Receptor Cross-talk and Neutrophil Function

Karin Granhagen Önnheim

UNIVERSITY OF GOTHENBURG

Department of Rheumatology and Inflammation Research,
Institute of Medicine, Sahlgrenska Academy,
University of Gothenburg

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karin.onnheim@gu.se

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Cover illustration: Crystal structure of beta-2 adrenergic receptor together with ligand and G-protein. Courtesy of Professor Brian Kobilka, Stanford University School of Medicne

Abstract

The chemotactic recruitment of neutrophils, the most abundant white blood cell in human circulation, to sites of infection and inflammation, is dependent upon a gradient of chemoattractants released from cells at the inflamed area. The chemoattractants, including formyl peptides, are recognized by G-protein coupled chemoattractant receptors (GPCRs) present on the neutrophil surface. Activation of GPCRs in neutrophils mediates in addition to chemotaxis, also granule mobilization and the generation of reactive oxygen species (ROS). ROS and granule constituents are not only essential for effective microbial killing, but may also account for unwanted tissue destruction. Stringent activation and termination of neutrophil GPCR signaling is therefore crucial for fine-tuning of inflammatory reactions. Two well-known control mechanisms are 1) receptor desensitization, a non-signaling state reached after termination of the agonist-induced GPCR signal, and 2) priming, a hyper-responsive state coupled to upregulation of surface receptors. The data presented in this thesis explore both of these control mechanisms, and in addition provide evidence for the existence of a novel receptor cross-talk mechanism whereby already desensitized receptors can be reactivated.

We first show that in analogy to what is known for human neutrophils, TNF-α is able to prime mouse neutrophils for FPR stimulation. Next we show that FPR desensitization can be broken by treatment with the β-galactioside binding human lectin galectin-3. This process is dependent upon ROS-mediated inactivation of the FPR agonist, which in turn relies on the carbohydrate-binding domain of the lectin and on the presence of the neutrophil peroxidase MPO. Most importantly, this thesis also discovers a novel cross talk mechanism whereby desensitized FPRs can be reactivated and turned into active signaling state. We show that stimulation of FPR desensitized neutrophils with 1) extracellular ATP (a damage-associated molecular pattern; DAMP) and 2) the platelet activating factor (PAF) transmit signals leading to reactivation of FPRs. This could be an important mechanism for amplification of cellular responsiveness during contact with multiple inflammatory mediators simultaneously. The signals leading to FPR reactivation were shown to be independent of intracellular calcium signaling, and an intact actin cytoskeleton, but required calyculinA-sensitive phosphatases. The data presented challenge the current view of actin-dependent FPR desensitization and the view of the desensitization process as a stable point-of-no-return.

List of publications

This thesis is based on the following papers referred to in the text by their roman numerals:

I Önnheim K, Bylund J, Boulay F, Dahlgren C, Forsman H

Tumour necrosis factor (TNF)-alpha primes murine neutrophils when triggered via formyl peptide receptor-related sequence 2, the murine orthologue of human formyl peptide receptor-like 1, through a process involving the type I TNF receptor and subcellular granule mobilization.

Immunology. 2008 Dec;125(4):591-600

II Forsman H, Salomonsson E, Önnheim K, Karlsson J, Björstad A, Leffler H, Bylund J, Karlsson A, Dahlgren C

The beta-galactoside binding immunomodulatory lectin galectin-3 reverses the desensitized state induced in neutrophils by the chemotactic peptide f-Met-Leu-Phe: role of reactive oxygen species generated by the NADPH-oxidase and inactivation of the agonist.

Glycobiology. 2008 Nov;18(11):905-12.

III Forsman H*, Önnheim K*, Andréasson E, Christenson K, Karlsson A, Bylund J, Dahlgren C

Reactivation of desensitized formyl peptide receptors by platelet activating factor: a novel receptor cross talk mechanism regulating neutrophil superoxide anion production.

*PLoS One. 2013;8(3):e60169 *Joint first authors*

IV Önnheim K, Christenson K, Amirbeagi F, Martner A, Bylund J, Dahlgren C, Forsman H

ATP triggers reactivation of desensitized formyl peptide receptors: A novel receptor cross talk mechanism regulates phagocyte superoxide anion production

In manuscript

Ligand: Definition noun, plural: ligands
(1) A molecule, ion or atom bonded to the central metal atom of a coordination compound.
(2) Any substance (e.g. hormone, drug, functional group, etc.) that binds specifically and reversibly to another chemical entity to form a larger complex.
*Supplement : A **ligand** may function as agonist or antagonist.*

Agonist: Definition noun, plural: agonists
(pharmacology) A molecule that combines with a receptor on a cell to trigger physiological reaction. An example is an acetylcholine being the **agonist** that combines with the cholinergic receptor.
(histology) A muscle that contracts while another muscle relaxes, e.g. when bending the elbow the biceps are the agonist.
Supplement: (pharmacology) There are different kinds of agonists:
Full agonists have an affinity for and activate a receptor.
Partial agonists bind and activate a receptor but have only partial efficacy at the receptor (compared with full agonists).
Inverse agonists reverse constitutive activity of receptors.
Co-agonists work with other co-agonists to produce the desired effect together.

Antagonist: Definition noun, plural: antagonists
A biological structure or chemical agent that interferes with the physiological action of another
Supplement: Examples of antagonists are drugs that bind to cell receptors that prevent the agonists from eliciting a biological response. Other biological antagonists are muscles that occur in pairs. An antagonist muscle opposes the action of the agonist muscle, thus, helps in regulating movements. Word origin: Greek antagonistes (an opponent)

Source: www.biology-online.org/dictionary

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Abbreviations

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
C5a	Complement factor 5a (split product from C5)
cAMP	Cyclic adenosine monophostphate
CGD	Chronic granulomatous disease
CHIPS	Chemotactic inhibitory peptide from *Staphylococcus aureus*
CRD	Carbohydrate recognition domain
DAG	Diacylglycerol
DAMP	Damage associated molecular patterns
ERK1/2	Extracellular signal-regulated kinase
fMLF	N-formyl-methionyl-leucyl-phenylalanine
FPR1	Formyl peptide receptor 1
FPR2	Formyl peptide receptor 2
FPR3	Formyl peptide receptor 3
FPR_{des}	Cells desenitized in FPR
GDP	Guanosine diphosphate
GPCR	G-protein-coupled receptor
GRK	G-protein-coupled receptor kinases
GTP	Guanosine triphosphate
H_2O_2	Hydrogen peroxide
HOCl	Hypochlorous acid
HSV2	Herpes simplex virus type 2
IL-8	Interleukin 8
IP_3	Inositol 1,4,5-triphosphate
LPS	Lipopolysaccharide
LTB_4	Leukotrine B_4
MAPKs	Mitogen-activated-protein kinases
MPO	Myeloperoxidase
NADPH	Nicotinamide adenine dinucleotide phosphate
O_2^-	Superoxide anion
OH ·	Hydroxyl radical
PAF	Platelet activating factor
PAFR	Receptor for platelet activating factor
PAMP	Pathogen-associated molecular pattern
PI3Kγ	Phosphoinositide 3-kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol myristat acetate
PSM	Proteasome (Phenol-soluble peptide)
PRR	Pathogen recognition receptor
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TNF	Tumour necrosis factor
UDP	Uridine diphosphate
UTP	Uridine triphosphate

Introduction

Although humans are continuously exposed to potentially harmful microorganisms, the frequency of microbial infections is not as high as might be expected. This is largely due to our innate immune system, which, as the name indicates, is present from birth and is the product of a long evolutionary process. Innate immune reactions protect us from different types of threats. The innate immune system acts very rapidly to block the entrance of pathogens and to recognize and kill invading microbes, thereby giving the adaptive immune system time to mobilize.

Phagocytic white blood cells, such as neutrophil granulocytes and monocytes, constitute an important part of the innate immune system. All blood cells are produced in the bone marrow and mature cells are released into the circulation to "scout" the body for infections or damaged tissues. The neutrophils, which are the most abundant leukocyte type in the peripheral blood, constitute our body's first line of defense. They are armed with an arsenal of bactericidal compounds, e.g., antimicrobial peptides and degradative enzymes, and they are equipped with an electron transport system that is designed to produce reactive oxygen species (ROS) (1). Together, these weapons are highly efficient at killing microbes. However, the antimicrobial weapons also have the capacity to harm host cells/tissues and if not properly regulated these systems are potentially dangerous to the host. While cells of the adaptive immune system can be "educated" to recognize new structures, neutrophils have to rely on receptors that primarily recognize preserved pathogen-associated molecular patterns (PAMPs), structures that are shared by many different microorganisms. A limited number of pattern recognition receptors (PRR) expressed by host cells constitute the basis for recognition of "danger signals" in the form of PAMPs.

The formyl peptide receptors (FPRs) expressed by neutrophils and monocytes are a prominent example of PPRs, and they exemplify how the recognition of a molecular pattern can be achieved by a cell of the immune system. The FPRs belong to the large group of G-protein-coupled, seven-transmembrane receptors (GPCRs) and they recognize peptide sequences that start with an N-formylated methionine, which is a hallmark of protein synthesis in bacteria (2). Mitochondria, which are proposed to have originated from free-living bacteria, resemble in some aspects prokaryotes. For example, the synthesis of proteins in these subcellular organelles also starts with a formylated methionine, unlike protein synthesis in the rest of the cell. Damaged host cells release endogenous "danger signaling" molecules, and mitochondrion-derived formyl peptides are one example of how host-derived molecules can be sensed by the FPRs (3,4). In addition to FPRs, neutrophils express many other surface receptors that are important in infection and inflammation. Given that many different agonists for these receptors are present in the body during both healthy and disease conditions, it is crucial that the different receptors communicate with each other, as to maintain proper cell function. Therefore, elucidation of the molecular mechanisms that underlie receptor cross-talk are fundamental to our understanding of receptor-mediated cellular functions.

Cell surface receptors, focusing on neutrophil GPCRs

Eukaryotic cells sense the environment through receptors that are expressed on the cell surface, while for the transmission of intracellular signals, receptors are also expressed in the nucleus, cytosol, and endoplasmic reticulum (ER). The neutrophil is a good example of a cell that expresses a large variety of different receptors in subcellular organelles (e.g., granules and secretory vesicles), and these receptors can be mobilized to the cell surface through secretion following exposure of the cell to a stimulus. In general, receptor activation is induced when a specific agonist binds to a specific binding site on/in the receptor, and depending on the receptor-agonist pairing, an explicit cellular response is induced through a signaling cascade that is initiated by the occupied receptor. The signal may modulate a specific biochemical pathway and/or regulate the function of another receptor. The diversity of receptors types and receptor ligands, together with the variety of intracellular second messengers, represents the framework for the many different and often fine-tuned cellular responses. The family of G-protein-coupled receptors (GPCRs) represents the largest group of transmembrane receptors, and these receptors regulate a large variety of biological functions. Unsurprisingly, GPCRs currently constitute major targets for drug development. This means that improving our knowledge of receptor structures and signaling will facilitate the development of new therapeutic agents in many clinical fields (5-7). In 2012, Robert Lefkowitz and Brian Kobilka were awarded the Nobel Prize in Chemistry for their work on GPCRs. Decades of research, which steadily increased our understanding of the molecular details of GPCR functions, was crowned with success when efforts to crystallize the β-adrenergic receptor in combination with both an agonist and the heterotrimeric G-protein bore fruit (8). To illustrate the common structural features of the GPCRs, I have received permission and have the privilege to use an image of the crystallized β-adrenergic receptor on the cover page of this thesis.

The GPCR family members recognize and respond to a diverse pool of agonists, ranging from neurotransmitters, hormones, odors, light, and inflammatory mediators, including endogenously derived danger signals and components of microbes (9-11). The agonists that signal through GPCRs range from large proteins to very small peptides, and even photons. Despite the number and diversity of substances that activate GPCRs, the members of this receptor family show many structural similarities and share many intracellular signaling pathways (11). For all of these proteins, the amino terminal is located on one side of the membrane, they transverse this membrane seven times, and the C-terminus/carboxyl tail is located on the membrane side opposite to the N-terminus (12). Despite the structural similarities of the seven-transmembrane regions, evolution has conferred upon the GPCR family the ability to adapt to the presence of diverse ligands, making it possible for ligands of different sizes and structures to bind perfectly to their specific receptors. Based primarily on similarities/differences in the amino acid sequences, a categorization of the GPCRs of both vertebrates and invertebrates has allowed categorization into five classes: Secretin; Glutamate; Frizzled; Adhesion; and Rhodopsin

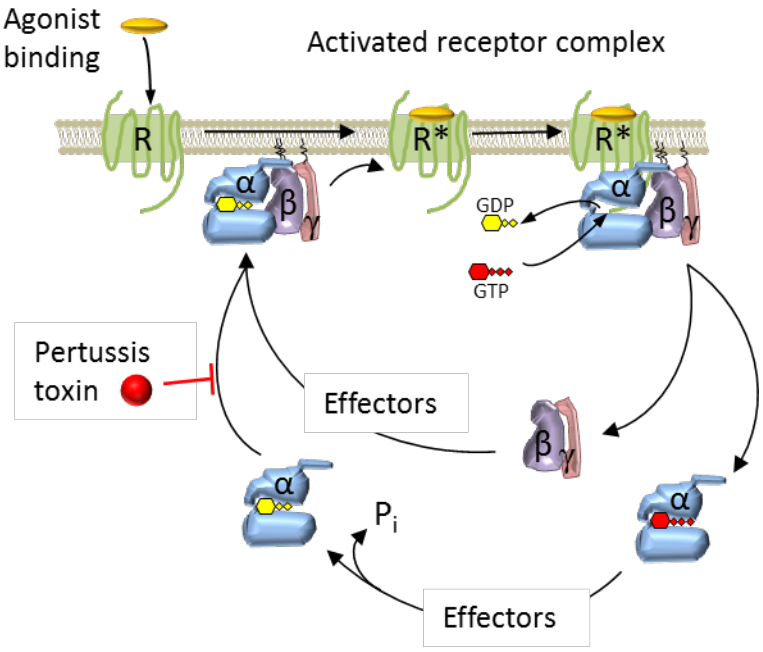

Figure 1. Upon GPCR activation by agonist binding, the conformation of the receptor changes, allowing the heterotrimeric Gαβγ-protein coupled to GDP to bind to the receptor. The exchange of GDP to GTP drives the release of the G-protein from the receptor and the dissociation into Gα and Gβγ subunits is the key event that leads to downstream signaling. Pertussis toxin can inhibit GPCR-signaling by modification of $G\alpha_i$ and thereby preventing association with the receptor.

(13). The Rhodopsin or the A family, which includes more than 700 GPCRs and is by far the largest subgroup, can be further divided into four subgroups (α, β, γ, and δ).

The chemoattractant receptors belong to the γ-group of GPCRs. The chemoattractant receptors, which are responsible for the recruitment of leukocytes from the bloodstream to sites of inflammation, share certain features, including many intracellular signaling pathways responsible for mediating the chemotactic signaling. The heterotrimeric G-protein, which contains an α-, a β-, and a γ-subunit, is normally responsible for the transduction of signals from the occupied/activated GPCR (Fig. 1). At least 16 unique mammalian α-subunits (classified into four α-families), 5 β-subtypes, and 14 γ-subtypes, have been identified to date, allowing for numerous different combinations (14). Some GPCRs have also been shown to interact with more than one G-protein, and can even induce G-protein-independent cellular responses (15). The chemoattractant receptors all use G-proteins that contain the $G\alpha_i$-subunit, as evidenced by the fact that the agonist-induce d response can be inhibited by treatment with pertussis toxin, a toxin from *Bordetella pertussis* that inhibits the function of $G\alpha_i$. These receptors are described in more detail below.

Receptors important in neutrophil recruitment to inflammatory sites

The process of neutrophil recruitment to sites of inflammation/infection starts with the release of cytokines and chemokines from the cells that border the site of inflammation/infection, which promotes endothelial cells in the blood vessels to up-regulate their expression of adhesion molecules, such as E-selectin and P-selectin. Selectins are single-chain glycoproteins that interact with carbohydrate structures (ESL-1, PSGL-1) expressed on leukocytes, and these initial interactions reduce the speed of the circulating cells, causing them to roll along the endothelium. Firmer adhesion, crawling, and transmigration across the wall of the blood vessel are mostly dependent upon other cell surface structures, such as heterodimeric transmembrane integrins that can signal through the cell membrane in both directions (16). Neutrophils contain numerous granules/ secretory vesicles, which are mobilized once the cell is activated. The secretory vesicles contain plasma proteins, as well as receptors and adhesion molecules that are up-regulated during extravasation from the blood to the tissue (17). During chemotaxis, neutrophils mobilize more granules, delivering to the cell surface additional receptors that become involved in the chemotactic process. The chemotactic recruitment of neutrophils is dependent upon the chemical gradient of agonists created, which comprise both proteins/peptides (e.g., formylated peptides, C5a, IL-8) and lipids (e.g., PAF, LTB_4) that are recognized by chemoattractant receptors on the plasma membrane, together with reorganization of the cytoskeleton, providing the cells with a polarized structure, with a defined front and rear ends (18).

The receptor for the lipid chemoattractant PAF (PAFR)

In 1970, "a soluble factor" identified as being released from leukocytes was found to stimulate platelets to release vasoactive amines, such as histamine and serotonin (19). However, it would be another 10 years until platelet activating factor (PAF) was isolated and its chemical structure determined (Fig. 2; 20). Over the last decades, knowledge of PAF has accumulated and many cells, including platelets, human endothelial cells, neutrophils, and macrophages, have been shown to release PAF upon stimulation (21). The receptor for PAF (PAFR) is a GPCR that is expressed by several human cell types, including neutrophils, platelets, monocytes, and eosinophils, as well as cells of the liver and lung (reviewed in 21). There are also indications that PAF has receptor(s) other than PAFR, as both human eosinophils treated with PAFR antagonist and eosinophils from *PAFR* $^{-/-}$

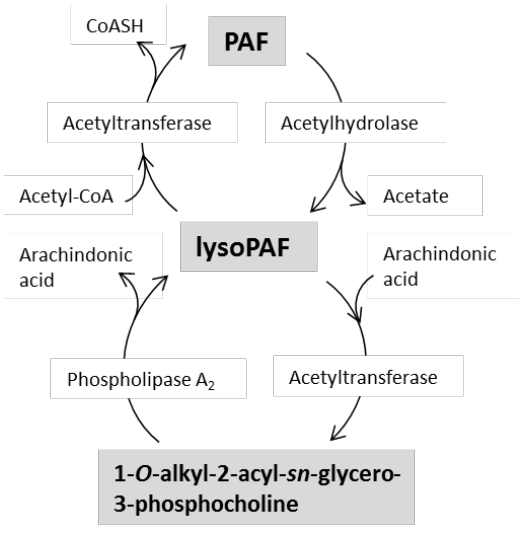

Figure 2. Metabolic pathways of PAF

mice retain the ability to degranulate when stimulated with PAF (22).

Pathophysiologic roles for PAF have been described in asthma, sepsis, and pancreatitis endotoxemia (23-25). The *PAFR*$^{-/-}$ mice show strong resistance to antigen-induced systematic anaphylaxis, bradycardia, circulatory shock, and lung edema (26). Phosphorylcholine, a molecule that structurally resembles PAF, is found in the cell membrane of *Streptococcus pneumoniae* and can bind to the PAFR, such that PAFR mediates the adhesion of *S. pneumoniae* to epithelial cells (27,28). In addition, this microbe uses the PAFR as a gateway, since ligand binding results in simultaneous internalization of the receptor and microbe. Accordingly, *PAFR* $^{-/-}$ mice are less susceptible to *S. pneumoniae* infections (29). The correlation between smoking and being more sensitive to *S. pneumoniae* infections has recently been linked to the up-regulation of PAFR in the lower airways (30). PAF activation through PAFRs triggers several intracellular signaling events, including activation of phospholipase C and A_2, increased intracellular levels of free Ca^{2+}, activation of protein kinase C, and phosphorylation of tyrosine residues in proteins (reviewed in 21). Moreover, PAF induces platelet aggregation, leukocyte recruitment by chemotaxis, and the activation of neutrophils, eosinophils, and macrophages. In neutrophils, PAF induces superoxide production, calcium mobilization, and actin polymerization (Paper III), and these responses are inhibited by the specific PAFR antagonist WEB 2086. In addition, the more stable PAF analogue mcPAF and the PAF precursor lysoPAF trigger the production of superoxide through their interactions with the PAFR. Most intriguingly, a novel receptor-mediated response has been disclosed, whereby PAFR reactivates desensitized FPRs through an as-yet-unidentified-molecular receptor cross-talk mechanism (Paper III**,** see details under 'reactivation').

The receptor for the complement component C5a (C5aR)

The complement system constitutes an important part of the innate immune system. In addition to the direct killing of microorganisms mediated by the membrane attack complex, activation of the complement cascade generates a number of soluble mediators, including anaphylatoxins C3a, C4a, and C5a. Inappropriate activation of the complement system can lead to various pathologies and inflammatory disorders. The complement component C5a is a 78-amino acid peptide that functions as a potent pro-inflammatory mediator. The receptor for C5a (C5aR or CD88) is a GPCR that is present on leukocytes, such as granulocytes, monocytes, and dendritic cells. Upon activation, this receptor triggers chemotaxis, degranulation, calcium mobilization, and the release of ROS. These functions are also triggered by other chemoattractant receptors that are occupied with their respective agonists. The gene for C5aR is localized to chromosome 19, proximal to the genes that encode the FPRs, and C5aR shares 34% similarity at the amino acid level with FPR1 (31,32). Activation of C5aR generates signals that are similar to those generated from agonist-occupied FPRs (reviewed in 32). In addition, C5a has been shown to bind C5L2, which is another GPCR that displays approximately the same affinity for the agonist as does C5aR (33). C5L2 is believed to be a decoy receptor that triggers a null or very weak cellular response. Interestingly, it has been suggested that C5aR and C5L2

have synergistic effects, as blockage of C5aR alone is not sufficient to protect mice from sepsis. In contrast, simultaneous blockage of C5aR and C5L2 increases dramatically the survival rate of these mice (34,35).

The C5aR has been shown to be involved in different types of receptor cross-talk, forming homodimers and oligomers of higher order (36), as well as heterodimers (37). The C5aR is also a strong neutrophil chemoattractant receptor and is capable of another form of receptor cross-talk by inducing heterologous desensitization of other chemotactic receptors of lower hierarchy (38), which is described in more detail in the section dealing with the desensitization phenomenon.

The receptors for the cytokine interleukin-8 (CXCR1/CXCR2)

The so-called CXC-group of inflammatory mediators represents a group of chemokines that induce neutrophil and monocyte migration through interactions with receptors that are named in accordance with their specific agonists (39). Thus, CXCR1 and CXCR2 are the receptors for CXCL8, a chemoattractant that is also known as IL-8 (40). This interleukin is produced and secreted by several cell types that are linked to inflammation, including endothelial cells (41,42). IL-8 is also synthesized and released by mature neutrophils upon stimulation (43). During inflammation, IL-8 is released at a very early stage from endothelial cells and it mediates the recruitment of neutrophils from the blood to the areas neighboring the inflammation/infection in co-operation with other end-type chemoattractants, such as bacterial-derived formyl peptides that are produced in the vicinity of a site of bacterial infection. Thus, IL-8 has been defined as an intermediate chemoattractant that is involved in neutrophil migration (38). The receptors for the intermediate chemoattractants, including those for IL-8 and LTB4, are heterologously desensitized by receptors of higher hierarchy with agonists that are considered to be end-target chemoattractants (e.g., FPRs, C5aR) (38,44,45). This is explained in more detail in the section in which the receptor desensitization phenomenon is discussed. Apart from chemotaxis, IL-8 induces granule mobilization and ROS production in neutrophils (44,46). Both CXCR1 and CXCR2 have been proposed to interact with different G-protein subclasses, although given that the free radical response is inhibited by pertussis toxin, it seems likely that the signals required for this response are mediated through G_i (47,48). The receptors for IL-8 have been shown to participate in different forms of receptor cross-talk (38). Dimerization is one such form of receptor communication and CXCR1 can form both homodimers and heterodimers (with CXCR2) (49).

The receptors for ATP on inflammatory cells

Adenosine triphosphate (ATP) synthesis takes place in all eukaryotic cells, and the normal concentration of ATP inside cells ranges from 1 nM to 10 nM. Extracellular ATP serves as an agonist for many purinergic receptors, some of which are ionotropic, ligand-gated ion channels ($P2X_n$), while others are metabotropic G-protein-coupled receptors ($P2Y_n$). ATP is rapidly hydrolyzed to adenosine in the extracellular milieu, and this metabolite

exerts its functions through the P1 adenosine receptors (50). The P2Y family of receptors has been shown to associate with several different G-proteins and one of these receptors, $P2Y_2$, interacts with both $G_{q/11}$ and $G_{i/0}$ (51). In addition to extracellular ATP, the P2Y receptors recognize other nucleotides, such as ADP, UTP, UDP, and UDP-glucose (50). ATP is secreted from almost all mammalian cells as a result of cell activation/stimulation, and the released ATP acts on cells in an autocrine fashion as it binds to many purinergic receptors expressed by virtually all cell types. Agonist occupation of neutrophil chemoattractant receptors triggers the cells to release extracellular ATP, and the autocrine loop affects the neutrophil chemotactic responsiveness (52). Neutrophils are thought to express several receptors for ATP, although this is based on assays of mRNA expression rather than on the identification of a functional receptor protein. Nevertheless, the $P2Y_2$ receptor has been shown to be expressed in neutrophils, as detected by the binding of specific antibodies ((53), Paper IV). This receptor is suggested to be responsible for the priming effects of ATP on the responses induced by various inflammatory mediators, resulting, for example, in the increased release of oxygen radicals (reviewed by (54)). ATP can also be passively released trough leakage from damaged or disturbed/stressed cells, which means that extracellular ATP works as a danger signal or a damage-associated molecular pattern (DAMP). Accordingly, extracellular ATP activates the NLRP2 inflammasome (3,55,56) but it is not directly chemotactic. Rather, it functions as a "find-me signal", since it potentiates the chemotactic signals induced by other chemoattractants, promoting the recruitment of leukocytes to the site of inflammation and the clearance of apoptotic or damaged cells (57). Numerous studies have shown that ATP can activate neutrophils, most probably *via* $P2Y_2$, and that this activation leads to granule mobilization, a proposed priming mechanism for several inflammatory mediators and increased production of ROS (58) (reviewed by (54)). $P2Y_2$ has also been implicated in receptor cross-talk through the formation of homodimeric (59) and heterodimeric receptor complexes with adenosine receptor A_1 (60), and non-purinergic receptors (thoroughly reviewed by (61)). In this thesis, I describe a novel form of receptor cross-talk that involves ATP and reactivation of FPRs (Paper IV). This is described in more detail in the section on receptor cross-talk.

The receptors for formyl peptides (FPRs)

The formyl peptide receptors, FPRs, constitute a receptor group with three members in humans (FPR1, FPR2, and FPR3), which belong to the GPCR family. In the middle of the 1970's, neutrophils were shown to migrate directionally in response to a gradient of N-formylated peptides derived from *Escherichia coli* (62,63). The responsible receptor (FPR1) was identified and cloned in 1990 (64). Although expression of the FPRs was initially thought to be restricted to leukocytes, it is now clear that these receptors are expressed in various cell types throughout the human body, including endothelial cells, astrocytes, microglial cells, and fibroblasts (65,66), and it was recently shown that intestinal epithelial cells also express these receptors, whereby they contribute to colonic epithelial homeostasis (67). Human neutrophils express two of the three members of the FPR family, namely FPR1 and FPR2, while monocytes and macrophages express also FPR3.

Following the initial observation that bacterial-derived formylated peptides are agonists for FPRs, it has become clear that both neutrophil receptors are promiscuous in terms of agonist recognition. Thus, the FPR1 and FPR2 recognize both formylated and non-formylated peptides/proteins derived from pathogens or the host, as well as synthetic molecules identified through screens of small-molecule libraries (2,68-70), and these substances have very little or no known structural similarities. In contrast to FPR1 and FPR2, the ligand for FPR3 is relatively uncharacterized (71).

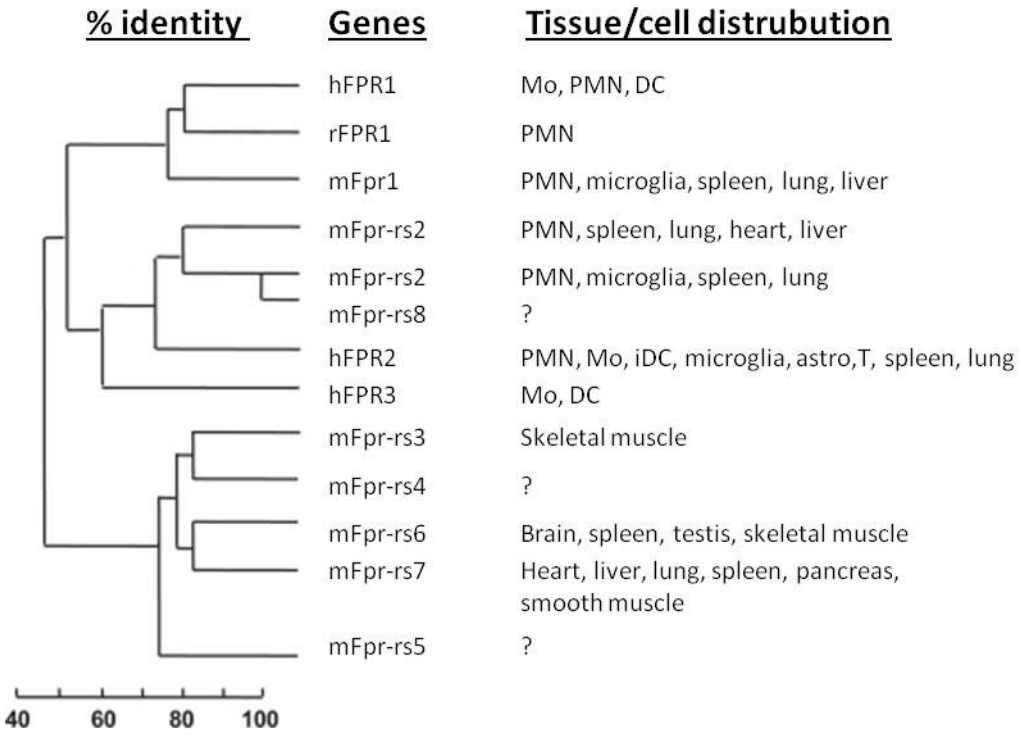

Figure 3. Sequence homology and cellular distrubution of formyl peptide receptors in mice and men. Adapted from (2).

Looking at the similarities at the amino acid level among the human FPRs, FPR1 and FPR2 share 69% sequence similarity, whereas FPR1 and FPR3 share 56% and FPR2 and FPR3 share 83% sequence similarity (**Fig. 3**) (72). Screening of the murine genome using probes for the three human receptors initially led to the identification of six genes, named *Fpr1* and *Fpr-rs1* to *Fpr-rs5*, all of which were localized to chromosome 17 ((73) **Paper I**). However, the presence of a stop codon in the gene for *Fpr-rs5* means that it is unlikely to yield a functional receptor. Comparing the human and murine FPRs, Fpr1 is believed to be the orthologue of human FPR1 (76% sequence homology), while Fpr-rs1 and Fpr-rs2 are more closely related to human FPR2 than to FPR3 (74% and 76% versus 63% and 60% identity at the amino acid level). We have identified Fpr-rs2 as the murine orthologue of human FPR2 (**Paper I**), and this finding is in agreement with the results reported by others (74). Based on mRNA analyses, it has been suggested that the three murine Fprs, i.e., Fpr1, Fpr-rs1, and Fpr-rs2, are expressed in murine leukocytes, spleens, and lungs, whereas Fpr-rs3 is expressed solely in skeletal muscle; neither Fpr-rs4 nor Fpr-

rs5 was detected in any of the tested tissues (73). Subsequently, two additional murine Fprs were discovered using low-stringency DNA hybridization, and they were named Fpr-rs6 and Fpr-rs7 (75). Recently, five of the seven members of the FPR family were found in the murine vomeronasal organ (an olfactory structure in the nasal septum that detects pheromones and other social cues), raising interesting questions regarding the factors that these receptors really recognize (76). An eighth member of the murine Fprs, Fpr-rs8, has been recently identified (77). While the function of this receptor remains unclear, *Fpr-rs8*$^{-/-}$ mice have a markedly shorter life span than their wild-type and heterozygous littermates (77). The differences between human and murine Fprs are apparent during stimulation of cells with fMLF, which is a highly potent agonist of human FPR1 but a weak agonist of murine Fpr1 (78). It is obvious from the differences between mice and men that the FPR gene family has a complex evolutionary history. This indicates differential gene expansion or extinction and suggests that different selective pressures have been imposed on the two species, possibly reflecting differences in exposure and sensitivity to infectious agents. The importance of FPRs for host defense has been demonstrated in both mice and men; *Fpr1*$^{-/-}$ mice are more susceptible to *Listeria monocytogenes* infections than wild-type littermates (79) and mutations in the gene that encodes FPR1 has been described in patients with juvenile periodontitis, a disease characterized by severe gingival infections (80,81). Furthermore, Fpr-rs2 in murine neutrophils is important for protection against *L. monocytogenes*, as evidenced by the finding that after intravenous injection of the pathogen 100% of the *Fpr-rs2*$^{-/-}$ mice died, as compared to 50% of the wild-type mice (82). The same study showed that both Fpr1 and Fpr-rs2 are needed for optimal protection against *L. monocytogenes,* since double-knockout mice had shorter survival times and higher bacterial loads in the liver, and were incapable of chemotaxis towards a *L. monocytogenes*-derived peptide, as compared to single-knockout mice (82).

While FPRs have not been described as participants in receptor dimerization, we describe how these receptors are involved in other types of receptor cross-talk, such as heterologous receptor desensitization and receptor reactivation (see the section dealing with receptor cross-talk).

FPR ligands are numerous and diverse

The list of FPR ligands (Table 1) is steadily growing, and they are various and diverse with few or no common features and they have diverse origins. Exogenous ligands from bacterial sources, both formylated and non-formylated peptides, as well as virulence factors have been shown to be potent agonists or antagonists of the FPRs (2,83-85). Some FPR agonists originate from endogenous sources, and synthetic agonists have been identified through extensive screening of peptide/small-compound libraries (70,86-88). The identification of the highly specific FPR2 agonist WKYMVM and the conformer WKYMVm provided valuable tools for the characterization of FPR2, and they are still the most potent agonists for this receptor identified to date (89,90). Highly effective and specific receptor ligand antagonists for FPRs have also been identified over the years, and they also originate from different source and show few structural similarities (91-96).

Table 1. FPR ligands, used by our laboratory, and their receptor specificity and origin.

FPR ligands	Receptor	Source
Exogenous		
fMLF	FPR1, Fpr1	*E. coli*
fMIFL	FPR1, Fpr1	*S. aureus*
PSMα2, PSMα3	FPR2	*S. aureus*
fMIVIL	FPR1, Fpr1	*L. monocytogenes*
Hp(2-20)	FPR2 > FPR3	*H. pylori*
gG-2p20	FPR1	HSV-2
Endogenous		
LL-37	FPR2	hCAP-18, Neutrophils
SAA	FPR2	Liver
Formylated peptides	FPR1, FPR2	Mitochondria
Annexin A1	FPR1, Fpr1	Leukocytes
Synthetic		
Pepducins	FPR2	
WKYMVM	FPR2, FPR3	
WKYMVm	FPR2, FPR1, FPR3, Fpr-rs2	
Small molecules[1]	FPR1, FPR2	
Antagonists		
Cyclosporin H	FPR1	*T. inflatum*
WRWWWW	FPR2	Synthetic
Boc-fMLF	FPR1	Synthetic
PBP10	FPR2	Synthetic
CHIPS	FPR1	*S. aureus*
FLIPr	FPR2	*S. aureus*

[1]For more information regarding small molecules, see (87)

Formylated peptide ligands for FPRs

Prokaryotes initiate protein synthesis with an N-formylated methionine, which is sequentially cleaved off by a peptide deformylase and a methionine aminopeptidase, to generate the mature protein (97). The formylation/deformylation of proteins is a hallmark of bacterial metabolism. The formylated peptide fMLF isolated from Gram-negative *E. coli* was the first described and characterized agonist for FPR1 (62). The formyl group is of great importance for the FPR to recognize the fMLF peptide, as the biological response is lost concomitant with removal of the formyl group. More recently, other peptides of microbial origin, such as fMIFL from *Staphylococcus aureus* and fMIVIL from *L. monocytogenes*, both of which are Gram-positive bacteria, have been identified as potent agonists of human FPR1 (Papers III and IV). These two formylated peptides have higher agonist potencies for murine Fpr1 than fMLF (78). This may reflect differences between

the species with respect to susceptibilities to different microbial pathogens (79). Until recently, none of the formylmethionyl peptides were defined as potent FPR2 agonists. However, a new group of formylated peptides derived from community-associated, methicillin-resistant *S. aureus* (CA-MRSA) were identified as potent neutrophil activators acting through interactions with FPR2 (98,99). In addition to their pro-inflammatory activities, these phenol-soluble modulin (PSM) peptides (PSMα2 and PSMα3) are FPR2 agonists and are cytotoxic, with the latter activity being mediated by the α-helical structure of the peptides. This portion of the PSM peptides is capable of permeabilizing cell membranes, primarily those of apoptotic neutrophils (98).

In contrast to the situation in prokaryotes, protein synthesis in eukaryotes encoded by nuclear DNA does not involve a formylated methionine as the starting residue. However, in mitochondrial protein synthesis, N-formylmethionyl is the initial amino acid, just like in prokaryotic synthesis. Human neutrophils migrate towards mitochondrial lysates, and more specifically towards the N-formylmethionyl-containing peptides/proteins found in mitochondria (100). It has been confirmed that endogenous peptides with N-formyl modifications are also ligands for FPRs (101,102). Rabiet *et al* investigated 13 formylated peptides of human mitochondrial origin for their abilities to induce chemotaxis, calcium mobilization, and superoxide generation in transfected HL-60 cell that expressed the three human FPRs. They identified three of these peptides as being equivalently potent agonists for both FPR1 and FPR2 (102). Formylated peptides derived from mitochondria that are released to the extracellular milieu from apoptotic cells or damaged tissues have been shown to function as DAMPs and are of importance for recruiting cells to sites of inflammation and tissues damage (3,4).

It remains unclear as to whether neutrophils are capable of distinguishing between pathogen-derived formylated peptides (PAMPs) and host-derived factors (DAMPs) and whether these agonists mediate the same responses *in vivo*. The overlap between a microbial infection and an aseptic injury regarding the role of released formylated peptides can be seen in sepsis and sepsis-like conditions, in which the contributions of inflammatory mediators of bacterial and mitochondrial origin are of importance (103,104). Accordingly, inhibition of formylated peptide receptor activity has been suggested as a potential therapy not only for profound bacterial infections, but also for aseptic injury (104).

Early on it was discovered that when the formyl group of fMLF was replaced by a butyloxycarbonyl group the properties of the peptide were changed in that the FPR1 agonist was transferred to an FPR1 antagonist (105). The Boc-MLF peptide binds the receptor and blocks agonist binding by direct steric hindrance. Following the description of the first antagonist, several other more potent agonists for FPR1 and FPR2 have been described and characterized. Cyclosporin H, which is a cyclic undecapeptide derived from the fungus *Tolypocladium inflatum*, has been shown to be one of the most potent and selective FPR1 antagonists (91). In addition to its inhibitory effect on ligand-induced activities, cyclosporin H reduces the basal activity of the non-agonist-occupied FPR1, demonstrating that cyclosporin H acts as an inverse agonist (106).

Receptor regulation

G-protein-mediated signaling and neutrophil activation

The binding of an agonist to its GPCR induces a conformational change in the receptor. The resulting stable receptor-ligand complex becomes active in relation to the heterotrimeric G-protein that binds in its GDP form. This binding further stabilizes the ligand-receptor complex (8). The G-protein receptor coupling drives the exchange of GDP for GTP, which in turn releases the G-protein from the receptor (107,108). The dissociation of the heterotrimer into Gα-GTP and Gβγ is the first step in a signaling cascade that involves intracellular mediators with positive or negative regulatory effector systems/enzymes (109). These include adenylate cyclases, phospholipases, different kinases, and ion channels, and the cascade results in the generation of small-molecule second messengers that control cellular functions (107,110). However, there are signaling pathways that are triggered by occupied GPCRs independently of the heterotrimeric G-protein. The β-arrestin and mitogen-activated kinase pathways are examples of such G-protein-independent signaling routes (111), and there is increasing evidence that the β-arrestins do more than just terminate GPCR signaling, as on their own they can induce signals that are clearly separated from the G-protein function (15). The nature of the G-proteins, i.e., the particular Gα-subunit together with the Gβγ combination involved, determines the specificities of the induced intracellular signaling and cellular responses (108). Chemoattractant receptors are usually coupled to $G\alpha_i$, which activates or inhibits adenylyl cyclase and thereby regulates intracellular ATP conversion to cAMP. With regard to the responses induced by both FPRs and C5aR, the Gβγ subunit activates phospholipase Cβ2 (PLCβ2), which subsequently hydrolyzes PIP_2 into diacylglycerol (DAG) and IP_3. IP_3 induces the release of Ca^{2+} from intracellular stores, and this together with DAG activates protein kinase C (PKC), which is involved in the activation of the NADPH-oxidase, as described below (Fig. 4) (32). The Gβγ subunit further activates phosphoinositide 3-kinase (PI3Kγ), which has been shown to be required for both the migration and generation of superoxide in murine neutrophils (112,113). Several other kinases, including extracellular signal-regulated kinase (ERK1/2) and p38 MAP kinase, are also activated by FPRs and C5aR, along with the activation of guanine-nucleotide exchange factors (GEFs), which in turn activate small G-proteins of the Rho family (Rac, Rho, and Cdc42). These Rho family proteins are key regulators of several functions, such as adhesion, migration, and superoxide production (32,114).

Both the concentration and binding affinity of a chemoattractant are of importance for the cellular response. It is fascinating that one ligand acting on one receptor is able to evoke distinct cellular responses depending on the agonist concentration. The induction of a chemotactic response usually requires a low concentration of agonist, whereas higher concentrations of agonist are needed to promote mobilization of the classical granules (115,116). Activation of the superoxide-generating NADPH-oxidase requires even higher concentrations of agonist (115,116). While the precise regulatory mechanisms underlying

this graded response remains to be clarified, it is highly relevant from a neutrophil function point-of-view that an agonist concentration that mediates chemotaxis of neutrophils does not elicit superoxide generation, which minimizes the risk for the host tissue during the recruitment of inflammatory cells.

The concentration dependency of neutrophil chemotaxis is usually bell-shaped, which means that there exists an optimal concentration of the chemoattractant, with the induced response being lower at concentrations higher and lower than the optimum. The Gα-subunit does not appear to be important for the chemotactic ability of neutrophils, and chemotaxis does not require either receptor internalization or redistribution (117). The chemotaxis of neutrophils is dependent upon the release of Gαβγ for subsequent Gβγ signaling, which is essential for the orientation of the cell (118). Chemotactic movement is a multistep process in which the neutrophils are able to distinguish and respond to multiple signals, whereby certain chemotactic agonists have a higher priority than others. This ability to discriminate chemotactic signaling is attributed to a hierarchical receptor system in which "end-target chemoattractants" predominate over "intermediate chemo-attractants" (38). This will be described in more details in the section on receptor desensitization (see below).

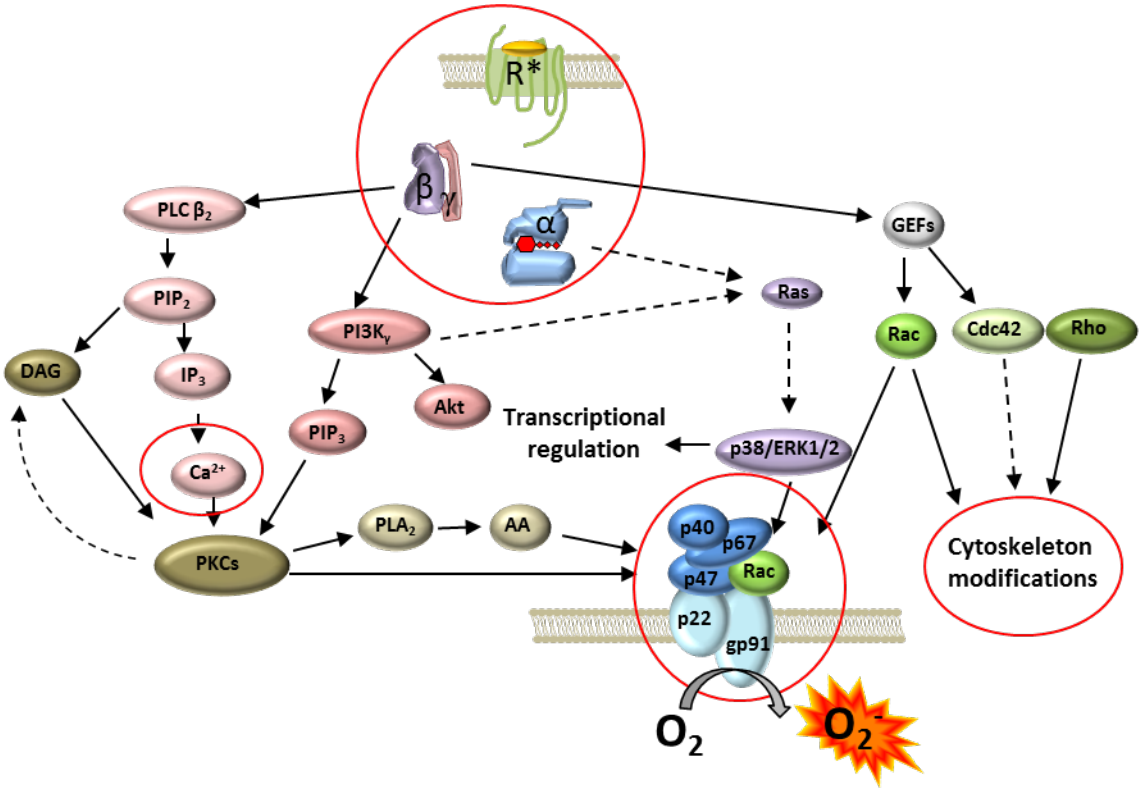

Figure 4. Schematic drawing of the main signaling pathways downstream of activated FPRs in myeloid cells. Agonist binding to the receptors results in dissociation of heterotrimeric G protein which activate downstream effectors and signaling cascades involved in the regulation of cellular functions (chemotaxis, superoxide production and degranulation). R* activated receptor-complex. *Figure adapted from (32).*

Activation of the NADPH-oxidase

Neutrophils have the capacity to phagocytose and kill microbial pathogens. The killing machinery comprises the combined activities of antimicrobial proteins/peptides, hydrolytic enzymes, and ROS (119-121). ROS are formed in an electron transport enzyme system that involves the neutrophil NADPH-oxidase. The functionally active oxidase is a protein complex that is built up from five subunits, two of which are membrane-bound ($gp91^{phox}$, also known as Nox2, and $p22^{phox}$), and the enzyme is located in the plasma membrane or in the membranes of intracellular granules, which can fuse with the phagosome or the plasma membrane (121). This dual location allows neutrophils to engage in both extracellular and intracellular production of reactive oxygen radicals (122). The two membrane-bound subunits together form cytochrome b, and upon cell activation, the remaining three subunits ($p67^{phox}$, $p47^{phox}$, and $p40^{phox}$, located in the cytosol of resting cells) are recruited to cytochrome b-containing membranes and combine to form an active enzyme complex that also contains Rac1 or Rac2, which are small G-proteins of the Rho family (123,124). This complex uses cytosolic NADPH as an electron donor to drive the reduction of molecular oxygen (O_2) to the superoxide anion (O_2^-). The electrons are transported from the cytosol to the oxygen present on the other side of the membrane. Superoxide anions can dismutate to hydrogen peroxide (H_2O_2), either spontaneously or enzymatically through superoxide dismutase (SOD) (125). Both catalase (which enzymatically converts H_2O_2 to O_2 and H_2O) and SOD are antioxidants that protect cells from harmful ROS, and these enzymes can be used as an experimental tool to remove extracellular ROS (Paper II). H_2O_2 can be further processed by myeloperoxidase (MPO) to form more reactive and toxic oxygen radicals. MPO is located in the neutrophil azurophilic granules, which fuse with microbe-containing phagosomes, and the enzyme participates in the generation of halogenated products, e.g., hypochlorous acid ($HClO^-$). These products are highly reactive and bactericidal. Persons who are born with an MPO deficiency have adequate antimicrobial protection, which suggests that the other killing systems together with a functional NADPH-oxidase are sufficient for dealing with microbial challenges (Paper II, 126). The importance of a functional NADPH-oxidase is underlined in patients who suffer from chronic granulomatous disease (CGD). These patients lack a functional NADPH-oxidase, which makes them highly susceptible to recurring infections with bacteria and fungi (127). In these patients, the lymph nodes, lungs, skin, and liver are the most common sites of infection, while prophylactic treatment with antibiotics reduces the frequency of bacterial infections. Although fungal infections have until recently been the leading cause of mortality for patient with CGD, the development of efficient antifungal drugs has greatly improved the treatment of fungal infections in patients with CGD and the mortality rate has diminished (124,127).

There are many chemoattractants and other mediators of inflammation that are capable of inducing activation of the NADPH-oxidase and the production of oxygen radicals. Several of these mediators are described in this thesis (i.e., fMLF, fMIFL, PAF, C5a, galectin-3, IL-8, LTB_4). Oxygen radicals are not only involved in direct microbial killing, but also function as immunomodulators. Oxygen radicals can alter cellular functions, such as phagocytosis, secretion, gene expression, and apoptosis (128).

The generation of radicals can also have regulatory effects. For example, fMLF can trigger its own inactivation mediated by the peroxide system, which oxidizes the methionine residue of fMLF (129). Other molecules such as protease inhibitors and bacterial toxins, are also believed to be sensitive to MPO-catalyzed inactivation though the oxidation of methionine residues (Paper II, (130)). It has been shown that the PSMα peptides, which contain methionine, also trigger their own inactivation, by the same mechanism that involves both H_2O_2 released from neutrophils and MPO (98).

Signaling termination and receptor desensitization

Appropriate termination of receptor signaling is crucial for limiting and resolving the inflammatory process. After activation and signaling, chemoattractant receptors become desensitized, i.e., non-responsive, to a new dose of the same agonist. This desensitization is probably linked to the phosphorylation of residues in the C-terminal region, leading to blockage of the G-protein interaction and subsequent termination of signaling. Phosphorylation of serine and threonine residues in the receptor intracellular domains is mediated by G-protein-coupled receptor kinases (131). Cells that are non-responsive to an agonist that they have already encountered are said to be homologously desensitized; this phenomenon is not limited to chemoattractant receptors but applies to all members of the GPCR family (132). Homologously desensitized cells may retain fully responsiveness to another agonist using a different receptor; i.e., FPR1-desensitized neutrophils are still capable of a response when exposed to the FPR2 agonist WKYMVM (89). Desensitization occurs when the signals from an occupied receptor decrease and ultimately stop, despite a high concentration of the agonist still being present. The biological effects triggered by receptor activation are thereby attenuated. Receptor desensitization (non-responsiveness) is a control mechanism for receptor function that is vital for limiting the release of ROS from activated neutrophils and it is also an important tool for restricting and resolving an inflammatory response (132). Heterologous receptor desensitization, which is a different type of chemoattractant receptor desensitization, will be discussed in detail in the section dealing with receptor cross-talk.

Phosphorylation of ligand-occupied GPCRs mediates high-affinity binding to β-arrestin (133). Arrestins are regulatory proteins that are expressed in all eukaryotic cells, and in general, they affect GPCR function in two different ways: 1) they inhibit signaling through the G-protein-dependent pathways; and 2) they promote receptor internalization. However, FPR1 and the chemoattractant receptor C5aR have the ability to be internalized by an arrestin/clathrin-independent process (134). This indicates that there are alternative means to target receptors for internalization. The binding of β-arrestin to phosphorylated GPCRs sterically blocks receptor/G-protein interactions, thereby terminating the signal (135). Arrestin binding also initiates the process, leading to removal of the receptor from the cell surface, through a clathrin-dependent receptor internalization process (135). It is clear that receptor phosphorylation is of great importance with respect to the termination of agonist-induced responses and receptor internalization. Cells that express FPR1 in which the phosphorylation sites have been mutated show normal chemotaxis towards fMLF but they do not internalize the receptor-ligand complex and

the receptors are not properly desensitized (136,137). The inappropriate desensitization in these mutated cells is characterized by prolonged responses and elevated levels of both calcium and oxygen radicals, as compared with cells that express the wild-type receptor (138,139). In contrast to FPR1 and C5aR, internalization of PAFR has been shown to be dependent upon associations with both arrestin-2 and arrestin-3 (140).

Regarding FPR1, phosphorylation is mediated mainly by GRK2, and the phosphorylation sites are restricted to serine and threonine residues in the C-terminus of the receptor (136). Phosphorylation is independent of PKA, PKC, and tyrosine kinase, as evidenced by the finding that inhibitors of these kinases have no effects on FPR1 agonist-mediated phosphorylation (2,32). Similar to FPR1, FPR2 is phosphorylated at the C-terminus upon ligand binding; the responsible kinase has not been identified. In contrast to the other FPR family members, FPR3 is highly phosphorylated also in the resting state in the absence of ligand binding (2,141).

Cytochalasin B (a fungal metabolite that inhibits reorganization of the actin cytoskeleton; (142)) both prolongs and enhances FPR-mediated responses (143). This suggests that chemoattractant-induced receptor signaling is terminated also through an interaction with the cytoskeleton. Accordingly, the ligand-receptor complex has been shown to bind to the cytoskeleton soon after ligand binding (143,144). This association blocks G-protein binding in a manner very similar to that exerted by β-arrestin. The association between the receptor-ligand complex and the cytoskeleton occurs also at lower (non-physiologic) temperatures (Paper III, (145,146)). The inhibition of cellular phosphatases by treatment with okadaic acid or calyculinA affects termination in a manner very similar to that seen after treatment with cytochalasin B, which suggests that the phosphorylation level is of importance for the interaction between the cytoskeleton and the receptor-ligand complex (147).

Priming

Priming is defined as the process that converts a cell from a low-responding state to a high-responding state. A priming agent (for neutrophils, this can be a receptor agonist, such as TNF-α) drives the cell to a state in which it will exhibit an increased response to another receptor agonist, as compared with the response induced when the same agonist is added to resting cells. The priming process is complex, and the difference between a primed cell and a resting, non-treated or "naïve" cell, depends not only on the type of cell that is primed but also on the priming agent and the triggering agonist. With respect to neutrophils, priming can be achieved through several very different treatments, and the effect of priming on superoxide production is often substantial with respect to both the amplification and duration of the response. Normal neutrophils isolated from the peripheral blood of healthy individuals are by definition non-primed, although priming can be initiated in circulation through systemic effects induced by infections or trauma. The extravasation process, whereby neutrophils are recruited from the blood to an inflamed tissue, normally converts the cells to a primed state (148-150). Investigations of neutrophil priming have mostly been performed *in vitro* using different priming agents (e.g., TNF-α, LPS, GM-CSF, ATP, latrunculin A, cytochalasin B, calyculinA, PAF, and

IL-8), as well as triggering agonists (e.g., formyl peptide receptor agonists, other chemoattractants, and lectins, such as galectins) (151-153).

Even though there are several effective neutrophil priming agents and extensive studies have been conducted on this subject, our knowledge regarding priming mechanisms remains rather limited. Our research group has proposed that granule up-regulation is an important priming mechanism, and an increase in the number of receptors on the cell surface explains (at least in part) neutrophil priming (150,151,154). FPR2 has been shown to be localized primarily to the specific and gelatinase granules of the neutrophils, with only small amounts of FPR2 being found in the plasma membrane and in the secretory vesicles (152). Both specific and gelatinase granules are mobilized during priming, providing the cell with an increasing number of FPR2s on the cell surface, which is in line with the notion that an increased number of receptors correlates with a primed response (152) and the findings presented in Paper I with regard to the priming of Fpr-rs2 responses in murine neutrophils. The sub-cellular distribution of FPR1 is roughly the same as that of FPR2, and mobilization of the specific/gelatinase granules together with the secretory vesicles increases the number of this receptor on the cell surface (152,155).

Changes at the level of the NADPH-oxidase have also been suggested as priming mechanisms. These alterations include phosphorylation of the subunits of the NADPH-oxidase (156), up-regulation (from the granules) or translocation (from the cytosol to the membrane) of the NADPH-oxidase complex (157,158), and increased PI3K activity (159). A common feature of many of the priming agents mentioned above, including TNF-α, is that they do not induce a radical response of their own. Latrunculin A and cytochalasin B both affect actin polymerization, while calyculinA and okadaic acid are phosphatase inhibitors. However, all four toxins are believed to interfere with coupling of the actin network to the receptor, which leads to disruption of termination/ desensitization. These types of agents are expected to prime the cells to many different chemoattractants (Papers III and IV). A novel priming mechanism that involves receptor cross-talk-mediated receptor reactivation, whereby FPR_{des} neutrophils are clearly primed in their responses to both PAF and ATP (Papers III and IV), is described in more detail in the section on receptor cross talk.

Receptor cross-talk

It has become increasingly clear that receptors are not just capable of inducing a single distinct signal, but that they are also capable of interacting with other receptors to create more complex signaling events. The receptor cross-talk discussed here can be defined as a co-ordination of signaling through simultaneous or sequential occupation of multiple cell surface receptors that bind the same or different ligands. This co-ordinated signaling event results in a different type of activation or suppression of cell function, as compared with the signals transmitted from the receptor alone. There is no single mechanism that accounts for all the different types of receptor cross-talk, and the number of possible mechanisms is high. While the receptor cross-talk phenomenon has no defined structural basis, it can involve direct or indirect interactions between receptors of the same or

different types where the activity of one receptor influences the signaling mechanism or conformation of another receptor. So as to avoid complicating this already complex topic, this section will not cover receptor cross-talk between/among cells but will deal primarily with receptor cross-talk in a single cell type, i.e., the neutrophil, with a focus on the GPCRs.

Receptor cross-talk through a direct receptor-receptor interaction

A direct physical interaction between receptors that results in the formation of receptor dimers or oligomers of higher order has emerged as an important topic in GPCR biology over the last decade. Examination of this phenomenon will most likely increase our knowledge and understanding of GPCR function in general. Hopefully, this knowledge can be applied to the development of improved therapeutic strategies and more efficient pharmaceutical agents that target receptor function. Albizu and colleagues have compiled an extensive article on the topic of heteromerization of GPCRs and its relevance to neurologic disorders (160). They describe how heteromerisation of GPCRs are implicated in many neurologic diseases, such as Parkinson's, schizophrenia, and addiction. They define receptor heteromerization as a direct interaction between at least two different functional receptors in which the formed complex possesses biochemical and/or functional properties that are different from the individual components. This physical interaction seems to rely mainly on the transmembrane helices, although the intracellular domains are also of importance. This type of GPCR cross-talk can directly change the function of the receptor complex and alter the regulatory properties or signaling mechanisms. Receptor dimerization also has been shown to affect the process of internalization of the receptor-ligand complex, and alter selectivity for the G-protein and β-arrestin, thereby modulating signaling (160).

The C5aRs has been shown to form homodimers as well as oligomers of higher order early in the biosynthetic process, and this could be important for both proper glycosylation of the terminal regions of the receptors and appropriate delivery of the receptors to the plasma membrane/storage organelles (36). The C5aRs has also been shown to form hetero-oligomers with the chemokine receptor CCR5 as a binding partner (37). This type of physical receptor cross-talk can lead to signaling cross-talk, as illustrated by the finding that binding of a C5aR agonist to its receptor leads to phosphorylation not only of the C5aR, but also of CCR5. The phosphorylated CCR5 no longer responds to receptor-specific agonists but is heterologously desensitized. Thus, activation of C5aR targets both C5aR and CCR5 for internalization, thereby regulating the function of the chemokine receptor (37).

The receptors for IL-8 have been shown to form receptor dimers. Both homodimers (CXCR1 with CXCR1) and heterodimers (CXCR1 with CXCR2) can be formed (49,161), although no studies have yet described this type of dimerisation in neutrophils or described the functional consequences of the formation of such receptor complexes.

A receptor cross-talk phenomenon has also been described for the $P2Y_2$ receptor, and this receptor has been shown to form both homodimers (59) and heterodimers with the adenosine receptor A_1 (60), as well as with non-purinergic receptors (thoroughly reviewed by (61)). While the biological effects of these dimers remain to be clarified, it has been shown for the $P2Y_2/A_1$ complex that simultaneous stimulation suppresses A_1 receptor activity while enhancing the effects of $P2Y_2$.

Unlike many other GPCRs, FPRs do not form homodimers (162). Studies using cell lines that over-express FPR show no differences in receptor signaling and function, as compared with primary cells in which FPRs are expressed together with other receptors, indicating that FPR does not form heterodimers. A recent study using single fluorescent molecule imaging methods revealed that under physiologic conditions, FPRs exist in a dimer-monomer equilibrium and that the dimers are rapidly disassociated into monomers (163). Whether the transient formation of FPR dimers has any impact on FPR functions is not known.

Receptor cross-talk leading to a primed response

Receptor cross-talk can also be more indirect. An individual receptor that lacks direct physical contact with another receptor may influence the signaling of the other receptor. An example of such indirect receptor cross-talk is the priming effect mediated by TNF-α (Paper I, (164)), which in murine cells is mediated by TNFRI, and the signaling events mediated by this receptor prime the cells for their response to an agonist that binds one of the murine FPRs (Fpr-rs2). As mentioned earlier, a previously proposed mechanism that leads to a primed response in human neutrophils involves the up-regulation of surface receptors through mobilization of granule-localized receptor pools (151,152,154). Basic knowledge of priming, as well as the granule structure/content of murine neutrophils is very limited, as compared with our knowledge of the corresponding structures in human cells. It is clear however that TNF-α induces mobilization of CR3 to the cell surface also in murine neutrophils (Paper I). This suggests that murine and human neutrophils share a priming mechanism that involves granule mobilization and up-regulation of Fpr-rs2. Neutrophils isolated from the peritoneal cavities of mice after injection of uric acid show increased levels of CR3 on their surfaces and show increased levels of radicals, as compared with neutrophils isolated from the bone marrow (149). This indicates that the extravasation of neutrophils induces priming, supporting the notion of a role for receptor mobilization in priming.

Many other receptors are able to mediate signals that prime neutrophils in terms of their responses to NADPH-oxidase-activating agonists. Many chemoattractant GPCRs are also potent priming agents, and the correlation between granule mobilization and enhanced superoxide production is strong. To this group of receptors belongs all of the receptors described earlier: PAFR, CXCR1/2, FPR1, FPR2, C5aR, and $P2Y_2$ (44,54,116,165-167).

Receptor cross-talk mediated by downstream signaling

The ability to communicate through receptor cross-talk has been demonstrated for various receptors, including GPCRs. The receptor for hepatocyte growth factor (HGF), termed Met, possesses tyrosine kinase activity and is involved in many biological responses, including epithelial growth, embryonic development, wound healing, tumorigenesis, and tumor metastasis (168). It has been shown that cross-talk between Met and several other receptors, such as the epidermal growth factor (EGF) receptor family members HER2 and HER3, is required for tumorigenesis (169). In gastric cancer cell lines, in which Met expression is amplified, the basal phosphorylation of both EGFR and HER3 is inhibited by inhibitors of Met (170), and Met and HER2 cooperate through downstream signaling by increasing the invasiveness of epithelial cells (171,172).

Cross-talk between hormonal receptors, e.g., G-protein-coupled estrogen receptor (GPER) and the membrane-localized ERα, has also been reported (173 ,174).

The role of cross-talk between integrins and GPCRs in the airway hyper-responsiveness (AHR) of asthmatic patients has recently been investigated and reviewed (175). Downstream cross-talk mechanisms have also been identified that regulate chemo-attractant receptor function. The following sections describe the cross-talk signals implicated in heterologous desensitization and a novel form of receptor cross-talk that involves receptor-dependent reactivation of desensitized FPRs.

Desensitization by receptor cross-talk

In addition to homologous receptor desensitization, there is the phenomenon of heterologous desensitization, which is receptor cross-talk leading to hierarchical desensitization of non-occupied receptors. This type of receptor cross-talk involves two different receptors: one that is occupied by a specific agonist and one unrelated receptor that is unoccupied. The signals generated by the occupied receptor desensitize both receptors, causing the second receptor to be non-responsive to its ligand. Receptor hierarchy and heterologous desensitization are believed to result from the prioritization of end-target chemoattractants over intermediate chemoattractants when several different chemoattractants are present. The C5a/C5aR and formylated peptides/FPRs are regarded as end-target receptor/ligand pairs, while IL-8/CXCR1/2 and LTB4/BLT1 are intermediate chemoattractant pairs (38). Receptor hierarchy and cross-talk through heterologous desensitization not only influence the chemotactic signals, but also the signals that are responsible for NADPH-oxidase activity, as exemplified by the finding that neutrophils activated with the FPR1 agonist fMLF are desensitized not only for further FPR1 stimulation (homologous desensitization) but also for stimulation with IL-8, which is a CXCR1/CXCR2 agonist (Paper III).

The hierarchy is illustrated by the discovery that changing the order in which the agonists are added to the cells does not lead to any desensitization of FPR1 (44,45). This phenomenon is believed to be a consequence of phosphorylation of the unoccupied receptor by second messengers, such as protein kinase A (PKA) and protein kinase C (PKC) (176). It has also been suggested that the PI3K pathway (which is important for

retaining polarity and navigation during neutrophil migration (104)) and the p38 MAPK pathway are used differently by end-target and intermediate chemoattractants for inducing migration (177), while both signal pathways are equally important terms of triggering the NADPH-oxidase (44). It can be speculated that these pathways have different levels of significance depending on which cellular function is used as the readout system.

Receptor reactivation – breaking desensitization

I have recently identified a novel form of receptor cross-talk in which the signals generated during the activation of one receptor reactivate another desensitized receptor. Two distinctively different GPCRs, the receptor for platelet activating factor (PAFR) and the receptor for ATP ($P2Y_2$), share the feature of having agonists (DAMPs) that are released during cellular stress or damage, along with the ability to generate reactivation signals that re-convert desensitized FPRs to the active signaling state. These findings are described in greater detail in Papers III and IV and below.

Actin-dependent and -independent reactivation – roles of receptor cross-talk

I have recently described a novel receptor cross-talk mechanism whereby two receptors, PAFR and $P2Y_2$, independently express the ability to reactivate desensitized FPRs (Fig. 5; Papers III and IV). I show that this reactivation is unidirectional, in that PAFR reactivates

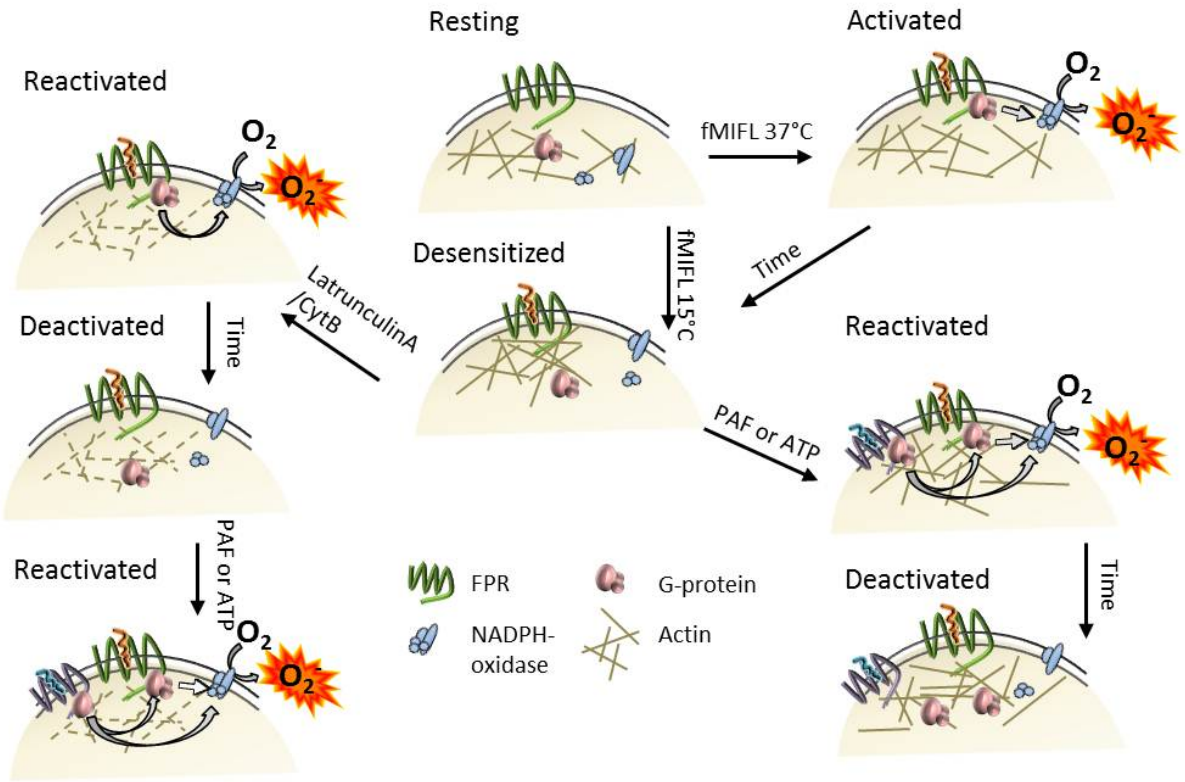

Figure 5. Schematic drawing of different cellular states regarding FPR activation, desensitization, reactivation and deactivation.

FPR_{des} but FPR is not capable of reactivating $PAFR_{des}$ (Paper III). Whether this unidirectional cross-talk also applies to $P2Y_2$/FPRs has not yet been clarified as we currently lack the tools (i.e., good specific inhibitors of $P2Y_2$) required to address this question. The reactivation of FPR_{des} is shown to be separate from receptor internalization and recycling (Paper III).

Some receptors (e.g., FPRs and C5aR) that are desensitized but not internalized can be reactivated by disruption of the cytoskeleton (46). This disruption can be achieved with drugs that interfere with the assembly of G-actin to the filamentous form present in the cytoskeleton network. Cytochalasin B and latrunculin A are examples of such drugs. Data have been presented showing the effects of these drugs and providing evidence for the involvement of the cytoskeleton in the regulation of receptor signaling and termination (178). There are some important similarities between receptor (PAFR or $P2Y_2$)-dependent reactivation of FPRs and reactivation induced by disruption of the cytoskeleton: 1) the addition of receptor antagonists specific for the desensitized receptor inhibit reactivation, indicating that the reactivation signal involves the desensitized receptor; 2) treatment of the desensitized cells with the phosphatase inhibitor calyculinA also clearly reduces reactivation; and 3) no Ca^{2+} transient is induced upon reactivation by either cytoskeleton-disrupting agents or PAFR/$P2Y_2$ (Papers III and IV). Interesting, reactivation of desensitized FPRs by cytoskeleton-disrupting drugs occurs also in differentiated HL-60 cells, which lack secretory granules for storing the reserve pool of mobilizable receptors in neutrophils (145). This suggests that receptor up-regulation is of minor importance in the receptor reactivation process induced by cytoskeleton disruption. An attractive model to explain this reactivation involves the physical removal of the cytoskeleton from the desensitized receptor-ligand complex, allowing the G-protein to bind to the receptor complex and thereby transduce the activating signal. Whether or not this is true for PAFR- and $P2Y_2$-dependent reactivation has not been investigated to date. It is difficult to evaluate the effect of pertussis toxin treatment, as the receptor-dependent reactivation is reliant on both the desensitized and reactivating receptors.

Our initial hypothesis was that PAF reactivates the FPR_{des} through a mechanism similar to that mediated by cytochalasin B/latrunculin A. However, the notion of breakage of the association between the receptor and the actin cytoskeleton was found to be invalid based on the results of the following two experiments: 1) using a technique that stains for polymerized actin, I showed that PAF stimulation did not reduce the total level of polymerized actin during reactivation of the $FPR1_{des}$ neutrophils (in contrast to the effect of latrunculin A); and 2) we showed that $FPR1_{des}$ neutrophils were reactivated by PAF and ATP even when the filamentous actin cytoskeleton was disrupted before activation (Papers III and IV).

The finding that the phosphatase inhibitor calyculinA primes the response induced by FPR agonists in naïve neutrophils but inhibits the cross-talk mechanism while priming the response of fMIFL and PAF in naïve neutrophils, is hard to explain and can only be speculated upon. The relationship between receptor-induced phosphorylation and receptor binding of β-arrestin has been investigated in other cell types (133). Regarding FPR1, it has also been shown that FPR1 phosphorylation is linked to the association with

arrestin-2 (134). Arrestins have long been thought to function exclusively as terminators of the GPCR signaling. However, it has been shown recently that arrestins have several different binding partners and can form scaffolding complexes with mitogen-activated protein kinases (MAPKs), Src, PI3K, Akt, cofilin, and many other factors (133), and that they are capable of signaling without involvement of the heterotrimeric G-protein. Nevertheless, it seems that the nature of the GPCR is still important for establishing the outcome from arrestin involvement. A number of actin assembly proteins have also been shown to interact with β-arrestin, strongly indicating roles for arrestins in actin cytoskeleton reorganization and cell migration (133,179,180). It would be of interest to investigate whether β-arrestin and arrestin scaffold signaling are involved in the reactivation and receptor cross-talk between PAFR and FPRs.

In an attempt to understand the downstream signaling involved in FPR_{des} reactivation, the Ca^{2+} response induced by PAF in FPR_{des} cells was investigated. Calcium mobilization is a part of the signaling pathway for most GPCRs, although the reactivation signals bypass this signaling pathway. There was no amplification of calcium signaling when FPR_{des} cells were stimulated with PAF, and the release of calcium was not inhibited by the FPR1 antagonist cyclosporin H.

Regarding the reactivation of FPRs, this receptor cross-talk mediated by the ATP receptor or the PAFR may help to clear microbes effectively during infections. Reactivation of the receptor may affect both the recruitment of effector cells through modification of the chemotactic response and direct bacterial clearance through enhanced NADPH-oxidase activity. In the case of sterile inflammation, reactivation of FPRs may represent a risk for exacerbation of the inflammation. Thus, reactivation may result in the release of harmful neutrophil substances into the extracellular environment, resulting in additional damage to tissues and cells.

Reactivation of an as-yet-unidentified receptor

Neutrophils that are treated with TNF-α, does not in itself induce radical formation, can be activated by cytochalasin B in a way similar to that observed for FPR_{des} neutrophils. This response most probably involves an as-yet-unidentified GPCR, as the response is pertussis toxin-sensitive (46). The receptor for TNF-α is a $G\alpha_i$-independent single-transmembrane glycoprotein that upon ligation induces the secretion of neutrophil granule constituents. The release of endogenous molecules that bind to an unknown GPCR on the neutrophil plasma membrane may be the basis for the activation potential of cytochalasin B. However, not all secretagogues are able to convert neutrophils to a state in which they can be re-activated by cytochalasin B, which suggests that the secreted products differ depending on the secretagogue used to induce granule mobilization. An alternative explanation is that TNF-α treatment triggers not only secretion, but also cross-talk that converts unknown GPCRs to a state that resembles that of desensitized receptors but without any involvement of an agonist (46).

Galectin-3 breaks the desensitization of FPR through activation of the NADPH-oxidase

Galectin-3, which is a member of the family of β-galactoside-binding lectins, has been described as an endogenous inflammatory mediator with the capacity to activate neutrophils (166). Galectin-3 is involved in several activities of the innate immune system, including the recruitment of inflammatory cells, binding of leukocytes to endothelial cells, and the recognition and killing of bacteria (181,182). Several cells, including neutrophils, produce galectin-3 (although macrophages represent the primarily source), and galectin-3 has the capacity to activate the NADPH-oxidase of human neutrophils, provided that the cells are primed (166,183). The receptor(s) responsible for galectin-3 responses in neutrophils are not fully established. Nonetheless, CD66, which is stored in the gelatinase and specific granules, has been proposed as a candidate receptor for galectin-3 (184), while several structurally and functionally diverse candidate receptors have also been described (185). It is known from earlier studies that fMLF can function as a priming agent and secretagogue to induce neutrophils to mobilize galectin-3 receptors (166). I have discovered a novel function for galectin-3 as an inflammatory mediator, whereby galectin-3 induces the inactivation of chemoattractants, thereby breaking the desensitized state of FPR1. This inactivation is achieved through oxidation of fMLF and is dependent upon reactive oxygen metabolites generated by the combined effects of the NADPH-oxidase and MPO (Paper II). The ability of galectin-3 to trigger fMLF inactivation is dependent upon the carbohydrate recognition domain (CRD) of the lectin and on both hydrogen peroxide and MPO (Paper II).

The interplay that occurs between the different inflammatory mediators during inflammatory processes needs to be considered to understand fully the course of events *in vivo*. Even if the individual actions of many inflammatory mediators are starting to be mapped out from *in vitro* studies, the complexity of multiple signaling ligands *in vivo* should not be underestimated. My finding that galectin-3 can mediate the inactivation of the neutrophil activator fMLF is an example of how the signals from one ligand-receptor complex influence the status of another receptor (Paper II), through indirect receptor cross-talk. Accumulating evidence implies that the low levels of radicals produced by the NADPH-oxidase serve as regulators of immunity, as well as autoimmunity, by suppressing lymphocytes involved in the elimination of tumor cells and autoreactive cell clones (186-188).

The oxidative activation and oxidative modulation activities that I demonstrate for galectin-3 are most likely shared by other inflammatory mediators, and this underlines the pathophysiologic significance of oxygen radicals.

Future perspectives

FPRs participate in different types of receptor cross-talk signaling with very different outcomes. On one hand, FPRs have the capacity to induce heterologous desensitization of intermediate chemoattractant receptors, such as CXCR1/CXCR2 and BLT1, owing to the fact that they are higher in the hierarchy of chemoattractant receptors. On the other hand, FPRs also have the ability to be reactivated by other inflammatory mediators upon binding to their cognate receptors. The roles of FPRs as regulators in immunity are therefore multifaceted and require further investigation. Additional studies and elucidation of the mechanisms underlying this reactivation may provide the insight needed for the development of treatment strategies for inflammatory disorders and new therapeutic drugs.

The future studies should attempt to examine whether the sub-cellular localizations of FPRs and PAF play a role in receptor reactivation. FPRs are located within mobilizable granules (152,155), whereas our preliminary data suggest that the PAFR is expressed solely in the plasma membrane, in agreement with earlier published data on the localization of the IL-8 receptor (43). As is the case for IL-8, PAFR cannot be reactivated by cytoskeleton-disrupting drugs (cytochalasin B/latrunculin A). Regarding the sub-cellular localization of the different chemoattractant receptors in murine cells, nothing is really known, and it would be of great interest to determine the differences/similarities between mice and men with respect to the processing of this group of GPCRs. Current knowledge regarding the presence/localization and functions of the receptors in neutrophils that recognize ATP is also rather limited, and it would be of interest to investigate this in more detail. However, good selective agonists/antagonist, as well as other tools (e.g., receptor-specific antibodies) are still lacking, making this type of work rather difficult at the moment.

It would also be interesting to determine the role of arrestin in the termination of GPCR signaling in neutrophils and in the receptor-dependent cross-talk that leads to reactivation. Is arrestin binding of importance for FPR-triggered responses? Neutrophil studies on this subject are lacking, and it is a challenge to develop techniques that make it possible to tackle experimentally this issue in primary neutrophils. Regarding receptor internalization and the role of arrestin, differences have been noted in that FPR1 can be internalized independently of arrestins (134), whereas PAFR internalization is dependent upon both arrestin-2 and arrestin-3 (140). It should be noted that these studies were performed with cloned receptors in different types of tissue cultured cells, and nothing is really known about the dependence/independence of arrestin in primary neutrophils.

The chemotactic receptor signaling dogma states that a very early signal following receptor activation triggers the PLC/Ca^{2+} signaling route, although it is obvious that the PAF- or ATP- induced FPR_{des} reactivation bypasses this signaling route. Thus, PAFR utilizes at least two different signaling pathways, and it would be interesting and challenging to determine the precise signaling pathway that leads to receptor reactivation. Understanding the inhibitory effect of calyculinA on the cross-talk that leads to reactivation would also provide insights into the molecular mechanism. The search for potent inhibitors that

affect the cross-talk signal downstream of the receptors (using receptor-specific inhibitors) would be of great interest for further *in vivo* investigations into the relevance of receptor cross-talk. Our laboratory has previously used a screening facility to identify receptor-selective small molecules and it would be possible to use the same strategy to screen for new molecules that affect specifically the receptor cross-talk. Another important question is whether murine neutrophil receptors talk to each other in the same way as human receptors. If so, the option to explore different gene knockout models could also be a way to investigate both the *in vivo* relevance of the cross-talk phenomenon, but also an opportunity to clarify the underlying molecular mechanism.

Future studies should aim to understand the biological significance of receptor cross-talk *in vivo. In vitro* studies to clarify the signaling mechanisms behind these phenomena will provide some clarification as to how different subsets of receptors communicate and affect each other. Further investigations are clearly needed to reveal the molecular mechanisms behind the regulation of FPR reactivation. The relevance of receptor cross-talk probably differs depending on the particular cause of the response and the inflammatory mediators present, as well as the involvement of other regulatory factors that act in combination.

Populärvetenskaplig sammanfattning

Miljön vi lever i är full av mikroorganismer som bakterier, virus och svampar som är potentiellt sjukdomsframkallande. Men vi har under evolutionens gång utvecklat bra system (immunförsvar) för hur vi kan skydda oss från infektioner. Till att börja med har vi fysiska barriärer i form av vår hud och slemhinnor som tillsammans med saliv, magsyra och sekret utgör kraftfulla och potenta hinder för mikroberna. Men ibland händer det att någon mikrob lyckas ta sig igenom den första barriären och det är då viktigt att vårt inre immunförsvar skyddar oss och avlägsnar hotet innan vi blir allvarligt sjuka. Vårt inre immunförsvar består till största delen av vita blodceller som hela tiden cirkulerar vårt blod, beredda att agera vid tecken på fara. Immunförsvaret kan delas upp i två delar, det medfödda och det adaptiva/förvärvade. Det adaptiva immunförsvaret är unikt för varje individ eftersom det lär sig och blir bättre och bättre på att försvara vår kropp för varje ny infektion. Det är också det som aktiveras vid en vaccination så att vi blir motståndskraftiga mot en viss typ av infektion. Vårt medfödda immunförsvar är däremot fullt utvecklat från födseln och ser väldigt likt ut mellan olika individer. Cellerna i det medfödda immunförsvaret är svåra att utbilda eller lära upp utan har genom evolutionen lärt sig känna igen potentiella hot genom gemensamma strukturer som uttrycks av bakterier och virus. Det medfödda immunförsvaret agerar fort och på samma sätt och kan inte effektiviseras. Neutrofilen tillhör vårt medfödda immunförsvar och utgör 60-70% av våra vita blodceller men antalet ökar kraftigt vid en infektion/inflammation. Neutrofilen är en fagocyterande cell, vilket betyder att den kan omsluta/äta upp mikroorganismer. Vid en skada, t.ex. ett sår, så producerar kroppen substanser som lockar till sig neutrofiler från blodbanan och de vandrar sedan genom vävnaden mot skadan mot en så kallad gradient av kemotaktiska faktorer. Dessa faktorer kan komma från skadade celler eller från bakterier i såret och känns igen av neutrofilerna genom speciella receptorer som de uttrycker på sitt cellmembran. Receptorerna är ansvariga för den kemotaktiska rörelsen mot inflammations/infektions härden, men kan också hjälpa till att fagocytera och döda av bakterier. Avdödningen sker med hjälp av syreradikaler men även andra antimikrobiella substanser. Men eftersom receptorerna också känner igen substanser från trasiga celler eller som produceras vid inflammation så finns det risk att neutrofilen frigör radikaler i omgivningen vilket kan vara direkt skadligt för den egna vävnaden. Därför är det av största vikt att neutrofilen har kontrollerande aktiverings- och avstängningsfunktioner.

I min avhandling har jag studerat neutrofilen, deras receptorer och hur de aktiveras och kontrolleras. Jag har fokuserat på en receptorfamij som heter FPR, som känner igen peptider (korta proteiner) som produceras av bakterier men också av mitokondrier, våra cellers kraftverk. FPR receptorerna förmedlar kemotaktiska signaler som hjälper cellen att hitta infektionen/inflammationen men också signaler som aktiverar produktionen av syreradikaler. Jag har funnit att FPR receptorerna är kapabla att samverka med andra receptorer som också uttrycks av neutrofiler och på så sätt skapas ett tämligen komplicerat signaleringsmönster som ser olika ut beroende på vilka faktorer som finns i cellens omgivning. I tre av de fyra arbeten som avhandlingen baseras på, studerar jag fenomenet receptordesensitisering. Det är en form av receptorinaktivering där receptorer som bundit

deras specifika agonist inte svarar på en ny stimulering med samma agonist. Jag visar i min avhandling att desensitiseringen kan brytas på olika sätt.

I arbete I så har jag studerat neutrofiler hos möss och kartlagt hur de aktiveras av peptiden WKYMVm. Vi visste sen tidigare att WKYMVm aktiverar humana neutrofiler genom FPR2 och vi fann att den också aktiverar musneutrofiler. Musneutrofiler uttrycker flera Fpr receptorer och för att närmare avgöra vilken receptor som aktiveras av WKYMVm så studerade vi en cellinje som transfekterats och uttryckte bara en av dessa, nämligen Fpr-rs2. Vi visar att den receptorn kan aktiveras av WKYMVm och också hämmas av den FPR2 specifika inhibitorn WRW_4. Vi föreslår därför att Fpr-rs2 är den murina motsvarigheten till den human FPR2. Vi konstaterar också att cytokinen TNF-α primar (förstärker) svaret medierat av WKYMVm hos musneutrofiler och att primingeffekten föremedlas av TNF-receptor typ I (TNFRI). Den slutsatsen drar vi genom att musneutrofiler som saknar TNFRI inte primas av TNF-α. Primingen leder både till en större produktion av syreradikaler och också en mobilisering av intracellulära organeller vilket resulterar i ett ökat antal receptorer på cellernas yta.

I arbete II undersöker jag hur humana neutrofiler som har interagerat med peptiden fMLF och därmed är icke-responsibla, desensitiserade, i fMLFs receptor FPR1, kan bli responsibla igen genom inkubering tillsammans med lektinet galectin-3. Jag visar att galectin-3 aktiverar neutrofilen att producera syreradikaler som i sin tur bryter ner fMLF. Den sockerbindande delen av galektin-3 är viktig för att nedbrytningen ska ske eftersom laktos (blockerar den sockerbindande delen) kan inhibera nedbrytningen. Likväl förlorar ett muterat galektin-3, som inte längre har en fungerad sockerbindning, förmågan att inducera nedbrytningen. MPO, ett peroxidas väsentligt för neutrofilens produktion av högreaktiva syreradikaler, visade sig också vara viktigt för nedbrytningen då neutrofiler från en patient som saknar MPO, inte kunde bryta ner fMLF.

I arbete III upptäcker jag en ny form av receptor kommunikation. Här visar vi att receptorn för lipiden PAF, PAFR, kan reaktivera desensitiserade FPR-receptorer. Vi vet fortfarande inte genom vilken molekylär mekanism reaktiveringen sker men vår initiala hypotes var att cytoskelettet var inblandat. Men hypotesen visade sig felaktig eftersom reaktiveringen kunde ske även om man slår ut cellens cytoskelett (som är viktigt för både cellens form, funktion och rörelse). Vi finner också att både FPR och PAFR är viktiga för reaktiveringen eftersom inhibitorer för båda receptorerna kan förhindra reaktiveringen. Vidare kan fosfatasinhibitorn calyculinA inhibera reaktiveringen men vi kan ännu inte dra några slutsatser hur det påverkar den molekylära mekanismen. Reaktiveringen är också en envägskommunikation eftersom FPR inte kan reaktivera desensitiserade PAFR.

Arbete IV är mycket likt arbete III men här studerar vi hur ATP delar PAFs förmåga att reaktivera desensitiserade FPR. ATP har många olika receptorer som kan förmedla signaler efter att ha bundit ATP. Vi försöker att kartlägga vilken receptor som är ansvarig för reaktiveringen av FPR men saknar bra verktyg i form av bra receptor-inhibitorer, antagonister. Men då vi kan ersätta ATP med UTP, en trifosfat som är mycket lik ATP, kan vi begränsa antalet receptorer. Vi tror därför att det är receptorn P2Y2 som är den receptorn som förmedlar signalen som reaktiverar FPR.

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