Granulocyte activation by danger signals and blocking of receptor responses

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// ABSTRACT

ORIGINAL PAPERS

This thesis is based on the following papers, referred to in the text by their Roman numerals.

I. **A.-L. Stenfeldt and C. Wennerås.** Danger signals derived from stressed and necrotic epithelial cells activate human eosinophils. *Immunology* 2004, 112:605-614

II. **A.-L. Stenfeldt, C. Wennerås and C. Dahlgren.** Epithelial cell components as danger signals to human neutrophils. *In manuscript*

III. **A.-L. Stenfeldt, J. Karlsson, C. Wennerås, J. Bylund, H. Fu and C. Dahlgren.** Cyclosporin H, Boc-MLF and Boc-FLFLF are antagonists that preferentially inhibit activity triggered through the formyl peptide receptor. *Inflammation* 2007, *In Press*

IV. **A.-L. Stenfeldt, J. Karlsson, C. Wennerås, J. Bylund, H. Fu and C. Dahlgren**. The non-steroidal anti-inflammatory drug piroxicam blocks ligand binding to the formyl peptide receptor but not the formyl peptide receptor like 1. *Biochemical Pharmacology* 2007, 74:1050-1056

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ABBREVIATIONS

Ac9-25	amino acid 9-25 at the aminoterminus of Annexin 1
APC	antigen presenting cell
Boc	tert-butoxycarbonyl
C5aR	complement fragment 5a receptor
Ca^{2+}	calcium ion
CCR	CC chemokine receptor
CD	Cluster of differentiation
CLC	Charcot-Leyden crystal
COX	cyclooxygenase
CR	complement receptor
CsH	cyclosporin H
Cyt b	cytochrome b
DC	dendritic cell
DC-SIGN	dendritic cell-specific ICAM-3-grabbing non-integrin
DNA	deoxyribonucleic acid
ECP	eosinophil cationic protein
EPO	eosinophil peroxidase
EPX	eosinophil protein X
FcR	receptor for the Fc-part of immunoglobulins
FLFLF	phenylalanyl-leucyl- phenylalanyl-leucyl-phenylalanine
fMLF	*N*-formyl-methionyl-leucyl-phenylalanine
FPR	formyl peptide receptor
FPRL1	formyl peptide receptor-like1
GDP	guanine diphosphate
GPCR	G-protein coupled receptor
GTP	guanine triphosphate
HMGB1	high-mobility group B1 protein
ICAM	intracellular adhesion molecule
Ig	immunoglobulin
LFA	leukocyte function associated antigen
LPS	lipopolysaccharide
LxA_4	lipoxin A_4
Mac-1	macrophage-1 antigen
MBP	major basic protein
MLF	methionyl-leucyl-phenylalanine
MPO	myeloperoxidase
NADPH	nicotinamide adenine dinucleotide phosphate
NSAID	non-steroidal anti-inflammatory drug
PRR	pattern recognition receptor
PSGL	P-selectin glycoprotein ligand
ROS	reactive oxygen species
TLR	toll-like receptor
VLA	very late antigen
WKYMVM	tryptophyl-lysyl-tyrosyl-methionyl-valyl-L-methionine
WKYMVm	tryptophyl-lysyl-tyrosyl-methionyl-valyl-D-methionine
WRWWWW	tryptophyl-arginyl-tryptophyl-tryptophyl-tryptophyl-tryptophan

PREFACE

The immune system is a complex mixture of molecules and cells that have evolved to protect us from infectious agents. The human immune system is divided into two parts, the innate (also called the naïve or natural) and the adaptive (also called specific) immune system. Although both subgroups of the immune system work together to protect against invading organisms, they act in different ways. The adaptive immune system requires some time to react to formulate an antigen specific, precise response to the intruder. The innate immune system on the other hand, responds quickly to infectious agents, but it has classically been thought to have a limited capacity to distinguish between different antigens and respond without distinction. Normally the immune system neglect healthy tissues but induces an inflammatory reaction in infected or injured tissues. During the years many different hypotheses of what underlying mechanisms cause this action have been proposed. Still, scientists do not know the correct answer, but as our knowledge of different immune cells increases, the knowledge about immune reactions increases correspondingly. This thesis will focus on the most common cell type in our immune system, the granulocyte, and its activation by damaged uninfected tissue cells. It will also discuss possible ways to block receptors that may contribute to the activation of granulocytes by damaged cells.

INNATE IMMUNITY

Most organisms can fight infections through innate immunity alone. It is only in vertebrates that an adaptive immune system has evolved[1]. Innate immunity is enormously broad, consisting of both cellular and humoral components and it is difficult to decide what should be included or not. The more knowledge obtained about human immune defences the harder it is to uphold the historical description of innate immune cells. For example, epithelial cells lining the lungs and intestinal tract were primarily thought to form only a mechanical barrier to the outside, but it has become evident that these cells are able to secrete both anti-microbial agents and recruit leukocytes[2]. However, classic active innate immune cells are limited to granulocytes (neutrophils, eosinophils, basophils), mast cells, macrophages (e.g. Küpffer cells, microglia and osteoclasts), dendritic cells (e.g. Langerhans cell and plasmacytoid dendritic cells) and natural killer (NK) cells. These cells express structures, often referred to as pattern recognition receptors (PRRs), that can interact with molecules common to many pathogens e.g. lipopolysaccharides (LPS), other bacterial cell wall components and formylated peptides[3]. Activation of innate immune cells through PRRs starts a cascade of events, including production and secretion of anti-bacterial and leukocyte attracting molecules. This leads to the recruitment of more immune cells (innate as well as adaptive) and an inflammatory response.

Apart from their role in host defence, innate immune cells have also been predicted to have a role in tissue remodelling. The secretion of pro-inflammatory cytokines and growth factors has been shown to contribute to, among other things, angiogenesis[4, 5].

GRANULOCYTES

Granulocytes are cells that are filled with membrane-sealed organelles called granules (thereby the name). These cells were probably first discovered by Hunter in 1774 who described movable cells at the site of inflammation[6]. Almost 100 years later, in 1882, Metchnikoff witnessed the engulfment of particulate dyes by movable cells. However, it was Paul Ehrlich who classified granulocytes and divided them further into neutrophil, eosinophils and basophils depending on their staining characteristics, **Fig.1** [7].

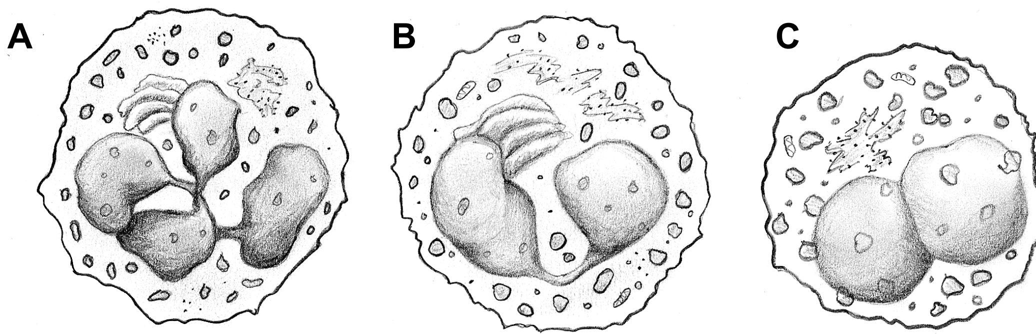

Fig. 1. *Granulocytes constitute three subpopulations of cells. **A** The neutrophil with its segmented nucleus. **B** The eosinophil with a bilobed nucleus and granules that can be stained with the acidic dye eosin. **C** The basophil with slightly fewer and larger granules then the other granulocytes*.

Granulocytes develop in the bone marrow by a process referred to as myelopoiesis [8]. During myelopoiesis, granulocytes are formed from pluripotent stem cells via a sequence of events starting with the proliferation and formation of granules and ending with changes in nuclear shape and cell size[9]. This maturation process in the bone marrow takes about 14 days[10]. In contrast to other leukocytes, granulocytes are fully mature, non-dividing cells when they leave the bone marrow and enter the blood circulation. Hence, they have synthesised most proteins and other molecules important for their function before they enter the blood stream. This cell type is therefore able to respond rapidly to stimuli; for example by releasing their granule content in the vicinity of the stimuli. Granulocytes are predominantly tissue dwelling cells and do not re-enter the circulation.

NEUTROPHILS

Neutrophils are the first type of immune cells that are recruited to the site of infection. They are the most abundant cell type in the blood of a healthy individual and constitute around 60-70% of the blood leukocytes. On average, a neutrophil spends 10-25 hours in the blood before it enters the tissue[10, 11]. In healthy subjects, neutrophils infiltrate most tissues in low numbers where they may live for several days before they undergo apoptosis and are cleared by macrophages or are lost via mucosal surfaces[12].

Circulating neutrophils are almost spherical and measure 10-12 μm in diameter. However, as soon as they are activated by adhesion to a surface, they flatten, and assume an amoeboid shape with extended pseudopodia. Mature neutrophils have a segmented nucleus typically

composed of two to four segments with the chromatin content coarsely clumped. Characteristically, they contain very few mitochondria, a small amount of Golgi and endoplasmic reticulum. Based on mature neutrophil morphology, it was formerly thought that protein synthesis in these cells was negligible. Today however, it is generally accepted that mature neutrophils are capable of rapidly starting biosynthesis during the inflammatory challenge process[13]. Neutrophils also contain membrane-sealed organelles called granules, which are filled with degradative enzymes and antimicrobial agents. On the basis of protein content, the granules are divided into four distinct populations: azurophil, specific, gelatinase and secretory granules. Azurophil granules are identified by the high myeloperoxidase (MPO) content. Specific granules are characterised by the high content of lactoferrin and vitamin B_{12}-binding protein. Gelatinase granules are distinguished by their content of gelatinase and lack of lactoferrin[14, 15]. The secretory vesicles are formed by endocytosis during neutrophil maturation and are therefore filled with plasma proteins. These organelle membranes are rich in receptors and other structures and their main function is thought to be to quickly change the expression of neutrophil cell surface structures[16]

Neutrophils are effective bacterial killers endowed with the capacity to engulf and eliminate pathogens (phagocytosis). Accordingly they express receptors for immunoglobulins (i.e. IgG receptors, FcγIR, FcγRII, FcγRIII) and complement receptors important for phagocytosis of particles opsonised with IgG and complement[17].
They also express other cell structures important for bacterial recognition e.g. formyl peptide receptors (FPRs, more thoroughly described below) and toll-like receptors (TLRs)[18, 19].

EOSINOPHILS

It was in the late 1870s that Paul Ehrlich noticed that a certain type of white blood cell was stained with the negatively charged dye, eosin, and that is presumably why he called them eosinophils. These cells are normally found in low numbers in the blood, constituting only 4-10% of the circulating leukocytes. Compared to neutrophils, eosinophils are recruited and infiltrate tissues during the later stages of inflammation. The peripheral blood mean transit time has, however, been estimated to be almost the same as for neutrophils (10-26

hours)[10, 11]. In contrast to neutrophils, eosinophils transmigrate towards distinct tissue areas. In normal healthy individuals eosinophils preferentially are reported to infiltrate the gastrointestinal tract but also other epithelial interfaces with the environment [11, 20].

Circulating eosinophils have a diameter of approximately 10-12 μm and like neutrophils they flatten and assume an amoeboid shape with extended pseudopodia when they attach to surfaces. Normally they have a bi-lobed nucleus with partially condensed chromatin, **Fig. 2**, but in some diseases the nucleus may be segmented into a larger number of lobes[21, A.-L. Stenfeldt unpublished observation]. Eosinophils contain four different types of granules

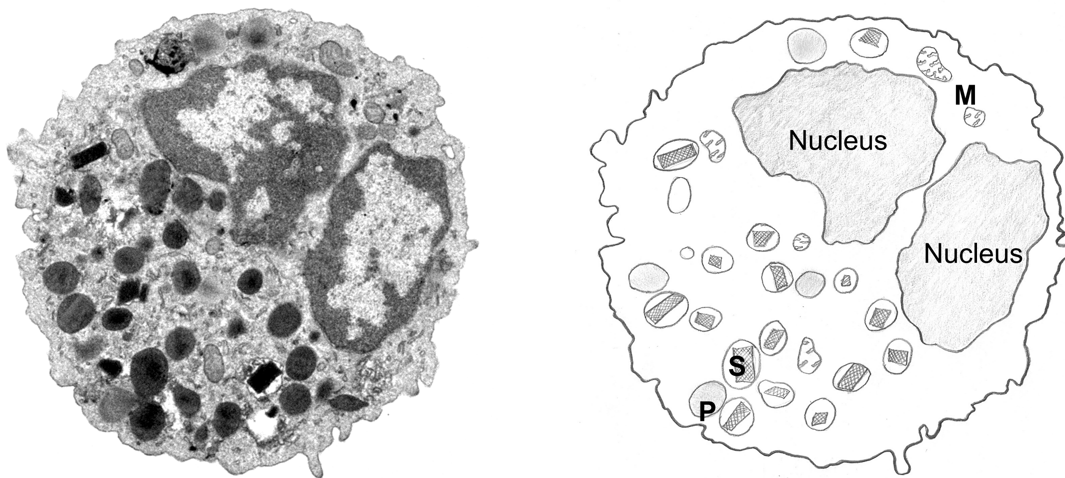

Fig 2. *A cross-section of a resting eosinophil. The two lobes of the neucleus are seen with partially condensed chromatin. The cytosol is filled with primary granules, mitochondria and the eosinophil characteristic specific granules. Transmission electron micrograph magnification ×8000 (A.-L. Stenfeldt, 2004). Intracellular structers marked in the sketch, (P) primary granule, (S) secondary granule, and (M) mitochondria.*

called primary, secondary, small granules and secretory vesicles. The primary granules are characterised by the content of Charcot-Leyden crystal (CLC) proteins, which have been classified as galectin-10 [22-24]. The secondary (also called specific) granules are the visual hallmark of the eosinophils. They contain the cationic proteins that are stained by negatively charged dyes and an electron dense angular core visable by electron microscopy. The core is mainly composed of major basic protein (MBP) and the less electron dense matrix consists of eosinophil peroxidase (EPO), eosinophil cationic protein (ECP) and

eosinophil-derived neurotoxin (EDN) also called eosinophil protein X (EPX)[25, 26]. Small granules have only been detected in tissue eosinophils and contain arylsulphatase and acid phosphates as well as ECP[25, 27]. Secretory vesicles are less well characterized but it might be that these compartments, as in neutrophils, contain membrane-bound receptors and proteins that can be rapidly mobilized during activation[28, 29].

Eosinophils are thought to be multi-functional cells and accordingly they express several different classes of membrane proteins and receptors on their cell surface: G-protein coupled receptors, important for chemotaxis such as CCR1 and CCR3, Fc receptors for immunoglobulin (Ig) A (CD89), E (CD23), and G (CDw32). β-integrins such as CD11a/CD18 (LFA-1) and CD11b/CD18 (CR3, Mac-1), just to mention a few[30-33]. In fact, eosinophils express most of the membrane proteins expressed by other leukocytes. Therefore, instead of a specific eosinophil marker, it is the lack of low affinity IgG receptors (FcγIII, CD16) in resting eosinophils that makes it possible to isolate these cells of high purity from blood[34].

BASOPHILS

Basophils are the third and rarest type of granulocytes in humans, comprising less than 1% of the blood leukocytes[35]. Compared to the other granulocytes, basophils are slightly smaller and contain larger and fewer granules[36]. Granule content is less well described compared to neutrophils and eosinophils, but granules are reported to contain CLC protein, histamine as well as MBP[37-39]. Activated basophils may also release interleukins such as IL-4 and IL-13[40, 41].

Similar to eosinophils, basophils express most of the cell surface proteins expressed by other leukocytes[35]. The lack of a unique cell surface marker and the low number of basophils in peripheral blood makes it difficult to purify and study these cells *in vitro*, thus this cell type is less investigated than other leukocytes. Basophils will not be included in the continuing discussion.

GRANULOCYTE ACTIVATION

Granulocytes activated by stimuli attach to the endothelium lining the blood vessel, transmigrate through it into the interstitial space where they start to mobilize their granules and migrate towards the infected or inflamed area. This is a complex process that is rigorously regulated and involves many different cell structures as well as inflammatory mediators derived from both granulocytes and other cells in their proximity.

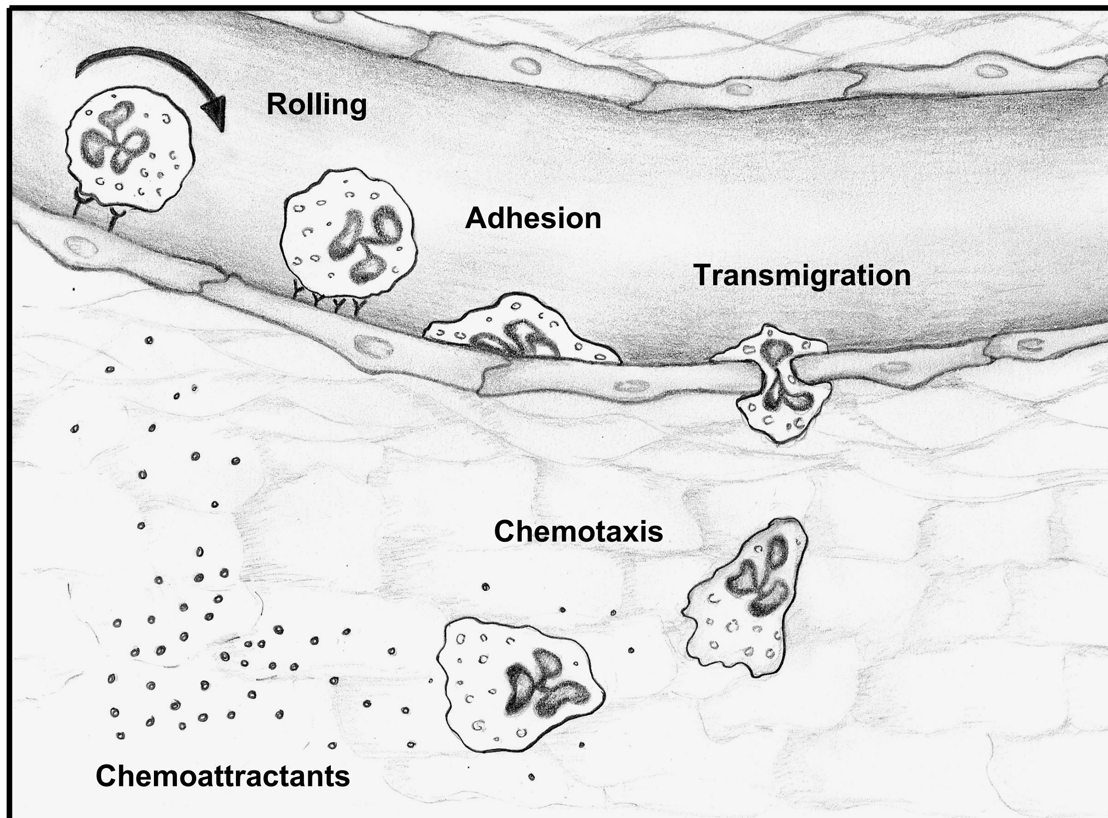

Fig. 3. *The recruitment of granulocytes towards infected or damaged tissues start in the blood stream. Endothelial cells lining the blood vessel are stimulated by infected/damaged tissues to upregulate their expresion of leucocyte adhesion molecules. Granulocytes express structers on their surface that interact with the adhesion molecules on the endothelial cells. The constant blood flow in the vessel, cause the attached granulocyte to role. This interaction stimulate granulocytes to express more adhesion molecules, which leds to a ferm adhesion to the endothelium. The granulocyte cross the endothelium (transmigration) and start to migrate towards a chemical gradient of molecules (chemotaxis) relaeased from the infected or injured tissue area.*

ENDOTHELIAL ADHESION

The migration of leukocytes from blood into tissue is roughly divided in to three steps: rolling, adhesion and transmigration, **Fig. 3**. In the blood stream, circulating granulocytes utilize P-selectin glycoprotein ligand 1 (PSGL-1), L-selectin and very late antigen-4 (VLA-4, CD49d/CD29) to attach to counter structures expressed on endothelia cells that line the blood vessel[42-46]. This makes the granulocytes roll and form tethers (long thin extensions of the granulocyte membrane formed during shear flow), which slow down the velocity of circulating cells. The loss of velocity allows the cells to sense molecules (chemoattractants) from the tissue surroundings and/or cell contact-mediated signals from the endothelium. The chemoattractants and/or cell contact-mediated signals functionally activate integrins such as CD11a/CD18 (LFA-1), CD11b/CD18 (CR3, Mac-1) and CD49d/CD29 (VLA-4) on the granulocyte surface[47]. The integrins in turn, bind to counter-structures expressed by endothelial cells, which results in a sustained, strong attachment.

ENDOTHELIAL TRANSMIGRATION

The firmly attached granulocytes leave the blood vessel in a process referred to as diapedesis or transendothelial migration, **Fig. 3**. The mechanism for transendothelial migration is less well understood than the adhesive interactions (tethering, rolling and adhesion). It is still controversial whether granulocytes prefer to use the route between endothelial cells (paracellular pathway) or through the endothelial cells (transcellular pathway) lining the blood vessel. Different experimental models that describe both these routes of transmigration have been studied, however, it is not clear which factors dictate the granulocytes choice of route[48-52]. In transmigration the integrins CD11a/CD18 and CD11b/CD18 also seem to play a crucial role as well as a heavily glycosylated 32-kD transmembrane protein, CD99[53-56].

MIGRATION

Granulocytes that have passed through the vessel wall continue to migrate through the extracellular matrix either by chemotaxis (locomotion towards an increasing gradient of chemoattractants) or by chemokinesis (non-directional movement)[57]. During chemotaxis granulocytes are able to sense a concentration difference as small as a few percent over the

length of the cell[58]. Cells move along the chemoattractant gradient by stretching out parts of the plasma membrane (pseudopodia). The pseudopodia tips are rich in adhesion molecules that interact with counter-structures on the extracellular matrix, and this interaction further propels the cell to move forward[59]. The formation of pseudopodia is dependent on cytoskeletal components called actin filaments, **Fig. 4**. These filaments are built up by polymerized actin monomers that continuously assemble at the leading end and dissociate at the posterior end of the filament, which in turn causes the directional membrane stretching[58, 60].

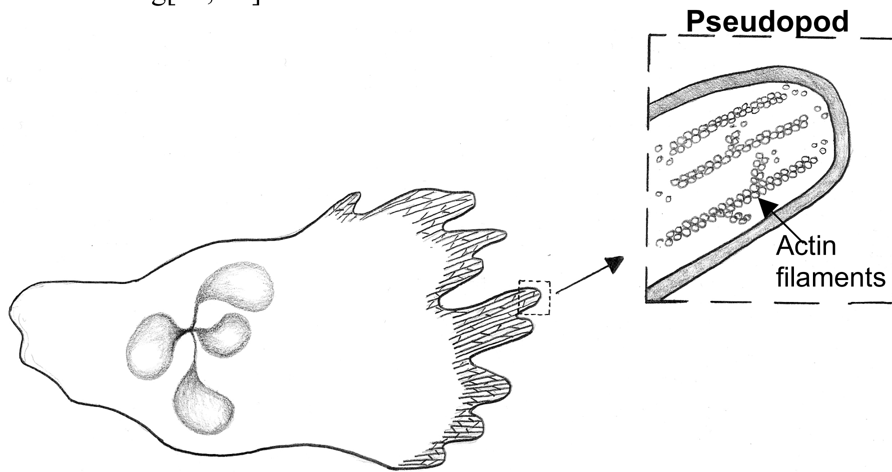

Fig. 6. *Granulocytes move by stretching out parts of the plasma membrane (pseudopodia). The formation of a pseudopod is dependent on actin filaments. These filaments are built up by actin monomers.*

DEGRANULATION

Activated granulocytes release granule constituents into the surroundings or into phagosomes. The most established way for cells to release granule constituents is by exocytosis. This is a rather well-defined process in which the granule membrane fuses with the plasma membrane, **Fig. 5A**[61-63]. In addition to exocytosis, eosinophils release their granule content in two non-classical ways, the so called piecemeal and cytolytic degranulation, **Fig. 5B, C**[64, 65]. During piecemeal degranulation vesicles bud of specific granules and fuse with the plasma membrane[64, 66]. This function makes it possible to partially empty granules. Cytolytic degranulation (when free granules are seen in association with necrotic eosinophils) is a phenomenon reported in subjects suffering from

atopic dermatitis and allergic inflammation of the upper airways [65, 67]. Whether this cytolysis is a regulated process or just an effect of milieu changes in the cell vicinity is not clear.

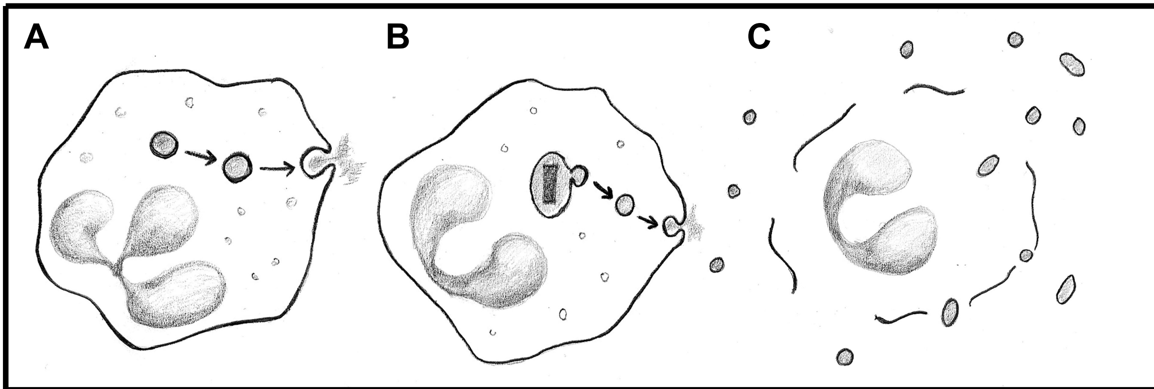

Fig. 5. *Three different ways for granulocytes to release their granule content.* ***A*** *Exocytosis, the most wellknown, when granules are transported to and fuses with the plasma membrane.* ***B*** *Piecemeal degranulation, when small vesicles bud of from the granula and fuses with the plasma membrane.* ***C*** *Cytolisis, the most debated one, when intact granules are released in the surroundings of a necrotic cell.*

Neutrophils mobilize their granules in an hierarchical order, starting with the secretory vesicles followed by gelatinase and specific granules[68, 69]. The azurophil granule population is the least prone to mobilization when it comes to extracellular release. Instead, fusion with and release of contents into phagosomes seems to be the main function of these granules[70]. There are no reports suggesting that eosinophils mobilize their granules in a hierarchical order.

Granule mobilization is not just a tool for release of granule content. The granule membranes are rich in proteins and upon activation these are translocated to the plasma membrane and exposed on the cell surface. This in turn makes it possible for the cell to facilitate new interactions with the surroundings[71].

SUPEROXIDE PRODUCTION

In addition to degranulation, activated granulocytes are able to produce and release reactive oxygen species (ROS). The production of ROS is associated with increased molecular oxygen consumption therefore it is often referred to as the respiratory burst[72]. In this

process, molecular oxygen is reduced to superoxide (O_2^-) by an enzyme called NADPH-oxidase (also called respiratory burst oxidase).

The NADPH oxidase is a membrane-bound, multi-component enzyme complex that is assembled only when cells are activated by appropriate stimuli (e.g. chemoattractants, chemokines and bacteria). Most studies of the NADPH oxidase are performed in neutrophils but the assembly and function of the enzyme in eosinophils is expected to operate in the same way. In resting neutrophils the enzyme is divided into a cytosolic heterotrimeric complex ($p40^{phox}$, $p47^{phox}$ and $p67^{phox}$), the cytosolic rac2 and a membrane bound heterodimer subunit flavo-protein, cytochrome b_{558} (cyt b_{558}, composed of $gp91^{phox}$ and $p22^{phox}$), **Fig. 6A**[73-75]. A few years ago, yet another cytosolic protein p29Prx was suggested as an oxidase-associated protein, but the role and requirement of this component needs to be further investigated[76]. In resting neutrophils most of the cyt b_{558} is localized to the specific granules, and only a minor portion of the protein is found in the plasma membrane [71, 77, 78]. It is well documented that neutrophil activation leads to the assembly of NADPH oxidase components in the plasma or phagosome membrane, but there are also reports suggesting a translocation of cytosolic components to the specific granules[79, 80]. The role of an active NADPH oxidase in specific granules is not known. The assembled and functional oxidase uses NADPH as an electron donor and transfers electrons across the membrane to molecular oxygen, thus generating superoxide either in the extracellular environment or the phagosomal cavity, **Fig. 6B**. Eosinophils have been reported to produce larger quantities of superoxide compared to neutrophils, and this phenomenon is simply explained by the fact that cytosolic components ($p40^{phox}$, $p47^{phox}$ and $p67^{phox}$) are more abundant in eosinophils[81-83].

Conventionally ROS have been considered to function as antimicrobial agents, even though in recent years the direct role in bacterial killing has been questioned[84]. There are also reports suggesting a roles for ROS in cell proliferation and differentiation[85, 86].

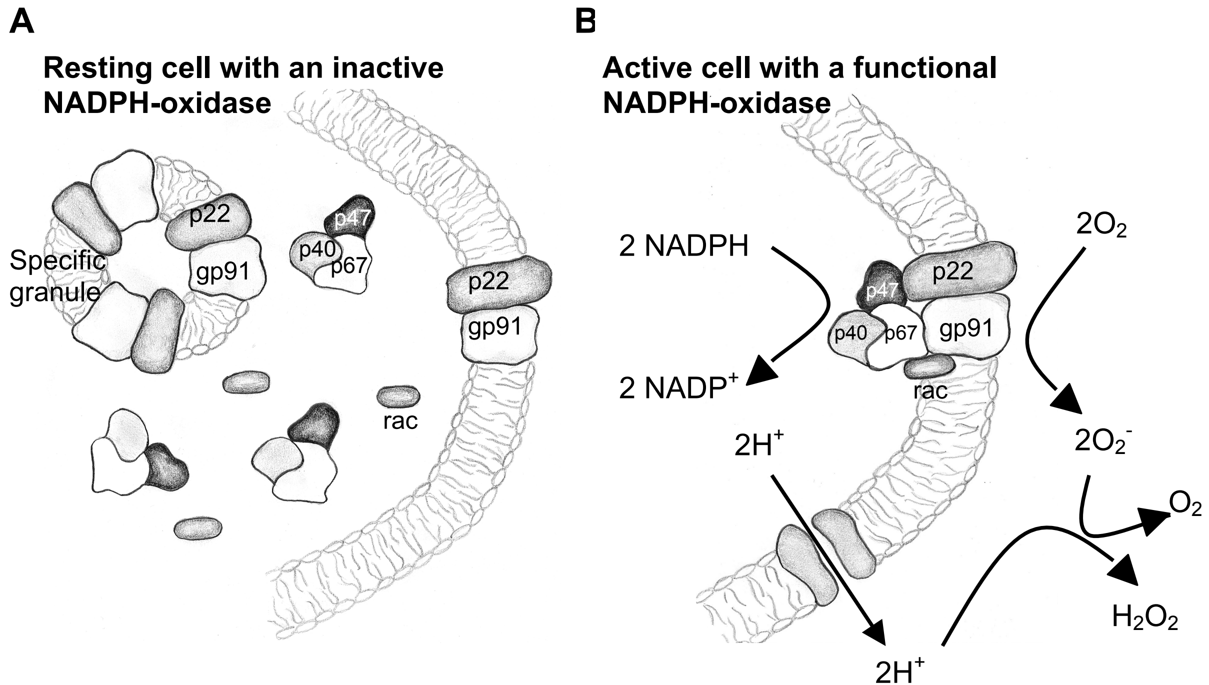

Fig. 6. *The NADPH-oxidase in the cell.* ***A*** *In a resting cell the NADPH-oxidase is divided into a cytosolic heterotrimeric complex ($p40^{phox}$, $p47^{phox}$ and $p67^{phox}$), a rac protein and a membrane bound heterodimer subunit ($gp91^{phox}$ and $p22^{phox}$) flavo-protein. Most of the $gp91^{phox}$ and $p22^{phox}$ is located to the specific granules, only a minor portion of these proteins is found in the plasma membrane.* ***B*** *In an activated cell the components of NADPH-oxidase assemble in the plasma or phagosome membrane to a functional enzyme. The functional NADPH-oxidase uses NADPH as an electron donor and transfers electrons across the membrane to molecular oxygen generating superoxide (O_2^-). Superoxide, in turn, is a reactive molecule that could react with protons (H^+) and form hydrogen peroxide (H_2O_2).*

THE DANGER THEORY

One fundamental question in immunology is how the immune system discriminates between different structures within the body. Why does it respond to some structures but neglect/tolerate others? In 1959, Lederberg proposed a tolerance model that was called self –nonself discrimination, **Fig. 7A**[87]. He suggested that cells of the adaptive immune system (lymphocytes) are responsible for the discrimination between bodily structures that are continuously present (self) or not (nonself). The theory was based upon the assumption that lymphocytes expressed multiple copies of one single surface receptor with specificity for one antigen epitope. Receptor-antigen (nonself) binding would activate these cells to produce antibodies and initiate an immune response. About ten years later, Bretcher and

Cohn further developed this model and proposed that adaptive immune cells were only activated to produce antibodies if two epitopes on the antigen were recognized by receptors from two different lymphocytes, **Fig. 7B** [88]. Since then, the self-nonself theory has gone through several modifications and in the late 1980s Medzhitov and Janeway claimed that cells within the innate immune system also were able to discriminate between self-nonself by expressing receptors (PRRs) towards highly conserved microbial structures, **Fig. 7C**[3, 89]. A few years later, Matzinger proposed to resume an old theory (postulated in 1890s by Ehrlich) to explain tolerance[90, 91]. The renewed model was called the danger hypothesis. According to this, the immune system is activated by molecules that are interpreted as threats to the body irrespective of endogenous (self) or exogenous (nonself) sources, **Fig. 7D**. Hence, stressed or injured cells (e. g. cells exposed to pathogens, toxins or mechanical damage) as well as cells that die necrotically are suggested to send out alarm signals whereas healthy cells or cells that die through normal programmed cell death (apoptosis) do not.

There are several hypotheses about what is sensed as danger and why. Molecules normally kept within the cell such as mitochondrial DNA and proteins, nuclear constituents (e.g. the high-mobility group B1 protein HMGB1) and heat shock proteins (Hsp) are some of the molecules that have been reported to activate immune cells[92-94]. Probably there are several other danger signals within cells that have yet to be characterized. The crystalline form of the extracellular molecule uric acid has also been suggested to act as a danger signal to dendritic cells (DCs)[95].

The danger model has focused on the activation of professional antigen presenting cells (APCs, DCs) but granulocytes are also directly activated by endogenous danger signals (Paper I and II). Necrotic epithelial cells stimulate eosinophils to migrate and release granule constituents (Paper I). Transmission electron microscopy (TEM) studies also show that eosinophils incubated with necrotic epithelial cells are markedly affected, **Fig 8**. The eosinophil granules are known to contain cationic proteins (such as ECP and EPO) that potentially damage tissues. It is hard to understand the advantage of releasing harmful

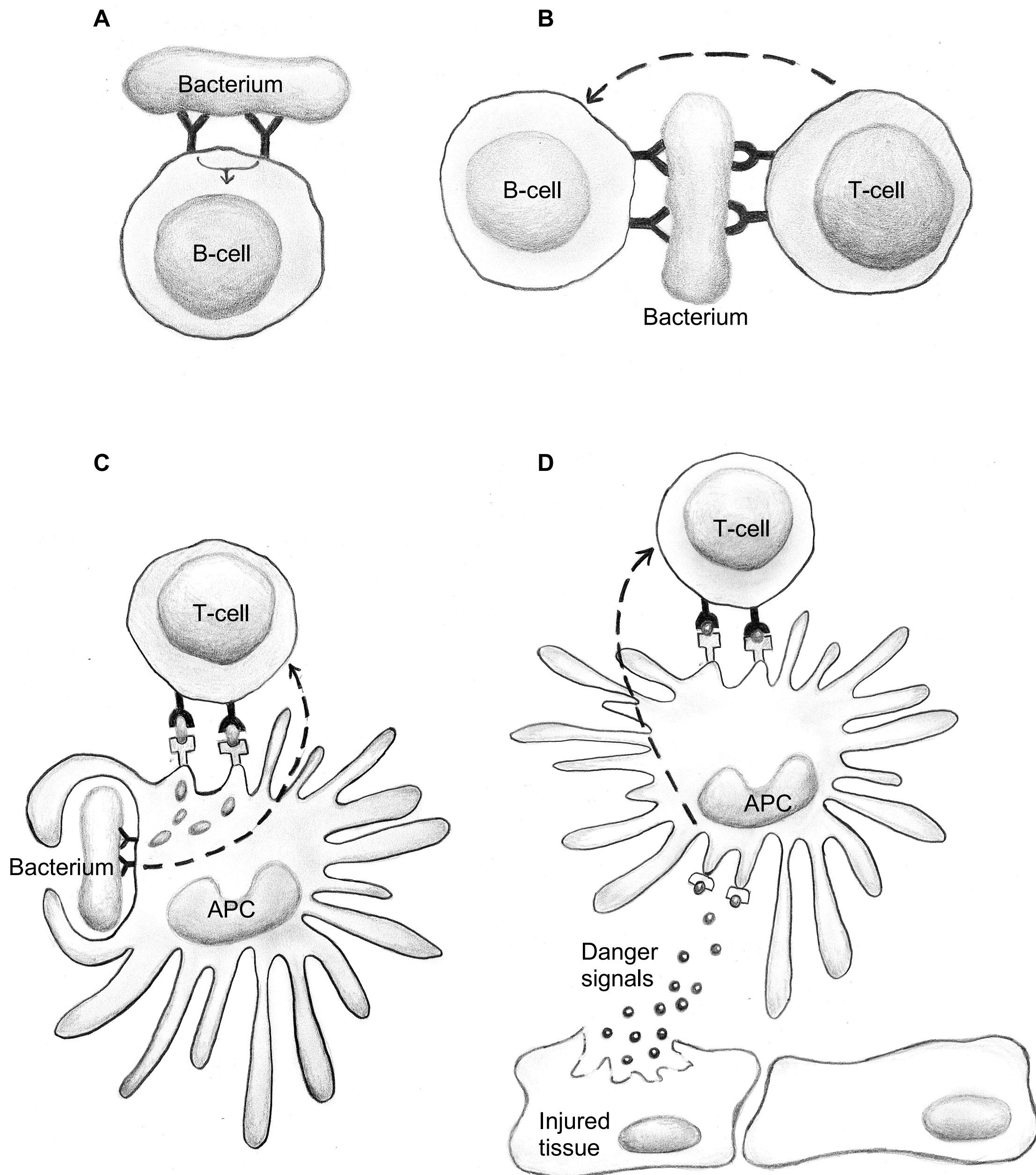

Fig. 7. *Historical overview of different tolerance models. **A** In 1959, it was thought that lymphocyte stem cells that did not recognize any antigen matured to antigen-sensitive cells (B-cells). These cells expressed multiple copies of one single surface receptor with specificity for one antigen epitope. Upon antigen recognition (e.g. bacterium) these cells became antibody-producing cells. **B** In the 1970s it was proposed that two different epitopes on one antigen had to be regognized by two different lymphocytes to induce an antibody production. **C** In 1989, it was suggested that antigen-presenting cells (APCs) recognize foreign structures via PRRs and activated T-cells by co-stimulatory signals. **D** In the mid-1990s it was proposed that molecules from injured tissues activate APCs to produce co-stimulatory factors for T-cells.*

substances into a milieu that already has damaged cells. However, it has been reported that healthy individuals may have at least two variants of ECP, one toxic and one markedly less toxic[96]. If the eosinophil cationic proteins (EPO and ECP) exist in more than one variant and these possess other functions than that already described, it would make sense that these molecules are released. Eosinophil accumulation and killing of helminthic parasites has been known for many years, but the accumulation of eosinophils in a variety of different diseases has continued to baffle scientists[97]. Most of these conditions have tissue damage in common. The capability of eosinophils to release toxic constituents (superoxide and cationic proteins) has therefore led to the conclusion that these cells induce tissue damage in different health conditions. However, it might also be the other way around, in that already damaged tissues attract eosinophils. With respect to this it is interesting to note that a recent study of eosinophil infiltration in solid tumours indicated that it is the necrotic tissue surrounding the growing tumour that attracts the eosinophils and not the tumour cells[98].

Neutrophils are also activated by necrotic epithelial cells (Paper II). The cell surface expression of the adhesion molecule CD11b is markedly increased in the presence of necrotic epithelial cells. As mentioned before, the CD11b/CD18 (CR3) molecule is important for neutrophil adhesion to endothelium as well as migration in tissues. However, this molecule is also involved in other important neutrophil functions such as phagocytosis, the regulation of neutrophil survival and apoptosis[99-102]. Lately, it has also been shown that CD11b/CD18 is important for neutrophil communication with DCs[103]. At the site of infection, neutrophils are reported to interact through CD11b/CD18 with the dendritic cell-specific ICAM-3-grabbing non-integrin (DC-SIGN) structure on immature DCs. This interaction induces DC maturation and as a result production of IL-12[103, 104]. It is therefore tempting to propose that neutrophil CD11b/CD18 expression induced by necrotic epithelial cells is one of the steps that initiate an immune response.

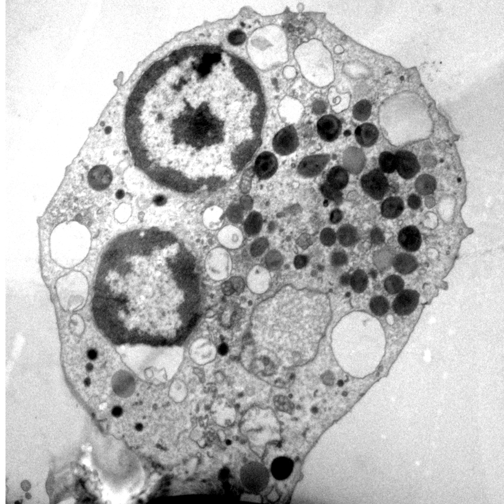

Fig. 8. *An eosinophil that has been incubated with freeze-pressed HT29 cells for 30 min in 37°C. The eosinophil is markedly affected and contains large empty vesicles. TEM picture, magnification ×5000 (A.-L. Stenfeldt, 2004).*

RECEPTORS INVOLVED IN DANGER SIGNALING

The huge variety of danger signalling molecules would suggest that many different receptors are involved. Conversely, the proposed main target for danger signals is a limited number of receptors within highly conserved receptor families like the toll-like receptor (TLR) and formyl peptide receptor family (FPR)[105]. These receptor families have developed during evolution and are expressed by different vertebrates (TLRs are also expressed in invertebrates and plants)[106, 107]. Both TLRs and FPRs bind molecules of extracellular as well as intracellular origin. The evolutionary reason for this is not known, but it is suggested that these receptors have evolved to protect hosts from microbes and viruses[3]. Mitochondria (intracellular organelles) are of bacterial origin and hence, proteins from this organelle also bind to these receptors. Others suggest that these receptors were produced to recognise damaged tissues and that microbes make use of these receptors for selfgain[90]. Irrespective of evolutionary background, the important thing is that receptors from both families are able to activate innate immune cells and initiate an immune response.

FORMYL PEPTIDE RECEPTORS

The human formyl peptide receptor family comprise three different receptors: the formyl peptide receptor (FPR), the formyl peptide receptor like 1 and 2 (FPRL1 and FPRL2). The FPR was first cloned in 1990 from differentiated HL-60 cells (myeloid leukemia cells) and shortly after both FPRL1 and FPRL2 were cloned by screening a cDNA library using FPR cDNA as a probe[108-110]. The three receptors possess a high degree of amino acid identity. It has been calculated that FPR/FPRL1 possess 69% identity, FPR/FPRL2 have 56% identity and FPRL1/FPRL2 are 83% identical at the amino acid level.

It was originally believed that only phagocytes expressed FPRs but these receptors are widely expressed by various cells of both hematopoietic and non-hematopoietic origin[111, 112]. The FPR has been observed in cells such as monocytes, immature DCs, neutrophils, eosinophils, platelets, fibroblasts, hepatocytes and endothelial cells. The FPRL1 is expressed for example by monocytes/macrophages, neutrophils, eosinophils, immature DCs, T- and B-cells, fibroblasts, epithelial cells and endothelial cells whereas FPRL2 has been observed in monocytes, macrophages, DCs, lung, liver and small intestine tissues. Human neutrophils and eosinophils express the FPR and FPRL1 but not the FPRL2[113, 114].

The FPRs belong to the seven transmembrane domain G-protein-coupled receptor (GPCR) family. Receptors within this family form loops, which span the membrane seven times and are intracellularly associated with a trimeric guanosine-triphosphate (GTP)-binding protein (G-protein), **Fig 9**. The receptor is orientated in the membrane with the N-terminus located extracellularly and the C-terminus within the cytosol. The C-terminus as well as other intracellular parts of the receptor contain binding sites for the heterotrimeric protein (G-protein). G-proteins are composed of three different polypeptide chains called α, β and γ[115, 116]. When an agonist binds to GPCR, the receptor changes conformation and alters the interaction with the G-protein. The altered interaction triggers the α-chain to hydrolyse GTP to guanosine-diphosphate (GDP) and dissociate from the βγ-complex. This in turn activates a cascade of downstream signalling molecules (referred to as second messengers) that leads to the release of Ca^{2+} from intracellular stores[117, 118]. The rise in cytosolic Ca^{2+} induces the opening of Ca^{2+}-channels in the plasma membrane, which results in a

sustained influx of Ca^{2+}. The high concentration of cytosolic Ca^{2+} activates Ca^{2+}-dependent proteins and starts the granulocyte functional repertoire[119-121]. The increase in cytosolic Ca^{2+} is one of the earliest detectable events in FPR-mediated neutrophil activation therefore Ca^{2+} measurement is often used as a tool for studying ligand-receptor interactions in granulocytes.

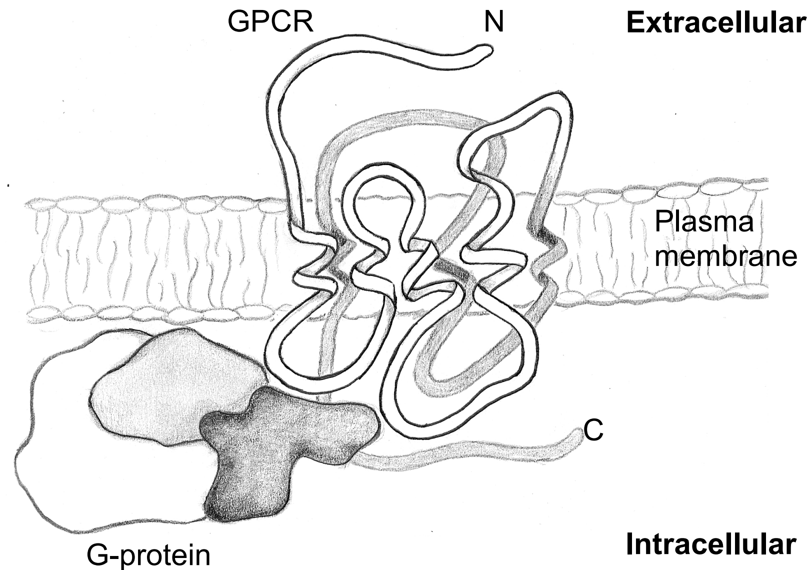

Fig. 9. *A model of a G-protein coupled receptor (GPCR). The protein spans the membrane with α-helical structures seven times. It is orientated in the membrane with the C-terminal end in the cytosol and the N-terminal facing the extracellular environment. In the cytosol a trimeric GTP-binding protein (G-protein) is in close contact with intracellular parts of the receptor.*

LIGAND RECEPTOR BINDING

The first described high affinity FPR ligand was a synthetic, trimeric, formylated peptide *N*-formyl-methionyl-leucyl-phenylalanine (fMLF). Thus, the biologically relevant agonists for this receptor are probably the *N*-formylated peptides formed in nature during bacterial and mitochondrial protein synthesis[122]. Results of binding studies with fMLF and its non-formylated counterpart led to the assumption that the formyl group was crucial for high affinity binding[123]. Later on, other studies revealed that the formyl group is less important than was first assumed. High affinity binding to FPR occurs with both non-

formylated peptides as well as peptides with no structural similarity to the prototype agonist fMLF[124, 125]. Low affinity ligands like Annexin 1 and its peptide fragments Ac2-12, Ac2-26 and Ac9-25 also bind to FPR[126, 127]

FPRL1 binds several non-formylated peptides with high affinity and formylated peptides with low affinity. The first described high affinity ligand for FPRL1 was the ecosanoid lipid, Lipoxin A_4 (LXA_4)[128]. LXA_4 is a potent anti-inflammatory molecule and in contrast to other FPRL1 ligands it inhibits neutrophil activity. Another FPRL1 specific agonist is the hexapeptide WKYMVM. It was identified by screening synthetic peptide libraries and initially shown to activate a human B-cell line[129]. Later on, it was shown that also neutrophils were activated by WKYMVM through the FPRL1[130]. It is of interest to note that one modification of the hexapeptide where the L-methionyl group (M) at the carboxy end of WKYMVM is replaced by a D-methionyl group (m) has broader receptor specificity[131]. Even though this peptide, WKYMVm, preferentially binds to the FPRL1, it also binds and activates granulocytes through FPR [132, 133].

The FPR and FPRL1 bind molecules from both endogenous and exogenous origin, **Table 1**. Although these receptors share a high degree of amino acid identity, the extracellular loops that are thought to be important for ligand binding have a lower degree of identity than other parts of the receptors[134]. This probably explains the difference in ligand specificity between FPR and FPRL1.

Even though FPR has been extensively studied for several years, no definite structure for FPR ligand binding has been identified. Studies with different chimera receptors have however led to the suggestion that the first, second and the third extracellular loop together with the second and fifth transmembrane regions are important for the formation of the ligand-binding pocket[135, 136]. The ligand-binding pocket is proposed to have a limited depth with room for as few as four to five amino acids[137]. FPRL1 ligand binding has also been studied using chimera receptors. According to this single study, the third extracellular loop and the sixth transmembrane region are the most important structures for agonist recognition[138].

Tabel 1. *Examples of FPR and FPRL1 ligands from both exogenous and endogenous origin.*

Receptor	Ligand	Origin	Reference
Human FPR	fMLF and analogues	Exogenous (bacteria)	[139]
	T20 (DP178)	Exogenous (HIV)	[140]
	T21 (DP107)	Exogenous (HIV)	[141]
	Formylated peptides	Endogenous (mitochondria)	[122]
	Cathepsin G	Endogenous (neutrophils)	[142]
	Ac9-25	Endogenous (Annexin 1)	[127]
	gG-2p20	Exogenous (HSV)	[143]
	WKYMVm	Synthetic peptide	[132, 144]
Human FPRL1	Hp2-20	Exogenous (bacteria)	[145]
	N36	Exogenous (HIV)	[146]
	F peptide	Exogenous (HIV)	[147]
	T21 (DP107)	Exogenous (HIV)	[141]
	SAA	Endogenous (serum)	[148]
	LL-37	Endogenous (neutrophil)	[149]
	WKYMVM	Synthetic peptide	[130]
	WKYMVm	Synthetic peptide	[150]
	MMK1	Synthetic peptide	[151]
	LxA_4	Endogenous	[128]
	PACAP27	Endogenous	[152]

Abbreviations and clarifications: Ac9-25, amino acid 9-25 in Annexin 1; fMLF, formyl-Met-Leu-Phe; F peptide, a 20 amino acid fragment of the HIV envelope protein gp120; gG-2p20, a peptide from herpes simplex virus type 2 glycoprotein g; Hp(2-20), 19-residues fragment of an *H. pylori* peptide; LL-37, a 37-residue fragment from cathelicidin; LxA_4, Lipoxin A_4, a lipid metabolite; MMK-1, Leu-Glu-Ser-Ile-Phe-Arg-Ser-Leu-Leu-Phe-Arg-Val-Met; N36, peptide domain on gp41 an HIV envelope protein; PACAP27, pituitary adenylate cyclase activating polypeptide, a neuropeptide; SAA, serum amyloid A, an acute phase protein; T20/T21, peptide domains of gp41 an HIV envelope protein; WKYMWM, Trp-Lys-Tyr-Met-Val-L-Met-NH_2; WKYMVm, Trp-Lys-Tyr-Met-Val-D-Met-NH_2.

RECEPTOR SPECIFIC ANTAGONISTS/INHIBITORS

The first specific antagonists reported to block FPR-mediated signals were the Boc-peptides, Boc-MLF (Boc1) and Boc-FLFLF (Boc2)[123]. They were developed in an attempt to determine the ligand-binding site in FPR. In both these peptides the formyl group is replaced by a tert-butoxycarbonyl (*t*Boc) at the N-terminus. The inhibitory effect achieved was, at that time, thought to be due to the loss of the formyl group, but probably it is due to the size and shape of the *t*Boc[153]. The *t*Boc is branched and hence bulky. If a small, unbranched, less bulky group replaces it, e.g. *n*-butyloxycarbonyl (*n*-Boc), the antagonistic effect is lost. The Boc-peptides specificity for FPR has later on been questioned and some have claimed that they also have an inhibitory effect on the FPRL1[154, 155]. However, the inhibitory effects of *t*Boc-peptides on neutrophil activity

reveal that they are fairly specific for FPR (Paper III). Cyclosporin (Cs) H, is another FPR specific antagonist, suggested to be the best choice for inhibition of FPR[156] (Paper III). CsH is a cyclic undecapeptide and an analog to CsA, a fungal peptide with T- and B-cell suppressing activity[157, 158]. CsH does not possess a lymphocyte inhibitory effect; its major immune suppressive effect seems to be the blocking of FPR.

The specific FPRL1 antagonist WRWWWW (WRW_4) was identified by screening hexapeptide libraries[159]. This is at present the only antagonist that specifically blocks signalling through FPRL1, (Paper III) [144]. The cell permeable rhodamine B-linked peptide (PBP10) derived from the polyphosphoinositide-binding region of the cytoskeleton protein gelsolin, has also been reported to inhibit cell activity mediated through FPRL1[160, 161]. The inhibitory effect achieved with PBP10 is due to intracellular interactions, but the exact inhibitory mechanism is not known.

Some natural inhibitors of FPR- and FPRL1-mediated activity have also been described. The bile-salts deoxycolic acid (DCA) and chenodeoxycholic acid (CDCA) as well as the bacteria derived proteins, chemotaxis inhibitory protein *Staphylococus aureus* (CHIPS) and FPRL1 inhibitory protein (FLIPr) have been reported to block FPR and FPRL1 ligand-binding[162, 163, 164, 165].

COX-INHIBITORS AND THEIR INTERACTION WITH FPRs

Non-steroidal inflammatory drugs (NSAIDs) are a group of drugs known for their anti-inflammatory and pain killing (analgesic) effect. These drugs are widely used to treat a variety of diseases ranging from the common headache to illnesses that cause chronic inflammation (e.g. rheumatoid arthritis). The use of NSAIDs goes back thousands of years in history when decoctions or preparation of plants containing salicylate were used to treat rheumatic pains[166]. Synthetic variants of salicylate were made at the end of 1850s and these were further developed into acetylsalicylic acid (aspirin) at the end of 1890s. Since then, several other drugs with antipyretic, anti-inflammatory and analgesic effects have been developed and produced. The main function for NSAIDs is thought to be the inhibition of the enzyme cyclooxygenase (COX)[167, 168]. This is a membrane bound

glycoprotein that is responsible for the formation of inflammatory mediators called prostanoids (prostaglandins, prostacyclin thromboxane). Since the beginning of the 1990s it has been known that at least two isoforms of COX exist, COX-1 and -2, but in recent years a third variant COX-3 has also been described[169, 170]. COX-1 is constitutively expressed in most cells whereas COX-2 is an inducible enzyme abundant in e.g. macrophages and neutrophils[171]. COX-3 on the other hand, has so far only been described in human brain tissues and the aorta[171].

Although NSAIDs share the same theraputic effect (antipyretic, anti-inflammatory and analgesic effect) as the original aspirin, they are a heterogeneous group of chemical compounds. Many NSAIDs have been synthesized in an attempt to minimize gastric damage, the most common side effect of these drugs. Accordingly the different NSAIDs have diverse selectivity for the three COX variants. The oxicams are one defined chemical group of NSAIDs, but even within this group there are differences in structure and function. For example meloxicam {4-hydroxy-2-methyl-N-(5-methyl-2-thiazolyl)-2H-1,2-benzothiazine-3-carboxamide 1,1-dioxide} and piroxicam {4-hydroxy-2-methyl-N-(pyridin-2-yl)-2H-1,2-benzothiazine-3-carboxymide 1,1-dioxide}have a similar "backbone" structure, but piroxicam has a pyridine attached to it while meloxicam has a methylated thiazol. This difference in structure makes meloxicam a potent COX-2 inhibitor and piroxicam the preferential inhibitor for COX-1 [172]. Piroxicam has been reported to affect neutrophil secretion and NADPH-oxidase activity in response to the FPR agonist fMLF, an effect achieved through blocking ligand receptor binding[173]. One report also suggests that piroxicam inhibits the G-protein GDP/GTP exchange, a function that would block not only FPR but GPCRs in general[174]. However, piroxicams effect on the neutrophil activity mediated by the FPRL1 ligand WKYMVM have been investigated (Paper IV). This study shows that piroxicam inhibits signalling through FPR but not through FPRL1 and an unrelated GPCR, namely C5aR.

CONCLUDING REMARKS

The danger theory introduced in the mid-1990s presented a new way of thinking about immune reactions and immune reactivity. The suggestion that tissue components released from damaged tissues may be the initiating factor for the triggering of an inflammatory reaction and immune response could be useful for a better understanding of the regulating mechanism behind the accumulation of immune cells in both health and disease. The precise role of eosinophils has baffled scientists for many years, since these cells accumulate in a variety of conditions with no direct common ground. In diseases eosinophils have been regarded as troublemakers that cause tissue destruction. The suggestion that these immune cells might be part of a response to damaged tissues could change how their role in inflammatory reactions are interpreted.

It still has to be determined which types of molecules are perceived as danger signals to immune competent cells. Presumably a large number of different molecules from damaged tissues orchestrate the immune response through different types of receptors. Much attention has been drawn to the pattern recognition receptors. These receptors have developed during evolution and bind molecules from both exogenous and endogenous origin and therefore it is tempting to believe that these receptors are the main target for danger signals.

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REFERENCES

1. Beutler, B. (2004) Innate immunity: an overview. *Mol Immunol* **40**, 845-59.
2. Eckmann, L., Kagnoff, M.F., Fierer, J. (1995) Intestinal epithelial cells as watchdogs for the natural immune system. *Trends Microbiol* **3**, 118-20.
3. Janeway, C.A., Jr. (1992) The immune system evolved to discriminate infectious nonself from noninfectious self. *Immunol Today* **13**, 11-6.
4. Schruefer, R., Lutze, N., Schymeinsky, J., Walzog, B. (2005) Human neutrophils promote angiogenesis by a paracrine feedforward mechanism involving endothelial interleukin-8. *Am J Physiol Heart Circ Physiol* **288**, H1186-92.
5. McCourt, M., Wang, J.H., Sookhai, S., Redmond, H.P. (1999) Proinflammatory mediators stimulate neutrophil-directed angiogenesis. *Arch Surg* **134**, 1325-31; discussion 1331-2.
6. Silverstein, A.M. (1979) History of immunology. Cellular versus humoral immunity: determinants and consequences of an epic 19th century battle. *Cell Immunol* **48**, 208-21.
7. Ehrlich, P., Lazarus, A. (1898) Die anämie. In *Specielle Pathologie und therapie* (N. H, ed) Hölder, Vienna.
8. Bainton, D.F., Ullyot, J.L., Farquhar, M.G. (1971) The development of neutrophilic polymorphonuclear leukocytes in human bone marrow. *J Exp Med* **134**, 907-34.
9. Bainton, D.F., Farquhar, M.G. (1970) Segregation and packaging of granule enzymes in eosinophilic leukocytes. *J Cell Biol* **45**, 54-73.
10. Cartwright, G.E., Athens, J.W., Wintrobe, M.M. (1964) The Kinetics of Granulopoiesis in Normal Man. *Blood* **24**, 780-803.
11. Steinbach, K.H., Schick, P., Trepel, F., Raffler, H., Dohrmann, J., Heilgeist, G., Heltzel, W., Li, K., Past, W., van der Woerd-de Lange, J.A., Theml, H., Fliedner, T.M., Begemann, H. (1979) Estimation of kinetic parameters of neutrophilic, eosinophilic, and basophilic granulocytes in human blood. *Blut* **39**, 27-38.
12. Savill, J.S., Wyllie, A.H., Henson, J.E., Walport, M.J., Henson, P.M., Haslett, C. (1989) Macrophage phagocytosis of aging neutrophils in inflammation. Programmed cell death in the neutrophil leads to its recognition by macrophages. *J Clin Invest* **83**, 865-75.
13. Cassatella, M.A. (1995) The production of cytokines by polymorphonuclear neutrophils. *Immunol Today* **16**, 21-6.
14. Kjeldsen, L., Bainton, D.F., Sengelov, H., Borregaard, N. (1993) Structural and functional heterogeneity among peroxidase-negative granules in human neutrophils: identification of a distinct gelatinase-containing granule subset by combined immunocytochemistry and subcellular fractionation. *Blood* **82**, 3183-91.
15. Dewald, B., Bretz, U., Baggiolini, M. (1982) Release of gelatinase from a novel secretory compartment of human neutrophils. *J Clin Invest* **70**, 518-25.
16. Borregaard, N., Christensen, L., Bejerrum, O.W., Birgens, H.S., Clemmensen, I. (1990) Identification of a highly mobilizable subset of human neutrophil intracellular vesicles that contains tetranectin and latent alkaline phosphatase. *J Clin Invest* **85**, 408-16.
17. Indik, Z.K., Park, J.G., Hunter, S., Schreiber, A.D. (1995) Structure/function relationships of Fc gamma receptors in phagocytosis. *Semin Immunol* **7**, 45-54.

18. Bylund, J., Christophe, T., Boulay, F., Nystrom, T., Karlsson, A., Dahlgren, C. (2001) Proinflammatory activity of a cecropin-like antibacterial peptide from Helicobacter pylori. *Antimicrob Agents Chemother* **45**, 1700-4.
19. Parker, L.C., Whyte, M.K., Dower, S.K., Sabroe, I. (2005) The expression and roles of Toll-like receptors in the biology of the human neutrophil. *J Leukoc Biol* **77**, 886-92.
20. Kato, M., Kephart, G.M., Morikawa, A., Gleich, G.J. (2001) Eosinophil infiltration and degranulation in normal human tissues: evidence for eosinophil degranulation in normal gastrointestinal tract. *Int Arch Allergy Immunol* **125 Suppl 1**, 55-8.
21. Sokol, R.J., James, N.T., Wales, J., Hudson, G. (1987) Morphometry of eosinophils in human blood. *Acta Anat (Basel)* **129**, 211-3.
22. Leonidas, D.D., Elbert, B.L., Zhou, Z., Leffler, H., Ackerman, S.J., Acharya, K.R. (1995) Crystal structure of human Charcot-Leyden crystal protein, an eosinophil lysophospholipase, identifies it as a new member of the carbohydrate-binding family of galectins. *Structure* **3**, 1379-93.
23. Dvorak, A.M., Letourneau, L., Login, G.R., Weller, P.F., Ackerman, S.J. (1988) Ultrastructural localization of the Charcot-Leyden crystal protein (lysophospholipase) to a distinct crystalloid-free granule population in mature human eosinophils. *Blood* **72**, 150-8.
24. Dyer, K.D., Rosenberg, H.F. (1996) Eosinophil Charcot-Leyden crystal protein binds to beta-galactoside sugars. *Life Sci* **58**, 2073-82.
25. Egesten, A., Alumets, J., von Mecklenburg, C., Palmegren, M., Olsson, I. (1986) Localization of eosinophil cationic protein, major basic protein, and eosinophil peroxidase in human eosinophils by immunoelectron microscopic technique. *J Histochem Cytochem* **34**, 1399-403.
26. Peters, M.S., Rodriguez, M., Gleich, G.J. (1986) Localization of human eosinophil granule major basic protein, eosinophil cationic protein, and eosinophil-derived neurotoxin by immunoelectron microscopy. *Lab Invest* **54**, 656-62.
27. Parmley, R.T., Spicer, S.S. (1974) Cytochemical and ultrastructural identification of a small type granule in human late eosinophils. *Lab Invest* **30**, 557-67.
28. Schaefer, H.E., Hubner, G., Fischer, R. (1973) [Specific microgranules in eosinophils. A comparative electronmicroscopic study on various mammals for the characterization of a special form of granulation in eosinophil granulocytes]. *Acta Haematol* **50**, 92-104.
29. Calafat, J., Kuijpers, T.W., Janssen, H., Borregaard, N., Verhoeven, A.J., Roos, D. (1993) Evidence for small intracellular vesicles in human blood phagocytes containing cytochrome b558 and the adhesion molecule CD11b/CD18. *Blood* **81**, 3122-9.
30. Capron, M., Capron, A., Dessaint, J.P., Torpier, G., Johansson, S.G., Prin, L. (1981) Fc receptors for IgE on human and rat eosinophils. *J Immunol* **126**, 2087-92.
31. Capron, M., Tomassini, M., Van der Vorst, E., Kusnierz, J.P., Papin, J.P., Capron, A. (1988) [Existence and function of a receptor for immunoglobulin A on human eosinophils]. *C R Acad Sci III* **307**, 397-402.
32. Fischer, E., Capron, M., Prin, L., Kusnierz, J.P., Kazatchkine, M.D. (1986) Human eosinophils express CR1 and CR3 complement receptors for cleavage fragments of C3. *Cell Immunol* **97**, 297-306.

33. Hartnell, A., Moqbel, R., Walsh, G.M., Bradley, B., Kay, A.B. (1990) Fc gamma and CD11/CD18 receptor expression on normal density and low density human eosinophils. *Immunology* **69**, 264-70.
34. Hansel, T.T., Pound, J.D., Pilling, D., Kitas, G.D., Salmon, M., Gentle, T.A., Lee, S.S., Thompson, R.A. (1989) Purification of human blood eosinophils by negative selection using immunomagnetic beads. *J Immunol Methods* **122**, 97-103.
35. Gibbs, B.F. (2005) Human basophils as effectors and immunomodulators of allergic inflammation and innate immunity. *Clin Exp Med* **5**, 43-9.
36. Dvorak, A.M. (2005) Ultrastructural studies of human basophils and mast cells. *J Histochem Cytochem* **53**, 1043-70.
37. Dvorak, A.M., Ackerman, S.J. (1989) Ultrastructural localization of the Charcot-Leyden crystal protein (lysophospholipase) to granules and intragranular crystals in mature human basophils. *Lab Invest* **60**, 557-67.
38. Dvorak, A.M., MacGlashan, D.W., Jr., Morgan, E.S., Lichtenstein, L.M. (1995) Histamine distribution in human basophil secretory granules undergoing FMLP-stimulated secretion and recovery. *Blood* **86**, 3560-6.
39. Ackerman, S.J., Kephart, G.M., Habermann, T.M., Greipp, P.R., Gleich, G.J. (1983) Localization of eosinophil granule major basic protein in human basophils. *J Exp Med* **158**, 946-61.
40. Ochensberger, B., Daepp, G.C., Rihs, S., Dahinden, C.A. (1996) Human blood basophils produce interleukin-13 in response to IgE-receptor-dependent and -independent activation. *Blood* **88**, 3028-37.
41. Schroeder, J.T., MacGlashan, D.W., Jr., Kagey-Sobotka, A., White, J.M., Lichtenstein, L.M. (1994) IgE-dependent IL-4 secretion by human basophils. The relationship between cytokine production and histamine release in mixed leukocyte cultures. *J Immunol* **153**, 1808-17.
42. Moore, K.L., Stults, N.L., Diaz, S., Smith, D.F., Cummings, R.D., Varki, A., McEver, R.P. (1992) Identification of a specific glycoprotein ligand for P-selectin (CD62) on myeloid cells. *J Cell Biol* **118**, 445-56.
43. Sriramarao, P., von Andrian, U.H., Butcher, E.C., Bourdon, M.A., Broide, D.H. (1994) L-selectin and very late antigen-4 integrin promote eosinophil rolling at physiological shear rates in vivo. *J Immunol* **153**, 4238-46.
44. Kitayama, J., Fuhlbrigge, R.C., Puri, K.D., Springer, T.A. (1997) P-selectin, L-selectin, and alpha 4 integrin have distinct roles in eosinophil tethering and arrest on vascular endothelial cells under physiological flow conditions. *J Immunol* **159**, 3929-39.
45. Patel, K.D., McEver, R.P. (1997) Comparison of tethering and rolling of eosinophils and neutrophils through selectins and P-selectin glycoprotein ligand-1. *J Immunol* **159**, 4555-65.
46. Conran, N., Gambero, A., Ferreira, H.H., Antunes, E., de Nucci, G. (2003) Nitric oxide has a role in regulating VLA-4-integrin expression on the human neutrophil cell surface. *Biochem Pharmacol* **66**, 43-50.
47. Worthylake, R.A., Burridge, K. (2001) Leukocyte transendothelial migration: orchestrating the underlying molecular machinery. *Curr Opin Cell Biol* **13**, 569-77.
48. Carman, C.V., Springer, T.A. (2004) A transmigratory cup in leukocyte diapedesis both through individual vascular endothelial cells and between them. *J Cell Biol* **167**, 377-88.

49. Feng, D., Nagy, J.A., Pyne, K., Dvorak, H.F., Dvorak, A.M. (1998) Neutrophils emigrate from venules by a transendothelial cell pathway in response to FMLP. *J Exp Med* **187**, 903-15.
50. Hoshi, O., Ushiki, T. (1999) Scanning electron microscopic studies on the route of neutrophil extravasation in the mouse after exposure to the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP). *Arch Histol Cytol* **62**, 253-60.
51. Shaw, S.K., Bamba, P.S., Perkins, B.N., Luscinskas, F.W. (2001) Real-time imaging of vascular endothelial-cadherin during leukocyte transmigration across endothelium. *J Immunol* **167**, 2323-30.
52. Cepinskas, G., Noseworthy, R., Kvietys, P.R. (1997) Transendothelial neutrophil migration. Role of neutrophil-derived proteases and relationship to transendothelial protein movement. *Circ Res* **81**, 618-26.
53. Yang, L., Froio, R.M., Sciuto, T.E., Dvorak, A.M., Alon, R., Luscinskas, F.W. (2005) ICAM-1 regulates neutrophil adhesion and transcellular migration of TNF-alpha-activated vascular endothelium under flow. *Blood* **106**, 584-92.
54. Lou, O., Alcaide, P., Luscinskas, F.W., Muller, W.A. (2007) CD99 is a key mediator of the transendothelial migration of neutrophils. *J Immunol* **178**, 1136-43.
55. Shaw, S.K., Ma, S., Kim, M.B., Rao, R.M., Hartman, C.U., Froio, R.M., Yang, L., Jones, T., Liu, Y., Nusrat, A., Parkos, C.A., Luscinskas, F.W. (2004) Coordinated redistribution of leukocyte LFA-1 and endothelial cell ICAM-1 accompany neutrophil transmigration. *J Exp Med* **200**, 1571-80.
56. Ebisawa, M., Bochner, B.S., Georas, S.N., Schleimer, R.P. (1992) Eosinophil transendothelial migration induced by cytokines. I. Role of endothelial and eosinophil adhesion molecules in IL-1 beta-induced transendothelial migration. *J Immunol* **149**, 4021-8.
57. Devreotes, P.N., Zigmond, S.H. (1988) Chemotaxis in eukaryotic cells: a focus on leukocytes and Dictyostelium. *Annu Rev Cell Biol* **4**, 649-86.
58. Wu, D. (2005) Signaling mechanisms for regulation of chemotaxis. *Cell Res* **15**, 52-6.
59. Bagorda, A., Mihaylov, V.A., Parent, C.A. (2006) Chemotaxis: moving forward and holding on to the past. *Thromb Haemost* **95**, 12-21.
60. Cicchetti, G., Allen, P.G., Glogauer, M. (2002) Chemotactic signaling pathways in neutrophils: from receptor to actin assembly. *Crit Rev Oral Biol Med* **13**, 220-8.
61. Ferro-Novick, S., Jahn, R. (1994) Vesicle fusion from yeast to man. *Nature* **370**, 191-3.
62. Denesvre, C., Malhotra, V. (1996) Membrane fusion in organelle biogenesis. *Curr Opin Cell Biol* **8**, 519-23.
63. Mellman, I. (1995) Enigma variations: protein mediators of membrane fusion. *Cell* **82**, 869-72.
64. Dvorak, A.M., Furitsu, T., Letourneau, L., Ishizaka, T., Ackerman, S.J. (1991) Mature eosinophils stimulated to develop in human cord blood mononuclear cell cultures supplemented with recombinant human interleukin-5. Part I. Piecemeal degranulation of specific granules and distribution of Charcot-Leyden crystal protein. *Am J Pathol* **138**, 69-82.
65. Cheng, J.F., Ott, N.L., Peterson, E.A., George, T.J., Hukee, M.J., Gleich, G.J., Leiferman, K.M. (1997) Dermal eosinophils in atopic dermatitis undergo cytolytic degeneration. *J Allergy Clin Immunol* **99**, 683-92.

66. Karawajczyk, M., Seveus, L., Garcia, R., Bjornsson, E., Peterson, C.G., Roomans, G.M., Venge, P. (2000) Piecemeal degranulation of peripheral blood eosinophils: a study of allergic subjects during and out of the pollen season. *Am J Respir Cell Mol Biol* **23**, 521-9.
67. Erjefalt, J.S., Greiff, L., Andersson, M., Matsson, E., Petersen, H., Linden, M., Ansari, T., Jeffery, P.K., Persson, C.G. (1999) Allergen-induced eosinophil cytolysis is a primary mechanism for granule protein release in human upper airways. *Am J Respir Crit Care Med* **160**, 304-12.
68. Sengelov, H., Follin, P., Kjeldsen, L., Lollike, K., Dahlgren, C., Borregaard, N. (1995) Mobilization of granules and secretory vesicles during in vivo exudation of human neutrophils. *J Immunol* **154**, 4157-65.
69. Sengelov, H., Kjeldsen, L., Borregaard, N. (1993) Control of exocytosis in early neutrophil activation. *J Immunol* **150**, 1535-43.
70. Borregaard, N., Cowland, J.B. (1997) Granules of the human neutrophilic polymorphonuclear leukocyte. *Blood* **89**, 3503-21.
71. Borregaard, N., Heiple, J.M., Simons, E.R., Clark, R.A. (1983) Subcellular localization of the b-cytochrome component of the human neutrophil microbicidal oxidase: translocation during activation. *J Cell Biol* **97**, 52-61.
72. Sbarra, A.J., Karnovsky, M.L. (1959) The biochemical basis of phagocytosis. I. Metabolic changes during the ingestion of particles by polymorphonuclear leukocytes. *J Biol Chem* **234**, 1355-62.
73. Fuchs, A., Dagher, M.C., Faure, J., Vignais, P.V. (1996) Topological organization of the cytosolic activating complex of the superoxide-generating NADPH-oxidase. Pinpointing the sites of interaction between p47phoz, p67phox and p40phox using the two-hybrid system. *Biochim Biophys Acta* **1312**, 39-47.
74. Lapouge, K., Smith, S.J., Groemping, Y., Rittinger, K. (2002) Architecture of the p40-p47-p67phox complex in the resting state of the NADPH oxidase. A central role for p67phox. *J Biol Chem* **277**, 10121-8.
75. Rotrosen, D., Yeung, C.L., Leto, T.L., Malech, H.L., Kwong, C.H. (1992) Cytochrome b558: the flavin-binding component of the phagocyte NADPH oxidase. *Science* **256**, 1459-62.
76. Ambruso, D.R., Cusack, N., Thurman, G. (2004) NADPH oxidase activity of neutrophil specific granules: requirements for cytosolic components and evidence of assembly during cell activation. *Mol Genet Metab* **81**, 313-21.
77. Segal, A.W., Jones, O.T. (1979) The subcellular distribution and some properties of the cytochrome b component of the microbicidal oxidase system of human neutrophils. *Biochem J* **182**, 181-8.
78. Wientjes, F.B., Segal, A.W., Hartwig, J.H. (1997) Immunoelectron microscopy shows a clustered distribution of NADPH oxidase components in the human neutrophil plasma membrane. *J Leukoc Biol* **61**, 303-12.
79. Borregaard, N. (1988) Subcellular localization and dynamics of components of the respiratory burst oxidase. *J Bioenerg Biomembr* **20**, 637-51.
80. Dahlgren, C., Follin, P. (1990) Degranulation in human neutrophils primes the cells for subsequent responsiveness to the chemoattractant N-formylmethionylleucylphenylalanine but does not increase the sensitivity of the NADPH-oxidase to an intracellular calcium rise. *Biochim Biophys Acta* **1052**, 42-6.
81. Petreccia, D.C., Nauseef, W.M., Clark, R.A. (1987) Respiratory burst of normal human eosinophils. *J Leukoc Biol* **41**, 283-8.

82. Sedgwick, J.B., Vrtis, R.F., Gourley, M.F., Busse, W.W. (1988) Stimulus-dependent differences in superoxide anion generation by normal human eosinophils and neutrophils. *J Allergy Clin Immunol* **81**, 876-83.
83. Someya, A., Nishijima, K., Nunoi, H., Irie, S., Nagaoka, I. (1997) Study on the superoxide-producing enzyme of eosinophils and neutrophils--comparison of the NADPH oxidase components. *Arch Biochem Biophys* **345**, 207-13.
84. Reeves, E.P., Lu, H., Jacobs, H.L., Messina, C.G., Bolsover, S., Gabella, G., Potma, E.O., Warley, A., Roes, J., Segal, A.W. (2002) Killing activity of neutrophils is mediated through activation of proteases by K+ flux. *Nature* **416**, 291-7.
85. Touyz, R.M. (2005) Reactive oxygen species as mediators of calcium signaling by angiotensin II: implications in vascular physiology and pathophysiology. *Antioxid Redox Signal* **7**, 1302-14.
86. Heneberg, P., Draber, P. (2005) Regulation of cys-based protein tyrosine phosphatases via reactive oxygen and nitrogen species in mast cells and basophils. *Curr Med Chem* **12**, 1859-71.
87. Lederberg, J. (1959) Genes and antibodies. *Science* **129**, 1649-53.
88. Bretscher, P., Cohn, M. (1970) A theory of self-nonself discrimination. *Science* **169**, 1042-9.
89. Janeway, C.A., Jr. (1989) Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb Symp Quant Biol* **54 Pt 1**, 1-13.
90. Matzinger, P. (1994) Tolerance, danger, and the extended family. *Annu Rev Immunol* **12**, 991-1045.
91. Hirsch, J.H., B. (1980) Paul Ehrlich and the discovery of the eosinophil. In *The Eosinophil in Health and Disease* (A. K. Mahmoud AAF, ed) Grune & Stratton, New York 3-23.
92. Rovere-Querini, P., Capobianco, A., Scaffidi, P., Valentinis, B., Catalanotti, F., Giazzon, M., Dumitriu, I.E., Muller, S., Iannacone, M., Traversari, C., Bianchi, M.E., Manfredi, A.A. (2004) HMGB1 is an endogenous immune adjuvant released by necrotic cells. *EMBO Rep* **5**, 825-30.
93. Chen, W., Syldath, U., Bellmann, K., Burkart, V., Kolb, H. (1999) Human 60-kDa heat-shock protein: a danger signal to the innate immune system. *J Immunol* **162**, 3212-9.
94. Vabulas, R.M., Ahmad-Nejad, P., Ghose, S., Kirschning, C.J., Issels, R.D., Wagner, H. (2002) HSP70 as endogenous stimulus of the Toll/interleukin-1 receptor signal pathway. *J Biol Chem* **277**, 15107-12.
95. Shi, Y., Evans, J.E., Rock, K.L. (2003) Molecular identification of a danger signal that alerts the immune system to dying cells. *Nature* **425**, 516-21.
96. Trulson, A., Bystrom, J., Engstrom, A., Larsson, R., Venge, P. (2007) The functional heterogeneity of eosinophil cationic protein is determined by a gene polymorphism and post-translational modifications. *Clin Exp Allergy* **37**, 208-18.
97. David, J.R., Butterworth, A.E., Vadas, M.A. (1980) Mechanism of the interaction mediating killing of Schistosoma mansoni by human eosinophils. *Am J Trop Med Hyg* **29**, 842-8.
98. Cormier, S.A., Taranova, A.G., Bedient, C., Nguyen, T., Protheroe, C., Pero, R., Dimina, D., Ochkur, S.I., O'Neill, K., Colbert, D., Lombari, T.R., Constant, S., McGarry, M.P., Lee, J.J., Lee, N.A. (2006) Pivotal Advance: eosinophil infiltration of solid tumors is an early and persistent inflammatory host response. *J Leukoc Biol* **79**, 1131-9.

99. Watson, R.W., Rotstein, O.D., Nathens, A.B., Parodo, J., Marshall, J.C. (1997) Neutrophil apoptosis is modulated by endothelial transmigration and adhesion molecule engagement. *J Immunol* **158**, 945-53.
100. Yan, S.R., Sapru, K., Issekutz, A.C. (2004) The CD11/CD18 (beta2) integrins modulate neutrophil caspase activation and survival following TNF-alpha or endotoxin induced transendothelial migration. *Immunol Cell Biol* **82**, 435-46.
101. Coxon, A., Rieu, P., Barkalow, F.J., Askari, S., Sharpe, A.H., von Andrian, U.H., Arnaout, M.A., Mayadas, T.N. (1996) A novel role for the beta 2 integrin CD11b/CD18 in neutrophil apoptosis: a homeostatic mechanism in inflammation. *Immunity* **5**, 653-66.
102. Wright, S.D., Levin, S.M., Jong, M.T., Chad, Z., Kabbash, L.G. (1989) CR3 (CD11b/CD18) expresses one binding site for Arg-Gly-Asp-containing peptides and a second site for bacterial lipopolysaccharide. *J Exp Med* **169**, 175-83.
103. van Gisbergen, K.P., Sanchez-Hernandez, M., Geijtenbeek, T.B., van Kooyk, Y. (2005) Neutrophils mediate immune modulation of dendritic cells through glycosylation-dependent interactions between Mac-1 and DC-SIGN. *J Exp Med* **201**, 1281-92.
104. Megiovanni, A.M., Sanchez, F., Robledo-Sarmiento, M., Morel, C., Gluckman, J.C., Boudaly, S. (2006) Polymorphonuclear neutrophils deliver activation signals and antigenic molecules to dendritic cells: a new link between leukocytes upstream of T lymphocytes. *J Leukoc Biol* **79**, 977-88.
105. Matzinger, P. (2002) The danger model: a renewed sense of self. *Science* **296**, 301-5.
106. Alvarez, V., Coto, E., Setien, F., Gonzalez-Roces, S., Lopez-Larrea, C. (1996) Molecular evolution of the N-formyl peptide and C5a receptors in non-human primates. *Immunogenetics* **44**, 446-52.
107. Beutler, B., Rehli, M. (2002) Evolution of the TIR, tolls and TLRs: functional inferences from computational biology. *Curr Top Microbiol Immunol* **270**, 1-21.
108. Ye, R.D., Cavanagh, S.L., Quehenberger, O., Prossnitz, E.R., Cochrane, C.G. (1992) Isolation of a cDNA that encodes a novel granulocyte N-formyl peptide receptor. *Biochem Biophys Res Commun* **184**, 582-9.
109. Bao, L., Gerard, N.P., Eddy, R.L., Jr., Shows, T.B., Gerard, C. (1992) Mapping of genes for the human C5a receptor (C5AR), human FMLP receptor (FPR), and two FMLP receptor homologue orphan receptors (FPRH1, FPRH2) to chromosome 19. *Genomics* **13**, 437-40.
110. Murphy, P.M., Ozcelik, T., Kenney, R.T., Tiffany, H.L., McDermott, D., Francke, U. (1992) A structural homologue of the N-formyl peptide receptor. Characterization and chromosome mapping of a peptide chemoattractant receptor family. *J Biol Chem* **267**, 7637-43.
111. Becker, E.L., Forouhar, F.A., Grunnet, M.L., Boulay, F., Tardif, M., Bormann, B.J., Sodja, D., Ye, R.D., Woska, J.R., Jr., Murphy, P.M. (1998) Broad immunocytochemical localization of the formylpeptide receptor in human organs, tissues, and cells. *Cell Tissue Res* **292**, 129-35.
112. Migeotte, I., Communi, D., Parmentier, M. (2006) Formyl peptide receptors: a promiscuous subfamily of G protein-coupled receptors controlling immune responses. *Cytokine Growth Factor Rev* **17**, 501-19.

113. Durstin, M., Gao, J.L., Tiffany, H.L., McDermott, D., Murphy, P.M. (1994) Differential expression of members of the N-formylpeptide receptor gene cluster in human phagocytes. *Biochem Biophys Res Commun* **201**, 174-9.
114. Svensson, L., Redvall, E., Bjorn, C., Karlsson, J., Bergin, A.M., Rabiet, M.J., Dahlgren, C., Wenneras, C. (2007) House dust mite allergen activates human eosinophils via formyl peptide receptor and formyl peptide receptor-like 1. *Eur J Immunol* **37**, 1966-77.
115. Gilman, A.G. (1987) G proteins: transducers of receptor-generated signals. *Annu Rev Biochem* **56**, 615-49.
116. Birnbaumer, L., Abramowitz, J., Brown, A.M. (1990) Receptor-effector coupling by G proteins. *Biochim Biophys Acta* **1031**, 163-224.
117. Camps, M., Carozzi, A., Schnabel, P., Scheer, A., Parker, P.J., Gierschik, P. (1992) Isozyme-selective stimulation of phospholipase C-beta 2 by G protein beta gamma-subunits. *Nature* **360**, 684-6.
118. Jiang, H., Kuang, Y., Wu, Y., Smrcka, A., Simon, M.I., Wu, D. (1996) Pertussis toxin-sensitive activation of phospholipase C by the C5a and fMet-Leu-Phe receptors. *J Biol Chem* **271**, 13430-4.
119. Kelher, M.R., Ambruso, D.R., Elzi, D.J., Anderson, S.M., Paterson, A.J., Thurman, G.W., Silliman, C.C. (2003) Formyl-Met-Leu-Phe induces calcium-dependent tyrosine phosphorylation of Rel-1 in neutrophils. *Cell Calcium* **34**, 445-55.
120. Andrews, P.C., Babior, B.M. (1984) Phosphorylation of cytosolic proteins by resting and activated human neutrophils. *Blood* **64**, 883-90.
121. Khalfi, F., Gressier, B., Brunet, C., Dine, T., Luyckx, M., Cazin, M., Cazin, J.C. (1996) Involvement of the extracellular calcium in the release of elastase and the human neutrophils oxidative burst. *Cell Mol Biol (Noisy-le-grand)* **42**, 1211-8.
122. Rabiet, M.J., Huet, E., Boulay, F. (2005) Human mitochondria-derived N-formylated peptides are novel agonists equally active on FPR and FPRL1, while Listeria monocytogenes-derived peptides preferentially activate FPR. *Eur J Immunol* **35**, 2486-95.
123. Freer, R.J., Day, A.R., Radding, J.A., Schiffmann, E., Aswanikumar, S., Showell, H.J., Becker, E.L. (1980) Further studies on the structural requirements for synthetic peptide chemoattractants. *Biochemistry* **19**, 2404-10.
124. Gao, J.L., Becker, E.L., Freer, R.J., Muthukumaraswamy, N., Murphy, P.M. (1994) A high potency nonformylated peptide agonist for the phagocyte N-formylpeptide chemotactic receptor. *J Exp Med* **180**, 2191-7.
125. Chen, J., Bernstein, H.S., Chen, M., Wang, L., Ishii, M., Turck, C.W., Coughlin, S.R. (1995) Tethered ligand library for discovery of peptide agonists. *J Biol Chem* **270**, 23398-401.
126. Walther, A., Riehemann, K., Gerke, V. (2000) A novel ligand of the formyl peptide receptor: annexin I regulates neutrophil extravasation by interacting with the FPR. *Mol Cell* **5**, 831-40.
127. Karlsson, J., Fu, H., Boulay, F., Dahlgren, C., Hellstrand, K., Movitz, C. (2005) Neutrophil NADPH-oxidase activation by an annexin AI peptide is transduced by the formyl peptide receptor (FPR), whereas an inhibitory signal is generated independently of the FPR family receptors. *J Leukoc Biol* **78**, 762-71.
128. Fiore, S., Maddox, J.F., Perez, H.D., Serhan, C.N. (1994) Identification of a human cDNA encoding a functional high affinity lipoxin A4 receptor. *J Exp Med* **180**, 253-60.

129. Baek, S.H., Seo, J.K., Chae, C.B., Suh, P.G., Ryu, S.H. (1996) Identification of the peptides that stimulate the phosphoinositide hydrolysis in lymphocyte cell lines from peptide libraries. *J Biol Chem* **271**, 8170-5.
130. Christophe, T., Karlsson, A., Dugave, C., Rabiet, M.J., Boulay, F., Dahlgren, C. (2001) The synthetic peptide Trp-Lys-Tyr-Met-Val-Met-NH2 specifically activates neutrophils through FPRL1/lipoxin A4 receptors and is an agonist for the orphan monocyte-expressed chemoattractant receptor FPRL2. *J Biol Chem* **276**, 21585-93.
131. Seo, J.K., Choi, S.Y., Kim, Y., Baek, S.H., Kim, K.T., Chae, C.B., Lambeth, J.D., Suh, P.G., Ryu, S.H. (1997) A peptide with unique receptor specificity: stimulation of phosphoinositide hydrolysis and induction of superoxide generation in human neutrophils. *J Immunol* **158**, 1895-901.
132. Le, Y., Gong, W., Li, B., Dunlop, N.M., Shen, W., Su, S.B., Ye, R.D., Wang, J.M. (1999) Utilization of two seven-transmembrane, G protein-coupled receptors, formyl peptide receptor-like 1 and formyl peptide receptor, by the synthetic hexapeptide WKYMVm for human phagocyte activation. *J Immunol* **163**, 6777-84.
133. Dahlgren, C., Christophe, T., Boulay, F., Madianos, P.N., Rabiet, M.J., Karlsson, A. (2000) The synthetic chemoattractant Trp-Lys-Tyr-Met-Val-DMet activates neutrophils preferentially through the lipoxin A(4) receptor. *Blood* **95**, 1810-8.
134. Sahagun-Ruiz, A., Colla, J.S., Juhn, J., Gao, J.L., Murphy, P.M., McDermott, D.H. (2001) Contrasting evolution of the human leukocyte N-formylpeptide receptor subtypes FPR and FPRL1R. *Genes Immun* **2**, 335-42.
135. Perez, H.D., Holmes, R., Vilander, L.R., Adams, R.R., Manzana, W., Jolley, D., Andrews, W.H. (1993) Formyl peptide receptor chimeras define domains involved in ligand binding. *J Biol Chem* **268**, 2292-5.
136. Quehenberger, O., Prossnitz, E.R., Cavanagh, S.L., Cochrane, C.G., Ye, R.D. (1993) Multiple domains of the N-formyl peptide receptor are required for high-affinity ligand binding. Construction and analysis of chimeric N-formyl peptide receptors. *J Biol Chem* **268**, 18167-75.
137. Sklar, L.A., Fay, S.P., Seligmann, B.E., Freer, R.J., Muthukumaraswamy, N., Mueller, H. (1990) Fluorescence analysis of the size of a binding pocket of a peptide receptor at natural abundance. *Biochemistry* **29**, 313-6.
138. Le, Y., Ye, R.D., Gong, W., Li, J., Iribarren, P., Wang, J.M. (2005) Identification of functional domains in the formyl peptide receptor-like 1 for agonist-induced cell chemotaxis. *Febs J* **272**, 769-78.
139. Schiffmann, E., Corcoran, B.A., Wahl, S.M. (1975) N-formylmethionyl peptides as chemoattractants for leucocytes. *Proc Natl Acad Sci U S A* **72**, 1059-62.
140. Su, S.B., Gong, W.H., Gao, J.L., Shen, W.P., Grimm, M.C., Deng, X., Murphy, P.M., Oppenheim, J.J., Wang, J.M. (1999) T20/DP178, an ectodomain peptide of human immunodeficiency virus type 1 gp41, is an activator of human phagocyte N-formyl peptide receptor. *Blood* **93**, 3885-92.
141. Su, S.B., Gao, J., Gong, W., Dunlop, N.M., Murphy, P.M., Oppenheim, J.J., Wang, J.M. (1999) T21/DP107, A synthetic leucine zipper-like domain of the HIV-1 envelope gp41, attracts and activates human phagocytes by using G-protein-coupled formyl peptide receptors. *J Immunol* **162**, 5924-30.
142. Sun, R., Iribarren, P., Zhang, N., Zhou, Y., Gong, W., Cho, E.H., Lockett, S., Chertov, O., Bednar, F., Rogers, T.J., Oppenheim, J.J., Wang, J.M. (2004) Identification of neutrophil granule protein cathepsin G as a novel chemotactic agonist for the G protein-coupled formyl peptide receptor. *J Immunol* **173**, 428-36.

143. Bellner, L., Thoren, F., Nygren, E., Liljeqvist, J.A., Karlsson, A., Eriksson, K. (2005) A proinflammatory peptide from herpes simplex virus type 2 glycoprotein G affects neutrophil, monocyte, and NK cell functions. *J Immunol* **174**, 2235-41.
144. Karlsson, J., Fu, H., Boulay, F., Bylund, J., Dahlgren, C. (2006) The peptide Trp-Lys-Tyr-Met-Val-D-Met activates neutrophils through the formyl peptide receptor only when signaling through the formylpeptide receptor like 1 is blocked. A receptor switch with implications for signal transduction studies with inhibitors and receptor antagonists. *Biochem Pharmacol* **71**, 1488-96.
145. Bylund, J., Karlsson, A., Boulay, F., Dahlgren, C. (2002) Lipopolysaccharide-induced granule mobilization and priming of the neutrophil response to Helicobacter pylori peptide Hp(2-20), which activates formyl peptide receptor-like 1. *Infect Immun* **70**, 2908-14.
146. Le, Y., Jiang, S., Hu, J., Gong, W., Su, S., Dunlop, N.M., Shen, W., Li, B., Ming Wang, J. (2000) N36, a synthetic N-terminal heptad repeat domain of the HIV-1 envelope protein gp41, is an activator of human phagocytes. *Clin Immunol* **96**, 236-42.
147. Deng, X., Ueda, H., Su, S.B., Gong, W., Dunlop, N.M., Gao, J.L., Murphy, P.M., Wang, J.M. (1999) A synthetic peptide derived from human immunodeficiency virus type 1 gp120 downregulates the expression and function of chemokine receptors CCR5 and CXCR4 in monocytes by activating the 7-transmembrane G-protein-coupled receptor FPRL1/LXA4R. *Blood* **94**, 1165-73.
148. Su, S.B., Gong, W., Gao, J.L., Shen, W., Murphy, P.M., Oppenheim, J.J., Wang, J.M. (1999) A seven-transmembrane, G protein-coupled receptor, FPRL1, mediates the chemotactic activity of serum amyloid A for human phagocytic cells. *J Exp Med* **189**, 395-402.
149. De, Y., Chen, Q., Schmidt, A.P., Anderson, G.M., Wang, J.M., Wooters, J., Oppenheim, J.J., Chertov, O. (2000) LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. *J Exp Med* **192**, 1069-74.
150. Bae, Y.S., Bae, H., Kim, Y., Lee, T.G., Suh, P.G., Ryu, S.H. (2001) Identification of novel chemoattractant peptides for human leukocytes. *Blood* **97**, 2854-62.
151. Klein, C., Paul, J.I., Sauve, K., Schmidt, M.M., Arcangeli, L., Ransom, J., Trueheart, J., Manfredi, J.P., Broach, J.R., Murphy, A.J. (1998) Identification of surrogate agonists for the human FPRL-1 receptor by autocrine selection in yeast. *Nat Biotechnol* **16**, 1334-7.
152. Kim, Y., Lee, B.D., Kim, O., Bae, Y.S., Lee, T., Suh, P.G., Ryu, S.H. (2006) Pituitary adenylate cyclase-activating polypeptide 27 is a functional ligand for formyl peptide receptor-like 1. *J Immunol* **176**, 2969-75.
153. Derian, C.K., Solomon, H.F., Higgins, J.D., 3rd, Beblavy, M.J., Santulli, R.J., Bridger, G.J., Pike, M.C., Kroon, D.J., Fischman, A.J. (1996) Selective inhibition of N-formylpeptide-induced neutrophil activation by carbamate-modified peptide analogues. *Biochemistry* **35**, 1265-9.
154. Perretti, M., Chiang, N., La, M., Fierro, I.M., Marullo, S., Getting, S.J., Solito, E., Serhan, C.N. (2002) Endogenous lipid- and peptide-derived anti-inflammatory pathways generated with glucocorticoid and aspirin treatment activate the lipoxin A4 receptor. *Nat Med* **8**, 1296-302.

155. Machado, F.S., Johndrow, J.E., Esper, L., Dias, A., Bafica, A., Serhan, C.N., Aliberti, J. (2006) Anti-inflammatory actions of lipoxin A4 and aspirin-triggered lipoxin are SOCS-2 dependent. *Nat Med* **12**, 330-4.
156. Wenzel-Seifert, K., Seifert, R. (1993) Cyclosporin H is a potent and selective formyl peptide receptor antagonist. Comparison with N-t-butoxycarbonyl-L-phenylalanyl-L-leucyl-L-phenylalanyl-L- leucyl-L-phenylalanine and cyclosporins A, B, C, D, and E. *J Immunol* **150**, 4591-9.
157. von Wartburg, A., Traber, R. (1986) Chemistry of the natural cyclosporin metabolites. *Prog Allergy* **38**, 28-45.
158. Borel, J.F., Baumann, G., Chapman, I., Donatsch, P., Fahr, A., Mueller, E.A., Vigouret, J.M. (1996) In vivo pharmacological effects of ciclosporin and some analogues. *Adv Pharmacol* **35**, 115-246.
159. Bae, Y.S., Lee, H.Y., Jo, E.J., Kim, J.I., Kang, H.K., Ye, R.D., Kwak, J.Y., Ryu, S.H. (2004) Identification of peptides that antagonize formyl peptide receptor-like 1-mediated signaling. *J Immunol* **173**, 607-14.
160. Cunningham, C.C., Vegners, R., Bucki, R., Funaki, M., Korde, N., Hartwig, J.H., Stossel, T.P., Janmey, P.A. (2001) Cell permeant polyphosphoinositide-binding peptides that block cell motility and actin assembly. *J Biol Chem* **276**, 43390-9.
161. Fu, H., Bjorkman, L., Janmey, P., Karlsson, A., Karlsson, J., Movitz, C., Dahlgren, C. (2004) The two neutrophil members of the formylpeptide receptor family activate the NADPH-oxidase through signals that differ in sensitivity to a gelsolin derived phosphoinositide-binding peptide. *BMC Cell Biol* **5**, 50.
162. Chen, X., Yang, D., Shen, W., Dong, H.F., Wang, J.M., Oppenheim, J.J., Howard, M.Z. (2000) Characterization of chenodeoxycholic acid as an endogenous antagonist of the G-coupled formyl peptide receptors. *Inflamm Res* **49**, 744-55.
163. Chen, X., Mellon, R.D., Yang, L., Dong, H., Oppenheim, J.J., Howard, O.M. (2002) Regulatory effects of deoxycholic acid, a component of the anti-inflammatory traditional Chinese medicine Niuhuang, on human leukocyte response to chemoattractants. *Biochem Pharmacol* **63**, 533-41.
164. Postma, B., Poppelier, M.J., van Galen, J.C., Prossnitz, E.R., van Strijp, J.A., de Haas, C.J., van Kessel, K.P. (2004) Chemotaxis inhibitory protein of Staphylococcus aureus binds specifically to the C5a and formylated peptide receptor. *J Immunol* **172**, 6994-7001.
165. Prat, C., Bestebroer, J., de Haas, C.J., van Strijp, J.A., van Kessel, K.P. (2006) A new staphylococcal anti-inflammatory protein that antagonizes the formyl peptide receptor-like 1. *J Immunol* **177**, 8017-26.
166. Vane, J.R., Botting, R.M. (1998) Anti-inflammatory drugs and their mechanism of action. *Inflamm Res* **47 Suppl 2**, S78-87.
167. Smith, J.B., Willis, A.L. (1971) Aspirin selectively inhibits prostaglandin production in human platelets. *Nat New Biol* **231**, 235-7.
168. Vane, J.R. (1971) Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nat New Biol* **231**, 232-5.
169. Xie, W.L., Chipman, J.G., Robertson, D.L., Erikson, R.L., Simmons, D.L. (1991) Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. *Proc Natl Acad Sci U S A* **88**, 2692-6.
170. Chandrasekharan, N.V., Dai, H., Roos, K.L., Evanson, N.K., Tomsik, J., Elton, T.S., Simmons, D.L. (2002) COX-3, a cyclooxygenase-1 variant inhibited by

acetaminophen and other analgesic/antipyretic drugs: cloning, structure, and expression. *Proc Natl Acad Sci U S A* **99**, 13926-31.
171. Hersh, E.V., Lally, E.T., Moore, P.A. (2005) Update on cyclooxygenase inhibitors: has a third COX isoform entered the fray? *Curr Med Res Opin* **21**, 1217-26.
172. Lazer, E.S., Miao, C.K., Cywin, C.L., Sorcek, R., Wong, H.C., Meng, Z., Potocki, I., Hoermann, M., Snow, R.J., Tschantz, M.A., Kelly, T.A., McNeil, D.W., Coutts, S.J., Churchill, L., Graham, A.G., David, E., Grob, P.M., Engel, W., Meier, H., Trummlitz, G. (1997) Effect of structural modification of enol-carboxamide-type nonsteroidal antiinflammatory drugs on COX-2/COX-1 selectivity. *J Med Chem* **40**, 980-9.
173. Colli, S., Colombo, S., Tremoli, E., Stragliotto, E., Nicosia, S. (1991) Effects of tenoxicam on superoxide anion formation, beta-glucuronidase release and fMLP binding in human neutrophils: comparison with other NSAIDs. *Pharmacol Res* **23**, 367-79.
174. Abramson, S.B., Leszczynska-Piziak, J., Clancy, R.M., Philips, M., Weissmann, G. (1994) Inhibition of neutrophil function by aspirin-like drugs (NSAIDS): requirement for assembly of heterotrimeric G proteins in bilayer phospholipid. *Biochem Pharmacol* **47**, 563-72.