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A Functional Role of Neutrophils in the Regulation of Innate and Acquired Immunity to Bacterial Infection

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Abstract: Neutrophils express receptors that specifically recognized microorganisms and viruses and efficiently ingest and destroy these pathogens. Moreover, antigen presenting cells as macrophages and dendritic cells present the microbial antigens via MHC class II molecules, resulting in the activation of specific CD4 T cells. Since neutrophils have a short lifespan and are highly susceptible to apoptosis, their role in antigen presentation has been questioned. However, various pro-inflammatory cytokines such as IL-1, IL-6, TNF-α and IFN-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 the ability of antigen presentation. Up until now, the role of neutrophils has been focused only on the phagocytosis-mediated microbial activity. However, neutrophils are as competent as dendritic cells and macrophages in antigen presentation. An updated review of the old concept regarding the roles of neutrophils in biodefense is necessary. In the present review on the role of neutrophils, we describe both the classic innate and acquired immunity.

Key words: Neutrophils, toll-like receptor, chemokines, cross- presentation

INTRODUCTION

The immune system of mammals can be broadly classified into innate immunity and acquired immunity. Neutrophil is the cell type that plays the major role in innate immunity. The mechanisms of recognition of pathogenic microorganisms and viruses in mammals are known to be similar to those found in invertebrates including insects[1,2]. So far, attention on the function of neutrophil as a member of the biodefense system has been directed mainly to its role in the 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, antimicrobial action, fragmentation and antigen presentation by neutrophils.

Table 1: Function roles of pattern recognition receptors on PMNs

Receptors	Ligand
1. Toll-like receptors (TLR)	
TLR-2 and TLR-6 / TLR-2 and TLR-1 / TLR-2 and TLR-10	*Paptidhlycan lipopeptide* (Microplasma, Measles virus)
TLR-3	ds RNA (poly (I:C)
TLR-4 , MD2 and Cd14	LPS (Chlamydia, RS virus)
TLR-5	Flagellin
TLR-7	R-88, Ioxoriboine (anti-virus drug)
TLR-8	R-848
TLR-9	CpG DNA
TLR-10 (human)	unknown
2. Opsonin receptors	
Immunoglobulin-Fc receptors (Fc) / Complement receptors (CR1, CR2, CR3, CR4)	Opsonization
2. Scavengers receptors	LPS, lipoteichoic acid
SR-A I/II	
CD36 (SR-B)	
4. C-type lectin receptors 1)	
Tupe-1: Mannose receptor (CD206)	C type lectin domain
DEC 205	
Tupe-2: CLECSF6(DCIR)	Ligand (unknown)
Dectin-1	β-glucan receptor (Zymozan)

1) N-terminal of type-1 receptors is expressed on cell surface memmbrane, but not type 2

Expression and function of toll-like receptors in neutrophil: Neutrophils, macrophages and dendritic cells express receptors that specifically recognize microorganisms and viruses. These receptors are called Pattern-recognition Receptors (PRRs). They are known not only to distinguish common molecular structures to the pathogens that invade the body, but also bind the ligands of pathogens to induce cell activation (Table 1). Toll-like Receptors (TLRs) are typical PRRs and 10 types of TLRs have been identified in human.

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Neutrophils express all types of TLR but TLR-3 and they possess a wider profile of TLRs compared to other granulocytes[3]. In fact, stimulation of human neutrophils by pathogen components such as LPS, peptidoglycan and CpGDNA activates multiple immune mediator genes. The characteristic components of pathogens recognized by the TLR family are called Pathogen-associated Molecular Patterns (PAMPs). In the recognition of each PAMP, interaction between members of the TLR family is observed. For example, TLR4 and TLR2 play particularly important roles than other TLRs in pyogenic bacterial infection. TLR2 recognizes the lipoproteins of various pathogens and also peptidoglycan that is the major component of Gram positive bacteria. In general, TLR1, TLR6, TLR10 and TLR2 form heterodimers and bind specifically with the respective ligands.

On the other hand, the extracellular domain of TLR4 associates with the MD-2 molecule, forming the TLR4-MD-2 complex. This complex recognizes the LPS specifically present in Gram negative bacteria as well as proteins derived from viruses. Through this recognition process, LPS for the first time forms a complex with the LPS-binding protein. This complex binds with CD14 on the neutrophil surface. Thereafter LPS binds with TLR4 and the signal is transmitted into the cytoplasm. During this process, binding does not occur between LPS and TLR4 directly and MD-2 is essential for the association with TLR4[4]. For the other TLRs, TLR5 is able to recognize flagellin; TLR9 with bacterial DNA and TLR3 with viral double stranded RNA. Even though neutrophils are deficient in TLR3, TLR4 is also associated with recognition of viral components and compensates the function of TLR3. Although the pathogen-derived ligands specific for TLR7 and TLR8 have not yet been identified, it is now known that TLR7 is essential for the recognition of the single stranded RNA derived from Human Immunodeficiency Virus (HIV)[5]. Heil *et al.*[6] have reported that the HIV-derived guanosine and uridine-rich single stranded RNA induces the activation of human and mouse dendritic cells and that this single stranded RNA is recognized by TLR7 and TLR in humans and by TLR7 in mice. These findings indicate that TLRs recognize DNA and double-stranded RNA derived from bacterial components as well as single-stranded RNA as one of the viral components, playing a part of the roles in body defense against bacteria and viruses. Novel TLRs may be discovered in the future. Zhang *et al.*[7] have provided evidence for the novel TLR11 that takes part in the defense against infections transmitted via the urogenital route.

Unlike antibodies and T cell antigen receptors that recognize diverse antigens, TLRs are specific receptors encoded in the germline DNA and do not require rearrangement of the encoding genes on the genome[8]. These receptors are limited in number. Although they recognize various microbial components via their extracellular domain, these molecules do not possess the function to activate intracellular signals by themselves. Rather, their intracellular domain binds with adapter molecules to induce the production of inflammatory cytokines and type 1 interferon to effect defense against infection. The extracellular domain of TLRs possesses a protein motif called leucine-rich repeat and is associated with specific binding with pathogen components[9].

On the other hand, each TLR recognizes a specific component of the pathogens and activates an intracellular signaling pathway. For this purpose, the intracellular domain of TLR possesses a Toll-interleukin 1 Receptor (TIR) site that resembles the IL-1 receptor and binds the adapter molecule which is crucial for initiating the intracellular signaling pathway. The signaling pathway starts with phosphorylation of the receptor protein, followed by structural changes that occur as a series of linked events. The adapter molecule plays a linkage role between the signaling molecules involved in the cascade reactions. MyD88 is a representative adapter molecule. Since MyD88-deficient mice are affected by severe bacterial infections, MyD88 is considered to be essential in the signaling pathways mediated by all the TLRs associated with bacterial infection and consequently producing inflammatory cytokines[10].

Unlike the case of bacterial infections, type I interferon (IFN-γ) which is a cytokine with antiviral activity is induced during viral infections. While TLR3 is a receptor that recognizes viral components such as double stranded RNA, TLR4 is known also to recognize viral components leading to the production of type I interferon. Especially, in neutrophils, TLR4 is important in the defense against viral infection. An interesting finding is that type I interferon production mediated by TLR3 and TLR4 is induced normally even in the absence of MyD88[10]. These findings suggest the presence of MyD88-dependent and MyD88-independent pathways among TLR-mediated intracellular signaling pathways. In support of this hypothesis, novel adapter molecules TIRAP and TRIF that mediate MyD88-independent pathway have been identified[10,11]. In TIRAP-knockout mice, TLR4 and TLP2-mediated signaling pathways are impaired, whereas the responses to pathogen components recognized by TLR3, TLR7 and TLR9 are normal[12,13]. These results prove that TIRAP is specifically associated with intracellular signaling mediated by TLR2 and TLR4.

Neutrophil trafficking and chemokine: When pathogenic microorganisms infect the host, neutrophils rapidly migrate to the site of infection at 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 and adhere to the vessel wall at sites of tissue damage and then transmigrate through endothelial cells to the site of infection. Activated vascular endothelial cells and fibroblasts at the site of inflammation produce chemokines that induce chemotaxis of leukocytes. This accumulation of leukocyte forms the first step in the immune surveillance and plays a key role in the infectious immunity. Chemokines mediate chemotaxis and activation of immune cells and their molecular mass approximately 7-15 kDa. They are classified into four subfamilies of CXC, CC, C and CX3C chemokines based on the arrangement of the cysteines within this signature motif[14]. 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/IL-8 are required for the chemotaxis of neutrophils. Leukotactin-1/CCL15 of CC chemokine can also behave as a chemotactic factor for neutrophils[15,16] with the exception of 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 is 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 PMN-I (IL-12/CCL3), PMN-II (IL-10/CCL2), PMN-N (no cytokine/chemokine) subsets, based on their pattern of production[17]. PMN-I subset was obtained from MRSA (Methicillin-resistant *Staphylococcus aureus*) resistant hosts, while 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 type 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 FLR arrangement[18]. The chemokine receptors are members of a superfamily of seven 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 fMLP (formyl-Met-Leu-Phe) exhibit attenuated chemotactic response to CXCL8/IL-8, showing that cross-talk occurs in the signaling pathways mediated by the two receptors[19]. This type of phenomenon is also observed with other cytokines. Treatment of neutrophils with TNF-α results in cross-talk between the NF-κB and PI3-kinase (PI3K) pathways. Thus, CXCL8/IL-8 produced through NF-κB activation acts in an autocrine manner to induce PI3-kinase-dependent survival, prolonging the survival of neutrophils[20]. Prolongation of the lifespan 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 adequate concentration of a chemokine is kept by diffusion away from secreting cells, this phenomenon can induce migration of neutrophils. Since neutrophils can effectively infiltrate the infection sites, production of chemotactic factors is not transiently augmented but they have to be continuously produced during the inflammatory period. It was reported that stimulation of neutrophils with IL-12 in the presence of LPS for 18 h increased IL-8 production via NF-κB activation[21]. In addition, plasma CC chemokine regakine-1 and tissue CXC chemokine ligand IL-8 act synergistically in order to enhance neutrophil chemotaxis[22]. Therefore synergistic action or cascade reaction of multiple chemotactic factors permits 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 on 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 result in accumulation of signaling molecules such as PI3K and chemokine receptors[23]. Migration of neutrophils induced by IL-8 is inhibited by PI3K inhibitor and PI3K deficiency affects not only the migration, but also the respiratory burst and expression of G-protein-coupled receptor kinase[24-26]. Interestingly, the migrated cell forms the intracellular concentration gradient of PIP3 toward the concentrated gradient of chemokine and the leading edge contains PI3K and Rho family molecules that control cytoskeleton elements[27]. ROCK and PAK of Rho family molecules mediate the LIM kinase that phosphorylates cofilin such as the depolymerizing factor of actin[28]. 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[29]. Swap 70 accumulates in the membrane raft formed by the stimulation of cell migration and is associated with the cell migration[29]. That neutrophil chemotaxis induced by IL-8 and fMLP is not completely abolished in PI3Kg-deficient mice suggests the presence of other pathways independent of PI3K[27].

Antigen-presentation by neutrophils: Science 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 produced from those cells, the enhanced phagocytosis of neutrophils can eliminate infective microorganisms in the infection site.

In dendritic cells and macrophages, the antigenic peptides derived from the digested pathogen bind to MHC class II molecules and those 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, but 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 IFN-γ and GM-CSF, IL-1, IL-6 and TNF-α, is necessary to express these molecules on the surface of resting neutrophils[30]. The activated neutrophils act as an antigen presentation cell. In addition, neutrophils can migrate more quickly and accumulate more than any other cells at the infection site.

Microorganisms and viruses that are phagocytosed via the TLRs are processed inside antigen-presenting cells. There are at least five pathways of antigen presentation that leads to antigen-specific immune responses. The first pathway, endogenous antigen in the antigen-presenting cell is presented via MHC class I antigen to CD8-positive cytotoxic T cells. The second pathway, exogenous antigen is presented via MHC class II antigen to CD4-positive cytotoxic T cells. The third pathway, exogenous antigen activates CD8-positive T cells via the MHC class I antigen presentation pathway. This phenomenon is called cross-presentation. The fourth pathway is mediated by CD1 molecule expressed on antigen-presenting cells. Unlike the MHC molecules, CD1 lacks polymorphism and the genes encoding the CD1a, b, c and d molecules are present in antigen presenting cells. Each type is associated with presentation of individual lipid and glycolipid antigens[31]. The fifth pathway is mediated by exosome secreted by dendritic cells.

As described previously, the resting neutrophils lack the expression of the surface molecules necessary for antigen presentation, but cytokine-activated neutrophils show upregulated expression of these molecules and degrade infective microorganisms and can then present CD4 T cells as a professional antigen presentation cells similar to dendritic cells and macrophages. Neutrophils have adequately reserved lysosomal proteases; cathepsins B and D, necessary for antigen processing[32]. In fact, antigen-pulsed neutrophils obtained from mouse peritoneal cavities are capable of activating T cells in a MHC restricting manner[33]. However, several investigators rule out the function of antigen presentation by neutrophils because neutrophils have a short lifespan. This is the major factor that raises doubt over the function of neutrophil as an antigen presenting cell[34]. 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[20,35,36]. There is an interesting report that cytokine-activated neutrophils with a prolonged lifespan can present superantigen of pathogens and processed antigenic peptides[37]. Moreover, there are reports that the Mcl-1 and A1 genes belong to the bcl-2 family and suppress apoptosis of neutrophils[38-40]. In addition, inflammatory cytokines and TLR4 activate the signal transduction pathway that suppresses apoptosis.

When neutrophils are cultured in the presence of IFN-γ or GM-CSF, the CD83 as a dendritic cell marker is expressed on the surface and acquires the characteristics of dendritic cells[41]. 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 a MHC class II-restricted manner has also been observed[42]. 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 principally can mediate this differentiation. Other noteworthy finding is that the mature B cell marker CD19.20.21 is present intracellularly in these dendritic-like neutrophils[43]. These results highlight the developmental program of dendritic-like neutrophils. Cytokine-activated neutrophils in 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 lifespan 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 immune response efficiently, these neutrophils have to migrate to lymph nodes to initiate a T cell response, in the same manner as migration of the dendritic cells and macrophages.

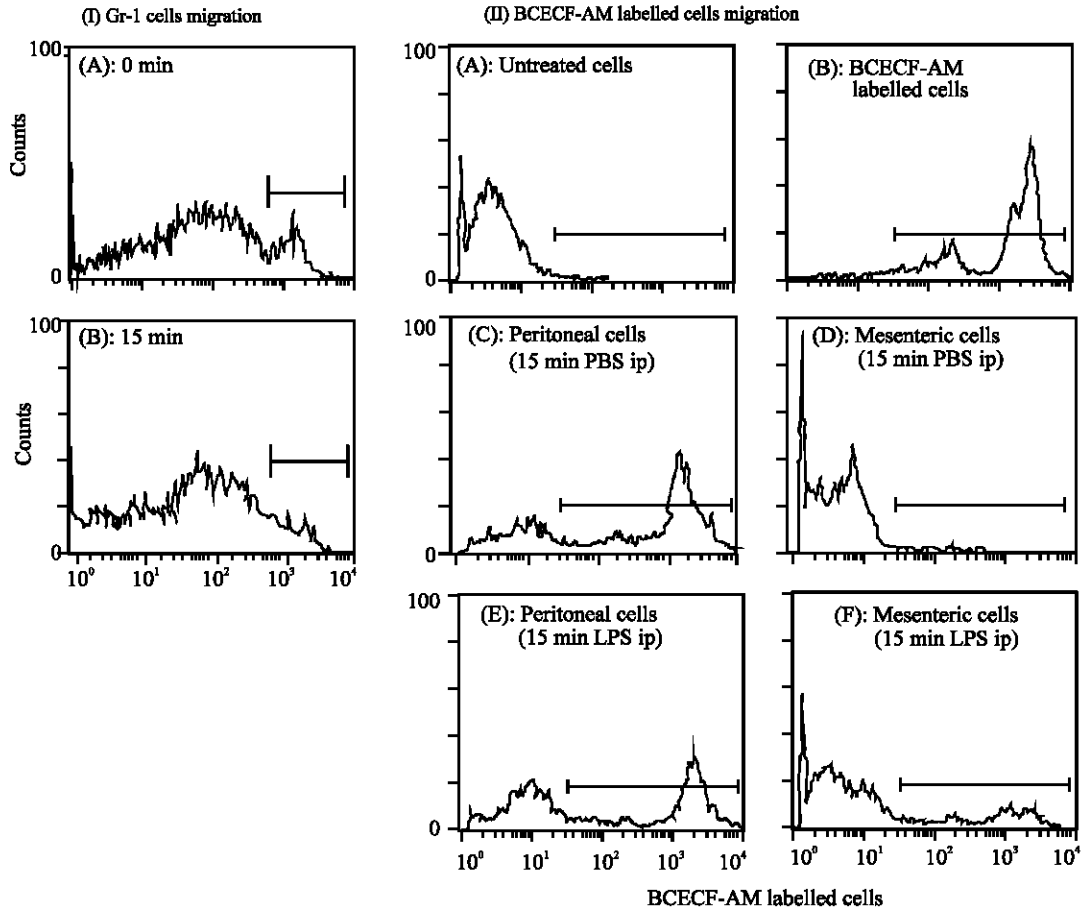

Fig. 1: Intraperitoneal Gr-1^{high} PMNs migrate immediately into mesenteric lympho nodes. BALB/c mice were injected ip with 3 mL of 3% Proteose Peptone (PP). After 3 h, peritoneal exude cells were harvested and were labelled BCECF-AM. Mice were injected ip with both labelled cells and LPS or PBS. Peritoneal cells and mesenteric lympho node cells were collected at the indicated times and were stained with FITC-conjugated Gr-1 mAb. These cells were analysed by FACStar. As a result, Gr-1^{high} PMNs were immediately disappeared in the peritoneal cavity after LPS-injections (I). Moreover, transferred BCECF-AM labelled PMNs migrated into mesenteric lympho nodes by LPS-injections (II)

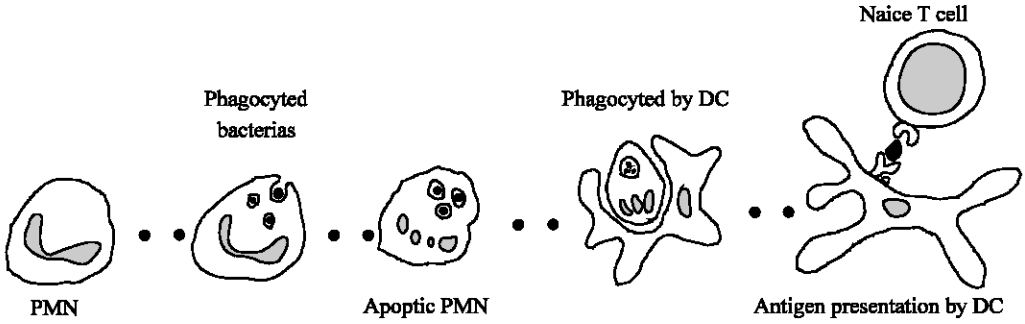

Fig. 2: Cross-presentation of phagocytosed bacteria-antigen by neutrophil

Miyazaki *et al.*[44] 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. 1). Furthermore, it is reported that CD157[45] and the newly identified integrin-associated GPI-anchored protein[46,47] 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 the location allows them to function at an advantage[48].

Finally, neutrophils that 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. Figure 2 is illustrating, neutrophils that phagocytosed infective pathogens undergo apoptotic cell death, but maintain their intracellular structures until the cell destruction occurs. Subsequently, dendritic cells process antigenic fragments of pathogens in apoptotic neutrophils to bind MHC molecules or CD molecules. Pathogen-derived protein antigens are presented to T cells by dendritic cells via MHC class I restricted CD8 T cells. Dendritic cells possess the ability of the cross-presentation[49]. The 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 presentation of antigens derived from dead bacteria[50]. 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[51]. Moreover, CD91 is the only common receptor for the HSP family such as HSP70 and gp96 and specific binding of HSP and CD91 in neutrophils results in cell activation[52,53]. 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 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[54].

A unique presentation pathway is that antigenic peptides generated by neutrophils are presented by bystander cells surrounding neutrophils. The interesting presentation mediated by this pathway is observed by an assay using OVA-expressing *E. coli* transfectant. OVA antigen peptide 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[55]. 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 antigen peptide from neutrophils uses a delivery carrier such as trafficking vesicles, in manner similar to the transport in intracellular organelles. The role of 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 molecules and costimulatory molecules. The antigen bound MHC molecules is presented in MHC-restriction as well as presentation by other professional antigen presentation 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.

Up until now, the role of neutrophils has been focused only on the phagocytosis-mediated microbial activity. However, neutrophils are as competent as dendritic cells and macrophages in antigen presentation. An updated review of the old concept regarding the roles of neutrophils in biodefense is necessary.

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