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Review

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New biodefense strategies by neutrophils

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Summary

Chemokines and other chemotactic factors induce neutrophils, macrophages, and dendritic cells to migrate to an inflammatory site and efficiently ingest and destroy infective microorganisms. Moreover, antigen-presenting cells, such as macrophages and dendritic cells, present the microbial antigens via major histocompatibility complex class II molecules, resulting in the activation of specific CD4 T cells. Since neutrophils have a short life-span and are highly susceptible to apoptosis, their role in antigen presentation has been questioned. However, various pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6, tumor necrosis factor α, and interferon γ, produced at the site of inflammation activate neutrophils and suppress apoptotic death. These cytokine-activated neutrophils show enhanced expression of cell surface molecules and become as competent as dendritic cells and macrophages in their ability of antigen presentation. Traditionally, neutrophils are known to be responsible for innate immunity, and recently they are also considered to be intimately associated with the establishment of acquired immunity. In the present review on the role of neutrophils we describe both classic innate and acquired immunity.

Key words: **neutrophils • chemotaxis • chemokines • antigen presentation**

Abbreviations: **TNF** – tumor necrosis factor, **IL** – interleukin, **IFN** – interferon, **PMN** – polymorphonuclear neutrophils, **PI3K** – phosphoinositide-3 kinase, **GM-CSF** – granulocyte macrophage-colony stimulating factor, **MHC** – major histocompatibility complex, **PIP3** – phosphatidylinositol-3,4,5-trisphosphate.

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INTRODUCTION

Neutrophils exhibit phagocytic activity similar to that of macrophages and dendritic cells and play a central role in innate immunity against bacterial and viral pathogens. So far, attention on the function of the neutrophil as a member of the biodefense system has been directed mainly to its role in classic innate immunity. However, recent studies have demonstrated that accumulated neutrophils at inflammatory sites are activated by pro-inflammatory cytokines and are closely associated with the establishment of acquired immunity. In this review we describe the mechanism of neutrophil trafficking to the sites of microbial invasion, capture and phagocytosis of microorganisms, anti-microbial action, and fragmentation and antigen presentation by neutrophils.

NEUTROPHIL TRAFFICKING AND CHEMOKINES

When pathogenic microorganisms infect the host, neutrophils rapidly migrate to the site of infection in 1–4 h post-infection. In general, neutrophils produced in the bone marrow enter the blood stream by the inflammatory signals originating from the infection sites, adhere to the vessel wall at sites of tissue damage, and then transmigrate through endothelial cells to the site of infection. In fact, activated vascular endothelial cells and fibroblasts at the site of inflammation produce chemokines that induce the chemotaxis of leukocytes. This accumulation of leukocytes forms the first step in immune surveillance and plays a key role in immunity to infection. Chemokines mediate the chemotaxis and activation of immune cells and have molecular masses of approximately 7–15 kDa. They are classified into the four subfamilies of CXC, CC, C, and CX3C chemokines, based on the arrangement of the cysteines within this signature motif[50]. There are two subtypes of CXC chemokine, with or without the arrangement of glutamic acid-leucine-arginine (ELR) preceding the CXC motif. ELR-positive CXC chemokines, such as CXCL1 to CXCL8/interleukin (IL)-8, are required for the chemotaxis of neutrophils. Leukotactin-1/CCL15 of the CC chemokines can also behave as a chemotactic factor for neutrophils[42, 49] with the exception of the CXC chemokines. Although CXCL8/IL-8 exhibits potent chemotactic activity for human neutrophils, the activity of CXCL2 and CXCL3 has been demonstrated in mice deficient in these genes.

A functional subclass of dendritic cells are known as critical effector cells in the immune response. Similarly, the existence of subsets of stab and segmented neutrophils is also well known based on nuclear morphology. Moreover, neutrophils produce various chemokines and cytokines, which are classified into polymorphonuclear neutrophils (PMN)-I (IL-12/CCL3), PMN-II (IL-10/CCL2), and PMN-N (no cytokine/chemokine) subsets, based on their pattern of production[44]. The PMN-I subset was obtained from methicillin-resistant *Staphylococcus aureus* (MRSA)-resistant hosts, while the PMN-II subset was found in MRSA-sensitive hosts, suggesting that different neutrophil subsets may affect the innate immunity against MRSA. The differences in the quality and quantity of surface antigen expression on neutrophils also indicate that the action of neutrophils is different between heterogeneous subsets.

Furthermore, the expression of chemokine receptors varies among neutrophils; each polarized expression plays an important role in the migration and homing of these cells in tissues. Two types of receptors to CXCL8/IL-8 chemokine, CXCR1 and CXCR2, have been cloned from human neutrophils, and these receptors show different affinity between the ligands. CXCLR1 binds with CXCL6 and CXCL8, while CXCLR2 binds with all CXC chemokines that have the FLR arrangement[26]. The chemokine receptors are members of a superfamily of 7 transmembrane-spanning G protein-linked receptors and transduce their signals through heterotrimeric G proteins mediated by chemokines. All chemokine receptors are structurally related to each other. For example, the receptors of C5a and C3a anaphylatoxin, which are chemotactic factors, also have the same structure. Neutrophils pretreated with C5a and formyl-Met--Leu-Phe (fMLP) exhibit attenuated chemotactic response to CXCL8/IL-8, showing that cross-talk occurs in the signaling pathways mediated by the two receptors[31]. This type of phenomenon is also observed with other cytokines. Treatment of neutrophils with tumor necrosis factor (TNF)-α results in cross-talk between the nuclear factor (NF)-κB and phosphoinositide-3 kinase (PI3K) pathways. Thus, CXCL8/IL-8 produced through NF-κB activation acts in an autocrine manner to induce PI3K-dependent survival, prolonging the survival of neutrophils[6]. Prolongation of the life-span of neutrophils by these cytokines and chemokines produced at the site of inflammation is important in biodefense during the early phase of infection.

One of the basic conditions for cell migration is the maintenance of a concentration gradient of chemokines produced at the site of inflammation. As an adequate concentration of a chemokine is maintained by diffusion away from secreting cells, this phenomenon can induce migration of neutrophils. Since neutrophils can effectively infiltrate infection

sites, the production of chemotactic factors is not transiently augmented, but must be continuously produced during the inflammatory period. In fact, it was reported that stimulation of neutrophils with IL-12 in the presence of lipopolysaccharide for 18 h increased IL-8 production via NF-κB activation[10]. In addition, the plasma CC chemokine regakine-1 and the tissue CXC chemokine ligand IL-8 act synergistically in order to enhance neutrophil chemotaxis[16]. Therefore, synergistic action or a cascade reaction of multiple chemotactic factors permits an efficient accumulation of neutrophils at the site of inflammation.

Although research on chemotactic factors and their receptors has developed in recent years, many questions remain unsolved as to how the cells respond to chemotactic factors. In *in vitro* experiments of chemotaxis, migrating cells are observed to extend cellular projections toward the concentration gradient of the chemotactic factor, and lipid rafts are detected in these projections, which also results in an accumulation of signaling molecules, such as PI3K and chemokine receptors[15]. The migration of neutrophils induced by IL-8 is inhibited by PI3K inhibitor, and PI3K deficiency affects not only migration, but also the respiratory burst and expression of G protein-coupled receptor kinase[11, 18, 35]. Interestingly, the migrated cell forms the intracellular concentration gradient of phosphatidylinositol-3,4,5--trisphosphate (PIP3) toward the concentrated gradient of chemokine, and the leading edge contains PI3K and Rho family molecules that control cytoskeleton elements[39]. ROCK and PAK of the Rho family molecules mediate the LIM kinase that phosphorylates cofilin, such as the depolymerizing factor of actin[1]. In a study of the effect of PI3K on the cytoskeleton, a novel protein, Swap 70, was found which binds the PI3K-induced phosphorylated PIP3 with polymerized actin through cross-linkage[43]. Swap 70 accumulates in the membrane raft formed by the stimulation of cell migration, and is associated with cell migration. That neutrophil chemotaxis induced by IL-8 and fMLP is not completely abolished in PI3K-deficient mice suggests the presence of other pathways independent of PI3K[39].

MECHANISMS OF ANTIGEN PROCESSING BY NEUTROPHILS

Neutrophils migrate to the site of inflammation and then rapidly phagocytose infective pathogens. Complement receptor, Fc receptor, mannose receptor, β glucan receptor, scavenger receptor, and Toll--like receptor (TLR) present on the surface of neutrophils play important roles in the trapping of infective pathogens. When neutrophils recognize the ligands of the pathogen via these receptors, the pathogen is progressively phagocytozed by neutrophils. The chemokines do not only stimulate neutrophil chemotaxis, but also elicit a variety of immune responses. For example, IL-8 induces respiratory burst mediated by CXCR1[22]. Furthermore, migrated neutrophils down-regulate the expression of CXCR1 and CXCR2 during phagocytosis in order to diminish the response to other chemokines. Among the signaling pathways, PI3K, which is associated with the migration of neutrophils, is also involved in the formation and maturation of the phagosome. In fact, enteropathogenic *Escherichia coli* organisms inhibit PI3K-dependent F-actin polymerization, thereby escaping the phagocytic action of neutrophils[5]. These findings imply that cell migration, intracellular movement of the phagosome, and the restructuring of the cytoskeleton are basically a common phenomenon.

Recent results indicate that neutrophils make extracellular traps to capture pathogens, as spiders make webs to trap insects[4]. The extracellularly extended matrix contains digestive enzymes associated with antigen processing, such as elastase, cathepsin G, myeloperoxidase, lactoferrin, and gelatinase. Extracellular extension of cellular projections is not an unusual phenomenon. Actually, this phenomenon is also observed in dendritic cells, macrophages, and lymphocytes. When B cells are stimulated with anti--IgM, a cytosome-like extension from the cell membrane is induced within 15 min. Within this extension are lipid rafts with tyrosine kinase and actin filaments, and these molecules facilitate effective signal transduction at the cell membrane[17]. In neutrophils, moreover, DNA is detected as a component of the matrix that extends extracellularly[4]. This finding indicates that the trap is not merely a passive structure for capturing pathogens, but is able to actively respond to outside stimuli.

The bacteria and viruses ingested by neutrophils are processed intracellularly, a process mediated by oxygen-dependent or oxygen-independent pathways. NADPH oxidase in the endosome is activated by internalization of bacteria and viruses, and respiratory burst accompanied by marked oxygen consumption is observed in phagocytic cells. This is a phenomenon generated during the process of superoxide anion synthesis, in which the membrane protein NADPH oxidase is activated and then receives electrons from intracellular NADPH and transfers them to extracellular enzymes for superoxide anion synthesis. NADPH oxidase is a complex composed of 5 glycoprotein subunits and contributes to cellular superoxide production. The main subunit of oxidase activity is cytochrome b558, localized in the lipid rafts of

the cell membrane[46]. In the process of superoxide anion synthesis, p47phox, p67phox, p40phox, and the low-molecular-size G protein Rac present in the cytoplasm respond to phagocytosis stimulation by translocating to the lipid rafts where the membrane-bound cytochrome b558 is localized and they finally form complexes. The lipid raft plays a role in physically juxtaposing the NADPH oxidase subunits, leading to efficient NADPH oxidase activation[40]. Neutrophils that phagocytose microorganisms eventually undergo apoptosis through a caspase-dependent pathway. However, in neutrophils with impaired ability to produce reactive oxygen species, phagocytosis-induced cell death does not occur. Therefore the reactive oxygen species acts as a molecule timer determining the life-span of the neutrophils[48].

On the other hand, the oxygen-independent anti-microbial actions involve anti-microbial peptides present in the azure granules, a low-molecular-size protein group, digestive enzymes, and acidification in the endosome. The anti-microbial activities of anti-microbial peptides and the low-molecular-size protein group require optimal conditions for *de novo* synthesis of proteins. In contrast, the acidification in the endosome is similar to the anti-microbial activity of gastric acid resulting in increased resistance against gastrointestinal infection, and it is extremely useful against all pathogenic microorganisms. The acidic environment is generated by proton transport mediated by vacuolar ATPase (V-ATPase), and acidification in the vesicles contributes to the activation of hydrolytic enzymes that have low optimal pH. Immediately after phagocytosis, the endosome contains a large amount of external fluid and the pH is therefore around neutral. However, during the process of transition from early endosome to late endosome and progressive transport into the cellular core, the endosome is acidified to a pH value of around 4.5–6.0 by V-ATPase and then fuses with the lysosomes. In the acidic environment, the S-S bond is cleaved and 3-dimensional structures cannot be maintained. While the intact structure is resistant to the enzymatic attack, breakdown of the structure renders microbial components susceptible to extensive enzymatic degradation, leading to death of the microorganism. Therefore, a defect in either component leads to loss of effective anti-microbial activity. In fact, may intracellular pathogens utilize various ways to inhibit the lowering of pH in the endosome, and consequently succeed to survive intracellularly.

The endosome system degrades pathogens, including bacteria, fungi, and extracellular toxin uptake, via pinocytosis or phagocytosis, and part of the processed fragments are transported to the intracellular organelles and cell surface membrane. The early endosomes are identified as vesicle-positive endosomes for early endosome-associated protein, but late endosomes are different from these early ones[41]. Usually, phagocytosed bacteria are completely killed and fragmented by transportation through the pathways of early endosomes, late endosomes, and then lysosomes. Exogenous peptides processed in the endosomal processing pathway act as antigen and bind with major histocompatibility complex (MHC) class II molecules. In this regard, the antigen uptake mediated by IgG Fc receptors and immunoglobulin receptors on the cell membrane is 1000--fold more effective than other pathways of the antigen presentation. The receptors associated with antigen uptake return to the cell membrane for recycling[41]. The binding compartments of the antigenic peptide and MHC class II molecules vary depending on the antigenic property, and the uptake pathway utilizes specific receptors, such as opsonizing pathogens, and the binding occurs by translocating the antigen peptide from the early or late endosome to intracellular MHC class II compartments (MIIC). To prevent random binding of intracellular peptides to MHC molecules in the endosome, the binding to variant chains is detached, and suitable antigenic peptides strongly bind to the empty MHC molecules, which are transported to the cell surface via trafficking class II vesicles (CIIV). To date, many aspects of the endosome pathway remain unclear, especially the mutual relationship between the late endosome, MIIC, CIIV, and lysosome. In any case, the most important role of the endosome pathway is that it not only processes and fragments pathogens, but delivers processed molecules to a delivery compartment as a concerted intracellular organelle network.

ANTIGEN PRESENTATION BY NEUTROPHILS

When neutrophils are in the company of macrophages and dendritic cells, they exhibit strong phagocytosis activity. As the activity of neutrophils is enhanced by chemokines and other chemotactic factors induced from those cells, the enhanced phagocytosis of neutrophils can eliminate infective microorganisms at the infection site. In dendritic cells and macrophages, the antigenic peptides derived from the digested pathogen bind to MHC class II molecules and the cells present them to T cells. This antigen presentation is the starting point of acquired immunity. In resting neutrophils, MHC class I molecules are expressed, while MHC class II molecules and costimulatory molecules such as CD80 and CD86 are not detected on the cell surface. However, these surface molecules exist intracellularly, and their induction by cytokines such as interferon (IFN)-γ and

granulocyte macrophage-colony stimulating factor (GM-CSF), IL-1, IL-6, and TNF-α is necessary to express these molecules on the surfaces of resting neutrophils. Activated neutrophils (neutrophils induced into the abdominal cavity by injection of proteose peptone) act as an antigen-presenting cell (Fig. 1). In addition, neutrophils can migrate more quickly and accumulate more than any other cells at the infection site.

The antigen presentation necessary for acquired immunity takes place via at least 5 different pathways (Table 1). As described previously, resting neutrophils lack the expression of the surface molecules necessary for antigen presentation, but cytokine-activated neutrophils show upregulated expression of these molecules, degrade infective microorganisms, and can then present to CD4 T cells as professional antigen-presenting cells, similar to dendritic cells and macrophages. Neutrophils have the adequately reserved lysosomal proteases, cathepsins B and D, necessary for antigen processing[23]. In fact, antigen-pulsed neutrophils obtained from mouse peritoneal cavities are capable of activating T cells in an MHC-restricting manner[28]. However, several investigators rule out the function of antigen presentation by neutrophils because neutrophils have a short life-span. This is the major factor that raises doubt over the function of neutrophils as antigen-presenting cells[20]. With regard to this point, various pro-inflammatory cytokines, such as IL-1, IL-6, TNF-α and IFN-γ, produced at the site of inflammation, are known to activate neutrophils and inhibit apoptotic death[6, 24, 47]. There is an interesting report that cytokine-activated neutrophils with a prolonged life-span can present superantigen of pathogens and processed antigenic peptides[9]. Moreover, there are reports that the Mcl-1 and A1 genes belong to the bcl-2 family and suppress apoptosis of neutrophils[7, 8, 14]. In addition, inflammatory cytokines and TLR4 activate the signal transduction pathway that suppresses apoptosis[33].

Table 1. Classification of antigen presentation pathway

Pathway	Natures of antigen	Antigen presentation ability by neutrophils	Target cells
MHC class I	intracellular	yes	CD8 T cells
MHC class II	extracellular	yes	CD4 T cells
Cross-presentation	extracellular/ /intracellular	no	CD8/CD4 T cells
CD1-dependent	lipid (α-galactosyl ceramide, glycosylphosphatidylinositol)	unknown	CD8 T cells, NKT cells
Exosome mediated	extracellular/ /intracellular	unknown	CD8/CD4 T cells

When neutrophils are cultured in the presence of IFN-γ or GM-CSF, CD83, as a dendritic cell marker, is expressed on the surface and acquires the characteristics of dendritic cells[21]. Dendritic-like neutrophils enhance not only CD83 expression, but also MHC class II molecules and costimulatory molecules, including CD80 and CD86 on the cell surface, and the presentation of foreign antigen and superantigen to T cells in an MHC class II-restricted manner has also been observed[12]. The acquisition of CD83 molecule in neutrophils is also induced by IFN-γ, GM-CSF, or a combination of GM-CSF, IL-4, and TNF-α, suggesting that GM-CSF can in principle mediate this differentiation[27]. Another noteworthy finding is that the mature B cell marker CD19, CD20, CD21 is present intracellularly in these dendritic-like neutrophils[34]. These results highlight the develop-

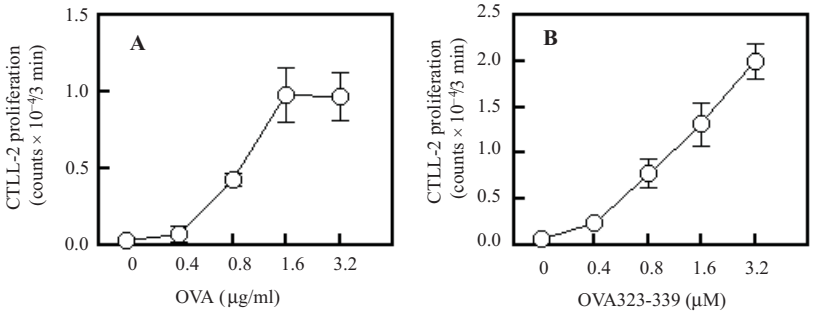

Figure 1. PMNs presented OVA or OVA323-339 peptide to DO 11.10 T cells. BALB/c female mice (6–12 weeks old) were injected intraperitoneal with 3 ml of 3% proteose peptone. After 3 h, the granulocyte-rich sedimentary cells were harvested by Ficoll-Hypaque, which was cultured in plates for 20 h in the presence of 100 U/ml GM-CSF. Non-adherent cells were collected and used as a source of PMNs. PMNs treated with GM-CSF (1×10^5/well) were cultured with DO 11.10 T cells (4×10^4/well) in the presence of OVA (**A**) or OVA323-339 peptide (**B**) at the indicated doses. After 20 h of incubation, 50 μl of culture supernatant were harvested from each well and assayed for IL-2 activity using IL-2 dependent proliferation of CTLL-2 cells. As a result, PMNs were used as antigen-presenting cells, IL-2 production reached the plateau at 1.6 μg/ml OVA. OVA323-339 peptide stimulated IL-2 production in a dose-dependent manner.

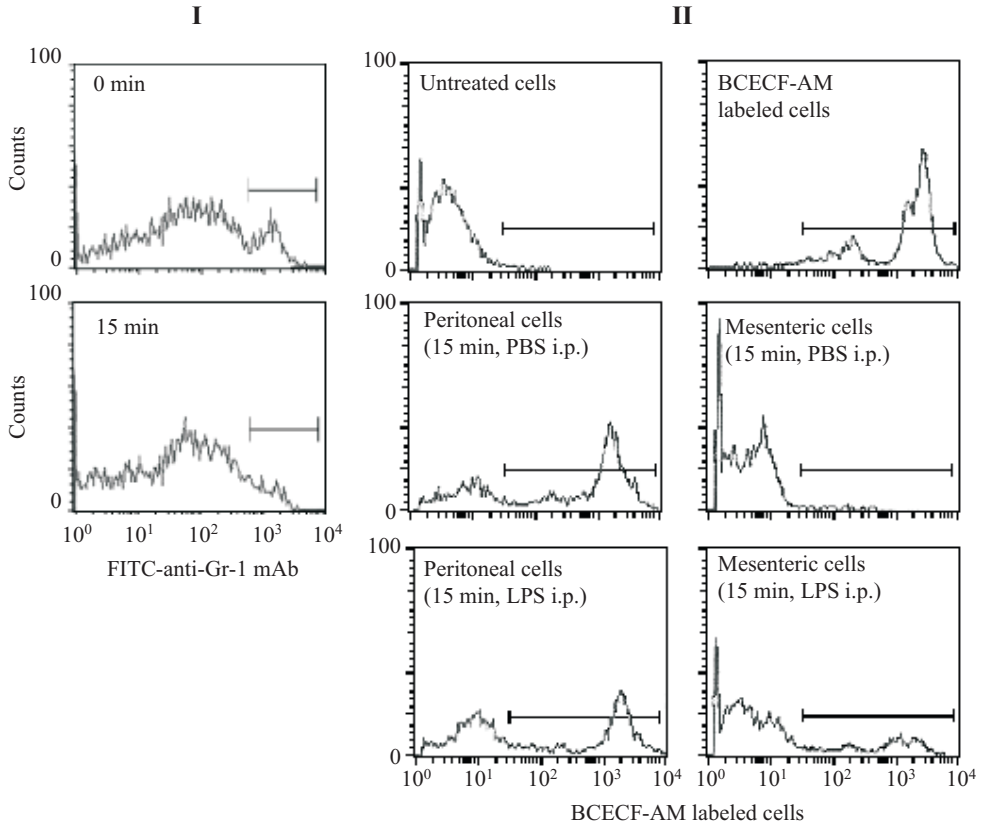

Figure 2. Intraperitoneal (i.p.) Gr-1^{high} PMNs migrate immediately into mesentric lymph nodes. BALB/c mice were injected i.p. with 3 ml of 3% proteose peptone. After 3 h, peritoneal exude cells were harvested and labeled with BCECF-AM. Mice were injected i.p. with both labeled cells and lipopolysaccharide (LPS) or PBS. Peritoneal cells and mesenteric lymph node cells were collected at the indicated times and stained with FITC-conjugated Gr-1 monoclonal antibody (mAb). These cells were analyzed by FACStar. As a result, Gr-1^{high} PMNs immediately disappeared in the peritoneal cavity after LPS injections (**I**). Moreover, transferred BCECF-AM-labeled PMNs migrated into the mesenteric lymph nodes by LPS-injections (**II**).

mental program of dendritic-like neutrophils. Cytokine-activated neutrophils at the inflammatory site can initiate acquired immunity.

The proliferation of antigen-specific T cells takes place in lymph nodes in the vicinity of the infection focus. Even though neutrophils acquire a prolonged life-span and the ability to present antigen to T cells infiltrating the inflammation site, the immune response elicited by these neutrophils is limited. In order to induce an immune response efficiently, these neutrophils have to migrate to lymph nodes to initiate a T cell response, in the same manner as the migration of the dendritic cells and macrophages. Miyazaki et al.[25] reported that a subset of neutrophils with strong expression of Gr-1 antigen (Gr-1^{high}) has high and rapid motility to lymph nodes in the vicinity of the inflammatory site (Fig. 2). Furthermore, it is reported that CD157[13] and the newly identified integrin-associated glycosyl phosphatidyl inositol (GPI)--anchored protein[37, 38] are important mediators of neutrophil adhesion and migration. However, how these GPI-anchored proteins mediate cell migration remains unknown at present. It is speculated that since the acyl chains of the GPI-anchored proteins are mostly saturated, they are preferentially inserted into the lipid raft, and that location allows them to function at an advantage[19].

Finally, neutrophils that have phagocytosed microorganisms finally undergo apoptosis through a caspase-dependent pathway. The susceptibility of neutrophils to apoptosis paradoxically plays a role in the establishment of acquired immunity. In other words, neutrophils that have phagocytosed infective pathogens undergo apoptotic cell death, but maintain their intracellular structures until cell destruction occurs. Subsequently, dendritic cells process antigenic fragments of pathogens in apoptotic neutrophils to bind MHC molecules or cluster differentiation molecules. Pathogen-derived protein antigens are presented to T cells by dendritic cells via MHC class I-restricted CD8 T cells. This phenomenon is called cross-presentation[3]. The dendritic cells are a heterogeneous

cell population; the $CD8^+$ dendritic cells mainly present antigens derived from apoptotic or necrotic cells, while the $CD4^+$ dendritic cells are associated with the presentation of antigens derived from dead bacteria[36]. It is known that cross-presentation is associated with infection immunity. For example, immunization of mice with the fusion protein of malaria antigen and heat shock protein (hsp)70 efficiently stimulates cross-presentation and induces antigen--specific cytotoxic T cells[45]. Moreover, CD91 is the only common receptor for the hsp family, such as for hsp70 and gp96, and specific binding of hsp and CD91 in neutrophils results in cell activation[2, 30]. It remains unclear how the uptake mediated by CD91 converges with the MHC class I pathway. In the late endosome, MHC class I is fractionated from the MHC class II pathway and transported into the cytoplasm. This fractional transport depends on the molecular size of the antigenic peptide and is an active transport pathway restricted only to dendritic cells[32].

A unique presentation pathway is that antigenic peptides generated by neutrophils are presented by bystander cells surrounding the neutrophils. The interesting presentation mediated by this pathway is observed by an assay using OVA-expressing *E. coli* transfectant. OVA antigen peptides are generated by neutrophils that have phagocytosed OVA-expressing *E. coli* transfectant and are captured by neighboring dendritic cells, which migrate to the lymph nodes to present the antigen peptides to T cells[29]. Since antigen peptides bound with MHC molecules bind under an acidic condition in the late endosome/lysosome and dissociate under a neutral condition, it is possible that the delivering of antigen peptide from neutrophils uses a delivery carrier such as trafficking vesicles, in a manner similar to the transport in intracellular organelles. The role of an exosome as a delivery carrier is speculated. Exosomes are uniform vesicles with a diameter of 60–90 nm, secreted by antigen-presenting cells. Exosomes express MHC class I, MHC class II, and costimulatory molecules. The antigen-bound MHC molecules are presented in MHC restriction as well as presentation by other professional antigen-presenting cells. Otherwise, the antigen-bound MHC molecule is detected in the extracellular matrix extension by neutrophils and can be transported to bystander dendritic cells without delivery. However, since these pathways have not been demonstrated, it is necessary to clarify these mechanisms in the future.

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