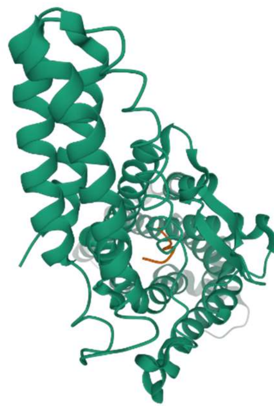
Neutrophil Function and Signaling Induced by Ligands for the Formyl Peptide Receptor 2



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“Once we accept our limits, we go beyond them”

Albert Einstein

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ABSTRACT

Neutrophil pattern recognition receptors belonging to the G-protein coupled receptor (GPCR) family play a role in the processes of initiation as well as resolution of inflammatory processes. Formyl peptide receptor 2 (FPR2) in neutrophils is such a receptor and plays an important role in inflammation.

This thesis focuses on the molecular basis for FPR2 ligand recognition, receptor signaling and activation of neutrophils. The experimental data generate new knowledge that is related specifically to FPR2 but also of general importance for GPCR function, knowledge possibly of importance also for future drug development. To characterize FPR2 mediated signaling, cell-based *in vitro* methods were used, including sensitive methods to measure i) production of reactive oxygen species (ROS), ii) the transient rise of intracellular calcium ions, iii) chemotactic migration, iv) β-arrestin recruitment and, v) the dynamic reorganization of the actin cytoskeleton. A new class of FPR2 ligands belonging to peptide mimetics (peptidomimetics) were identified and characterized as functional selective (biased) agonists triggering ROS release but not chemotaxis, a neutrophil function linked to receptor recruitment of β-arrestin. A novel receptor crosstalk-signaling pathway is also disclosed, a pathway leading to a reactivation of desensitized FPRs and involve a Gαq containing G-protein downstream of the receptor for platelet activating factor (PAFR). Data obtained with Barbadin, an AP2 inhibitor able to impair endocytosis of many GPCRs, clearly show that internalization of ligand-bound FPR2 occurs independently of β-arrestin. In addition, a lipopeptide (pepducin)

suggested to be a putative Gαq-inhibitor, is shown to lack inhibitory effect of the neutrophil response mediated by Gαq linked PAFR, but instead distinctly modulates the function of both FPR2 and the free fatty acid receptor FFAR2, two Gαi-coupled neutrophil GPCRs.

In conclusion, this thesis adds new knowledge and novel insight into FPR2 signaling in neutrophils and GPCR regulation mechanism in general. Hopefully this knowledge will contribute to future drug development for treating inflammatory diseases.

SAMMANFATTNING PÅ SVENSKA

Vi träffar dagligen mikroorganismer som kan orsaka sjukdom och i värsta fall död, men trots det är vi sällan allvarligt sjuka, det beror på ett effektivt immunförsvar som har utvecklats för att skydda oss mot sjukdomsframkallande mikroorganismer som bakterier, virus, svamp och parasiter. De vita blodkropparna som bildas i benmärgen och när de mognat rekryteras till blodbanan där de utgör basen för immunförsvaret. Immunförsvaret är uppbyggt av många olika försvarsmekanismer, med funktion att hitta, döda och om det behövs lagra information om den specifika mikroorganism som invaderat värden. Immunförsvaret består av två delar, det medfödda och det förvärvade. Immuncellerna känner igen specifika strukturer som mikroorganismerna uttrycker och de har förmåga att särskilja dessa strukturer från egna celler och vävnader. Det förvärvade immunförsvaret är specifikt riktad mot mikrogranismen som aktiverade det. Det har också en minnesfunktion som ger ett långvarigt skydd mot den specifika mikroben. Den här processen tar dock dagar till veckor att initiera, vilket innebär att är beroende också av ett snabbare försvar. Detta medfödda immunförsvarets celler känner igen ett begränsat antal strukturer som finns hos många olika mikroorganismer men ibland också hos skadade kroppsegna celler och vävnader. De här molekylära ”fingertrycken” talar om att immunförsvaret snabbt skall aktiveras. Den så kallade neutrofila granulocyten är en immuncell som finns i stort antal i vårt blod och som är mycket viktig i det medfödda immunförsvaret. När mikroorganismer bryter igenom skyddande barriärer som hud och slemhinnorna får neutrofilerna en larmsignal i form av molekyler som bildas av invaderande mikroorganismer

och/eller skadad vävnad. Dessa signaler gör att neutrofiler lämnar blodet och beger sig till stället där de invaderande mikroorganismerna/vävnadsskadan finns. Väl där börjar neutrofilerna med hjälp av sina effektiva ”vapen” att döda de oönskade mikroorganismerna, och när detta är avklarat ansvarar de också för att påbörja själva läkeprocessen. De syreradikaler, som är ett av neutrofilernas ”vapensystem”, är väldigt reaktiva och kan skada den också egna vävnaden så det är kritiskt att produktionen regleras noga. Neutrofilerna känner igen de molekylära ”fingeravtrycken” från mikroorganismer med hjälp av proteiner som sitter på cellens utsida och kallas receptorer. Dessa upplyser (signalerar) cellen om faran som hotar och talar om vad den skall göra. En receptorgrupp kallas för sjutransmembran eller G-protein kopplade receptorer (GPCRs) beroende på att de har en gemensam struktur (de passerar det membran de sitter i sju gånger) och de vidarebefordrar informationen med hjälp av ett speciellt signalprotein (G-protein). Receptorer som tillhör den här gruppen eller familjen reglerar bland annat hur neutrofiler hittar och dödar mikroorganismerna. Molekyler som aktiverar en receptor kallas för agonister och de som blockerar funktionen kallas för antagonister. Dessa aktiverande eller inhiberande molekyler kan komma såväl från mikroorganismer som från oss själva, eller vara syntetiska i form av läkemedel. Målsättningen med avhandlingsarbetet har varit att undersöka hur receptorer som uttrycks av neutrofiler och som tillhör GPCR familjen, känner igen agonister/antagonister och sedan för information vidare till cellen. Fokus i arbetet har varit en receptor som fått namnet formylpeptid receptor 2 (FPR2), beroende på att den tillhör

en grupp av receptorer som känner igen så kallade peptider (en kedja av olika aminosyror) som har en formylgrupp (en CHO-grupp). Jag har i arbetet använt mig av celler isolerade ur blod från friska blodgivare. De resultat som presenteras visar hur olika slags agonister som aktiverar samma receptor, sätter igång olika signalvägar i cellen. En ny klass av agonister/antagonister är också karaktäriserade som är lämpliga för studier i djurmodellerna och kan bli framtida läkemedel. Jag visar också hur receptorer kan ”prata” med varandra och hur FPR2 signaleringen stängs av. De nya kunskaperna kan förhoppningsvis vara till hjälp vid framtida utveckling av läkemedel för behandling av inflammatoriska sjukdomar, men också bidra till förståelsen av hur andra receptorer som tillhör GPCR-familjen fungerar och hur aktiviteten hos dessa kan styras.

LIST OF PAPERS

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

I. **Reactivation of Gαi-coupled formyl peptide receptors is inhibited by Gαq selective inhibitors when induced by signals generated by the PAF receptor**

   André Holdfeldt, Agnes Dahlstrand Rudin, Michael Gabl, Zahra Rajabkhani, Gabriele M. König, Evi Kostenis, Claes Dahlgren, and Huamei Forsman. *Published in: J Leukoc Biol.102(3):871-880, 2017*

II. **Structure–Function Characteristics and Signaling Properties of Lipidated Peptidomimetic FPR2 Agonists: Peptoid Stereochemistry and Residues in the Vicinity of the Headgroup Affect Function**

   André Holdfeldt, Sarah Line Skovbakke, Michael Gabl, Christina Nielsen, Claes Dahlgren, Henrik Franzyk, and Huamei Forsman. *Published in: ACS Omega 4 (3), 5968–5982, 2019*

III. **The PAR4-derived pepducin $P4Pal_{10}$ lacks effect on neutrophil GPCRs that couple to Gαq for signaling but distinctly modulates function of the Gαi-coupled FPR2 and FFAR2**

   André Holdfeldt, Simon Lind, Camilla Hesse, Claes Dahlgren, Huamei Forsman. *Published in: Biochemical Pharmacology 180, 114143, 2020*

IV. **Barbadin selectively modulates FPR2-mediated neutrophil functions independent of receptor endocytosis**

   Martina Sundqvist*, **André Holdfeldt***, Shane C Wright, Thor C Møller, Esther Sia, Karin Jennbacken, Henrik Franzyk, Michel

Bouvier, Claes Dahlgren, Huamei Forsman. *Published in: Biochim Biophys Acta Mol Cell Res. 867(12):118849, 202*0. * These authors contributed equally

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ABBREVIATIONS

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
C5aR	Component 5a receptor
$Ca2^+$	Calcium ion
CR	Complement receptor
DAG	Diacylglycerol
DAMP	Danger associated molecular pattern
dsRNA	Double-stranded RNA
ERK	Extracellular signal-regulated kinase
FPR	Formyl peptide receptor
G-CSF	Granulocyte colony-stimulating factor
GDP	Guanosine diphosphate
GPCRs	G-protein coupled receptors
GRK	G-protein coupled receptor kinase
GTP	Guanosine triphosphate
HEK	Human embryonic kidney cell line
Hsp27	Heat shock protein 27
HL-60	Human promyelocytic leukemia cell line
IL	Interleukin

ILR	Interleukin receptor
IP3	Inositol trisphosphate 3
LPS	Lipopolysaccharides
MAPK	Mitogen-activated protein kinase
NADPH-oxidase	Nicotinamide adenine dinucleotidephosphate oxidase
NFκB	Nuclear factor κ-light-chain-enhancer of activated B-cells
O_2^-	Superoxide anion
PAF	Platelet activating factor
PAFR	Platelet-activating factor receptor
PAMP	Pathogen associated molecular pattern
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol 4, 5-bisphosphate
PIP3	Phosphatidylinositol (3, 4, 5)-trisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PMN	Polymorphonuclear leukocyte
PRR	Pattern recognition receptor
PSM	Phenol-soluble modulin
Rac	a GTPase
ROS	Reactive oxygen species
SAA	Serum amyloid A

TLR	Toll-like receptor
TNFR	Tumor necrosis factor receptor
TNFα	Tumor necrosis factor α

INTRODUCTION

The inflammatory response is initiated when microorganisms or trauma inflicts tissue injury. It is a vital multifaceted cellular response aimed to kill invading microbes and repair damaged tissue. However it is critical that this process is tightly controlled; immune system disorders occurs when the response is diminished or excessive but also in form of autoimmune conditions when the response is aimed at host cells/tissue. Neutrophils are innate immune cells that are key players in the inflammatory responses and the first cell type to arrive at the affected tissues. Receptors are proteins that receive and then transduce signals, which are integrated into the cell and play a critical part in the regulation of the inflammatory process. To exert proper functions, neutrophils rely on surface expressed G-protein coupled receptors (GPCRs), a group of membrane-spanning receptors that regulate many different functions in almost all of our cells. Formyl peptide receptor 2 (FPR2) is a GPCR, and it is a critical regulator of inflammation. FPR2 has been proposed to trigger both pro- and anti-inflammatory responses depending on the ligand that activates the receptor. This is in line with the ability of GPCRs to induce biased signals, a response induced by receptor specific ligands that trigger one receptor-signaling pathway over another, which will lead to a distinct cellular response. The focus of this PhD thesis is to uncover the molecular basis for FPR2 recognition and signaling in neutrophils, with the aim to generate new knowledge about FPR2 but also about GPCRs in general.

THE INNATE IMMUNE SYSTEM

Overview

The inflammatory reaction constitutes an important part of the innate immune defense system evolved to eliminate microbes, initiate clearance of damaged/necrotic cells/tissues and to start the tissue repair mechanisms leading to wound healing. However, sometimes an inflammatory reaction does not resolve properly, and instead either causes or increases the destruction of the inflamed tissue. In the worst-case scenario, this leads to a local or systemic acute or chronic inflammation that may cause/lead to a serious disease [1]. It is thus of outmost importance that the inflammatory process is tightly regulated.

Pathogenic microbes are constantly challenging the human body in a variety of ways. Despite this, severe infections are relative rare, and we have to thank a remarkable efficient immune system for this. The innate immune system is an ancient defense system with key immune mechanisms being shared between mammals, plants and invertebrates. It phylogenetically appeared around 750 million years ago and is remarkably conserved [2]. Attacks by a significant part of potentially harmful microorganisms that pass skin and mucous membranes are terminated by mechanisms of the innate immune system. Only a small portion of highly virulence strains of bacteria and viruses require activation of the adaptive immune system, and this activation relies on a tight coordination with the innate part of our immune system [1].

The concept of immunity

The immunity concept relies on the ability of the defense systems to distinguish between “self” (body constituents) and “non-self” (foreign materials) as well as danger signals, and to direct the response towards elimination/killing and ultimately healing. A critical feature to discriminate between “self” and “non-self” are receptors for pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). PAMPs are conserved structures expressed by microorganisms such as lipopolysaccharides (LPS) from Gram-negative bacteria, cell wall teichoic/lipotechoic acids and peptidoglycans from Gram-positive bacteria and, formylated peptides generated by all bacteria. These PAMPs will activate the innate immune system when they are recognized by different receptors expressed by host cells. DAMPs are host-derived molecules such as adenosine triphosphate (ATP), formylated peptides of mitochondrial origin and so called heat-shock proteins that are recognized by cells of the innate immune system as a signal of necrotic cell death/tissue destruction [3].

Receptors expressed for recognition of these DAMPs and PAMPs includes the membrane-localized pattern recognize receptors (PRRs) e.g., some of the cell surface and endosomal compartment expressing Toll-like (TLRs), C-type lectin receptors (CLRs) and the formyl peptide receptors (FPRs). TLRs is a super family of membrane spanning receptors and there are 10 active members in humans; TLRs are high affinity receptors for a diverse set of PAMPs including LPS (TLR4) and dsRNA from viruses (TLR3). Activation of TLRs will induce production of different pro-inflammatory cytokines such as IL-1, IL-6,

IL12 and TNF-α [4]. CLRs recognize different microbial carbohydrate structures, which enable the receptor expressing cells to induce immunity through activation of pro-inflammatory mediators as NFκβ [5]. FPRs are classical chemoattractant receptors belonging to the GPCR family and they are key participants in innate immunity, with the role to guide receptor expressing leukocytes to sites of inflammation [6]. In addition to the membrane-localized receptors, there are also cytosolic receptors of importance for regulation of cell functions. Cytoplasmic PRRs include NOD-like nucleotide-binding oligomerization domain-like receptors (NOD-like receptors) that recognize microbes that in one way or another have entered the cell, DNA sensor cyclic GMP-AMP synthase (cGAS) [7] as well as RNA sensor retinoic acid-inducible gene I (RIG-I)-like receptors that recognize virus infections [8].

Neutrophils are the most abundant (50-70%) leukocyte in human blood and play critical roles in the elimination of pathogenic microorganisms. Neutrophils have a characteristic segmented nucleus and a large number of cytoplasmic granules. Their critical role in immunity is well illustrated by the enhanced susceptibility to opportunistic fungal and bacterial infections linked to defects in neutrophil maturation or functions [9-11]. Neutrophils are innate immune cells that together with basophils and eosinophils form the subgroup polymorphonuclear (PMN) leukocytes or just granulocytes. Epithelial cells serve as a barrier between outer surfaces and the endothelial lining cells of the blood vessels are the first to react to pathogens breaching the epithelial surface and initiate the immune/inflammatory response. This response is characterized by rapid accumulation of neutrophils and other innate

immune cells like macrophages, monocytes and dendritic cell, at the site of injury, a process coordinated by released PAMPs and DAMPs [3]. At the site of injury, the recruited cells initiate the killing of the invading microbes. This process is achieved by the effects of antimicrobial peptides, proteolytic enzymes, and reactive oxygen species (ROS) generated and released in the environment or inside enclosed intracellular vesicles (phagosomes) containing engulfed (phagocytosed) microbes. Optimally, the process is finalized by a removal of microbes and necrotic/apoptotic host cells and tissue debris.

A powerful killing system of its own and an important supplement to enhance the immune response, is the complement system, built-up by around 30 plasma proteins that together have the capacity to trigger a powerful pro-inflammatory response. The system is activated through an amplification cascade, and there are three main pathways that initiate and lead to an activation of the complement system: i) the classical pathway, initiated by antibodies bound to a target or by one of the complement components (C1q) bound to the microbial surface; ii) the lectin pathway, triggered by binding of a host-protein with sugar binding capacity to bind carbohydrates or glycoproteins present on bacterial and fungal surfaces; iii) the alternative pathway is triggered by the activation and binding of one of the complement proteins (C3/C3b) directly by/to the surface structures on a microbial pathogen. All three pathways will give rise to split products (protein fragment) that facilitate the engulfment of the microbes (opsonization) and participate in the recruitment of immune cells to the site of infection [12].

The human innate immune system also consists of endogenous antimicrobial peptides that can act both as weak broad-spectrum antibiotics and as immune-modulators. Such peptides constitute a defense line in the skin, at epithelial and mucosal layers and are also part of the killing arsenal in neutrophils [13]. Prominent examples of such antimicrobial peptides include the defensin group of short peptides, as well as the human cathelicidin LL-37. Their importance is shown in the immune compromised phenotype of patients with a deficiency/lack of affecting defensins or LL-37 [14, 15].

THE NEUTROPHIL

The Neutrophil Life Cycle

Hematopoietic stem cells in the bone marrow produce around $5x10^{10}$-10^{11} neutrophils daily and the mature cells are released into the blood where they constitute the circulating as well as the marginating pool [16]. The blood neutrophils have three different cytosolic granule types and one membrane vesicle that are all mobilizable. These granules/vesicles are formed during maturation/differentiation in the bone marrow and they contain stores of bactericidal enzymes, proteolytical enzymes, the membrane components of the radical producing NADPH-oxidase and a reserve pool of membrane receptors. These granules/vesicles are mobilized when neutrophils go from a resting state to a primed or active state [17]. A breakthrough in the understanding of neutrophil granule biology was when experimental separation of granules through a subcellular fractionation technique was described [18-20].

During granulopoiesis (Figure 1), myoblasts differentiate to promyelcytes and then to myelocytes that will mature to metamyelocyte, band cells and finally segmented neutrophilic cells that are released to the blood as mature neutrophils. Azurophilic granules are formed during the promyelocyte stage, specific granules during the myelocytes/ metamyelocyte stage, gelatinase granules as band cells, and finally the secretory vesicles are formed from the plasma membrane through an endocytic process in segmented neutrophilic cells [21].

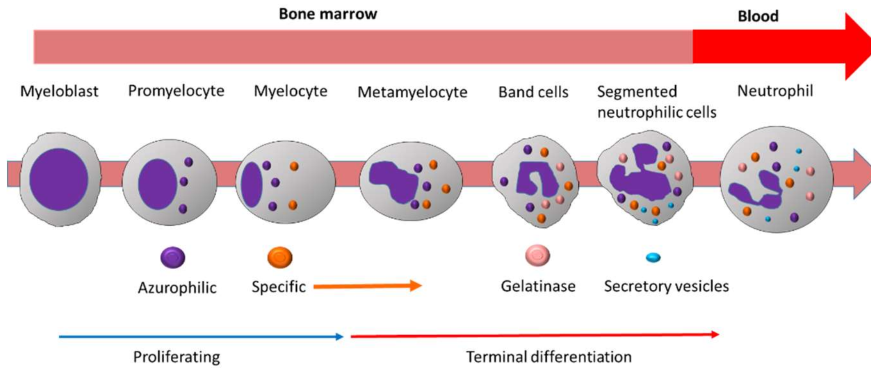

Figure 1. Schematic illustration of neutrophil maturation and granulopoiesis. *Myeloblasts differentiate to promyelocytes (where Azurophil granules are formed) followed by differentiation to myelocytes and metamyelocyte (where specific granules are formed). The subsequent maturation steps includes band cells (where gelatinase granules are formed), and segmented neutrophilic cells (where secretory vesicles are formed), which ends with mature neutrophils released to the peripheral blood.*

A number of diseases are associated with impaired neutrophil maturation and granulopoiesis. Severe congenital neutropenia 1 (SCN1) is a rare disease characterized by decrease in circulating neutrophils. The disease is caused by mutations in the gene ELANE, that codes for the protein neutrophil granule serine protease elastase [22]. The granulocyte population of SCN1 patients is skewed towards eosinophils and patients presents with eosinophilia both in bone marrow and blood [23]. This clearly shows that this neutrophil granule serine protease is important for neutrophil maturation in the bone marrow.

Specific granule deficiency (SGD) is a rare primary immunodeficiency characterized by neutrophils with diminished granules and lack of granule proteins in specific and gelatinase granules and proteins expressed in azurophilic granules during the late promyelocyte proliferation phase. The disease is caused by mutations in myeloid transcription factor CCAAT/enhancer-binding-protein-ε. Neutrophils from SGD patients are impaired in chemotactic migration and bacterial killing [24, 25].

Neutrophils in peripheral blood have generally been considered short-lived with a half-life of only 6-8h [16]. However a study with stable isotope labeling with heavy water shown neutrophil life length of 5.4 days [26], however this study has been disputed [27]. The lifetime of neutrophils remains a controversial topic that yet has to be solved.

The number of neutrophils in the blood can be increased through a rapidly recruitment from a considerable storage pool in the bone marrow [28]. Accordingly, during an infection/sterile inflammation, neutrophils are rapidly mobilized from the bone marrow to the blood for further transmigration to the infected tissue. The release from the bone marrow to the blood is regulated by chemokine receptors [28, 29]. Stromal cell express CXCL12, the endogenous ligand for the receptor CXCR4 that is the retaining signal for neutrophils in the bone marrow. Granulocyte colony-stimulating factor (G-CSF) is the main protein that induces the release of neutrophils from the bone marrow and it is also of main importance for the proliferation from precursor to mature neutrophils [28]. The release is probably to large extent mediated through endothelial cells expression of CXCL2, the endogenous ligand

for CXCR2. These observations are consistent with the neutropenia phenotype associated with “gain of function” of CXCR4 or “loss of function mutations of CXCR2 [30]. Senescent neutrophils circulating in the blood have increased expression of CXCR4, which allows them to return back to the bone marrow for clearance. Tissue neutrophils undergo apoptosis and is cleared by macrophages and dendritic cells in a process called efferocytosis [31]. Neutrophils isolated from peripheral blood undergoes apoptosis spontaneously, this process can be altered by pro-survival and pro-apoptosis signals such as LPS and Fas ligand respectively. Neutrophils that have transmigrated to a tissue have undergone fundamental functional changes. This can be illustrated by comparing *in vivo* transmigrated neutrophils using a skin chamber technique to neutrophils isolated from peripheral blood. The transmigrated tissue neutrophils were completely unaffected by anti-apoptotic stimulation as compared to their blood counterpart [32].

A feedback mechanism to ensure the resolution of inflammation is the negative feedback control of macrophages and reduction of granulopoiesis. Data obtained from an *in vivo* model in mice, indicate that macrophage phagocytosis of apoptotic neutrophils gives rise a more anti-inflammatory macrophage phenotype producing reduced amounts of the pro-inflammatory cytokines IL-23 and IL-17 and reduced G-CSF production, which in turn lead to reduced granulopoiesis [33].

Neutrophil recruitment to sites of infection

Recruitment of neutrophils to a site of infection or trauma is a multifaceted process and there are four major steps that are of prime importance when circulating blood neutrophils are recruited to extravascular tissues. The major steps are termed: neutrophil rolling, endothelium adhesion, crawling into the interstitum, and transmigration to the site of infection/trauma. Invading microbes recognized by host tissue cells initiate a production and release of pro-inflammatory cytokines, and together with activated complement components and microbial derived PAMPs, they will act as chemoattractant and guide neutrophils to the site of infection. During a non-microbial inflammation (sterile inflammation), neutrophils will instead be guided by DAMPs released by host cells [34]. The production/release of pro-inflammatory cytokines will lead to an expression of P and E-selectins on the endothelial wall, and allow an interaction with L-selectin that is exposed on the neutrophil surface, an interaction that slow the speed down and allow the neutrophil "roll" along the vascular endothelium. This interaction will induce cytoskeleton rearrangement, fusion of secretory vesicles with the neutrophil plasma membrane and an upregulation of membrane receptors and β2-integrins originating also from mobilized specific and gelatinase granules. Shedding of L-selectin and the increased expression of β2-integrins will allow a firm adhesion of the neutrophil to the endothelium. Finally the neutrophils will migrate through/over the endothelium (diapedesis) to reach the site of infection and the process is guided by gradients of the different chemoattractants generated in the infected tissue (Figure 2) [35].

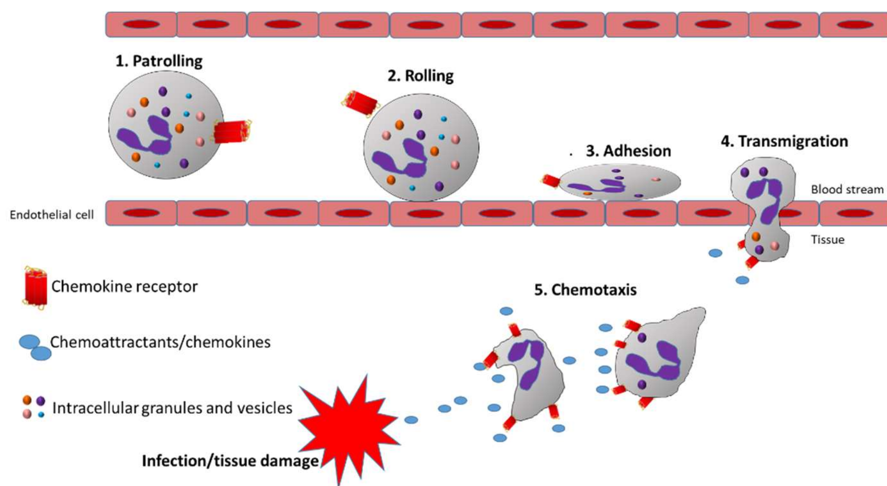

Figure 2. Schematic figure of neutrophil transmigration. *Neutrophils react to inflammatory stimuli, and adhesion molecules are upregulated on neutrophils and endothelial cells, this will cause shedding of L-selectin and neutrophils will roll along the endothelial wall. This is followed by neutrophils binding through integrins to the endothelium. Subsequently, neutrophils transmigrate through the endothelium to reach the site of inflammation guided by gradients of chemoattractant released by the site of infection.*

A new concept that neutrophils instead of undergoing apoptosis at the site of injury leaves the inflammatory site through reversed transmigration, i.e., returns to the bloodstream, was first described in a sterile inflammation model in zebrafish embryo [36]. At the receptor level the chemokine receptor CXCR2 seems to be important for the reverse transmigration [37]. Reverse transmigration also occurs in mice; in a thermal hepatic injury model, neutrophils transmigrate to the injury site and “clean up” damaged vessels and organize the milieu for new

vascular growth, and instead of succumbing to death when their task is done, some neutrophils transmigrate to the blood vessels, then further to the lungs, where the chemokine receptor CXCR4 is upregulated before finally returning to undergo apoptosis in the bone marrow [38].

Microbial killing by neutrophils

In addition to the direct killing of microbes mediated by the complement system and antimicrobial peptides, neutrophils have a diverse set of antimicrobial killing tools at their disposal. The classical killing process is initiated when a microbe is engulfed through phagocytosis, and following this, antimicrobial effector molecules stored in neutrophil granules are delivered into the phagosome containing the engulfed microbe. In the phagolysosome formed when the granules fuse with the phagosome, also the oxygen radical forming NADPH-oxidase is activated, and the reactive oxygen species (ROS, see below) formed should ultimately together with the other antimicrobial systems kill and degrade the phagocytosed microbe [39, 40]. Professional phagocytes regulate the engulfment process by recognizing microbes with specific membrane receptors. Opsonization is a process used to enhance phagocytosis, and as mentioned earlier, activation of the complement system by microbes will dress the surface of the microbe with complement components recognized by receptors (CR1/CR3) on the phagocyte. Antibodies that specifically recognize a microbe will also tag this microbe with “an eat me signature” that facilitates Fc receptor (FcR)-mediated phagocytosis and enable the immune system to specifically target and kill invading microbes [41].

The "non-classical" killing mechanism, the formation of neutrophil extracellular traps (NETs), is a process by which neutrophils release decondensed nuclear chromatin and granular proteins to the extracellular space and these NETs not only trap but also kill captured bacteria [42].
It is clear, however, that virulent bacteria have evolved a number of different factors to outmaneuver the immune system such as inhibitors of chemotaxis and granule release and toxic peptides that lyse neutrophils. These secreted factors may in one way or another counteract the defense mechanisms used to find and kill invading bacteria [43-47]. Intracellular bacteria use diverse strategies to regulate phagosome formation and maturation, in order to survive and replicate within the host [48]. The development of these virulence factors to evade the immune system is a fascinating testimony to the evolutionary arms race between microbes and the immune system.

The phagocyte NADPH-oxidase

Reactive oxygen species (ROS) play an important role in many biological processes including immunity, but because of their reactivity and toxicity, many diseases are also connected to an excess ROS production [49]. Neutrophils and other phagocytes are equipped with a protein, NOX2 that belongs to a class of proteins responsible for transmembrane electron transfer. NOX2 ($gp91^{phox}$) form together with another protein ($p22^{phox}$) a membrane localized heterodimer (cytochrome b_{558}) and this is the catalytic center of the phagocyte NADPH-oxidase. The b cytochrome contains two heme groups and the

redox coenzyme, flavin adenine dinucleotide (FAD), and together with some cytosolic components, $p40^{phox}$, $p47^{phox}$, $p67^{phox}$ and Rac1/2 (a small GTPase) an NADPH-oxidase heteromeric complex is formed [50]. In resting neutrophils, a small fraction (≈5%) of the b cytochrome is present in the plasma membrane, the rest being stored in the intracellular vesicles/granules. During phagocytosis and activation/priming of neutrophils, the vesicles/granules fuse with the plasma and/or phagosome membranes where an activation of the NADPH-oxidase complex is achieved [51]. The NADPH-oxidase can assemble and be activated in different cellular locations, when the complex is assembled in the plasma membrane such as upon stimulation of cells with chemoattractants, O_2^- formed by the NADPH-oxidase will be secreted extracellularly (Figure 3), and when the complex is assembled in the phagolysosmal membrane, the ROS will be retained intracellularly. The oxidase can also be assembled in membranes of intracellular vesicles also in the absence of phagocytosis, and recent published data imply that ROS generated in intracellular compartments are of prime importance for NETosis and for controlling inflammatory signaling [52, 53].

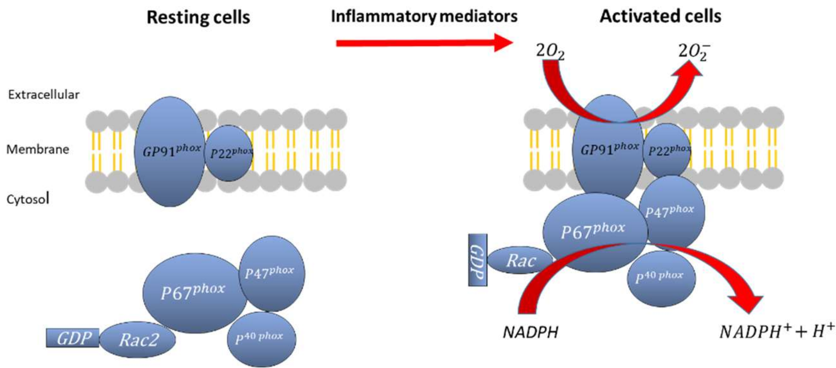

Figure 3. Activation of the NADPH-complex in the plasma membrane. *In resting cells, the catalytic subunit, gp91phox, together with p22phox form the flavocytochrome b558 (NOX2) in the cell membrane, p47, p67 and p40 subunits are localized in the cytosol. Upon activation the cytosolic parts together with the small G-protein Rac in its GDP form will translocate to the membrane and form the active complex. The enzyme will now produce* O_2^- *by reduction of molecular* O_2 *by using electrons donated from NADPH.*

The critical role of ROS in immunity is obvious through studies of the primary immunodeficiency disorder, chronic granulomatous disease (CGD). The disease is caused by mutation in one of the subunits of the NADPH-oxidase, which lead to a non-functional NADPH-oxidase complex and the inability to produce ROS. CGD neutrophils are also deficient in PMA-triggered neutrophil formation of extracellular traps (NETosis), apoptosis and autophagy [54] and most patients are characterized by high sensitivity to fungal and bacterial infections, an increased susceptibility thought to be due to impaired ROS dependent

killing of phagocytosed microbes. These patients are also hyperinflammatory due to redox dysregulation in phagocytic cells [55]. It should be noticed, however, that mutations affecting the function of the cytosolic oxidase subunit p40^{phox} seems to on the one hand be critical solely for the intracellular superoxide production and on the other hand patients with this deficiency, suffer from mild, atypical, form of CGD when it comes to susceptibility to infections [56]. Before prophylactic antimicrobial drugs were introduced as part of clinical practice, children with CGD often died before the age of ten, but with proper diagnosis and the monetary resources for the treatment the mean life expectancy is now around 40 years [57].

ROS as a regulators of cell function

NADPH-oxidase derived ROS play a more diverse role than just the killing of microbes, i.e., ROS regulate numerous proteins such as kinases, phosphatases and ion channels [49]. Tyrosine phosphatases has a catalytic center containing cysteine that is sensitive to oxidative stress and accordingly an increased ROS production may inhibit the enzymatic activity of these enzymes, leading to increased activation of cellular signaling pathways that are promoted by phosphorylation [58]. The activities of kinases might also be affected, illustrated by the fact that hydrogen peroxide activates the p38 mitogen-activated protein (MAP) kinase in endothelial cells [59]. In addition role of NADPH-oxidases in activation of kinases has been suggested in several of other cell types [49]. ROS can also function as a negative feedback control

mechanism that together with the enzyme myeloperoxidase (MPO) inactivate peptides by oxidation of the sulfur of methionine, which reduce neutrophil infiltration [60-62]. Defects in ROS production is also connected to multiple autoimmune diseases, a missense of the gene NCF1, encoding for p47phox subunit of the NADPH-oxidase complex leads to reduced ROS production and predispose to numerous of autoimmune diseases [63]. This has also been shown in animal disease models, where defects of p47phox subunit is strongly connected to the severity of arthritis in rats and mice [64].

ROS is also involved in cell-cell communication. Moreover, the ROS generated by the phagocyte NADPH-oxidase inhibit the ability of NK-cells and T-cells to suppress tumor growth, a mechanism critical in chronic myelogenous leukemia (CML). The NADPH-oxidase in the un-mature leukemic cells produce sufficient amounts of ROS to induce apoptosis in NK cells and by that promote their own survival. This process can be reversed by ROS scavengers and by histamine that limits activation of the phagocyte oxidase reducing through activation of the histamin-H_2 receptor [65]. The fact that CGD patients are not only immunocompromised but also suffer from different inflammatory conditions as systemic lupus erythematosus (SLE), strongly suggest that low levels of ROS or absent production of ROS is linked to an hyper-inflammatory phenotype, a suggestion that has been confirmed in studies using different models [66-68].

ROS are also involved as regulators of genome stability, a numerous of gene transcriptions factor and DNA repair enzymes are susceptible to intracellular redox status [69].

RECOGNITION OF RECEPTOR LIGANDS

Cell surface receptors

For life to exist, cells must be able to sense information about the environment and properly respond to new information about changes, transmitted by external signaling molecules. The largest and most important protein family that recognize and regulate the responses needed are receptors, and this type of sensors are ubiquitously expressed in all cells in all forms of life. Receptors can be localized both inside cells or localized in the plasma membrane. Their role is to recognize and transduce signals that are integrated parts of the communication systems in an organism. The number of receptors and the biological functions they regulate are extremely diverse and involve all manner of cellular functions including growth, metabolism, immune response and gene expression. Membrane receptors transduce signals that regulate cellular responses to extracellular stimuli. There are multiple classes of membrane receptors that are integrated in the cell membrane and exposed on the surface of the cell. They transduce the intracellular signals in a number of different ways including regulation of enzymatic activities, change in membrane potential regulating by ion channels, and they couple to heterotrimeric G-proteins that regulate signaling systems further down-stream in the signaling cascade. The family of G-protein coupled receptors (GPCRs) is the largest and most diverse class of membrane receptors in eukaryotes and the focus of this thesis.

Structure and function of G-protein coupled receptors (GPCRs)

GPCRs, also known as seven-transmembrane receptors, constitute the largest class of membrane receptors and their roles as regulators in different aspect of human cell/organ physiology are incredibly diverse. Cell surface expressed GPCRs recognize a large number of different external stimuli belonging to different groups, including peptides, proteins, lipids, lipoproteins and glycoproteins. The receptor binding ligand can be endogenous, the hormone dopamine is a good example, recognized by a group of dopamine GPCRs expressed in the central nervous system and important regulators of different neurological processes. Receptor ligands can also be exogenous as illustrated by different microbial specific molecules recognized by GPCRs expressed in cells of the innate immune system [70].

GPCRs are by far the largest membrane receptor family with more than 800 genes in the human genome, and they display a remarkable diversity both with respect to recognition of ligands and in intracellular signaling capabilities [71, 72]. Due to the role of different GPCRs in broad variety of diseases, members of this group of receptors have been/are attractive drug targets [73], and the understanding of how GPCRs recognize specific signaling molecules and how the down-stream signals generated by ligand occupied receptors are transduced, has significantly affected modern drug development. Accordingly,

around 35% of all marketed drugs act through a GPCR and the numbers are steadily increasing [74].
Out of the six classes of the GPCR family defined based on function and sequence [73], the Rhodopsin family is by far the largest with more than 680 members in humans [75] and all the receptors focused on in this thesis belong to this subgroup of GPCRs.

Receptor signaling

The peptide chain of a GPCR passes the plasma membrane that expresses the receptor seven times, leaving three peptide loops and the N-terminus tail exposed on the surface and three loops together with the C-terminus tail on the cytosolic side of the membrane. The largest homologies are found in the transmembrane domains. Ligands are recognized by the domains that can be reached from the surface of the receptor expressing cell, whereas the down-stream signaling depends on the domains reached from the cytosolic side of the membrane [76]. Accordingly, the cytosolic domains possess the binding site for a heterotrimeric G-protein, a group of proteins composed of an α-subunit combined with a heterodimer β/γ complex. Such a protein complex transduces a downstream signaling cascade from the ligand occupied receptor [77]. The receptor conformation change is transmitted to the G-protein in which the GDP (guanosine diphosphate) bound is exchanged for a GTP, and this in turn leads to dissociation of the Gα subunit from Gβγ. The two separated subunits will activate an array of different secondary messengers and evoke a cellular response.

The heterotrimeric G-proteins family consists of 16 Gα, 5Gβ and 12 Gγ subunits [78] and are divided into four main classes: Gαi, Gαq, Gα12/13, and Gαs, these classes are defined by their amino acid sequence and distinct (sometimes overlapping) signaling properties as well as sensitivity to different bacterial/plant toxin. Bacterial toxins have been important tools to define subclasses of G-proteins; the classical *Bordetella pertussis* toxin has been shown to inhibit Gαi whereas the *Vibrio cholera* toxin inhibits Gαs [79]. More recently, data show that two structurally similar cell permeable depsipeptides produced by the bacterium *Chromobacterium sp. QS3666* the plant *Ardisia crenata*, respectively, specifically inhibit G-proteins containing a Gαq subunit [80, 81]. Both these depsipeptides pseudo-irreversibly block the exchange between GDP and GTP and by that, the activation of downstream signaling is blocked.

In relation to a non-signaling receptor with no ligand bound, the Gα monomer is bound to the Gβγ dimer, and due to intrinsic GTPase activity of the Gα subunit, the background receptor activity is very low. When an activating ligand (agonist) binds to the surface exposed orthosteric site, a change in receptor conformation is induced. Signaling by an activated GPCR is turned off by mechanisms that physically inhibit the heterotrimeric G-protein to bind to the receptor, a process called receptor desensitization [82].

The arrestin protein family has four isoform members of which two (β-arrestin 1/β-arrestin 2), are ubiquitously expressed in most cell types in vertebrates. These proteins can interact with over 800 GPCRs but also other proteins and induce non-canonical receptor signaling [83, 84].

GPCR activation and subsequent phosphorylation of signaling receptor domains by G-protein-coupled receptor kinases (GRK) will allow a recruitment of β-arrestin to the cytosolic part of the GPCR and this binding physically blocks G-protein binding and terminates G-protein dependent signaling. The recruited β-arrestin then binds to AP2 and the heavy chain of clathrin, which start clathrin-coated pit formation, and initiate the endocytic process. GPCRs will then either be recycled to the cell membrane or degraded in a lysosomal compartment. The fate of the endocytosed receptor is determined by the strength in the interaction between the receptor and β-arrestin [85]. β-arrestin will also induce downstream signaling, most commonly described is activation of extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathway; this pathway regulates a diverse set of cellular processes [86, 87].

Activation: the two state model and beyond

Activating endogenous GPCR ligands that bind to the orthosteric site are usually termed full "orthosteric agonist" or partial "orthosteric agonists" if they only partly activate signaling. Antagonists bind to the orthosteric site and blocks the interaction between agonist and orthosteric site but have no direct effect on receptor conformation. Inverse agonists transfer a receptor from a basal signaling level to a non/low signaling state. In the classical two state activation model, a receptor exists in two states that are in equilibrium. The receptor is either "off" or "on" and when an activating agonist binds to the orthosteric site, the equilibrium will shift towards the "on" conformation (Figure 4).

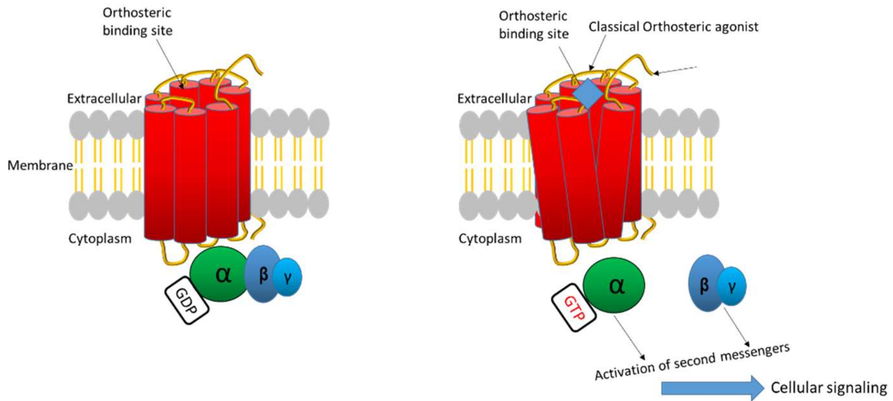

Figure 4. Structure and activation of GPCR. *GPCRs share a common structure of seven transmembrane spanning domains, with an extracellular amino terminus tail and an intracellular carboxyl terminus. The extracellular domain includes the orthosteric binding site. Agonist binding will induce a conformation change that is followed by dissociation of the heterotrimeric G-protein from its receptor into the cytoplasm, where the Gα subunit and the β/γ complex will initiate signaling events by activation of different secondary messengers.*

This basic on/off canonical signaling model was for long the established model for how GPCR signaling was regulated. This is however now considered an over-simplification and GPCRs seems to be able to exist in multiple receptor conformations. Some ligands bind receptor sites structurally distinct from the orthosteric site, and depending on the outcome of such an interaction this type of ligands are termed positive, negative or neutral allosteric agonist or modulators [88].

As mentioned, the two-state activation model is an over-simplification and we know now that GPCRs are able to exist in multiple receptor

conformations. Depending on the panel of activating/modulating ligands bound to a receptor it can adopt different conformations by which the signals generated may be biased and functionally selective; for example one agonist might favor the G-protein signaling pathway and one the β-arrestin signaling pathway, leading to different cellular responses through the same receptor (Figure 5).

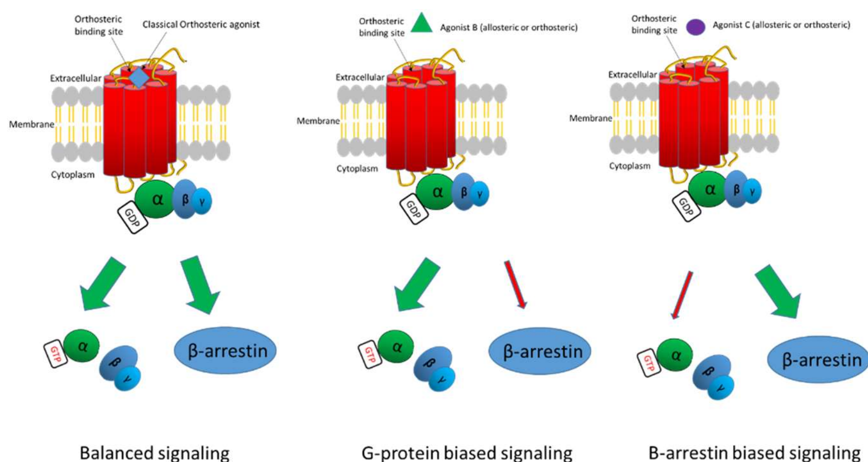

Figure 5. Bias signaling. *A conventional "balanced" orthosteric agonist activates both the G-protein and the β-arrestin-mediated signaling pathways (left panel). A bias agonist will predominantly activate the G-protein or β-arrestin mediated signaling pathway (middle and right panel respectively).*

This means that the activated receptor can induce one signal over another, and this will lead to a distinct a cellular response [89]. Based on the fact that this makes it possible to increase the desired cellular signals and (if possible) limit the signaling pathways leading to

unwanted side effects) [90], the phenomenon is of particular interest when designing new drugs. For example, side effects in drugs that causes analgesia through the mu-opioid receptor (MOR) is associated with β-arrestin interactions and novel drugs might benefit to be biased towards G-protein signaling [91].

Allosteric ligands, both synthetic and endogenously produced, might also be non-activating but either act as positive allosteric modulators (PAM) or negative allosteric modulators (NAM), increasing and decreasing signaling, respectively, of an orthosteric agonist. Some examples of GPCRs that are functionally changed by allosteric modulators include adenosine and adrenergic receptors, chemokine receptors, FFARs, opioid receptors, and 5-HT receptors [92]. Allosteric modulators are interesting for the potential use as therapeutic agents, and a phase II trial with a NAM that modulates GPR84, a receptor highly expressed in neutrophils associated with inflammatory and fibrotic diseases, is currently ongoing [93].

Neutrophil GPCRs

To exert proper functions, neutrophils rely on surface expressed GPCRs, receptors that share a large degree of structural similarity but recognize and are activated/inhibited by different set of ligands [9]. Accordingly, neutrophils express numerous of different GPCRs, some of the important receptors for regulation of inflammation in neutrophils will be described below. Formyl peptide receptors 1 and 2 (FPR1/2), the two FPRs are high affinity receptors for formylated peptides, danger signals originated from bacterial or mitochondrial protein synthesis (for

more details see below), are the best characterized receptors [6, 94]. They have also been implicated in some forms of cancer [95].

Neutrophils express, also the chemoattractant receptors for leukotriene B4 (BLT1/2), platelet activating factor (PAFR), the complement component C5a (C5aR) and the chemokine receptors CXCR1, CXCR2, CCR1 and CCR2 [96]. Another pattern recognition GPCR that is highly expressed in neutrophils is the Gαi/q coupled free fatty acid receptor 2 (FFAR2; earlier named GPR43) that recognizes short chain fatty acids (SCFA; acetate, propionate and butyrate) generated by fermenting gut microbes [97]. The exact roles of the FFARs in inflammation are not known, but the level of circulating SCFAs are high during some inflammatory diseases, indicating a role for SCFAs/FFAR2 in inflammation [98]. Neutrophils also express GPR84 and this receptor was suggested to recognize medium chain fatty acids when deorphanized. The two FFARs can, when properly activated, trigger the cells to produce and secrete O_2^- generated by neutrophil NADPH-oxidase [99]. Neutrophils also express $P2Y_2R$, a receptor for ATP, a danger signal for necrotic cell death and tissue destructing activities. $P2Y_2R$ regulates neutrophil migration and ROS production through different mechanisms and one is through reactivation of desensitized FPRs (see below) [100, 101].

The PAFR is predominantly expressed in immune cells but can be found in other cells/tissues and recognizes the endogenous phospholipid agonist PAF, a potent pro-inflammatory mediator produced by several cell types including platelets, endothelial cells and immune cells such as monocytes and neutrophils [102-104]. The PAF

receptor has been shown to couple to G-proteins containing either a Gαi subunit or a Gαq subunit, G-proteins that can utilize distinct as well as overlapping downstream signals [104-108]. However as shown (see **Paper I)**, in neutrophils, the PAFRs couple primarily to G-proteins of the Gαq subtype [108]. Similar to the prototypical FPR1 agonist fMLF, PAF is highly chemotactic, and also activate neutrophils to produce O_2^- and mobilize granules [109]. Moreover, PAFR can reactivate desensitized FPRs and this is achieved through a receptor cross-talk signaling mechanism [101, 108, 110] described in **Paper I and II.**

There are many similarities between the PAFR and the FPRs with respect to neutrophil responses including migration, ROS production and secretion, but contrary to the latter that is expressed on the surface as well as in granule membranes, the PAFRs seems to be exclusively expressed in the plasma membrane [109].

Neutrophils express GPCRs that mostly couple to G-proteins that contain either Gαi or Gαq suggesting that it should be possible to determine the precise coupling by the sensitive to Gαi inhibitor pertussis toxin and by the depsipeptides shown to inhibit Gαq containing G-proteins [111]. However, as shown in **Paper I** pertussis toxin inhibits both the Gαi coupled FPRs and the Gαq coupled PAFRs [108]. The depsipeptides also selectively inhibit signaling by the ATP receptor $P2Y_2R$ [112] , but to be able to dissect the direct link between the different G-proteins and the different neutrophil GPCRs, new selective inhibitors for the different Gα subunits are eagerly awaited.

THE FORMYL PEPTIDE RECEPTORS

Formyl peptides

Due to a similar evolutionary background, not only newly synthesized proteins of bacterial origin, but also the proteins encoded for by the mitochondrial DNA, have a formylated methionine (fMet) as the first amino acid. Compared to eukaryotic protein-translation, this is a pattern unique for prokaryotes and mitochondria. In 1975, Schiffmann and co-worker showed that formylated peptides were potent chemoattractants for neutrophils and macrophages, whereas non-formylated analogs were not [113]. A couple of years later, mitochondrial derived formylated peptides were also shown to be chemotactic [113, 114]. The hypothesis put forward by Schiffmann and others was that formyl peptides that are products of prokaryotic/mitochondrial activities, constitute a molecular pattern that is recognized by receptors expressed on phagocytic immune cells, a hypothesis that later has been confirmed [6, 94].

Receptors that recognize formylated peptides

Shortly after the original publication showing that N-formylated methionyl-peptides are chemotactic for phagocytes, binding experiments using a radioactively labeled peptide, fMLF (at that time wrongly named fMLP that rapidly became the prototype chemotactic peptide) were performed and the presence of a stereospecific binding site with criteria corresponding to a receptor, was suggested to be expressed in neutrophils [115]. In 1990, this receptor was cloned using

an expression library from HL60 cells [116, 117] and the year after this receptor was named FPR1. The background to this was that two other very closely related receptors, located on chromosome 19, were identified and named FPR2 (initially named FPRL1; 69% amino acid sequence similarity to FPR1) and FPR3 (initially named FPRL2; 56% amino acid sequence similarity to FPR1) [118, 119]. Despite the high amino acid sequence similarly between FPR1 and FPR2 the two receptors recognize different sets of ligands. To generalize, FPR1 recognizes primarily short bacterial/mitochondrial derived formylated peptides, such as the prototype peptide fMLF and fMIFL, peptides released from growing *Escherichia coli* and *Staphylococcus aureus bacteria,* respectively [6]. FPR1 recognizes also formylated peptides originating from proteins encoded for by the mitochondrial DNA such as cryptic peptides derived from the ND3 and ND6 proteins [120]. The molecular background to why FPR1 prefers short formylated peptides might be that the binding pocket is fairly small/narrow and fit a maximum of five amino acids. FPR2 has a much larger binding pocket [121, 122] and mutagenesis studies have shown that the negative charge of Asp-281 (replaced with Gly in FPR1) aggravates interactions between FPR2 and shorter formylated peptides [123]. This might help to explain the differences in ligand recognition profiles for FPR1 and FPR2. The prototype peptide fMLF is a weak FPR2 agonist, whereas formylated cryptic mitochondrial peptides originating from the proteins ND4, ND5, and cytochrome b, are together with phenol-soluble modulin (PSM) peptides released from virulent *S. aureus*, potent activators of FPR2 [120, 124]. Molecular modeling and site-directed

mutagenesis has shown that FPR2 prefers longer formylated peptides and that shorter peptides bind to the supposed smaller binding pocket of FPR1 [123, 125]. The *S. aureus* generated PSMs are formylated cytotoxic peptides secreted from highly virulent bacterial strains. It is clear that the formyl group is not involved in the cytolytic effect and structurally the peptides are amphipathic α-helical chains with lytic activity preferentially for apoptotic neutrophils [62, 126]. In addition, PSMs and PSMα2 in particular are also potent agonists for FPR2 [62, 124]. Studies using shorter PSMα2 peptide variants show that the C-terminus is critical for the cytolytic activity of PSMα2, and that a receptor shift from FPR2 to FPR1 is obvious with shorter peptides that have the same amino acids in the formylated N-terminus. It is thus clear, that short and flexible peptides prefer FPR1 and longer less flexible prefer FPR2 [127]. The N-terminal formylated methionyl group is of outmost importance for the interactions with both FPR1 and FPR2 [125].

FPRs are primarily expressed in myeloid cells but the expression pattern differs; neutrophils express FPR1 and FPR2 but not FPR3. FPR1 and FPR2 are expressed after the promyelocyte phase of differentiation. They are sorted to the plasma membrane but also foremost to the easily mobilized secretory vesicles and to the gelatinase granules [6]. All three FPRs are expressed in monocytes and FPR3 is expressed also by macrophages and dendritic cells [128]. Not much is known about FPR3 because selective ligands that activate the receptor are still lacking [6]. Although almost all our knowledge are based on experiments with cells with myeloid origin, FPRs have been shown to

be expressed also in other cells such as epithelial cells, endothelial cells, NK cells, fibroblasts and neural stem cells [129]. FPR1 polymorphisms has been associated with juvenile periodontitis, possibly linked to impaired signaling through the coupled G-protein [130]. FPR1 polymorphism has also been linked to poor prognosis for survival prognosis after chemotherapy to treat breast and colorectal cancer [131, 132]. No polymorphisms have been described in the coding regions of FPR2 or FPR3. FPRs are also functionally expressed in other mammalian species but divergences are obvious especially between humans and rodents [133]. The mouse genome has eight FPR homologs, Fpr1 and Fpr-rs1 and are believed to be the orthologous of the two human FPRs expressed by neutrophils [134], but the ligand recognition profiles between the receptors are not identical. No clear orthologue of FPR3 can be found in mice [135].

This discrepancy between the human and the mouse receptors is important, especially since murine disease models commonly are used to determine the role of a given protein in a more complex *in vivo* system and to test the effect of new drug candidates targeting this protein. Ligands that activate/inhibit/modulate human receptors might not work in mouse and vice versa. It has been known for long that the prototypical FPR1 agonist fMLF is a poor activator of murine neutrophils [136]; the potent and selective antagonist CysH and PBP_{10} that inhibit FRP1 and FPR2 respectively, are without effect on the corresponding mouse receptors and activating/inhibiting FPR2 lipopeptides (pepducins; see below) has shown different affinity across species [137, 138]. However, there are potent cross species agonists

described, such as PSMα peptides, fMIFL and peptidomimetic agonists/antagonists [137].

Recently the structure of an FPR2-Gαi complex with a bound peptide agonist was solved with cryo-EM [121], and at about the same time, a high-resolution structure of FPR2 with the potent peptide agonist WKYMVm bound was disclosed when the complex was successfully crystalized [139]. Both structures identified an open extracellular region with a large binding pocket. This large pocket is probably the reason why different ligands that belong to different chemical classes and vary in size can be recognized by FPR2. As of today, there are no such structural information available for FPR1 or FPR3.

FPR activation and signaling

Overview of the initiation of signaling

The FPRs couple to heterotrimeric G-protein of the Gαi subtype, and accordingly, signaling down-stream of the Gαi protein is abolished by pertussis toxin [94]. Notably the effector functions induced through ligand bound FPR1 and FPR2 are very similar in neutrophils [6]. Ligand-induced structural change of the receptor is transmitted to the G-protein on the cytosolic side of the plasma membrane resulting in a dissociation of the Gαi subunit from the Gβγ heterodimer. Generally, the Gαi subunit will bind and inhibit the enzyme adenylyl cyclase that increases the cytosolic concentration of cAMP [6, 94] but the role of this signaling pathway downstream of the FPRs is not known in detail but it has been suggested to regulate the transcriptional regulator NFκB

and activate ERK signaling pathway [140-142]. It has been shown that FPR1 induced activation of the ROS producing NADPH-oxidase is negatively regulated by increased cAMP levels achieved through an activation of the Gαs coupled histamine receptor [143], or through the effect of an adenylate cyclase toxin-hemolysin (CyaA), a toxin produced by *Bordetella pertussis* [144].

Possibly of more importance in FPR signaling is the signaling activity by Gβγ part of the G-protein. This heterodimeric subunit will activate two distinct signaling pathways; **i)** activation of the membrane bound enzyme phospholipase C (PLC) that will cleave the membrane anchored phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol triphosphate (IP_3) [145] and, **ii)** activation of phosphoinositide 3-kinases (PI3Ks) that catalyze the transformation of PIP_2 into phosphatidylinositol (3,4,5)-trisphosphate (PIP_3). The PLC product DAG will remain in the plasma membrane and activate protein kinase C (PKC) that in turn will phosphorylate a number of different proteins including the NADPH-oxidase components $p47^{phox}$ and $p22^{phox}$ [146]; PKC is, thus, a key kinase in the assembly and activation of the NADPH-oxidase.

The other PLC product, IP_3, will enter the cytosol and bind to specific receptors present on the Ca^{2+} storing endoplasmic reticulum (ER), and the activated IP_3 receptors trigger a release of Ca^{2+} from the ER. The emptying of the stores is a signal that open store-operated Ca^{2+} channels (SOCs) in the plasma membrane. Together these two processes give rise to a transient increase in the cytosolic concentration free Ca^{2+}, leading to an activation of another phospholipase, phospholipase A2

(cPLA2). When active, this enzyme will generate arachidonic acid originating from phospholipids localized in the cell membrane. Arachidonic acid will activate different secondary messengers leading to a binding of Rac2 (a GTPases) to one of the cytosolic oxidase components $p67^{phox}$, a key event in the assembly of the NADPH-oxidase complex [147].

The PI3K product PIP_3 activates different secondary messenger including different kinases, guanine nucleotide exchange factors (GEFs) and GTPase activating G-proteins (GAPs) and also this signaling pathway has been suggested to be critical for the activation of the NADPH-oxidase complex [148]. This is illustrated by the inhibitory effects of the PI3K pan inhibitor Wortmannin [148, 149] and isoform specific inhibitors [150] that inhibit both FPR1 [151] and FPR2 [152] induced oxidative burst. In summary, all these activated secondary messengers will participate in the regulation of different neutrophil functions such as degranulation, chemotaxis and superoxide production (Figure 6) [6].

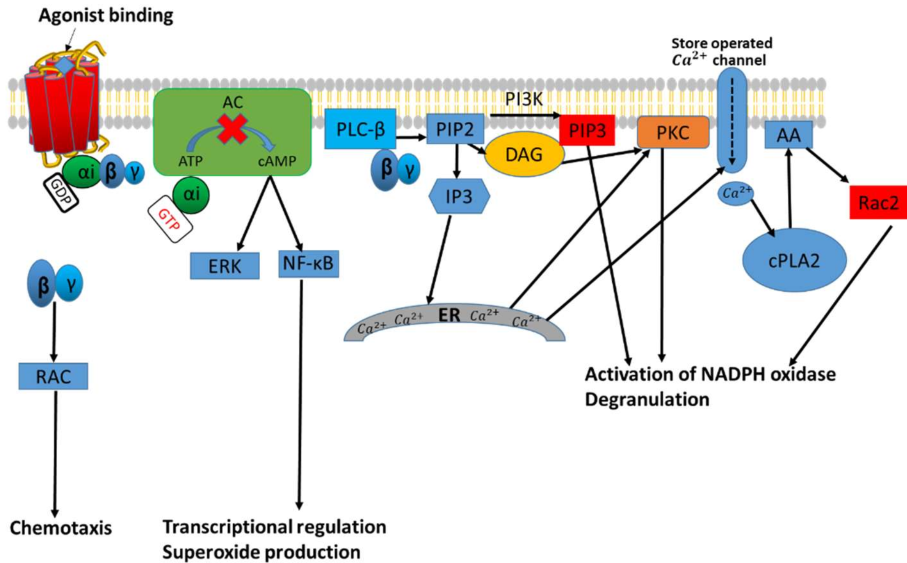

Figure 6. Schematic and simplified signaling pathways of FPR1/2. *Agonists binding induce dislocation of Gαi from Gβγ, Gαi will inhibit the enzyme adenylyl cyclase leading to reduced cAMP levels in the cell, followed by activation of different secondary messengers as possibly ERK and the transcriptional regulator NFκB will also be activated and induce production of pro-inflammatory cytokines. The Gβγ-subunit will induce intracellular calcium transient and other signaling pathways leading to activation of the NADPH-oxidase complex (ROS production), degranulation and chemotaxis.*

Receptor specific agonists that lack the N-formylated methionine

FPR2 is a promiscuous receptor that in addition to formyl peptides recognize a diverse set of ligands with different molecular structures,

including eicosanoids, proteins, peptidomimetics, and lipopeptides that all lack the formylated methionine [6, 153]. The first peptide agonist shown to activate FPR2 was WKYMVM that is a potent agonist that strongly prefers FPR2 over FPR1, and the D-methionine analog WKYMVm that is a potent dual agonist recognized by both FPR1 and FPR2 [154-157]. When bound to their receptor, these hexapeptides induce neutrophil superoxide production, granule mobilization, trigger a transient rise in intracellular Ca^{2+} and are chemotactic just as the prototypical FPR1 agonist fMLF [6].

Serum amyloid A (SAA) exists in four isoforms1and 2 are acute phase proteins that can be used as a diagnostic marker for inflammation [158]. The plasma concentration of SAA can increase 1000-fold during acute bacterial infections [159]. Patients with autoimmune disease like RA have often chronically elevated SAA levels in both blood and joints, and this elevation has been implicated in the pathogenesis of the disease [160]. SAA has been shown to interact with several receptors including FPR2 and Toll-like receptor 2/4 (TLR2/4), scavenger receptor SR-BI and the ATP receptor P2X7 [161-163]. However, the protein used in these studies is a recombinant hybrid of two isoforms that are not present *in vivo* and interactions with TLR has been attributed to contamination of bacterial TLR ligands in some studies [164]. More importantly, studies with the SAA1 endogenously protein isolated from patients with profound inflammation, has shown that this SAA has no modulatory effect through FPR2 [165, 166]. Others studies using purified SAA1 isoforms have shown that the ability to interact with FPR2 differs between isoforms [161]. These data questions the

biological relevance of SAA studies in neutrophils and all results generated by utilizing the recombinant hybrid proteins should be interpreted cautiously.

In order to investigate the endogenous and not the recombinant SAA, transgenic mice expressing human SAA1 were studied in both a lung injury and a peritonitis model. Interestingly, the supposed pro-inflammatory effects induced by SAA1 were absent, and on the contrary the transgenic mice showed reduced inflammation mediated by SAA1 through binding and clearance of the pro-inflammatory mediator LPS [167].

To investigate the structure activity for the pro-inflammatory effects of SAA1 mediated through different receptors, N and C terminal truncated version of SAA1 produced in yeast (*P.pastoris*) were studied in macrophages. Contrary to the pro-inflammatory effects observed in earlier studies, the amino acid 11-58 fragment lost the ability to induce a transient rise in calcium ions and chemotaxis through FPR2, instead showed potent anti-inflammatory properties mediated through TLR2 induced IL-10 production and suppression of the pro-inflammatory mediator LPS [168].

Amyloid beta 42 (Aβ42), one of the components of the plaques found in the brain of patients with Alzheimer´s disease has shown pro-inflammatory properties through an activation of FPR2 [169], and this receptor can also bind to the endogenous antimicrobial cathelicidin LL-37, a cleavage product of the neutrophil granule protein CAP18 [170, 171]. FPR2 also recognizes non formylated peptides of bacterial origin, e.g. cecropin-like antibacterial peptide from *H. pylori*, Hp (2-20),

induces pro-inflammatory activities in human neutrophils mediated through FPR2 [172, 173]. Pro-resolving lipids have also been described as FPR agonists and will be discussed in detail in below.

Small compound agonists

A number of small-compound FPR agonists have been identified through screening of chemical libraries using cells overexpressing FPR1 or FPR2. Promising leads have then been optimized with structure-activity-relation (SAR) analysis, and computer-aided drug design [174]. There are also a number of natural products and synthetic analogs that have been shown to inhibit FPR1-mediated responses [175]. Small molecule ligands are desired because of their higher stability and bioavailability combined with a reduced susceptibility to oxidation, characteristics making them more promising as therapeutic agents [61]. By the use of screening strategies, more than 100 small compound FPR ligands have been identified, belonging to 10 different chemical classes and these FPR interacting compounds are extensively reviewed elsewhere [174]. To give an example, the first FPR2 agonist of the kind that described was Quin-C1 (4-butoxy-N-[2-(4-methoxy-phenyl)-4-oxo-1,4-dihydro-2H-quinazolin-3-yl]-benzamide) and this agonist induces a transient Ca^{2+} response, mobilizes granules, activates the ERK1/2 phosphorylation pathway and is chemotactic for neutrophils. No superoxide production was, however, induced which classifies Quin-C1 as a biased FPR2 agonist [176]. The nitrosylated pyrazolone derivative compound 43 was initially also described as a potent FPR2 agonist [177], but later this agonist was shown to be a

potent dual agonist for both FPR1 and FPR2 with preference for FPR1 [178, 179]. Compound 43 also activate the corresponding mice receptors Fpr1/2 but with much higher affinity for Fpr1 [180]. Yet another very potent FPR agonist is Act-389949, and the receptor preference and signaling characteristics resemble the FPR2 peptide agonist WKYMVM [181]. Act-389949 is one of the few compounds that has undergone a phase I clinical trial and found to be nontoxic and well tolerated in healthy human subjects. The outcome of the Act-389948 study was, however, not the expected [182]. Despite this, Act-389949 is an excellent tool-compound for further mechanistic studies of FPR2-regulated effects both *in vitro* and *in vivo.* The response induced by RE-04-001, a small compound identified in a library screen looking at the ability of the compounds to activate ROS producing NADPH-oxidase in differentiated neutrophil-like HL-60 cells, was when characterized in more detail, found to be a selective FPR1 agonist. Interestingly the response was functional selective in favor of ROS production and away from chemotaxis as compared to the formyl peptide counterparts [183]. See table 1 for selected FPR1/2 agonists.

Table 1. Overview of selected FPR1/2 agonists available in the group or that I have been used in my research.

Name	Origin	Receptor selectivity	Reference
fMLF	*E. coli*	FPR1	[113]
fMIFL	*S. aureus*	FPR1	[184]
PSMα2	*S. aureus*	FPR2	[124]
$F2Pal_{10}$	FPR2 pepducin	FPR2	[101]
Act-389949	Small compound	FPR2	[181]
RE-04-001	Small compound	FPR1	[183]
Compound 43	Small compound	FPR1/FPR2	[178]
Quin-C1	Small compound	FPR2	[176]
Compound 14	Peptidomimetic	FPR2	[110]
SAA	Host-derived	FPR2	[185]
Lipoxin A4	Host-derived	FPR2	[186]
WKYMVM	Peptide library	FPR2	[155]
WKYMVm	Peptide library	FPR1/FPR2	[154]

Peptide antagonists/inhibitors with specificity for the FPRs

To be able to properly dissect FPR1 and FPR2 signaling, the availability of molecular tools such as specific and potent antagonists is a necessity. The first antagonist described for FPR1 was derived from the prototypical peptide agonist fMLF, with the formyl group that is critical for agonistic activity was exchanged for a tert-butyloxycarbonyl group (tBoc). This new peptide t-Boc-MLF (Boc-1) displayed antagonistic effects towards FPR1, later a more potent analog was described and named Boc-2 [187]. Both peptides lose, however, their antagonistic selectively towards FPR1 at higher concentrations > 5 μM [188]. The Boc-2 peptide antagonizes also murine Fpr1 (the lower case "pr" is used to differentiate mouse from human receptor) [137]. The inverse agonist (antagonist that also inhibit low spontaneous receptor activity) cyclosporine H is also a potent inhibitor of FPR1 signaling but at very high concentrations the receptor selectivity is lost [188]. Further, murine Fprs are not sensitive to cyclosporine H, and by that, cyclosporine H is not suitable in murine models [137].

The peptide WRW_4 was identified in a peptide library screen as a selective FPR2 antagonists [189] and has been shown to be a fairly potent and selective antagonist also for the murine orthologue Fpr2 [137]. PBP_{10} is rhodamine containing peptide (RhoB-QRLFQVKGRR), which is a potent FPR2 antagonist [190]. The peptide sequence corresponds to one of the PIP_2-binding domains of gelsolin (cytoskeletal protein) and the rhodamine group is required for PBP_{10} to transverse the cell membrane, but this is most probably not the important function for

the antagonistic effect. Truncation studies show that shorter peptides inhibit also FPR1 [191]. PBP_{10} has also shown to inhibit effects of an unidentified receptor expressed in monocytes when activated by the gG-2p20 peptide derived from the HSV-2 glycoprotein G [192]. More importantly, PBP_{10} has no effect on mouse neutrophils [137]. This together with the potential toxicity of rhodamine [193] make PBP_{10} inappropriate for *in vivo* studies. It should also be noticed that microbial virulence factors may in fact function as FPR antagonists; *Staphylococcus aureus* strains produce/release such peptide inhibitors that affect FPR1/2 as well as C5aR functions [6].

Compound FPR antagonists

High-throughput screening (HTS) of different compound libraries has been used to identify novel and potent small compound antagonist for FPR1. A screening of a small molecule library, identified 3,5-dichloro-N-(2-chloro-5-methyl-phenyl)-2-hydroxy-benzamide (BVT173187) as a selective inhibitor for FPR1, with similar properties as the Boc-1 and Boc-2 antagonists but with lower potency [194]. Another FPR1 small molecule antagonist is a compound (10(6-hexyl-2-methyl-3-(1-methyl-1H-benzimidazol-2-yl)-4-oxo-4H-chromen-7-yl acetate), identified in a study to optimize chromones and related isoflavones compounds, that has shown promising antagonistic properties towards FPR1. SAR studies revealed that the isoflavone scaffold represents a promising backbone for new FPR1 antagonists [195]. Among different chemical classes as inhibitors of the human FPR1, the most promising ones are compounds with a pyrazole scaffold. Two inhibitors were identified

with high-throughput screening (HTS) has provided two novel FPR1 antagonist series. These compound selectively inhibited FPR1>FPR2 in a calcium assay system with receptor overexpressing cells [196]. Further optimization of these pyrazole inhibitors lead to compound 20 and 22, these compounds inhibit fMLF induced calcium transient in HEK cells that overexpress FPR1, with IC_{50} < 100 nM. It should be noticed, however, that the effect on FPR2 was not determined in the initial characterization studies [197]. Later, the effect of the FPR1 inhibitor compound 20 (also called ICT12035) was evaluated in a cancer cell line and in an *in vivo* model. ICT12035 was able in a dose dependently inhibit the fMLF (FPR1 agonist) induced calcium transient and migration of U87-MG cells. Treatment of mice with xeno transplanted tumors with ICT12035 reduced the growth of the tumors, with no obvious toxic side effects [198]. Thus, ICT12035 or analogs that targeting FPR1 might be promising compounds for future cancer therapy.

For FPR2, the first and most potent small compound antagonists identified in a ligand-based virtual screen was Quin-C7 (Table 2). The structural difference between this antagonist and the biased FPR2 agonist Quin-C1 (mentioned above) is very small, the only difference being a hydroxy group instead of a methoxy in one of the phenol rings [199]. In another screening of more than five million small molecules, a potent FPR2 antagonist (1754-31) was identified with an EC_{50} of 81 nM in an intracellular Ca^{2+} assay [200].

Table 2. Overview of selected FPR1/2 antagonists available in the group or have been used in my research.

Name	Origin	Receptor selectivity	Reference
CysH	*T. inflatum*	FPR1	[188]
WRWWWW	Peptide library	FPR2	[189]
Boc-1 and Boc-2	fMLF N-terminus modification	FPR1	[188]
PBP_{10}	Gelsolin protein	FPR2	[190]
Quin-C7	Small compound	FPR2	[199]
ICT12035	Small compound	FPR1	[198]
RhB-(Lys-βNphe)$_6$-NH2	PBP10/peptidomimetic hybrid	FPR2	[201]
$F1Pal_{16}$	FPR1 pepducin	FPR2	[202]

Lipidated peptidomimetics – a new class of FPR regulators

Recently a new class of potent and highly selective FPR2 antagonists belonging to the peptidomimetic class with host defence activities have been characterized. Host Defense Peptides (HDPs) are important anti-microbial peptides in innate immunity having direct killing effects.

Unfortunately, few HDPs derived drugs has reached clinical trial, probably due to the low bioavailability of peptides *in vivo* [203]. Peptidomimetics mimic the structure/properties of peptides but they have unnatural residues incorporated in their backbone (Figure 7).

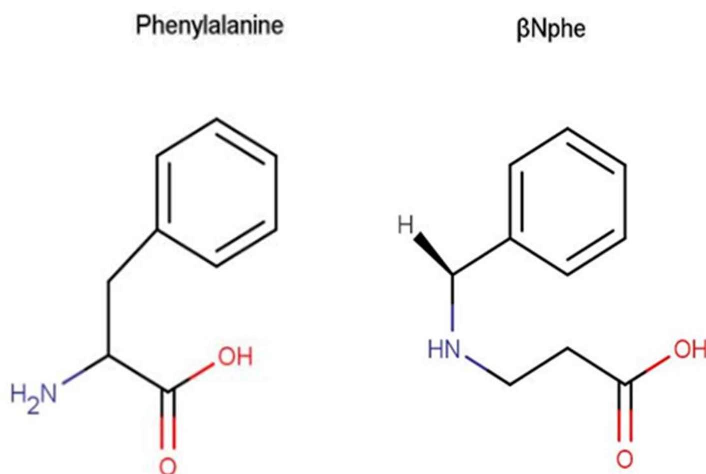

Figure 7. Example of an "unnatural" amino acid variant of phenylalanine. *The side group is attached to the nitrogen instead of the α-carbon.*

The advantages of peptidomimetics for future studies in *in vivo* animal models is that they are proteolytically stable when compared to their peptide counterparts [204]. In addition to the proteolytic stability, peptidomimetics possess direct microbial killing activity [205, 206]. The fact that both FPR1 and FPR2 have been shown to be targeted by anti-microbial peptides of both synthetic and natural origin, opens for an ability to design peptidomimetics as FPR ligands [203].

Peptidomimetics designed to mimic the formyl peptide agonist fMLF, induce a transient rise in the cytosolic concentration of free Ca^{2+} in FPR transfected rat basophilic leukemic cells and induce chemotaxis and superoxide production in neutrophil like HL-60 cells [207]. We have previously characterized a class of lipidated α-peptide/β-peptoid peptidomimetics that have been shown to possess both activating and inhibitory activities towards FPR2 [137, 208]. The peptidomimetic Pam-(Lys-βNSpe)$_6$-NH_2, potently reduced the superoxide production induced by FPR2 agonists [209]. The potency is comparable to PBP_{10} and the inhibitory effect was very rapid (<1 min. incubation required) and reversible. SAR studies show that both the conjugated fatty acid and the design of the alternating cationic/hydrophobic backbone are critical for inhibition of FPR2 [209]. Synthesis of hybrid of Pam-(Lys-βNSpe)$_6$-NH_2, and PBP_{10} gave rise to the most potent FPR2 antagonist to date with an IC_{50} in the low nanomolar range, and this was achieved by connecting the rhodamine group to the peptoid backbone forming RhB-(Lys-βNphe)$_n$-NH_2 [201]. It is interesting to note, that when the peptide part of PBP_{10} was conjugated with a palmitic acid that replaced rhodamine, this construct was a very weak antagonist, indicating that rhodamine might do more for the antagonistic activity besides allowing PBP_{10} to interact with the cell membrane and it is not interchangeable with another hydrophobic moiety as a fatty acid [201]. In a study to determine species selectivity, the peptidomimetic Lau-(Lys-βNSpe)$_6$-NH_2 was found to antagonize not only FPR2 but also its mouse orthologue Fpr2 with high potency [137].

The first generation of proteolytically stable lipidated α-peptide/β-peptoids being FPR2 agonists were found to be active in low μM concentrations and activate not only FPR2 but also its mouse orthologue Fpr2. Structure wise the lead compound has a headgroup of a 2-aminooctanoic acid (Aoc) residue acylated with lauric acid (12 carbon fatty acid), which is linked to a peptide/peptoid 6x-repeat (Lys-βNphe)$_6$-NH_2) (Figure 8). SAR studies showed that the 2-aminooctanoic acid (Aoc) residue and the linked fatty acid were critical for the agonist effects [208].

In paper II we identified structure determinants in lipidated α-peptide/β-peptoid hybrid peptidomimetics that are of importance for FPR2-dependent activation of neutrophils. We synthesized analogs of the first-generation of the lipidated α-peptide/β-peptoid agonist Lau-[(S)-Aoc]-[Lys-βNPhe]$_6$-NH_2 (Figure 8) [208], and show that the two residues adjacent to the N-terminal hydrophobic headgroup are critical for receptor interaction as is the presence/absence of α-chirality in the peptoid residues of the tail region.

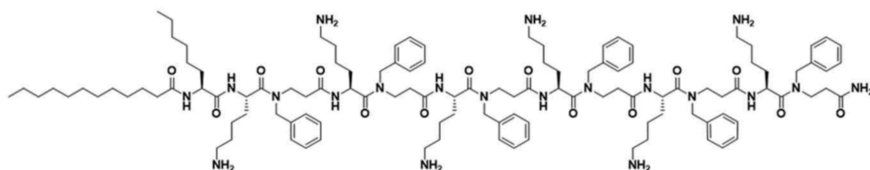

Figure 8. Structure of the first-generation FPR2 Peptidomimetic agonist.

A chiral version of the parent compound with a single βNrpe residue in the vicinity of the N-terminus (i.e., Lau-[(S)-Aoc]-Lys-βNrpe-[Lys-βNPhe]$_5$-NH_2) proved to increase the agonist potency, and this compound displayed high potency and selectively towards FPR2, and

also show biased signaling in neutrophils by activating the calcium and NADPH-oxidase pathways but not induce β-arrestin recruitment or chemotactic activity [110]. These proteolytically stable peptidomimetics with biased signaling capacity might be excellent tools for elucidating FPR2-mediated signaling as well as for disclosing its immunoregulatory function *in vivo* models of inflammation/inflammatory diseases. These compounds are also potent and selective in mouse neutrophils [137], making them potentially valuable tool compounds for *in vivo* studies in disease models. Accordingly, two of the antagonistic peptidomimetics were tested for anti-inflammatory activity in a PMA induced acute mouse ear inflammation model. Both peptidomimetics showed anti-inflammatory effects by reducing the ear edema, reduce production of pro-inflammatory cytokines, decrease neutrophil infiltration and diminish local ROS production [210]. This indicates that this class of peptidomimetic might have promising therapeutic potential in targeting FPR2 in disease models *in vivo* and as future therapeutics.

Pepducins - novel regulators of GPCR functions

In 2002 a novel class of GPCR modulators, called pepducins were introduced. Pepducins are synthetic lipidated peptides with a lipid connected to the N-terminus of the amino acid chain that should have a sequence corresponding to one of the intracellular loops of the GPCR to be targeted [211]. The pepducins were claimed to act as cell permeable modulators selective for of their cognate receptors, and the concept was shown to be valid for the PAR1 and PAR2 receptors [212].

The fact that the amino acid sequence present in the pepducins was identical to that in one of the cytosolic domains, was anticipated to determine receptor selectivity. Pepducins will then either inhibit or activate their cognate receptor. The suggested mechanism is that the hydrophobic lipid part in a pepducin binds to the cell membrane and allows the peptide part to transverse the membrane; when on the cytoplasmic side of the membrane, the peptide part interacts with its cognate GPCR, and this interaction induces a conformation change that either activates or inhibits receptor signaling (Figure 9). According to the pepducin dogma the lipid part should be critical for activity, the pepducins interact solely with their cognate receptor, and the effects of activating pepducins should be insensitive to conventional receptor antagonists.

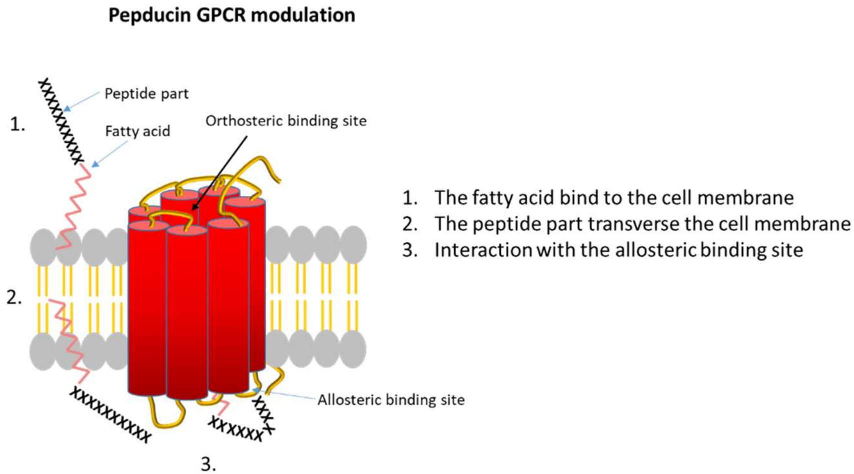

Figure 9. Proposed pepducin mode of action. *The pepducin attach to the cell membrane through interaction with the hydrophobic fatty acid followed by translocation of the peptide parts through the cell membrane. Inside the cell the pepducin will interact with its cognate receptor and signaling by the targeted receptor is either activated or inhibited.*

FPR2 hijacking and questioning of the pepducin dogma

Although an FPR2 derived pepducin is a selective agonist for FPR2 [213], the results obtained are not in total agreement with the concept. It is clear from the data presented in **Paper I** as well as in another published study [101], that FPR2 specific antagonists completely inhibit the response induced by the pepducin $F2Pal_{10}$ which is a lipidated peptide with an amino acid sequence identical to that in the third intracellular loop of FPR2. Moreover, pepducins designed for

several other GPCRs (i.e., $P2Y_2R$, CXCR4, FPR1 and PAR4) lack effects on their cognate receptor but "hijack" FPR2 [202, 214-216]. The amino acid sequences in the intracellular domains of $P2Y_2R$, CXCR4, and PAR4 lack direct sequence similarities with FPR2 (see table 3. for summary). Only two amino acids differ in the third intracellular loops of FPR1 and FPR2, yet the pepducin with an amino acid sequence identical to that in FPR1, has no effect on that receptor but modulates the function of FPR2, and an exchange of only one amino acid can change the activity, but when active, FPR2 is always the preferred receptor. Interestingly, the potent FPR2 activating pepducin $F2Pal_{10}$ [101] activates FPR2 overexpressing cells also when the third intracellular loop was changed to be identical to that in FPR1 [213]. This clearly shows that the amino acid sequence identity between the pepducin and the third intracellular loop is not what determines the ability for the pepducin to activate FPR2.

The lack of pepducin selectivity has been shown to be valid also for other GPCRs in other cell types; a pepducin derived from the β2 adrenergic receptor interacts with the β1 receptor [217] and a PAR1 derived pepducin activates PAR2 [218]. In addition, the $P4Pal_{10}$ derived from PAR4 that originally was described as a PAR4 antagonist with some affinity also for PAR1 [218], was later described as inhibitor for several other GPCRs. The common denominator for these GPCRs was that they mediate their signaling through a Gαq containing G-proteins, and the mechanism of action was suggested to be an inhibition of the recruitment of this group of G-proteins to the activated receptors [219]. Our results (**Paper III;** [216]) clearly show that this mechanism of

action does not apply for GPCRs expressed in human neutrophils. The $P4Pal_{10}$ pepducin was without effect on the responses mediated by Gαq-coupled neutrophil GPCRs whereas the response mediated by the Gαi-coupled receptor FPR2 was inhibited, and in addition, this pepducin activated FFAR2 when the receptor was allosterically modulated. Taken together, the data on pepducin effects on neutrophils put the interaction dogma in question, and instead suggest that lipopeptides might be a molecular pattern, recognized by FPR2 and allosterically modulated FFAR2. Nevertheless, irrespectively of the mechanism of action, pepducins can be useful tool compounds for mechanistic studies of neutrophil pattern recognition receptors such as FPR2 and FFAR2.

Table 3. Selected list of pepducin with modulatory effects on FPR2.

Name	Receptor origin	Pepducin sequence	FPR2 modulation	Ref
$F1Pal_{16}$	FPR1 3rd ICL	Pal-KIHK**Q**G**L**IKSSRPLRV	Inhibitory	[202]
$F2Pal_{16}$	FPR2 3rd ICL	Pal-KIHK**K**G**M**IKSSRPLRV	Activating	[213]
$F2Pal_{10}$	FPR2 3rd ICL	Pal-KIHKKGMIKS	Activating	[101]
ATI-2341	CXCR4 1st ICL	Pal-MGYQKKLRSMTDKYRL	Activating	[215]
P2Y2PalIC2	P2Y2R 2nd ICL	Pal-HRCLGVLRPLRSLRWGRARYARR	Activating	[214]
$P2Y_2Pal_{IC3}$	$P2Y_2R$ 3nd ICL	Pal-MARRLLKPAYGTSGGLPRAKRKSVRT	Activating	[214]
$P4Pal_{10}$	PAR4 3nd ICL	Pal-SGRRYGHALR	Inhibitory	[216]

FPR modulation and biased agonism

FPR1, and to a large extent also FPR2, interacts with a diverse array of ligands that trigger distinct activation signals and by that different functional responses. Basically, so far all FPR1-activating formylated peptides described are balanced agonist that activate both the G-protein dependent signaling route as well as GPCR signaling routes leading to recruitment of β-arrestin. Functionally this leads to activation of the ROS producing NADPH-oxidase and chemotaxis. In contrast, the small molecule RE-04-001 is an FPR1 agonists that very potently induces superoxide production, activates the PLC-PIP2 -Ca^{2+} and ERK1/2 phosphorylation pathways, but is a poor inducer of β-arrestin recruitment and a poor chemoattractant [183]. This exemplifies a biased agonist that is functionally selective. Another example, is the agonist Cmp17b, that when compared to the dual FPR1/2 agonist Cmp43 was a poor inducer of the PLC-Ca^{2+}-pathway, whereas both agonists activate the ERK1/2 and Akt pathway. The biased signaling induced by Cmp17b was shown to be linked to a reduced myocardial injury in an animal disease model [220].

Similar to FPR1 agonists, basically all described FPR2 activating peptides (formylated or not) are balanced agonists [6]. However, the potent FPR2 specific PSMα peptides are biased and functionally selective as they are unable to recruit β-arrestin and to induce neutrophil chemotaxis. It should be noticed, that despite the suggested link between β-arrestin recruitment, activation of ERK1/2 phosphorylation system and endocytosis of agonist occupied receptors, PSMα peptides

induce a rapid FPR2 mediated activation of the ERK1/2 system and the receptors are internalized [221]. These results are consistent with that the ERK signaling system can be activated downstream of the G-protein and not necessarily downstream of β-arrestin [82, 222]. Similar to PSMα peptides, most of the FPR2 activating peptidomimetics and pepducin variants are biased agonist that lack the ability to recruit β-arrestin (Figure 10) [110, 223].

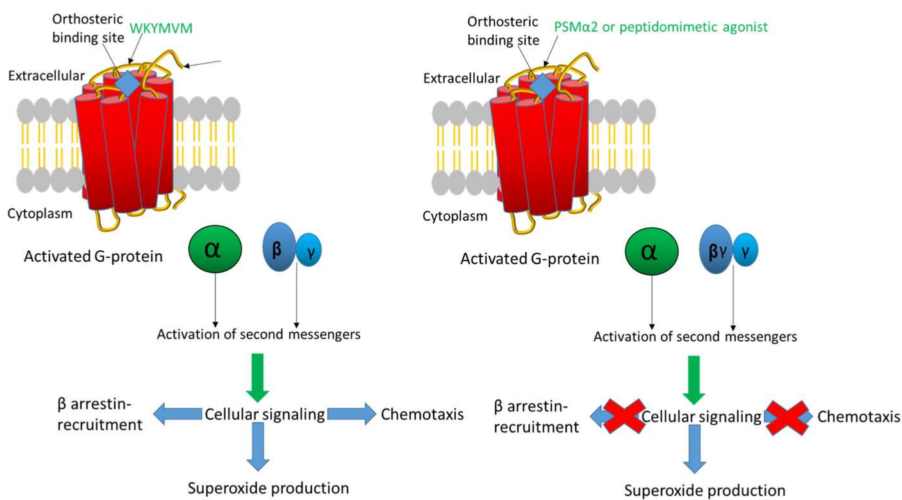

***Figure 10. FPR2 biased signaling**. The orthosteric agonist WKYMVM activates both the G-protein and the β-arrestin-mediated signaling pathways and induce chemotaxis. Peptidomimetic agonists and PSMα2 do not induce β-arrestin-translocation and are not chemotactic.*

Pro-resolving ligand with anti-inflammatory effects

Different mediators such as annexin I, lipoxin A4 (LXA_4) and resolvin D1 (RvD1) have been shown to have anti-inflammatory and pro-resolving effects, and they have all been suggested to transmit their pro-

resolving activities via FPR2 [94, 224]. Annexin I is a phospholipid binding-protein regulated by the Ca^{2+} concentration, and the full length protein as well as N-terminal peptide fragments have anti-inflammatory properties possibly mediated through FPR2 [94, 225, 226]. LXA_4 is bioactive metabolite derived from arachidonic acid [227].

Annexin and LXA_4 are reported to induce FPR2 homodimerization, leading to production of the anti-inflammatory cytokine IL-10 through activation of a signaling pathway involving β-arrestin, p38 MAPK, and Hsp27. The pro-inflammatory FPR2 agonist SAA did not activate that pathway, indicating that FPR2 can induce both pro-inflammatory and anti-inflammatory signaling [228]. LXA_4 could also reduce the FPR2 agonist SAA induced production of the pro-inflammatory cytokine IL-8 [185]. To test the hypothesis that lipoxin and the peptide agonist WKYMVm induce different conformation changes in FPR2, a sensitive FRET assay was recently used, and a potent anti-inflammatory compound 15-epi-lipoxin A4 (ATL) showed allosteric modulating effects towards FPR2. Pre-treating cells with pM concentrations of ATL (epimer of LXA4), induced a receptor conformation change and reduced both the concentration needed to induce β-arrestin translocation and inhibited the pro-inflammatory cytokine IL-8 secretion induced by WKYMVm, while treating the cells with WKYMVm induced different changes in FPR2 conformation [229].

Recently, also an annexin derived peptide was identified as an allosteric modulators, and to induce two distinct conformation changes in FPR2; pre-incubation with annexin peptide Ac2−26 altered WKYMVm signaling, in that the calcium transient was weakened, whereas

signaling leading to β-arrestin translocation and p38 MAPK phosphorylation was potentiated, pathways suggested to be associated with anti-inflammatory functions [230].
The pro-resolving activities and the importance of different phagocyte receptors have been described in recent reviews [186, 227, 231] but it should be noticed that some studies have questioned the coupling to FPR2 [178, 232].

TERMINATION OF RECEPTOR SIGNALING

Receptor desensitization and endocytosis

Receptor desensitization is the process whereby signaling is turned off and the receptor becomes non-responsive to activating agonists. In order to avoid prolonged signaling, the ability to terminate signaling and desensitize receptors is of outmost regulatory importance. Receptor endocytosis is the process by which agonist occupied receptors are removed from the cell surface. Interestingly for some GPCRs that bind strongly to β-arrestin as Vasopressin receptor 2 (V2R), the receptor will continue signaling after the internalization in endosomes in so called megaplexes, that constitute a complex of receptor, β-arrestin, and G-protein [87].
Homologous desensitization is by definition when an agonist occupied receptor, is turned into a state that is non-responding to the same agonists and to other agonists specific for the same receptor. After a period of active signaling, the FPRs will be homologously desensitized, and neutrophils desensitized with an FPR1 agonist are non-responsive

to further FPR1 stimulation, but fully responsive when activated with an FPR2 agonist.

Heterologous desensitization is the process by which a receptor, in addition to being homologously desensitized, also desensitizes another receptor that has no affinity for the desensitizing agonist. This phenomenon is clearly illustrated by neutrophil responsiveness to IL8, a neutrophil chemoattractant recognized by the receptors CXCR1/2; FPR agonists desensitize also the IL8 receptors and make cells unresponsive to IL-8 [233]. Importantly, heterologous desensitization is subjected to hierarchical regulation as IL-8 is not capable of desensitizing FPRs. This process is thought to be of importance for neutrophil tissue recruitment, allowing the cells to migrate directionally towards a "strong" end-target chemoattractant (i.e. FPR agonists) despite the presence of disturbing "weaker" intermediary chemoattractants such as IL-8. The hierarchy is defined at the signaling level; end target chemoattractant such as formyl peptides activate p38 MAPK, which inhibits the PI3K signaling pathway that is used by intermediary chemoattractant [234]. No cross desensitization is observed between FPR1 and FPR2, suggesting that the receptors are hierarchically equally strong [6].

At the molecular level, the desensitization mechanisms are not fully understood; GRK phosphorylation of specific sites in the intracellular loops of activated FPRs, leading to increased affinity and binding of β-arrestin. This process has been proposed to initiate GPCR desensitization and endocytosis (Figure 11) [86].

Another suggested mechanism is that a physical coupling of FPRs to the actin cytoskeleton desensitizes the receptor (see below). Results

obtained on the link between FPR-signaling and β-arrestin recruitment and receptor endocytosis, using cell-lines with overexpressed FPRs as model system, suggest that there are differences between FPR1 and FPR2; FPR1 was internalized also in cells lacking β-arrestin whereas FPR2 internalization was severely compromised [235].

In neutrophils, FPR agonists unable to recruit β-arrestin, will despite this desensitize their specific receptor [110, 221, 223], and these agonists also activate the ERK signaling pathway [221]. This clearly shows that recruitment of β-arrestin is not needed for receptor desensitization and for an initiation of the ERK signaling pathway.

A new tool to study receptor desensitization and the endocytosis that follow, was recently introduced when it was shown that Barbadin, an inhibitor that blocks the interaction between AP2 and β-arrestin, impair receptor endocytosis through the clathrin dependent pathway (Figure 11) [236, 237].

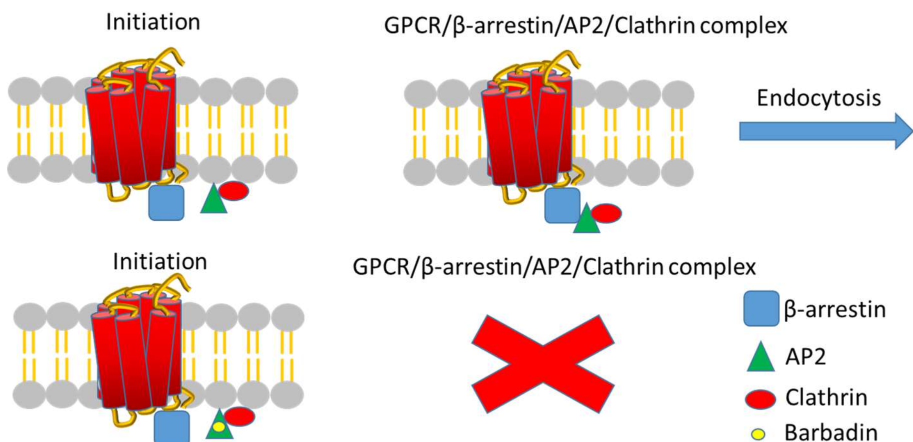

Figure 11. GPCR endocytosis is blocked by Barbadin. *The interaction between AP2 and β-arrestin is blocked by Barbadin and by that the clathrin dependent pathway leading to endocytosis is blocked.*

The AP2 protein is a heterotetramer, structure-wise consisting of a core domain composed by four adaptin (α, β, μ and σ), and this is attached to two appendage domains. The core domain binds the membrane and the protein targeted for internalization, while the appendage domains bind to clathrin and other accessory proteins. This will lead to the formation of clathrin-coated vesicles, which will undergo endocytosis [238].

In **Paper IV,** we applied Barbadin as a tool compound in order to elucidate the role of β-arrestin in FPR2 endocytosis. Using a using BRET technique, we show that Barbadin inhibits AP2/β-arrestin interaction, when stimulated with the FPR2 peptide agonist WKYMVM. Interestingly, Barbadin did not block FPR2 induced endocytosis in β-arrestin KO cells or in primary neutrophils (Fig 12). Our data clearly show that FPR2 endocytosis occurs independent of β-arrestin recruitment. This is true both for peptide agonist WKYMVM

that recruit β-arrestin and agonists that are poor inducer of β-arrestin. Interestingly Barbadin primed FPR2 induced superoxide production in neutrophils and reactivated FPR2 desensitized neutrophils, regardless if the agonist was strong or very poor inducer of β-arrestin recruitment.
This effect was selective for FPR2 induced superoxide production, shown by the fact that Barbadin had no effect on neutrophil chemotaxis, the FPR2 triggered transient rise in the intracellular concentration of Ca^{2+} or phagocytosis of opsonized yeast particles [239]. The fact that AP2 has been shown to bind also to AP180, ARH and Scr [236], opens for the possibility that the priming effect on superoxide, might be indirect through one of these binding partners.

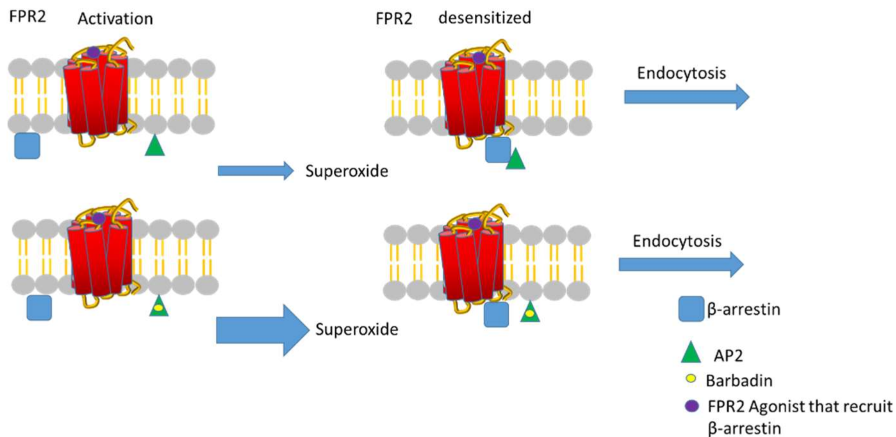

Figure 12. FPR2 can undergo endocytosis through a β-arrestin/AP2-independent pathway and Barbadin primes neutrophil superoxide production. *Neutrophils pre-treated with Barbadin, shows augmented FPR2 mediated superoxide production and undergo endocytosis, indicating a mechanism independent of AP2 interactions with β-arrestin for FPR2 endocytosis.*

The actin cytoskeleton and receptor desensitization

It has been suggested that the actin cytoskeleton has the ability to interact with FPRs and by that terminate G-protein signaling [240-242]. This suggestion gains support from the fact that in neutrophils with their FPRs desensitized, disruption of the actin cytoskeleton leads to a reactivation of the desensitized FPRs [6, 240]. This type of receptor reactivation is achieved also when the agonist used to induce the desensitized state is unable to recruit β-arrestin, showing that β-arrestin is not involved in this type of receptor reactivation [223, 240]. Interestingly, no transient rise in the concentration of cytosolic Ca^{2+} accompanies receptor reactivation [240]. This type of reactivation phenomena is not exclusive to FPRs, as the C5aR seems to be regulated in a similar way, whereas FFAR2, PAFR and CXCR2 (the IL-8 receptor) are not be reactivated/resensitizated by cytoskeleton disrupting drugs [243, 244].

Taken together these data suggest that the actin cytoskeleton plays important roles in receptor desensitization in neutrophils, and in contrast to what have been shown for other GPCRs and cells, there is no direct link between β-arrestin and desensitization or ERK signaling in neutrophils [245].

Our data strongly suggest a link between the ability of an agonist to induced β-arrestin recruitment and to induce neutrophil chemotaxis; agonist unable to recruit β-arrestin are not (or are very poorly) chemotactic [110, 221, 223]. This link has also been observed in other immune cells, i.e., β-arrestin deficient lymphocytes, show impaired

chemotaxis towards CXCL12 [246] and a biased agonist for GPR84 that do not recruit β-arrestin, is not chemotactic for macrophages while balanced GPR84 agonists are [247].
Experimental data strongly suggest that different cytoskeletal proteins and polymerized actin localized in the cytosol interacts and regulates FPR1/2 and other GPCRs in neutrophils, and accordingly, the response induced by many different GPCR specific agonists is largely increased and prolonged in neutrophils with a disrupted actin cytoskeleton [99, 111, 248, 249].

Receptor cross talk

It was for long thought that desensitized receptors were unable to return to an actively signaling state, but experimental evidence has shown that FPRs desensitized with receptor-specific agonists can be reactivated not only by disruption of the actin cytoskeleton (as outlined above), but also by a novel receptor cross-talk mechanism [6]. An example of such a cross-talk induced receptor reactivation is that between the ATP receptor $P2Y_2R$ and desensitized FPRs; no activation of the NADPH-oxidase is induced by ATP in naïve neutrophils, but when neutrophils with their FPR2s desensitized are activated by ATP, a pronounced production of O_2^- is achieved, and this response is inhibited not only by a $P2Y_2R$ antagonist but more importantly also by an FPR2 antagonist [250]. This receptor cross-talk is not a mechanism exclusive for $P2Y_2R$, but the same type of reactivation of desensitized FPR2 is induced by the PAFR (Figure 13) [6, 101, 108, 250, 251].

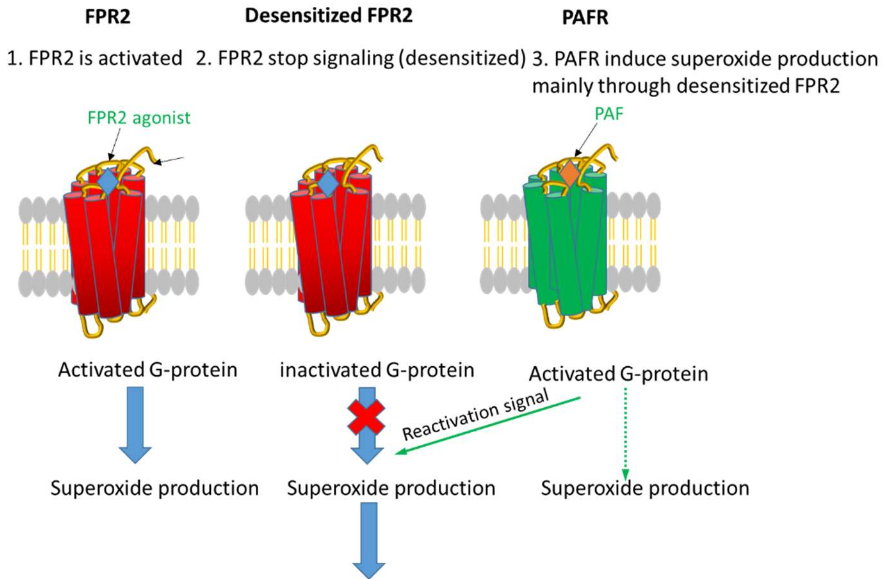

Figure 13 PAF induced receptor cross talk-induced reactivation desensitized FPR2. *When FPR2 is non-responsive to further stimulation with any FPR2 agonist (homologously desensitized), FPR2 can be reactivated and produce superoxide anion with subsequent stimulation of PAFR. This superoxide production is augmented and inhibitable by both FPR2 and PAFR antagonists.*

In Paper I we characterized this cross talk-induced reactivation process by using a recently described inhibitor the α-subunit Gαq containing G-proteins. As mentioned above, most neutrophil GPCRs signal through one of two distinct subclasses of G-proteins defined as Gαi (defined historically through the sensitivity to a toxin from *Bordetella pertussis*) or Gαq [79, 111, 252]. As previously described, the two peptides YM-254890 and FR900359 [80, 253] have been shown to selectively inhibit Gαq signaling upon receptor activation [254]. Our data show that direct activation of FPRs was insensitive to Gαq inhibition, whereas responses

generated directly by PAF was inhibited. Also, the PAF-induced reactivation of desensitized FPRs was inhibited by the Gαq inhibitor. This clearly shows that the signals from the PAFR that reactivate the desensitized FPRs are generated downstream of the Gαq containing G-protein coupled to the PAFR. Furthermore, the effect of FPR2 agonists on the PAF triggered reactivation, show that the signals leading to an activation of the NADPH-oxidase in FPR desensitized neutrophils are transmitted almost exclusively through FPRs [108]. It should also be noticed that the PAF or ATP-induced reactivation are most pronounced in neutrophils desensitized by FPR2 specific pepducins or peptidomimetics [101, 110], agonist unable to recruit β-arrestin. There is, however, no direct correlation between the level of β-arrestin recruitment and receptor reactivation, illustrated by the fact that there is no substantial difference between PSMα peptides (unable to recruit β-arrestin) and WKYMVM (a potent inducer of β-arrestin), in cross-talk induced receptor reactivation [221]. In agreement with the signaling pattern of FPR reactivation when induced by a disruption of the cytoskeleton, the receptor cross-talk activation signals are also functionally selectively; there is no transient rise in intracellular Ca^{2+} following the reactivation leading to neutrophil ROS production. This clearly shows that the signaling pathways leading to activation of the ROS-producing NADPH-oxidase and a rise in the cytosolic concentration of Ca^{2+}, respectively, are not necessarily intertwined [111, 240]. The exact intracellular signals that lead to reactivation of desensitized FPRs have not yet been identified, but there are differences between direct/naïve receptor activation and receptor reactivation. The

signals that activate the ROS generating system upon direct activation of FPRs and PAFR are inhibited by PI3K-δ inhibition, whereas the receptor cross talk activation signals largely bypass PI3K-δ [152]. A similar difference in sensitivity is obvious for the phosphatase inhibitor calyculinA that primes the response induced by direct/naïve FPR activation, whereas the receptor cross-talk reactivation signals are inhibited, suggesting that the phosphorylation status is important in discriminating between direct/naïve and receptor cross-talk reactivation [101].

FPR activation mediated through receptor cross talk and allosteric receptor modulation

The orthosteric FFAR2 agonist propionate is a poor activator of the neutrophil NADPH-oxidase, but pre-treating cells with allosteric FFAR2 modulators turns propionate into a potent activating agonist [111]. Our research also shows that non-activating concentrations of FPR agonists can activate neutrophils through a receptor cross-talk mechanism [181, 255, 256]. We show that non-activating concentrations of FPR agonists activate the NADPH-oxidase in neutrophils when pre-treated with the non-activating allosteric FFAR2 modulator Cmp58. This response is inhibited by the proper FPR antagonists as well as by a specific FFAR2 antagonist.

This illustrates that receptors that primarily couple to Gαi coupled G-proteins also can "talk" with each other in the same way as the Gαq

coupled receptors for PAF (PAFR) and ATP ($P2Y_2R$) cross-talk activate desensitized FPRs as described above [111].

FUTURE PERSPECTIVES

GPCRs have ability to induce biased signals leading to selective functional responses [89]. In line with this, FPR2, a key player in the inflammation process, has functions as initiator of inflammatory processes as well as for resolution of the same processes; the receptor triggers both pro- and anti-inflammatory responses depending on the ligand that activates the receptor [94, 186]. The signaling pathways downstream of the activated FPR2, triggered by pro-inflammatory agonists have been extensively studied and is the focus of my study. Regarding the anti-inflammatory signaling downstream FPR2, one suggested pathway activated by the pro-resolving agonists, induces receptor dimerization followed by β-arrestin dependent signaling leading to production of an anti-inflammatory cytokine [228]. The experiments in the study showing this, were, however, performed in HEK293 cells and the results have not yet been confirmed in primary neutrophils, and it is clear that that a lot of work remains [178, 257] to identify not only the precise down-stream receptor signals needed for resolution of inflammatory reactions but also the precise functional response leading to resolution. The role of β-arrestin in FPR signaling and neutrophil function is an important area for future research as peptide agonists unable to recruit β-arrestin are not chemotactic [110, 221, 223], and this finding might have some advantage in selectively modulating inflammatory responses *in vivo*. In addition, the ROS released by activated neutrophils have in addition to destructive effects also an anti-inflammatory properties [63], suggesting that future work should determine the precise mechanisms for the protective/anti-

inflammatory effects. Hopefully, recent and future molecular docking studies [186] aiming to determine different conformation changes and signaling induced by different FPR ligands as well as structural information made available from crystal structures of the FPRs [139] will be of help to determine FPR2 structures in complex with other regulatory partners, knowledge that could increase our understanding of FPR2-induce biased signaling and functional selectivity.

Inflammation is a critical pathological process in many diseases in which FPR2 plays an important role. Conventionally, the therapeutic strategy has been to reduce the pro-inflammatory processes; RA patients have successfully been treated with TNFα inhibitors for more than two decades, effectively reducing the pro-inflammatory cytokine mediated destruction on bone and cartilage [258]. Targeting FPR2 biased signaling could be a novel strategy for the resolution of inflammation and might be a complementary approach, instead of lowering the non-resolving inflammation with antagonists the resolution process is accelerated with biased agonists [259]. Indeed, targeting FPR2 with this approach has been studied in pathological conditions that leads to heart failure and arthritis [260, 261].

In addition to targeting FPR2 through the concept of biased signaling, administration of FPR1/2 antagonists might also be a possibly strategy to decrease pro-inflammatory response in different inflammation-associated disease conditions.

Our identification of biased peptidomimetic ligands and potent antagonists targeting FPR2 should provide tools not only for further

elucidating FPR2-mediated signaling but also for disclosing its therapeutic potential in animal models of inflammatory diseases.

Finally, knowledge gained from the studies of FPR2 and other GPCRs in this thesis should increase our understanding about GPCR biology in general. Our identification of biased agonists and potent antagonists targeting FPR2 will provide tools not only for further elucidating FPR2-mediated signaling but also for disclosing its therapeutic potential in animal models of inflammatory diseases.

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