycosaminoglycans (heparin, heparan sulfate, chondroitin sulfate, etc.) located on cell surface and also present in the extracellular matrix. Thus, once released, the chemokines are immobilized on the components of extracellular matrix, and tend to remain concentrated locally, forming stable gradients. Chemokines act on all types of leukocytes including hematopoietic precursors, mature leukocytes of nonspecific immunity, and various types of lymphocytes. Concentration gradients of



Chemokine families. Based on the location of cysteine residues close to the N-terminus, chemokines are classified into four families: CXC, CC, C, and CX_3C. The CXC chemokines are further subdivided into two groups: ELR^+CXC chemokines containing the ELR motif immediately before the CXC motif and ELR^-CXC chemokines lacking such motif. TD, transmembrane domain

Scheme 2

various chemokines between various organs and tissues determine preferential localization of certain types of leukocytes in particular compartments of the organism.

Receptors mediating biological effects of chemokines belong to the family of G-protein coupled receptors (receptors coupled to heterotrimeric guanosine triphosphate binding proteins also known as G-proteins) [10]. Chemokine receptor molecules contain seven transmembrane domains and share 25-80% identity in primary structure. About 20 chemokine receptors have been found in humans (CCR1-10, CXCR1-7, XCR1, and CX_3CR1). Usually each chemokine can interact with several receptors, and each chemokine receptor can bind several ligands. Activation of chemokine receptors results in dissociation of Gβγ-subunits of the heterotrimeric G-protein and subsequent activation of phospholipase Cβ, phosphatidylinositol-3-kinase-γ (PI3Kγ), and c-Src family nonreceptor tyrosine kinases. This results in activation of FAK and Pyk2 kinases regulating cytoskeleton rearrangement during chemotaxis [12]. There are chemokine receptors that bind a ligand without subsequent signal transduction into cells; these receptors act as scavenger receptor for chemokines [13]. Three such receptors have been found to date: DARC (Duffy antigen-related chemokine receptor), CCX-CKR (ChemoCentryx chemokine receptor), and D6. They can bind various chemokines. It is suggested that these receptors are involved in regulation of chemokine homeostasis in tissues, and they negatively regulate inflammatory responses.

Chemokines can negatively regulate activation of their own receptors (homologous desensitization) and also receptors of other cytokines (heterologous desensitization and competitive inhibition of receptors by chemokine antagonists). Receptor desensitization is a consequence of activation of mechanisms uncoupling the receptor and associated G-protein. Homologous desensitization is mediated by G-protein-coupled receptor kinases (GRKs), phosphorylating Ser/Thr residues in intracellular C-terminal domains of the ligand-bound receptor. Such phosphorylation results in binding of β-arrestin with this receptor. This binding sterically inhibits the interaction of the receptor with G-protein and causes clathrin-dependent internalization of the ligand–receptor complex [14]. Thus, the existence of a chemokine concentration gradient results in polarization of distribution of this chemokine receptor on the plasma membrane and directed migration of cells along the gradient of this chemoattractant.

The heterologous desensitization of chemokine receptors involves phosphorylation of the ligand-unbound receptors by protein kinases, which are not GRKs [15]. Heterologous desensitization was demonstrated not only for chemokine receptors, but also for other G-protein coupled receptors. This represents a mechanism by which chemokines can influence physiological processes unrelated to cell chemotaxis and, vice versa, ligands of non-chemokine G-protein coupled receptors can regulate chemokine-induced migration of leukocytes. Recent studies have shown that some chemokines act not only as agonists of certain type receptors, but they also can function as physiological antagonists of receptors for other chemokines [16]. Chemokine antagonists bind to chemokine receptors but do not induce their activation, and they competitively inhibit activation of these receptors by their agonists. Such chemokines can differentially regulate migration and activation of various cell populations by recruiting and/or activating population of cells expressing one type of receptor (for which these chemokines are agonists) and inhibiting migration and/or activation of the population by means of another receptor (for which these chemokines are antagonists).

Besides the classification of chemokines that is based on features of the polypeptide chain structure, chemokines are functionally subdivided into homeostatic and inflammatory ones [17]. The homeostatic chemokines are constitutively expressed by certain cell type(s) in certain compartments of the body; they control homeostatic migration of leukocytes and provide functional compartmentalization in lymphoid organs. The inflammatory chemokines are synthesized in tissues in response to proinflammatory stimuli (LPS, TNF-α, IL-1β); they control leukocyte recruitment into the sites of tissue damage and infection entry. Cells of the nonspecific immune system (neutrophils, monocytes, basophils, etc.), various types of lymphocytes, and dendritic cells are the targets for these chemokines. Some chemokines exhibit properties of both homeostatic and inflammatory chemokines. Under certain experimental conditions *in vitro* and also *in vivo*, neutrophils can synthesize and secrete some inflammatory chemokines. It should be noted that expression of many chemokines (e.g. RANTES/CCL5, MCP-2/CCL8, MCP-3/CCL7, I-309/CCL1) is not detected in activated neutrophils [6]. Below we consider inflammatory CXC and CC chemokines, which are characterized by induction of expression in activated neutrophils.

ELR^+CXC chemokines. In contrast to most of other chemokines, ELR^+CXC chemokines act as chemoattractants for neutrophils; they also exhibit angiogenic properties. The main function of ELR^+CXC chemokines in the inflammatory response consists of attraction of neutrophils to sites of inflammation (first phase of innate immune response), induction of granule exocytosis, and activation of NADPH-oxidase in these cells. The inflammatory mediators such as IL-1β and TNF-α, as well as bacterial cell components (e.g. lipopolysaccharides (LPS)) induce expression of ELR^+CXC chemokines in damaged tissues or at sites of infection entry. This results in rapid infiltration of the sites of inflammation with neutrophils. Activated neutrophils can also express some chemokines of this group. Human neutrophils synthesize

and secrete ELR$^+$CXC chemokines: IL-8/CXCL8 and GRO-α (growth related oncogene alpha)/CXCL1. These cells can also express GRO-β/CXCL2 and GRO-γ/CXCL3 genes, but the possibility of secretion of these proteins has not been demonstrated in direct experiments so far [6, 18].

IL-8 was the first identified chemokine secreted by neutrophils [19]. Initially, expression of this protein by neutrophils was demonstrated during cell activation by LPS and during phagocytosis of IgG-opsonized *Saccharomyces cerevisiae* [19]. Subsequently, many other stimuli inducing synthesis and secretion of IL-8 by neutrophils have been recognized. These include: cytokines and growth factors (TNF-α, IL-1β, IL-15, granulocyte macrophage colony stimulating factor (GM-CSF), and thrombopoietin), chemoattractants (N-formyl-methionyl-leucyl-phenylalanine (fMLP), leukotriene B_4, and anaphylotoxin C5a), corpuscular agents (microcrystals of sodium urate and potassium pyrophosphate dihydrate), various bacteria, fungi, and viruses (see references in [7]). Synthesis and secretion of IL-8 are likely to represent a stereotype neutrophil response to various activating treatments. Besides neutrophils, IL-8 is also expressed and secreted by monocytes, macrophages, T lymphocytes, and by many non-leukocyte cell types including endothelial and epithelial cells, hepatocytes, keratinocytes, and fibroblasts [20]. As in the case of neutrophils, IL-8 expression by these cells is not constitutive and it is induced in response to such proinflammatory stimuli as LPS, TNF-α, and IL-1β.

GRO-α is synthesized and secreted by neutrophils activated by LPS, TNF-α, GM-CSF, and fMLP and also during phagocytosis of IgG-opsonized *S. cerevisiae*, *Mycobacterium tuberculosis*, and *Helicobacter pylori* antigens [18, 21, 22]. Interestingly, extracellular secretion of GRO-α does not necessarily correlate with changes in expression of mRNA of this chemokine. This inconsistency can be attributed to the fact that GRO-α production by neutrophils can be regulated at posttranscriptional, translational, and posttranslational levels [21]. Like IL-8, GRO-α is expressed by other cell types (monocytes, fibroblasts, endothelial cells) during their activation by LPS, TNF-α, or IL-1β.

IL-8 interacts with two types of receptors (so-called IL-8 receptors): CXCR1 and CXCR2. GRO-α interacts only with CXCR2 [23]. In *in vitro* experiments, IL-8 and GRO-α exhibit similar biological activity, but IL-8 usually causes more potent responses. These chemokines exert chemotactic effects on neutrophils, basophils, and T and B lymphocytes [24-27]; they also suppress apoptosis in neutrophils and therefore extend the period of their functional activity in inflamed tissues [28]. IL-8 and GRO-α are inducers of neutrophil degranulation: they cause rapid translocation of an intracellular pool of membrane receptors from secretory vesicles to plasma membrane [29] as well as exocytosis of primary and secondary granules in cytochalasin B-primed cells [30]. IL-8 and GRO-α *per se* cannot induce superoxide production by neutrophils, but they exhibit a priming effect on the induction of superoxide production by the classical neutrophil activator fMLP. The mechanism of such priming effect can involve IL-8 and GRO-α-induced rapid translocation of formyl peptide receptors from secretory vesicles onto neutrophil plasma membrane [29].

In *in vivo* experiments subcutaneous administration of IL-8 and GRO-α induced accumulation of neutrophils at the site of injection 2-4 h after administration of the proteins [24, 30]. In other experiments IL-8 and GRO-α have been shown to be the first chemokines that are induced at the sites of acute inflammation caused, for example, by tissue damage. Time course and spatial mode of secretion of these chemokines coincided with neutrophil accumulation in the inflamed tissue [31]. Moreover, neutrophils attracted to a site of inflammation secrete IL-8 and GRO-α [32, 33]. Summarizing all these data, we propose the following scenario for the initial step of leukocyte recruitment into the forming site of inflammation. Tissue damage and/or infection entry induce synthesis of IL-8 and GRO-α by various cell types presented in this region. These chemokines attract neutrophils, which become activated; the activated neutrophils secrete IL-8 and GRO-α, thus potentiating their own infiltration into the inflamed tissue.

The key role of ELR$^+$CXC chemokines in initiation of the innate immune response has been demonstrated *in vivo* using various experimental models in mice. It should be noted that in mice the ELR$^+$CXC chemokine system significantly differs from that of in humans because mice lack human IL-8 and CXCR1 orthologs [34]. It is suggested that in mice CXCR2 functions as the ELR$^+$CXC chemokine receptor. Activated mouse neutrophils can secrete an ELR$^+$CXC chemokine, MIP-2/CXCL2 [35]. At sites of acute inflammation in mice induced by tissue damage or infection entry, there is induction of expression of the ELR$^+$CXC chemokines KC and MIP-2, which are functional homologs of human IL-8 and GRO-α [36-38]. At sites of inflammation induced by tissue damage, fibroblasts and endothelial cells express KC, whereas leukocytes, particularly neutrophils, migrating to the inflamed tissue express MIP-2 [37].

CXCR2 knockout mice are characterized by decreased ability of neutrophils to be accumulated at experimentally induced sites of inflammation. For example, in the case of the experimental model of acute peritonitis induced by thioglycolate, neutrophil infiltration into the site of acute inflammation was 5 times lower in CXCR2$^{-/-}$ mice than in wild type mice [34]. These results are even more demonstrative if we take into consideration that CXCR2$^{-/-}$ mice are characterized by marked neutrophilia, and the concentration of circulating neutrophils is 12 times higher in these mice than in wild type mice. Decreased ability of neutrophils to migrate toward

sites of inflammation in CXCR2$^{-/-}$ mice has also been demonstrated in the experimental model of acute pyelonephritis induced by *E. coli* infection [39] and during mechanical tissue damage [40]. In all these cases, suppression of neutrophil infiltration into inflamed tissue corresponded to more severe and longer duration of the inflammatory process in CXCR2$^{-/-}$ mice compared with wild type mice.

The role of particular ELR$^+$CXC chemokines in the immune response was also evaluated by means of neutralizing antibodies using the experimental model of acute inflammation caused by subcutaneous administration of TNF-α [41]. Such injection caused rapid accumulation of neutrophils into the forming site of inflammation (maximal accumulation of neutrophils was observed within 2-4 h after TNF-α injection). However, TNF-α-induced recruitment of neutrophils into the site of inflammation was significantly suppressed by passive immunization of the mice with antibodies neutralizing biological activity of MIP-2 and KC chemokines. Quantitative evaluation of the synthesized MIP-2 and KC and their mRNA demonstrated that TNF-α injection resulted in a sharp increase in these chemokines and corresponding mRNAs in the inflamed tissue; this increase obviously resulted from the enhanced expression of genes encoding these chemokines. Thus, formation of the site of acute inflammation causes expression of ELR$^+$CXC chemokines, which recruit neutrophils into inflamed tissue.

Thus, we conclude that ELR$^+$CXC chemokines play a key role in the formation of sites of acute inflammation; they provide rapid recruitment of neutrophils into damaged tissue and to regions of infection entry. Expression of ELR$^+$CXC chemokines by activated neutrophils in inflamed tissue can be considered as a positive feedback loop, which results in formation of higher concentration gradient of these chemokines between the site of inflammation and uninflamed tissue followed by subsequent accumulation of higher number of neutrophils at the site of inflammation.

ELR$^-$CXC chemokines. In contrast to ELR$^+$CXC chemokines, ELR$^-$CXC chemokines are not chemoattractants for neutrophils and exhibit angiostatic effects. Activated human neutrophils can synthesize and secrete two chemokines of this group: MIG (monokine induced by IFN-γ)/CXCL9 and IP-10 (IFN-γ inducible protein 10 kD)/CXCL10 [42]. Neutrophils can also express the gene encoding ELR$^-$CXC chemokine I-TAC (IFN-inducible T cell alpha chemoattractant)/CXCL11, but possibility of secretion of the protein has not been demonstrated [42]. Induction of ELR$^-$CXC chemokine synthesis and secretion *in vitro* requires a certain combination of activators. Secretion of MIG and IP-10 was demonstrated only during costimulation of neutrophils by interferon-γ (IFN-γ) and one of the proinflammatory mediators (LPS, TNF-α, or IL-1β), but not during separate activation of cells by any one of these agonists [42, 43]. Expression of ELR$^-$CXC chemokines by activated neutrophils was demonstrated *in vivo* using experimental models of various inflammatory processes in mice: cerebral malaria induced by *Plasmodium berghei* [44], delayed type hypersensitivity induced by repeated administration of herpes simplex virus type 1 (HSV-1) [45], and using the model of lung granuloma induced by *M. tuberculosis* [46].

IP-10, MIG, and I-TAC are ligands of the same receptor, CXCR3. This receptor is expressed by activated T lymphocytes (preferentially of Th1 phenotype), small populations of monocytes and natural killer cells (NK), dendritic cells, and some non-leukocyte cell types—endothelial cells, microglial cells, neurons, etc. [47]. A structural variant of CXCR3 formed as the result of alternative splicing, CXCR3-B, has also been found. The latter binds IP-10, MIG, I-TAC, and also PF4 (platelet factor 4)/CXCL4, but activation of this receptor does not induce chemotaxis.

The role of IP-10, MIG, and I-TAC in mononuclear leukocyte recruitment has been demonstrated in numerous experimental models of acute inflammation in mice. For example, the model of acute encephalomyelitis induced by intracranial administration of mouse hepatitis virus (MHV) has shown that most T lymphocytes that migrate to the site of inflammation express CXCR3 [48]. Passive immunization of such mice with anti-CXCR3 neutralizing antibodies resulted in significant decrease in CD4$^+$ T lymphocyte infiltration into the inflamed tissue (by 92% on the ninth day of infection). CXCR3-dependent recruitment of liver leukocytes was studied in mice infected with murine cytomegalovirus (MCMV) [49]. Immunoneutralization of IP-10 and MIG decreased MCMV-specific CD8$^+$ T lymphocyte accumulated in the liver by 79%. Similar suppression of infiltration of virus-specific CD8$^+$ T lymphocytes was observed in CXCR3 knockout mice. Panzer et al. studied recruitment of T lymphocytes into inflamed kidney tissue in nephrotoxic nephritis induced by administration of nephrotoxic sheep serum [50]. The development of nephrotoxic nephritis was accompanied by significant increase in IP-10 (8.6-fold), MIG (2.3-fold), and I-TAC (4.9-fold) in kidney. CXCR3$^{-/-}$ mice were characterized by significantly lower (by about 50%) number of T lymphocytes accumulated in inflamed kidneys.

In various inflammatory processes, ligands of the CXCR3 receptor play a key role in attraction of CD4$^+$ T lymphocytes of the Th1 phenotype to the site of inflammation. Naive T lymphocytes are activated by antigen-presenting cells followed by subsequent differentiation into antigen-presenting clones of effector T helpers (CD4$^+$ T lymphocytes) [51]. These T helpers are subdivided into T helpers of type 1 and type 2 (Th1 and Th2 cells, respectively); a new phenotype defined as Th17 has also been found recently, but it is still poorly studied. As a

rule, immune responses are "polarized": they are characterized by preferential accumulation of either Th1 or Th2 cells (causing Th1 and Th2 immune responses, respectively) at the site of inflammation [52]. The T helpers Th1 and Th2 express different sets of chemokine receptors. Th1 cells preferentially express CXCR3 and CCR5 receptors, whereas Th2 cells express CCR3, CCR4, and CCR8 [53]. In this connection, it is significant that ligands of CXCR3 receptor are antagonists of CCR3 receptor. Thus, recruiting Th1 cells, IP-10, MIG, and I-TAC suppress migration of Th2 cells; this results in polarization of T cell response.

The delayed type hypersensitivity (DTH) reaction induced in experimental mice was a useful model to study the role of neutrophils in Th1-dependent immune responses and attraction of CXCR3-expressing leukocytes into inflamed tissue. DTH is an allergic reaction mediated by Th1 cells. In this form of immune response, primary administration of an antigen into the body induces accumulation of antigen-specific clones of Th1 cells. Repeated antigen administration causes recruitment of preexisting antigen-specific Th1 cells into an antigen entry site and formation of Th1-dependent immune response. It has been shown that neutrophils play a significant role in the formation of the DTH site: in mice with experimentally induced neutropenia and also in the case of suppression of neutrophil recruitment into inflamed tissue by anti-MIP-2 antibodies, there was attenuation of immune responses after repeated exposure to the antigen causing DTH [54, 55].

Molesworth-Kenyon et al. investigated the role of neutrophils in formation of the DTH site in mice induced by repeated subcutaneous injections of HSV-1 virus [45]. In mice with experimental neutropenia, accumulation of leukocytes expressing CXCR3 at the site of inflammation was decreased by 73%. Such mice were characterized by significant decrease in production of IP-10 and MIG at the DTH site (by 53 and 75%, respectively). Immunohistochemical analysis of IP-10 expression in an inflamed tissue (MIG expression was not investigated) demonstrated that neutrophils were the main source of this chemokine at the site of inflammation. The authors concluded that activated neutrophils accumulated at the DTH site secrete chemokines (IP-10 and MIG), recruiting CXCR3-expressing leukocytes to the site of inflammation.

Involvement of neutrophils in the recruitment of CXCR3-expressing leukocytes to sites of inflammation was also demonstrated using experimental models of other inflammatory processes. Seiler et al. studied involvement of CXCR3 ligands in granuloma formation in mice under conditions of chronic inflammation induced by *M. tuberculosis* [46]. Granuloma formation is usually considered as a protective mechanism preventing pathogen dissemination and isolating the site of chronic inflammation. In mice with experimental neutropenia, granuloma formation in lungs was significantly suppressed. Analysis of chemokine gene expression in lungs of mice infected with the pathogen also showed that granuloma formation is accompanied by significant increase in expression of ELR$^-$CXC chemokine genes in the inflamed tissue; the highest (8.29-fold) increase was observed in expression of the MIG gene. Immunohistochemical analysis of MIG expression in the forming granulomas revealed neutrophils as the main source of secreted MIG. Passive immunization of mice with antibodies neutralizing biological activity of MIG caused significant suppression of granuloma formation. These data suggest that ELR$^-$CXC chemokines secreted by activated neutrophils play an important role not only in acute inflammatory responses, but also in chronic inflammation.

Chemokines of the CC family. Chemokines of the CC family exhibit chemotactic effects (of various selectivity) on monocytes, lymphocytes, basophils, eosinophils, and dendritic cells, but they are not chemoattractants for neutrophils. In humans, 28 chemokines of this family have already been described. Activated human neutrophils can secrete five CC-chemokines: MCP-1 (monocyte chemoattractant protein-1)/CCL2, MIP (macrophage inflammatory protein)-1α/CCL3, MIP-1β/CCL4, MIP-3α/CCL20, and MIP-3β/CCL19.

MCP-1 is the main chemokine regulating monocyte recruitment to a site of inflammation. Numerous studies have demonstrated that in various inflammatory responses neutralization of biological activity of this chemokine by anti-MCP-1 antibodies results in significant decrease in monocyte infiltration into an inflamed tissue [56], whereas subcutaneous injection of MCP-1 causes rapid directed movement of monocytes into the injection sites [57]. An important role of MCP-1 in monocyte recruitment to a site of inflammation has also been demonstrated using an experimental model of inflammation in MCP-1 knockout mice [58] and also CCR-2 (the receptor of this cytokine) knockout mice [59]. MCP-1 also acts as a chemoattractant for T lymphocytes; it is involved in recruitment of these leukocytes into sites of inflammation [60].

Human neutrophils synthesize and secrete MCP-1 during their *in vitro* costimulation by IFN-γ and LPS or IFN-γ and TNF-α, and also in response to GM-CSF [61, 62]. Neutrophil secretion of MCP-1 *in vivo* at sites of inflammation was demonstrated using DTH in rats [60]. Repeated subcutaneous administration of an antigen (hemocyanin of *Megathura crenulata*) resulted in formation of a site of inflammation characterized by rapid neutrophil infiltration followed by subsequent accumulation of mononuclear leukocytes. Immunohistochemical analysis of MCP-1 expression in the inflamed tissue showed that neutrophils and cells of epidermal and follicular epithelium are the main source of MCP-1.

Involvement of neutrophils in the MCP-1-dependent recruitment of leukocytes into an inflamed tissue was

demonstrated in experiments employing immunization of mice with apoptotic cancer cells [63]. Intraperitoneal administration of allogenic murine cells into mice caused standard biphasic accumulation of leukocytes in the forming site of inflammation. The maximum of neutrophil accumulation observed 3 h after cell injection represented the first phase, which was accompanied by a second phase of accumulation of mononuclear leukocytes, particularly cytotoxic $CD8^+$ T lymphocytes. In mice with experimental neutropenia, the infiltration of cytotoxic $CD8^+$ T lymphocytes into the site of inflammation was totally suppressed, and this was accompanied by significant (by about 90%) decrease of MCP-1 production in the inflamed area. Intraperitoneal administration of recombinant MCP-1 to such mice restored recruitment of $CD8^+$ T lymphocytes to the site of inflammation. These results show that MCP-1 acts as a link between neutrophils accumulated in an inflamed tissue and $CD8^+$ T lymphocytes recruited into this tissue.

In vitro experiments have shown that MIP-1α and MIP-1β are expressed by neutrophils in response to various agonists such as LPS and zymosan [64, 65]. The LPS-induced secretion of these chemokines is suppressed by the anti-inflammatory cytokine IL-10, reducing stability of MIP-1α and MIP-1β mRNA [66]. Expression of MIP-1 chemokines is also induced by outer membrane vesicles of *Neisseria meningitides* (a microorganism causing meningitis; IFN-γ significantly potentiates this effect) [67] and by *Toxoplasma gondii* antigens [68]. MIP-1α interacts with CCR1 and CCR5 receptors, whereas MIP-1β acts only on CCR5. These chemokines exhibit overlapping biological activity: they act as potent chemoattractants for monocytes, macrophages, natural killers, and immature dendritic cells. Granulocytes are much less sensitive to the chemotactic effect of MIP-1 chemokines: MIP-1α can recruit only IFN-γ-activated neutrophils and a small population of eosinophils expressing CCR1 [69]. MIP-1α and MIP-1β also exhibit a chemotactic effect on Th1 but not on Th2 lymphocytes [70].

Von Stebut et al. directly demonstrated involvement of MIP-1α and MIP-1β secreted by neutrophils in macrophage recruitment into inflamed tissue [71]. They studied involvement of neutrophils in formation of sites of acute inflammation using an experimental model of cutaneous granuloma (CG) formation in mice. Subcutaneous administration of polyacrylamide gel resulted in formation of a site of inflammation characterized by rapid infiltration of neutrophils followed by subsequent accumulation of mononuclear leukocytes, particularly macrophages. In mice with experimental neutropenia, macrophage infiltration into the forming CGs was significantly suppressed (by about 90%). Reconstitution of CGs in these mice with supernatants derived from *in vitro* cultured neutrophils restored macrophage infiltration into these sites of inflammation. However, neutralization of biological activity of the MIP-1α and MIP-1β chemokines in the injected supernatants (by means of corresponding neutralizing antibodies) abolished their ability to restore macrophage migration. Thus, MIP-1α and MIP-1β secreted by activated neutrophils are the main chemokines recruiting macrophages into inflamed tissue (at least in this model of inflammation).

Neutrophils activated by LPS and the inflammatory cytokine TNF-α [72, 73] and Gram-positive (*Staphylococcus aureus* and *S. epidermidis*) and Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) bacteria [74] can synthesize and secrete MIP-3α and MIP-3β. MIP-3α and MIP-3β interact with CCR6 and CCR7 receptors, respectively. In contrast to MIP-1α and MIP-1β, these chemokines exhibit a chemotactic effect on different types of leukocytes: MIP-3α is a chemoattractant for T cell memory and immature dendritic cells, whereas MIP-3β attracts naive and activated T lymphocytes and mature dendritic cells.

The chemokines MIP-1 and MIP-3 produced by activated neutrophils attract dendritic cells to a site of inflammation and therefore play an important role in coupling of innate and adaptive immune responses. Immature dendritic cells are localized in peripheral tissues, and they effectively capture antigens; however, activation of naive T lymphocytes and initiation of the adaptive immune response require their maturation and differentiation of the immature cells into mature dendritic cells [75]. Mature dendritic cells migrate from peripheral tissues into lymph nodes (through the afferent lymphatic system) and present the antigens to naive T lymphocytes, which then differentiate into effector cells.

Immature dendritic cells constitutively express several chemokine receptors including CCR1, CCR5, and CCR6 [76] and therefore they can be localized at a site of inflammation where activated neutrophils secrete ligands interacting with these receptors (MIP-1α, MIP-1β, and MIP-3α). Activation and maturation of dendritic cells in sites of inflammation suppresses expression of these receptors but induces expression of CCR7, the main receptor involved in homing of T and B lymphocytes and mature dendritic cells to secondary lymphoid organs. The ligands of this receptor, SLC (CCL20) and MIP-3β, are constitutively expressed by endothelium of the afferent lymphatic system and induce migration of mature dendritic cells to secondary lymphoid organs.

Thus, MIP-3β is an inflammatory (secreted by activated neutrophils and other cell types) and homeostatic (constitutively expressed in secondary lymphoid organs) chemokine. It is suggested that local secretion of MIP-3β at sites of inflammation suppresses migration of mature dendritic cells into lymph nodes and directs cells expressing CCR7 (B lymphocytes, effector T lymphocytes, and memory T cell) [75] to sites of chronic inflammation followed by formation of ectopic lymphoid tissue [73].

CHEMOTACTICALLY ACTIVE PROTEINS OF NEUTROPHIL GRANULES

Neutrophils contain four intracellular compartments that are able to secrete their contents during cell activation: primary (azurophilic) granules, secondary (specific) granules, tertiary (gelatinase) granules, and secretory vesicles [77, 78]. Besides differences in their contents, neutrophil secretory organelles exhibit various propensity for exocytosis in response to cell activation. Secretory vesicles represent the most readily mobilized compartment; the ability of granules for exocytosis decreases in the following order: tertiary, secondary, and finally primary granules (primary granules are the most difficult to mobilize cell compartment). Granules are formed during all stages of neutrophil maturation in bone marrow, and they are filled with secretory proteins that are expressed at a particular stage of differentiation. Primary and secondary granules are formed at the stages of promyelocyte and myelocyte, respectively, whereas tertiary granules are formed at the stages of metamyelocyte and band. Secretory vesicles are then formed by endocytosis.

Primary granules contain the main cytotoxic components of the neutrophil microbicidal system. The secretion of these granules occurring during phagocytosis of pathogenic microorganisms is highly localized to the regions of plasma membrane involved in phagosome formation [79]. However, besides the intraphagosomal secretion, extracellular exocytosis of primary granules also occurs. Secondary and tertiary granules contain a wide range of proteins including those involved in cell migration and protease inhibitors; during neutrophil activation they are secreted both intraphagosomally and extracellularly [80]. Secretory granules represent a store of plasma membrane receptors; they are subjected to extracellular exocytosis at the stages of rolling and adhesion of leukocytes on vascular walls [78].

For identification of proteins exhibiting chemotactic activity, granule contents from non-activated neutrophils were tested for their ability to induce chemotaxis of mononuclear leukocytes. Such experiments revealed chemotactic activity of serine protease cathepsin G, antimicrobial proteins azurocidin/CAP37, LL-37, and defensins HNP1 and HNP2 [81-83]. Below we consider the neutrophil granule proteins exhibiting chemotactic activity.

Cathelicidins. Cathelicidins are a family of antimicrobial proteins containing highly conservative cathelin domains of ~100 amino acid residues [84]. Isolated cathelin domain (or cathelin) was originally characterized as an inhibitor of a cysteine protease, cathepsin L, and its name reflects this property (cathelin, cathepsin L inhibitor). In the cathelicidin molecule the cathelin domain has central position, and it is flanked by N-terminal signal peptide and C-terminal antimicrobial peptide. Although about 20 proteins of this family have been described in mammals, only one cathelicidin, human cationic antimicrobial protein of 18 kD (hCAP18) has been found in humans. Cathelicidins can be considered as protein precursors of antimicrobial peptides that are formed during proteolytic processing. For example, cleavage of hCAP18 by proteinase 3 results in formation of the antimicrobial peptide LL-37 (its name reflects the fact that it consists of 37 amino acid residues and contains two N-terminal leucine residues) [85]. In contrast to the cathelin domain, antimicrobial peptides formed by cathelicidins significantly differ in primary and secondary structures.

Besides antimicrobial activity, the cathelicidin-derived antimicrobial peptides exhibit chemotactic activity for certain types of leukocytes. Chertov et al. showed that the antimicrobial peptide LL-37 is a chemoattractant for monocytes, T lymphocytes, and neutrophils. LL-37 exhibits chemotactic and antimicrobial effects at concentrations 10^{-5}-10^{-6} M [82, 86]. Within the scope of this review, it is important that increased concentration of LL-37 is detected in inflamed tissue [86]. A putative scenario for formation of the chemotactic gradient of LL-37 at sites of inflammation can be as follows: the LL-37 precursor, hCAP18, is localized in the secondary granules of neutrophils; it is also expressed by monocytes, natural killers, mast cells, epithelial cells, and keratinocytes. In neutrophils, proteinase 3 cleaving hCAP18 with formation of LL-37 is localized in primary and secondary granules and secretory vesicles [85]. Neutrophils recruited into inflamed tissue become activated and secrete hCAP18 and proteinase 3. Extracellular proteolytic processing of hCAP18 by proteinase 3 results in formation of LL-37 and causes local increased concentration of this chemoattractant in the inflamed tissue.

FPRL1 (formyl peptide receptor-like 1) is a receptor mediating the chemotactic effect of LL-37 on monocytes, T lymphocytes, and neutrophils. It belongs to the group of formyl peptide receptors. In humans, three such receptors have been found; these include one high affinity receptor FPR (formyl peptide receptor) and two low affinity receptors (FPRL1 and FPRL2). The formyl peptide receptors mediate the chemotactic effect of some other chemoattractants formed by activated neutrophils (see below). Other cathelicidins also exhibit chemoattractant properties. For example, porcine antimicrobial peptide PR-39 (consisting of 39 amino acid residues and containing N-terminal proline and arginine residues) is a chemoattractant for porcine neutrophils [87].

Defensins. Mammalian defensins represent a family of antimicrobial proteins that contain six highly conservative cysteine residues forming three disulfide bonds [88]. Based on disulfide bond topology, mammalian defensins have been subdivided into three families: α-, β-, and θ-defensins. Three disulfide bonds of α-defensins are formed by three pairs of cysteines (C1-C6, C2-C4, and C3-C5), whereas disulfide bond formation in β-defensins

involves different pairs (C1-C5, C2-C4, and C3-C6). The only representative of the θ-defensin family has cyclic structure and disulfide bonds formed by C1-C6, C2-C5, and C3-C4. In mammals, more than 50 proteins of this family have been identified. In human neutrophils, four defensins have been found: HNP (human neutrophil peptide) 1-4. They are referred to the family of α-defensins. HNP1-4 are localized in primary granules of neutrophils, where they are the main protein component of these granules (representing 25-30% of primary granule protein and 5% of total protein of neutrophils) [78]. The antimicrobial and cytotoxic effects of these short peptides (29-30 amino acid residues) are attributed to their pore forming ability: they can form multimer transmembrane pores permeabilizing microbial membranes. The antimicrobial effect of defensins is observed in the micromolar concentration range. Nanomolar concentrations of HNP1 and HNP2 exhibit chemotactic effect on resting $CD4^+$ CD45RA and $CD8^+$ T lymphocytes and also on immature dendritic cells [83, 89]. Chemotactic properties have recently been demonstrated also for HNP3 [90]. In should be noted that reports on defensin-induced chemotaxis of monocytes [91] has not been reproduced in other laboratories [83].

Chemotactic activity of neutrophil α-defensins *in vivo* has been demonstrated in mice. Subcutaneous injection of 1 μg of the defensin HNP1 resulted in accumulation of neutrophils and mononuclear leukocytes at the injection site [83]. The experimental model of acute peritonitis in mice induced by *Klebsiella pneumoniae* infection provided experimental evidence that involvement of α-defensins in immune response is not limited to their antimicrobial properties, but is also associated with their chemotactic activity [92]. Injection of the defensin HNP1 simultaneously with pathogen administration into the peritoneal cavity of mice caused a marked decrease in the number of bacteria in the peritoneal cavity. This antibacterial effect of HNP1 was associated with significant increase in the number of granulocytes, macrophages, and monocytes recruited into the site of inflammation in the peritoneal cavity. Leukocyte infiltration was the ultimate precondition for the antibacterial effect of HNP1 because the antibacterial effect of HNP1 was not observed in mice with leukocytopenia induced by cyclophosphamide.

Being chemoattractants for T lymphocytes and immature dendritic cells and attracting these types of leukocytes to the site of inflammation, neutrophil α-defensins are obviously involved in coupling of innate and adaptive immune responses. This conclusion is supported by results of *in vivo* experiments demonstrating that neutrophil α-defensins markedly increased antigen-specific immune responses during their co-administration with an antigen inducing the inflammatory process [93, 94]. Tani et al. investigated the effect of HNP1, HNP2, and HNP3 on intraperitoneal immunization of mice with the hemocyanin of *M. crenulata* [93]. Administration of defensins (accompanying such immunization) significantly increased production of hemocyanin-specific IgG antibodies. Moreover, defensins significantly increased adaptive immune response to other administered antigens. In another study, the effect of neutrophil defensins on adaptive immune response induced by a foreign antigen was evaluated using a model of intranasal immunization of mice [94]. It was demonstrated that during induction of adaptive immune response caused by intranasal administration of ovalbumin, defensins HNP1, HNP2, and HNP3 significantly increased formation of ovalbumin-specific IgG antibodies. Thus, defensins can act as immune adjuvants potentiating antigen-specific immune responses.

Cathepsin G. Cathepsin G is one of three serine proteases of the chymotrypsin family that are present in neutrophil granules [95, 96]. In neutrophils, this protein is localized in primary granules and is synthesized at the stage of promyelocyte; segmented neutrophils do not synthesize cathepsin G. The small amount of membrane-bound cathepsin G is present on the plasma membrane of segmented neutrophils. Weak expression of cathepsin G was also detected in monocytes and mast cells. *In vitro* cathepsin G is rapidly secreted into the extracellular space in response to activators that can induce exocytosis of primary granules. The extracellular concentration of catalytically active cathepsin G significantly increases in inflamed tissues.

Cathepsin G is synthesized as a precursor of 255 amino acids that contains an N-terminal signal peptide of 18 residues (which is removed during cotranslational processing) followed by activation prodipeptide and C-terminal propeptide (11 residues) [95]. Cathepsin G is localized in primary granules of neutrophils as a catalytically active protease (after removal N-terminal prodipeptide Gly-Glu). N-Terminal protein processing precedes cleavage of the Ser244–Phe245 peptide bond and removal of the C-terminal propeptide (the function of which remains unknown). Cathepsin G is a cationic protein, and in primary granules it is associated with negatively charged sulfated proteoglycans. Since the pH optimum of this enzyme is at neutral pH values, cathepsin G is inactive in primary granules characterized by acidic pH.

Chemotactic properties of cathepsin G were found in experiments on identification of chemotactically active proteins of neutrophil granules [81]. This protein exhibits chemoattractant properties with respect to monocytes and macrophages [81, 97]. It was demonstrated that the high affinity formyl peptide receptor FPR is a cathepsin G receptor in leukocytes [98]. Interestingly, manifestation of the chemotactic effect of cathepsin G requires its enzymatic activity, because modification of cathepsin G with serine protease inhibitors (diisopropyl fluorophosphate or phenylmethylsulfonyl fluoride) also inhibits its chemotactic activity [99]. The cathepsin G-induced

chemotaxis of monocytes was also effectively inhibited by a physiological inhibitor of cathepsin G, α_1-antichymotrypsin [98]. The serpin α_1-antichymotrypsin is an acute phase protein. Its concentration in biological fluids is in the micromolar range (5-7 μM in plasma), and at various inflammatory states its plasma concentration can be increased 3-4-fold. Although α_1-antichymotrypsin is present in blood plasma and interstitial fluid, subcutaneous administration of cathepsin G to mice results in infiltration of mononuclear leukocytes and neutrophils into the injection site [81]. This suggests that the concentration of α_1-antichymotrypsin in biological fluids is not sufficient for suppression of chemotactic activity of cathepsin G in the early stages of inflammatory response. Consequently, this protein can contribute to leukocyte recruitment to a site of inflammation *in vivo*.

Azurocidin/CAP37. Azurocidin, also known as CAP37 (cationic antimicrobial protein of 37 kD), is a member of a protein group of catalytically inactive homologs of serine proteases [100]. The azurocidin molecule contains only one of three conservative amino acid residues (Asp89) of the catalytic triad of the active site of serine proteases (histidine, aspartate, and serine). His41 and Ser175 of the catalytic triad are replaced by serine and glycine residues, respectively. The primary structure of azurocidin/CAP37 has 45% identity with neutrophil elastase, 42% identity with proteinase 3, and 32% identity with cathepsin G.

Azurocidin is a single chain glycoprotein that is synthesized as a precursor of 251 amino acid residues. Subsequent proteolytic processing results in removal of N-terminal peptide (of 26 residues) and C-terminal tripeptide and formation of the mature form of 222 residues. Azurocidin is preferentially located in primary granules of neutrophils. However, studies have shown the existence of easily mobilizable intracellular pool of this protein located in secretory vesicles of neutrophils [101, 102]. Azurocidin is also expressed by endothelial cells during their activation by proinflammatory mediators (LPS, TNF-α, IL-1) [103]. The proinflammatory cytokines TNF-α and IL-1β induce azurocidin expression in some types of endothelial cells [104]. Azurocidin was also found in platelets; however, its transcription was not detected in these cells. This suggests that azurocidin is presumably taken up by platelet precursors from blood plasma [105].

As in the case of neutrophil α-defensins, micromolar concentrations of azurocidin exhibit microbicide effects, and at concentrations of 10^{-8}-10^{-10} M it exhibits chemoattractant properties. Azurocidin is a potent chemoattractant for monocytes; it also exhibits a chemotactic effect on T lymphocytes, neutrophils, and fibroblasts [81, 83, 106]. In *in vivo* experiments, azurocidin was subcutaneously injected into BALB/c mice, and then cell composition at the site of injection was analyzed. Four hours after subcutaneous administration of 1 μg of azurocidin there was significant infiltration of neutrophils and mononuclear cells into the site of injection. After 24 h the number of accumulated cells of innate immune response was even higher in response to lower doses of 100 and 10 ng [83].

Recently another mechanism responsible for involvement of azurocidin in monocyte recruitment to a site of inflammation has been recognized. It is known that migration of leukocytes circulating in blood to an inflamed tissue includes several stages: formation of labile selectin-dependent adhesive contacts with blood vessel endothelium (rolling), tight integrin-dependent adhesion of cells on endothelium, transendothelial migration, and chemotaxis to the site of inflammation. Leukocyte adhesion on vascular endothelium of inflamed tissue is a consequence of exposure of some cell proteins on the activated endothelial cells (selectins, integrins, and their ligands); these proteins form adhesion contacts with leukocytes circulating in blood.

In *in vitro* experiments, Soehnlein et al. demonstrated that azurocidin might be one of these proteins [107]. Like chemokines, azurocidin is a heparin-binding protein that can be immobilized on components of the extracellular matrix, particularly on endothelial cells. Using the flow chamber model of leukocyte adhesion on the activated endothelium, it was shown that azurocidin immobilization on endothelial cells results in significant increase in the number of monocytes tightly adhered to the endothelial monolayer. Interestingly, this effect was specific for monocytes and immobilized azurocidin did not influence adhesion of neutrophils and T lymphocytes.

How can preferential immobilization of azurocidin occur on vascular endothelium of inflamed tissues *in vivo*? First, we have already mentioned that activation of endothelial cells by proinflammatory mediators induces expression of azurocidin [103]. Second, *in vitro* experiments have shown that neutrophil adhesion on endothelial cells results in secretion of a readily mobilized azurocidin pool from secretory granules followed by azurocidin binding to endothelial cell surface [107]. It is reasonable to suggest that during the first phase of innate immune response, neutrophils migrating to the site of inflammation "leave" azurocidin of their secretory granules on the surface of vascular endothelium of inflamed tissue, and this potentiates subsequent monocyte recruitment to the site of inflammation.

Urokinase. Urokinase (or urokinase-type plasminogen activator, uPA) is a key component of fibrinolysis; it plays an important role in directed cell migration [108]. This protein also exhibits a chemotactic effect on many cell types including leukocytes of the innate immune response. Urokinase is synthesized by vascular endothelial and smooth muscle cells, epithelial cells, fibroblasts, monocytes and macrophages, and also by malignant tumor cells of various origin. Neutrophils contain an intracellular pool of urokinase (localized in secondary

granules), which is secreted during cell activation [109, 110].

Urokinase is synthesized in cells as a single polypeptide of 411 amino acid residues. The polyfunctional properties of this protein are associated with the presence in its structure of three domains: N-terminal domain, which is homologous to epidermal growth factor (residues 1-45); central kringle domain (residues 46-143); and C-terminal proteolytic domain containing the active site of a serine protease of the trypsin family (residues 144-411). Cleavage of the Lys158–Ile159 peptide bond in the urokinase polypeptide chain transforms single-chain urokinase into a double chain form exhibiting higher proteolytic activity.

The chemotactic effect of urokinase is determined by the interaction of N-terminal growth factor domain of this protein with the urokinase receptor uPAR/CD87. This receptor is a membrane protein devoid of a transmembrane domain and anchored on the plasma membrane via glycosylphosphatidylinositol (GPI). It was shown that formyl peptide receptors play a role of adaptor transmembrane proteins required for signal transduction from the urokinase–uPAR complex and induction of chemotaxis [111]. *In vitro* experiments have shown that urokinase exhibits a chemotactic effect on monocytes [112], neutrophils [113], basophils [111], and mast [114] and smooth muscle cells [115].

An important role of urokinase in innate immune response has been demonstrated in experiments on urokinase knockout mice [116]. In the experimental model of lung inflammation induced by *Cryptococcus neoformans*, such mice demonstrated higher sensitivity to inoculation of this pathogen compared with control mice. At the same time, the number of monocytes, neutrophils, and T lymphocytes attracted to the inflamed tissue was significantly decreased. Decreased number of accumulated leukocytes cannot be attributed to decreased migration ability of these cells in urokinase knockout mice because urokinase plays an insignificant role in chemotaxis of monocytes and neutrophils [117, 118], and it is not expressed by T lymphocytes. Thus, urokinase is apparently one of the chemoattractants recruiting leukocytes to the inflamed tissues.

It should be noted that activated neutrophils are not the only source of urokinase in sites of inflammation. Bacterial products, such as LPS, and also antiinflammatory cytokines (e.g. IL-1β and TNF-α), the expression of which is increased in inflamed tissues, induce synthesis and secretion of urokinase [119, 120]. Thus, urokinase exhibits properties of inflammatory chemokine. Interestingly, urokinase potentiates LPS-induced activation of neutrophils [121]. This suggests that acting in auto- and paracrine manner at sites of inflammation, urokinase secreted by neutrophils and other cell types can increase immune response by stimulating activation of neutrophils.

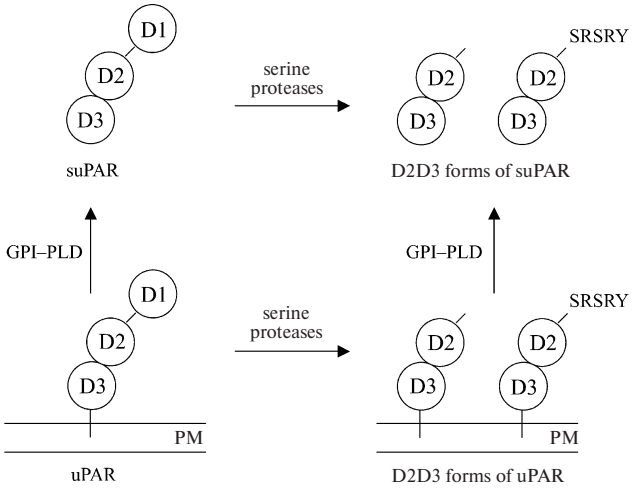
CHEMOTACTICALLY ACTIVE PROTEINS—PRODUCTS OF MEMBRANE PROTEIN SHEDDING

We have recently demonstrated a new pathway for generation of chemotactically active proteins during neutrophil activation: shedding and concomitant proteolytic processing of a membrane-bound protein with the subsequent release of chemotactically active form of the soluble protein [122, 123]. We showed that membrane-bound uPAR is shed from the surface of human neutrophils in response to some activating stimuli and the released soluble form is the chemotactically active truncated D2D3 form of the receptor [122, 123].

uPAR is a multiligand receptor playing an important role in pericellular proteolysis of migrating cells, cell adhesion, and chemotaxis [124]. uPAR is expressed by myeloid cells (monocytes and granulocytes) and vascular endothelial and smooth muscle cells as well as epithelial cells and many types of tumor cells. A mature receptor inserted into plasma membrane consists of 283 residues; it lacks transmembrane regions and is anchored on the membrane via GPI. Three homologous domains are recognized in uPAR: D1, D2, and D3. The N-terminal D1 domain contains a urokinase-binding site; the two other domains bind the extracellular protein vitronectin. uPAR also interacts *in cis* with various integrins [125]. A linker region joining the D1 and D2 domains can be cleaved by many proteases such as neutrophil elastase, plasmin, some metalloproteinases, urokinase, etc. [126]. Thus, plasma membrane contains both full-length uPAR, which can bind urokinase, and the D2D3 form of this receptor, which does not interact with urokinase.

Membrane-bound uPAR can be cleaved by GPI-specific phospholipase D followed by generation of the soluble form of this receptor (Scheme 3). Soluble uPAR (suPAR) has been found in biological fluids, and its concentrations in these fluids significantly increase under such pathological states as inflammation or various forms of cancer. As in the case of the plasma membrane-bound form of this receptor, suPAR can exist in biological fluids as full-length and D2D3 forms. Cleavage of the linker region between the D1 and D2 domains can occur at various sites. The D2D3 form of suPAR with exposed SRSRY fragment (residues 88-92) of the linker region is a chemoattractant for basophils, monocytes, and $CD34^+$ hematopoietic stem cells (HSCs). This form of suPAR can chemoattract basophils by interacting with the low-affinity formyl peptide receptors FPRL1 and FPRL2 [111] and monocytes and HSCs by activating the high-affinity formyl peptide receptor (FPR) and FPRL1, respectively [127, 128].

Human neutrophils contain an extensive intracellular pool of uPAR localized in primary and tertiary granules and secretory vesicles [129, 130]. Neutrophil activation results in rapid translocation of uPAR from intracel-



Shedding and proteolytic processing of uPAR. Membrane-bound uPAR can be shed from the plasma membrane to produce soluble form, suPAR. In tumor cells, uPAR shedding is catalyzed by GPI-specific phospholipase D (GPI-PLD). The linker region between domains D1 and D2 in both uPAR and suPAR is susceptible to cleavage by some serine proteases with the generation of the D2D3 form of the receptor. Cleavage of the linker region can occur at various sites; this results in formation of a D2D3 form of suPAR containing the epitope SRSRY and exhibiting chemoattractant properties or a D2D3 form of suPAR lacking both this epitope and chemoattractant properties. PM, plasma membrane

Scheme 3

lular compartments onto the plasma membrane [129]. Since activated neutrophils also translocate to the cell surface the serine proteases (neutrophil elastase and urokinase), which have previously been shown to be able to cleave the linker region between domains D1 and D2, we hypothesized that if neutrophil activation results in secretion of suPAR (uPAR shedding) the resultant form of the protein might represent a chemotactically active D2D3 form of the soluble receptor.

Indeed, we have demonstrated that neutrophils activated by ionomycin or primed by TNF-α and then stimulated by fMLP or IL-8 rapidly released the D2D3 form of suPAR [122, 123]. To demonstrate the chemoattractant properties of this form, we tested supernatants harvested after stimulation of TNF-α-primed neutrophils by IL-8 for their ability to induce chemotaxis of FPRL1-transfected HEK293 (human embryonic kidney 293) cells. We demonstrated that the immunodepletion of the D2D3 form of suPAR from these supernatants using monoclonal antibodies directed to D2 domain significantly decreased chemotactic response of these cells. Antibodies to D1 domain and antibodies with irrelevant specificity did not influence the chemotactic response. Chemotaxis was totally blocked by the hexapeptide WRW^4, an antagonist of FPRL1. Consequently, we conclude that activated neutrophils secrete the chemotactically active D2D3 form of suPAR, which acts as a ligand for the receptor FPRL1.

The characteristics of many chemotactically active proteins secreted by neutrophils in inflamed tissues and considered in this review draw a rather complicated picture of chemoattractants recruiting leukocytes into sites of inflammation. Synthesis and secretion of numerous chemoattractants can cause reasonable uncertainty about their biological functions. As in the case of any physiologically important mechanism, there is likely to be functional redundancy of some components of the chemoattractant system directing leukocytes to sites of inflammation. However, we believe that the existence of numerous chemoattractants for the recruitment of imflammatory cells into particular compartments of the body has also other physiological reasons. Secretion of many chemoattractants in inflamed tissues provides: first, specificity of immune response; second, possibility of leukocyte migration to extended areas inside tissues.

Specificity of immune response, particularly its cellular component, can be illustrated by numerous examples. For example, in rheumatoid arthritis Th1 cells are the main population of $CD4^+$ T lymphocytes in synovial fluid of inflamed joints, whereas in the case of asthma leukocyte infiltrates are characterized by accumulation of $CD4^+$ T lymphocytes of Th2 phenotype [52]. Preferential accumulation of certain types of leukocytes in various inflammatory processes might be reached by secretion of various sets of chemoattractants (specific for certain inflammatory response) in inflamed tissues. Indeed, as it was considered in detail above, neutrophils express most chemoattractants in response to certain sets of activating treatments. Moreover, *in vitro* experiments have shown that some stimuli (including proinflammatory ones) can inhibit expression of some chemokines by activated neutrophils. For example, phagocytosis of microcrystals of sodium urate and potassium pyrophosphate dihydrate causing inflammatory processes in joints in gout and pseudogout (respectively) stimulates expression of IL-8 but inhibits synthesis and secretion of MIP-1α by neutrophils activated by TNF-α [131]. Thus, secretion of a certain set of chemoattractants occurs in response to certain activating treatments. Differential secretion of chemoattractants by activated neutrophils in various inflammatory processes can result in recruitment of certain types of leukocytes (and at certain ratios); this determines specificity of inflammatory responses.

Another physiologically relevant explanation of secretion of numerous chemoattractants in inflamed tissues consists of the following. For attraction of distant cells, an "attracting compartment" should secrete the necessary quantity of a chemoattractant for its detection by attracted cells. However, during migration into a powerful stream of this chemoattractant a cell may be exposed to a saturating concentration, after which the cell loses its ability to respond to this chemotactic factor. The latter might represent a consequence of receptor saturation and/or desensitization. In such case, the existence of gra-

dients of other agonists generated by the same compartment might cause a switch of the cell for chemotaxis via gradients of a second, third, etc. chemoattractant. The ability of leukocytes to respond sequentially to various chemoattractants and also to sequentially employ various receptors during migration via the concentration gradients of particular chemoattractants provides the possibility for multistep navigation of leukocytes via multiple chemoattractant gradients *in vivo* [132, 133]. The multistep model of leukocyte recruitment represents a combinatorial mechanism by which many chemoattractants can be involved in attraction of certain types of leukocytes into certain compartments in tissues. The model of multistep navigation of leukocyte chemotaxis also explains the expression of numerous chemoattractant receptors on different types of leukocytes.

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