Modulation of Receptor Signaling and Functional Selectivity in Neutrophils

Michael Gabl

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

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michael.gabl@gu.se

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"*A witty saying proves nothing.*"
Voltaire

Abstract

Neutrophils are important effector cells of the innate immune system and in the regulation of inflammation. Many of their functions, such as chemotactic migration, secretion of granule constituents and activation of the oxygen radical-producing NADPH-oxidase, are regulated by cell surface receptors. The formyl peptide receptors (FPRs), the ATP receptor ($P2Y_2R$) and the receptor for platelet activating factor (PAFR) belong to the large family of G-protein coupled receptors (GPCRs) and, amongst other receptors, enable neutrophils to sense and respond to host- and pathogen-derived danger signals. Therefore, any regulatory imbalance in GPCR signaling can potentially contribute to the development of severe infections or autoimmune/inflammatory diseases.

The work presented in this thesis is focused on basic GPCR-signaling mechanisms in human neutrophils with the aim to generate new knowledge that could be of value for future GPCR-based drug development. To answer the scientific questions raised, numerous cell-biology-based experimental methods were applied, including measurements of neutrophil intracellular calcium release, superoxide production, degranulation, cell migration and cytoskeleton-mediated receptor regulation.

The functional responses triggered by GPCRs expressed by neutrophils can be modulated in various ways at the level of receptors/ligand interaction, in dependence of other GPCRs, as well as at the signaling level. Both FPR2 and $P2Y_2R$ have been shown to be able to exert functional selective signaling through distinct regulatory mechanisms. An FPR2-specific synthetic lipopeptide allosteric modulator was identified as a biased agonist that does not induce recruitment of β-arrestin or chemotactic migration and exhibits oppositional efficacies for direct FPR2 activation and receptor cross-talk-mediated signaling. Functional selectivity liked to the $P2Y_2R$ is not related to biased agonism but instead emerges from an endogenous actin cytoskeleton-dependent regulatory mechanism which selectively inhibits the signals that lead to the generation of oxygen radicals, while leaving other signaling pathways unaffected.

In conclusion, this thesis adds new knowledge to the field of neutrophil receptor biology and provides novel insights into the modulation of basic GPCR signaling mechanisms with intend to contribute to strategies for future drug design and treatment of inflammatory disorders and disease.

Populärvetenskaplig Sammanfattning

Vårt immunförsvar är till för att skydda oss från sjukdomar som orsakas av det stora antalet mikroorganismer som finns i vår omgivning och som vi ständigt träffar. Vi har, precis som andra ryggradsdjur, ett immunförsvar som består av både en medfödd del och en förvärvad del. Cellerna i det förvärvade immunförsvaret känner igen många olika strukturer som finns hos mikrober och denna igenkänning leder till ett mycket specifikt försvar riktat mot just den struktur som satte igång försvarsreaktionen. Det tar ganska lång tid (dagar) från igenkänning till att det finns ett fungerande försvar, och eftersom mikroorganismer förökar sig mycket snabbt behöver vi ett försvar som kan mobiliseras fort. Cellerna i det medfödda immunförsvaret har förmågan att reagera snabbt men för att detta skall vara möjligt kan de bara känna igen ett begränsat antal strukturer. Dessa strukturer eller mönster uttrycks av många mikroorganismer; de är alltså konserverade molekylära strukturer som härstammar från mikrober, men vissa av dessa har mycket stor likhet med det som frisätts från våra egna celler eller vävnader när dessa av någon anledning skadas. Frisättningen av denna typ av signaler från skadade celler/vävnad/ eller mikrober talar om för det medfödda immunsystemet att någonting inte är som det skall och att den akuta faran kräver en snabb mobilisering av ”försvarstyrkorna”.
En av de viktigaste cellerna i det medfödda immunförsvaret är de neutrofila granulocyterna, en celltyp som i dagligt tal brukar kallas neutrofiler. Normalt finns ett stort antal neutrofiler i vårt blod, där de patrullerar med sikte på mikroorganismer som bryter sig igenom de yttre försvarsmurarna i form av slemhinnor och hud och försöker etablera sig i någon vävnad. De larmsignaler som frisätts känns igen av neutrofilerna som rekryteras genom att de lämnar blodbanan och kryper till den plats där koncentrationen av larmsignaler är stor. När cellerna hittat de invaderande mikroberna initieras en rad olika funktioner som har till uppgift att döda mikroberna, städa undan de det som skadats och att sätta igång en läkningsprocess. Neutrofilerna kan äta upp (fagocytera) mikroberna och de är också utrustade med flera olika system som kan avdöda inte bara de inkräktare som

ätits upp utan också de som undkommit själva fagocytosprocessen. Det enzymsystem dessa celler är utrustade med och som har till uppgift att producera syreradikaler är mycket effektivt när det gäller döda mikrober, men om det bildas för mycket radikaler eller om de bildas på fel plats eller vid fel tidpunkt så kan dessa kraftigt toxiska molekyler skada på våra egna celler. Det är därför mycket viktigt att neutrofilernas funktion/aktivitet noga regleras.

Såväl neutrofilernas förmåga att hitta de invaderande mikroberna som deras förmåga att döda, städa och läka är beroende av igenkänningsstrukturer (receptorer), och en viktig grupp av receptorer kommunicerar med cellens inre genom s.k. G-proteiner, och de kallas därför allmänt för G-proteinkopplade receptorer och GPCRs som förkortning. När en sådan receptor känner igen larmsignal aktiveras den och talar om för cellen vad den skall göra. Molekyler som känns igen av en receptor och aktiverar den kallas agonister men det finns också molekyler som blockerar receptorers funktion och dessa kallas vanligtvis antagonister. Förutom naturligt förekommande agonister och antagonister finns också en rad syntetiska sådana, t.ex. i form av läkemedel.

Syftet med denna avhandling var att undersöka hur GPCR-signalering regleras i neutrofiler. Vi har använt oss av blod från friska blodgivare och de celler vi isolerat från detta blod har utsatts för olika agonister och och antagonister och andra substanser som direkt eller indirekt påverkar signaleringen från receptorerna och cellernas funktion. Vi har undersökt hur många olika funktioner och som exempel kan nämnas att vi mätt förmågan att svara på larmsignaler genom att krypa mot högre koncentrationer (kemotaxi) och deras förmåga att producera syreradikaler. I denna avhandling visas att vi genom att använda olika typer av agonister/antagonister och andra substanser som på något sätt påverkar receptorfunktion, kan styra cellernas funktion. De resultat som presenteras i avhandlingen kan vara användbara vid en framtida utveckling av läkemedel för behandling av inflammatoriska sjukdomar där neutrofilers funktion är av central betydelse för uppkomst eller sjukdomsförloppets svårighetsgrad. Det är helt klart att den familj av receptorer (GPCRs) vars funktioner undersökts i avhandlingen, är mycket viktiga för reglering av många vitala funktioner i våra celler och vävnader, och de nya kunskaper som avhandlingsarbetet genererat kommer förhoppningsvis i förlängningen också att kunna användas för att förstå och kunna reglera GPCR-singalering i andra sammanhang än just immunförsvaret.

List of Papers

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

I Michael Gabl, Malene Winther, Sarah Line Skovbakke, Johan Bylund, Claes Dahlgren, Huamei Forsman
A Pepducin Derived from the Third Intracellular Loop of FPR2 Is a Partial Agonist for Direct Activation of This Receptor in Neutrophils But a Full Agonist for Cross-Talk Triggered Reactivation of FPR2
PLoS One, 2014, 9(10):e109516

II Michael Gabl, Malene Winther, Amanda Welin, Anna Karlsson, Tudor Oprea, Johan Bylund, Claes Dahlgren, Huamei Forsman
$P2Y_2$ receptor signaling in neutrophils is regulated from inside by a novel cytoskeleton-dependent mechanism
Experimental Cell Research, 2015, 336(2):242-52

III Michael Gabl, André Holdfeldt, Malene Winther, Tudor Oprea, Johan Bylund, Claes Dahlgren, Huamei Forsman
A pepducin designed to modulate $P2Y_2R$ function interacts with FPR2 in human neutrophils and transfers ATP to an NADPH-oxidase-activating ligand through a receptor cross-talk mechanism
Biochimica et Biophysica Acta (BBA) – Molecular Cell Research, 2016, 1863(6 Pt A):1228-37

IV Michael Gabl, Andre Holdfeldt, Martina Sundqvist, Jalal Lomei, Claes Dahlgren, Huamei Forsman
FPR2 signaling without β-arrestin recruitment alters the functional repertoire of neutrophils
Biochemical Pharmacology, 2017, 10.1016/j.bcp.2017.08.018 *(in press)*

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Abbreviations

5-HT$_3$R	Serotonin receptor
7TMR	Seven-transmembrane receptor
ADP	Adenosine diphosphate
Akt	Protein kinase B
AMP	Adenosine monophosphate
AP-1	Activator protein 1
AP-2	Adaptor protein 2
ATP	Adenosine triphosphate
BTK	Burton's tyrosine kinase
C5aR	Component 5a receptor
Ca^{2+}	Calcium ion
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
c-FLIP	Cellular FADD-like IL-1β-converting enzyme inhibitory protein
CHIP	Chemotaxis inhibitory protein
cIAP1/2	Cellular inhibitor of apoptosis proteins 1 and 2
Cl^-	Chloride ion
CR	Complement receptor
CREB	cAMP response element-binding proteins
CXCR	Chemokine receptor
DAG	Diacylglycerol
DAMP	Danger-associated molecular pattern
DCs	Dendritic cells
DISC	Death-inducing signaling complex
dsRNA	Double-stranded RNA
Dyn	Dynamin (GTPase)
E/DRY	Glutamic acid/aspartic acid- arginine-tyrosine
ECD	Extracellular domain
EPAC	Exchange proteins directly activated by cAMP
ERK	Extracellular signal-regulated kinase
FAD	Flavin adenine dinucleotide

FADD	Fas-associated death domain
FAK	Focal adhesion kinase
FAS	First apoptotic signal (cytokine)
Fc receptor	Fragment crystallizable receptor
fMIFL	formyl-Methionine-Isoleucine-Phenylalanine-Leucine
fMLF	formyl-Methionine-Leucine-Phenylalanine
FPR	Formyl peptide receptor
GABA receptor	Gamma-aminobutyric acid receptor
G-CSF	Granulocyte colony-stimulating factor
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GPC	Glycerophosphocholins
GRK	G-protein coupled receptor kinase
GTP	Guanosine triphosphate
H_2O_2	Hydrogen peroxide
HEK cell line	Human embryonic kidney cell line
HL-60 cell line	Human promyelocytic leukemia cell line
HOCl	Hypochlorous acid
ICAM-1	Intercellular adhesion molecule-1
ICD	Intracellular domain
ICL	Intracellular loop
IFN	Interferon
IFNAR	Type I interferon alpha/beta receptor
IFNGR	Type II interferon gamma receptor
IKK-γ	Inhibitor of nuclear factor kappa-B kinase subunit gamma
IL	Interleukin
IL8R	Interleukin 8 receptor
ILR	Interleukin receptor
IP_3	Inositol trisphosphate
IRAK	Interleukin-1 receptor-associated kinase
IRF	Interferon regulatory factor
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
JAK	Janus kinase
JNK	c-Jun N-terminal kinase

K^+	Potassium ion
LPS	Lipopolysaccharides
LPS	Lipopolysaccharides
LRR	Leucine-rich repeats
LTB4R	Leukotriene B4 receptor
MAL	MYD88-adaptor-like protein
MAPK	Mitogen-activated protein kinase
Mg^{2+}	Magnesium ion
MLCP	Myosin light chain phosphatase
MYD88	Myeloid differentiation primary-response protein 88
Na^+	Sodium ion
nAChR	Nicotinic acetylcholine receptor
NADPH-oxidase	Nicotinamide adenine dinucleotide phosphate-oxidase
NFAT	Nuclear factor of activated T-cells
NFκB	Nuclear factor κ-light-chain-enhancer of activated B cells
PAF	Platelet activating factor
PAF-AH	PAF-acetyl hydrolase
PAMP	Pathogen-associated molecular pattern
PI3K	Phosphatidylinositol-4,5-bisphosphate 3 kinase
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PIP_3	Phosphatidylinositol-4,5-trisphosphate
PKB	Protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C
pLGIC	Pentameric ligand-gated ion channel
PMN	Polymorphonuclear leukocyte
PRR	Pattern recognition receptor
PSM	Phenol-soluble modulin
Raf	Rapidly accelerated fibrosarcoma (kinase)
Rap	Ras-related protein (small GTPase)
Ras	Retrovirus-associated DNA sequences (small GTPase)
RGS	GTPase-activating regulators of G-protein signaling
RhoA	Ras homolog gene family member A (GTPase)
RhoGDI	Rho guanine nucleotide dissociation inhibitor
RIP	Receptor interacting protein

ROCK	Rho-associated protein kinase
SAA	Serum amyloid A
Src	Sarcoma kinase
SRF	Serum response factor
STAT	Signal transducers and activators of transcription
STAT3	Activator of transcription 3
Syk	Spleen tyrosine kinase
TAK1	TGFβ-activated kinase 1
TIR domain	Toll-IL-1-resistence domain
TLR	Toll-like receptor
TMD	Transmembrane domain
TNFR	Tumor necrosis factor receptor
TNFα	Tumor necrosis factor α
TRADD	TNFR-1-associated death domain protein
TRAF2/3/6	TNFR-associated factors 2/3/6
TRAIL	TNF-related apoptosis-inducing ligand
TRIF	TIR domain-containing adaptor-inducing IFNβ
UDP	Uridine diphosphate
UTP	Uridine triphosphate
VCAM-1	Vascular adhesion molecule-1
WKYMVM/m	Tryptophan-lysine-tyrosine-methionine-valine-methionine (L/D chiral)

Introduction

Receptors are protein molecules that are expressed in all living cells and organisms, ranging from bacteria and fungi to plants and animals including humans. Depending on their structure, function and expression, they are divided into different types and classes. Receptors may be localized in the cytoplasm of a cell as well as in the nucleus and these are collectively termed intracellular receptors, or they may be expressed in/on the cytoplasmic membrane and belong to the group of membrane receptors. The signals (often chemical) sensed by membrane receptors enable cells or organisms to react to changes in their surrounding environment with a physiological response. As receptors are numerous and heterogeneous they commonly only interact with a limited number of molecules, termed ligands. Endogenous ligands, for example hormones, originate from within an organism and exogenous ligands, like photons or drugs, are derived from foreign sources. Receptors are implemented in most biological processes including smell, vision and other aspects of our sensory system, reproduction and growth, behavior, emotions and pain. But they are also critical regulators of our immune system and are directly involved in host defense against invading pathogens. Therefore, any dysregulation or imbalance in receptor activity or receptor-mediated responses can potentially lead to inflammatory disorders, autoimmunity or illness. G-protein-coupled receptors represent the largest group of membrane receptors and to date they are the number one target for drug-based therapeutics. The content of this PhD thesis is focused on regulatory aspects of G-protein-coupled receptors expressed by human neutrophils and on the physiological consequences of receptor modulation in these cells, which execute important functions in our innate immune system.

The Human Immune System

The human body is constantly exposed to a large number of microorganisms including the commensal microflora but also to directly or potentially harmful pathogens. Pathogenic microbes are diverse in nature and comprise viruses, bacteria, fungi, unicellular eukaryotic organisms (Protista) and parasitic worms, also known as helminths. Threads by such organisms are antagonized by our immune system which is able to distinguish between self- and non-self-molecules. In vertebrates the immune system consists of innate (inborn) components as well as of adaptive (acquired) components. Monocytes/macrophages are cells of the innate immune system that are able to release cytokines and inflammatory mediators which typically initiate the immune response aiming to kill invading microbes and to clear the organism from pathogens and cell debris. Together with dendritic cells (DCs), these cells also establish the link between the innate and the adaptive immune system. In contrast to the B and T cells of the adaptive immune system which need to undergo time consuming clonal expansion to execute their highly specific functions, the cells of the innate immune system are equipped with preformed molecules that detect so-called pathogen/microbial-associated molecular patterns (PAMPs/MAMPS). The innate immune system thus mediates swift responses to infections or damaged tissue and thereby relies on the recognition of conserved structures that can either be of microbial origin which includes lipopolysaccharides (LPS), double-stranded RNA (dsRNA) and peptidoglycans, or they are regarded as host-derived danger-associated molecular patterns (DAMPs), like adenosine triphosphate (ATP), heat shock proteins and mitochondrial DNA. Peptides with a formylated methionine at the N-terminus represent a molecular pattern that belongs both to the PAMP/MAMP and the DAMP group of danger molecules as they may originate either from microbes or from damaged host cells. PAMPs/MAMPs and DAMPs are recognized by so-called pattern recognition receptors (PRRs) that are expressed by all cells of the innate immune system, including neutrophils. Sentinel cells, i.e. tissue-resident macrophages and dendritic cells, initiate an inflammatory response through activation of their PRRs [1-3]. Recognition of pathogenic surface structures by soluble innate immune components, as well as recognition of antibody-

opsonized pathogens/antigens leads to activation of the complement system, i.e. proteins present in tissue and blood which will mediate and assist in phagocytosis, recruitment of leukocytes and cell lysis and apoptosis [4]. Further, endothelial cells in close proximity to the infection/inflammation increase cell expression of adhesion molecules (ICAM-1, VCAM-1, selectins) that bind activated integrins (adhesion receptors) and other structures present on immune cells and thereby aid them to leave the blood stream and enter the afflicted tissue [5]. Neutrophils are the first cells to arrive to an infected area, guided by chemoattractant receptors that allow for directed movement towards chemical gradients originating either from the invading pathogens (e.g. formylated peptides) or as a consequence of the host's immune response (e.g. IL8, C5a, LTB4) and their prime function is to neutralize pathogens, a process achieved through phagocytic killing and secretion of antimicrobial/cytotoxic substances.

The Neutrophil

The neutrophil granulocyte is the most abundant type of immune cell in peripheral blood with a concentration range of 3 – 7 million cells/ml in adults. Due its multilobular nucleus, a feature shared with the basophil and the eosinophil, the neutrophil is classified as a polymorphonuclear leukocyte (PMN). Neutrophils mature from hematopoietic stem cells in the bone marrow over a time span of two weeks, during which they undergo six developmental stages (myeloblast, promyelocyte, myelocyte, metamyelocyte, band, mature PMN), followed by a release into the blood stream where they form a circulating and a marginating (resident in tissue/organs) pool [6-8]. Under normal conditions the life span of neutrophils is estimated to range from a few hours to one or two days, after which they are cleared primarily by Kupffer cells in the liver [9, 10].

Neutrophil Granules

Neutrophils contain four types of intracellular membrane-enclosed vesicles (three granule types and the secretory vesicle) that are formed and filled with specific components during different developmental stages. This process for synthesis and sorting is referred to as "targeting by timing". Granules can be mobilized or fused as a consequence of neutrophil activation and during phagocytic processes. Experimental separation of these granules is done via subcellular fractionation and density gradient centrifugation [11-14]. In the 1960s it was found that one of the subtypes of granules, the peroxidase-positive (primary) granules, also known as the azurophil granules, are formed during the promyelocyte differentiation stage and that these organelles are fairly large and high in density. Another, (secondary) granule type, known as peroxidase-negative specific granules, is smaller and lower in density and these organelles are formed during the myelocyte stage [15, 16]. Azurophil granules contain, amongst others, myeloperoxidase, α-defensins and serine proteases (elastase and cathepsins). Specific granules typically contain high levels of lactoferrin, cytochrome b_{558}, and collagenase [17, 18]. In 1982 it was discovered that the peroxidase-negative granules contained two metallo-proteinases (collagenase and gelatinase) which

did not necessarily co-localize in the same subcellular compartments. This led to the identification of the gelatinase granules (tertiary granules) that were even lighter and could easily be mobilized relatively to the cell membrane through a secretion/membrane fusion process [19]. Contents typical of gelatinase granules include matrix metallo-proteinase (gelatinase), acetyltransferase, cytochrome b_{558}, adhesion proteins and β_2-microglobulin [17, 18]. In the early 1990s, yet another mobilizable organelle (the so-called secretory vesicles) was discovered. These vesicles are endocytic in origin and are formed through an invagination of the plasma membrane, a process occurring at a very late stage of neutrophil maturation. The secretory vesicles are very easily mobilized and fuse with the cell membrane already by mild stimulation. The secretory vesicles contain serum proteins (an effect of their endocytic origin) but also other molecules, such as alkaline phosphatase, cytochrome b_{558} and various immune and chemoattractant receptors [17, 20, 21]. Upon mobilization of these molecules to the cell surface, naïve neutrophils will enter a primed state of responsiveness. As the different granules are formed continuously during neutrophil maturation it is noteworthy that their contents are partially overlapping, especially between secondary and tertiary granules. Contrary to the tertiary granules and the secretory vesicles the primary and secondary granules predominantly fuse with the phagosome after microbial uptake to release their bactericidal and cytotoxic contents and are therefore hard to mobilize.

Priming

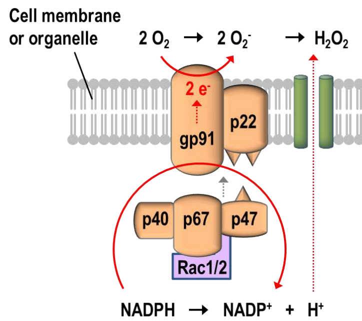
Neutrophils contain numerous proteolytic and toxic substances and have the potential to generate large amounts of reactive oxygen species, which not only kill pathogens but can also be destructive towards the host him/herself. Hence, it is necessary to maintain and control their activities accordingly to the given situation. Under healthy conditions, circulating neutrophils retain a resting state, meaning that they express only low amounts of adhesion molecules and receptors implemented in infection/inflammation in order to limit the strength in their response to inflammatory mediators. When neutrophils are exposed to proinflammatory stimuli (cytokines, chemokines, pathogenic metabolites and host-derived danger signals) they undergo certain morphological and functional changes and they are transferred from a resting to a primed state, characterized

by an ability to respond more strongly [22]. Initially, priming was defined by an invigorated respiratory burst activity mediated by the NADPH-oxidase in response to a secondary activating stimulus, usually the formylated peptide fMLF [23]. An increased NADPH-oxidase activity as a result of priming by agents like LPS, PAF or TNFα is largely dependent on mobilization of intracellular granules. This hallmark of neutrophil priming [24], achieved through fusion of secretory vesicles and gelatinase granules with the cell membrane increases the amounts of cytochrome b_{558}, the membrane-bound component of the NADPH-oxidase [12, 21] and the expression of cell surface receptors, such as FPRs and complement receptors CR1 and CR3 [25-27]. Priming of resting blood neutrophils *in vivo* leads to a state of increased adhesion and to neutrophil rolling on the vascular endothel, mediated by surface-exposed L-selectin. Subsequently, L-selectin is shedded from the cell surface and CD11/CD18 integrins provide firm adhesion to endothelial cells [28-30]. In addition, priming agents can alter the rate of neutrophil apoptosis [31], increase neutrophil chemotactic migration (directed movement in response to a chemical stimulus) [32-34] and phagocytosis [35]. Since priming agents are diverse in origin and properties, they differ in their effects on neutrophils but in an inflammatory environment cells of the immune system are typically exposed to multiple stimuli at the same time which complement each other to mediate appropriate cellular responses. It is also worth mentioning that there is no sharp line between priming and activating stimuli, as demonstrated by the fact that low concentrations of the bacteria-derived formyl peptide fMLF induce chemotactic migration, fusion of easily mobilized vesicles/granules and prime neutrophil superoxide production, whereas high concentrations directly mobilize other subsets of granules and activate the oxygen radical-producing NADPH-oxidase [36].

The Phagocyte NADPH-Oxidase

In general, so-called NOX proteins are conserved structures responsible for transmembrane electron transfer and exist in several different forms, i.e. NOX1 to 5 and DUOX1 and 2. These proteins are expressed throughout eukaryotes and regulate various biological processes [37]. The NOX2 protein is part of an electron transporting NADPH-oxidase in neutrophils, and this enzyme system comprises five different subunits, two of which are membrane bound and three that in

resting cells are present as a complex in the cytosol. All five subunits are needed for complete assembly of the oxidase and induction of enzyme activity. The activated oxidase enzyme generates superoxide radicals (O_2^-) through electron transport from the cytosolic substrate NADPH, either across the plasma membrane or across the membrane of granules or phagosomes. The membrane component of the NADPH-oxidase, the cytochrome b_{558}, is a protein heterodimer formed by the subunits gp91^{phox} (NOX2) and p22^{phox}. In resting neutrophils only a small fraction of the cytochrome b_{558} is readily present in the plasma membrane and the lion's share is localized within granules and secretory vesicles, to be mobilized either during phagocytosis or through priming-mediated secretion/degranulation [38, 39]. Cytochrome b_{558} is an electron (e^-) transporter that delivers electrons to molecular oxygen (O_2). The gp91^{phox} subunit contains a flavin adenine dinucleotide (FAD) and two heme molecules serving as the catalytic center of the enzyme. Two electrons are conveyed from NADPH to FAD, followed by single-electron reductions of the two heme groups which then reduce two O_2 to two O_2^- in another single-electron reduction process [40, 41]. Although p22^{phox} is not directly involved in electron transportation, its association with the gp91^{phox} subunit is required for proper expression and function of the membrane-bound cytochrome b_{558} [42]. The heterotrimeric cytosolic complex consists of i) the p47^{phox} subunit, an adaptor protein with autoinhibitory function and essential for interaction with membrane-bound p22^{phox}, ii) the p67^{phox} subunit with a regulatory domain for reduction of FAD from the substrate NADPH and iii) the p40^{phox} subunit for which several regulatory roles have been reported [43-46]. In addition, the NADPH-oxidase system requires a small Rho GTPase, Rac1 or Rac2, which, in its active GPT-bound form, interacts with the p67^{phox} subunit and catalyzes the electron transfer from the NADPH [47, 48] (Figure 1). Voltage-gated ion channels compensate for the charge differences across the membrane created by NADPH-oxidase activity [49]. The produced superoxide can further dismutate into hydrogen peroxide (H_2O_2), either spontaneously or catalyzed by superoxide dismutase, and this secondary oxygen metabolite serves as substrate for generation of hypochlorous acid (HOCl) by myeloperoxidase, the enzymatic active granule-localized peroxidase in neutrophils [50] (Figure 1). In human neutrophils a large number of stimuli, specific to various receptors, can induce respiratory burst activity.



Chemical reactions of oxygen radical formation

1) $2\,O_2 + 2\,e^- \rightarrow 2\,O_2^-$
2) $2\,H^+ + 2\,O_2^- \rightarrow H_2O_2 + O_2$
3) $H_2O_2 + Cl^- \rightarrow OHCl + H_2O$

Figure 1) Subunits of the NADPH-oxidase enzyme complex and chemical reactions of oxygen radical formation. Activation of the NADPH-oxidase leads to recruitment and interaction of the cytosolic complex, consisting of $p40^{phox}$, $p67^{phox}$ and $p47^{phox}$, with the membrane-bound heterodimer formed by the $gp91^{phox}$ and $p22^{phox}$ (also known as cytochrome b_{558}). The NADPH-oxidase enzyme mediates the reduction of two O_2 molecules to two O_2^- molecules that can subsequently dismutate into H_2O_2, which serves as a substrate for the formation of HOCl.

Membrane Receptors

Basic Characteristics of Recognition Proteins

Receptors interact with their specific ligands to mediate cellular responses to chemical signals. Membrane receptors are exposed on the surface of the plasma membrane, whereas intracellular receptors are present in the cytosol or nucleus. Ligands able to cross the cell membrane and bind directly to intracellular receptors are typically small and hydrophobic, like corticosteroids and sex hormones. Some cytosolic receptors are specific for activated second messengers as a consequence of prior membrane receptor activation. Through membrane receptors cells recognize and react to a large variety of substances present in the extracellular environment. Such ligands are commonly hydrophilic, do not cross the cell membrane and include growth factors, hormones, neurotransmitters, photons, PAMPs/MAMPs, DAMPs, and cytokines. All transmembrane receptors have one or more extracellular domain(s) that recognizes the ligand, transmembrane domain(s), and cytoplasmic signaling domain(s). Depending on their structure and function, receptors are divided into different types/classes (see below).

Non-G-Protein Coupled Receptors

Ligand-gated Ion Channels

Pentameric ligand-gated ion channels (pLGIC) are used to passively transport ions, such as calcium (Ca^{2+}), potassium (K^{+}), sodium (Na^{+}), magnesium (Mg^{2+}) and chloride (Cl^{-}), across the cell membrane in response to a specific ligand. Receptors of this type comprise five identical and symmetrically placed subunits. The extracellular domains form the orthosteric ligand binding sites, α-helix motifs in the membrane domains create the actual ion channel and the cytoplasmic domain can interact with proteins such as kinases and might be subject to post translational modifications [51, 52] (Figure 2). Ligand-gated ion channels are predominantly expressed in neurons and are involved in functions of the central nervous system, like motoric, sensory processes and emotions. Prominent examples are the serotonin receptor ($5\text{-}HT_3R$), the nicotinic acetylcholine recep-

tor (nAChR) and one member of the gamma-aminobutyric acid receptor ($GABA_AR$). Purinergic $P2X_{1-7}$ receptors are ATP-gated ion channels of which some are expressed on cells of the immune system. $P2X_{1,4,7}$ have been suggested to be present on human neutrophils, and to play a role in cell migration but these results have been regarded as controversial [53, 54]. In addition to pLGICs also voltage/ion gated ion channels exist that do not rely on ligand binding for function but operate in dependence of the membrane potential (i.e., differences in electric potential across the cell membrane).

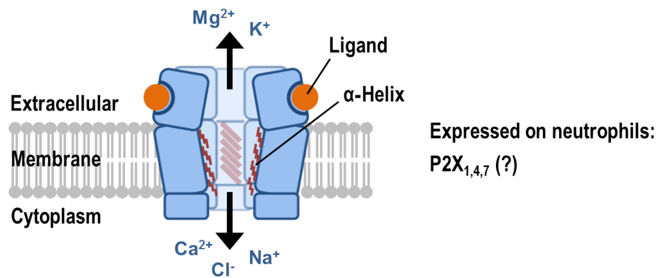


Figure 2) Pentameric ligand-gated ion channel.
These receptors consist of five identical subunits. Alpha-helices in their respective transmembrane regions form a cannel/pore structure and in the presence of an extracellular ligand conformational changes enable for passive transport of ions across the plasma membrane.

Fc Receptors

Fc receptors bind the constant region (i.e. fragment crystallizable, or Fc region) of antibodies on opsonized material and mediate their phagocytosis and clearance by immune cells. Fc receptors are divided in three classes depending on their binding preference and affinity; i.e. Fc-alpha (α), Fc-epsilon (ε) and Fc-gamma (γ). Naïve neutrophils express high amounts of FcγRIIIb (CD16) and also FcγRIIa/b (CD32). Expression of FcγRI (CD64) requires initial priming and has been shown to be upregulated as a consequence of bacterial infections [55, 56]. FcαRI (CD89) is involved in regulating neutrophil viability and can promote apoptosis in an inflammatory environment [57] and FcεRI has been suggested to have implications in allergic conditions [58]. Upon antibody binding, FcγRIs cross-link and their receptor class characteristic immunoreceptor tyrosine-based activation/inhibition motifs (ITAM or ITIM) get phosphorylated by sarcoma kinase (Src). This causes binding and phosphorylation of spleen tyrosine kinase (Syk) which activates phosphatidylinositol-4,5-bisphosphate 3 kinases (PI3K). This activates Burton's tyrosine kinase (BTK) and phospholipase C

(PLC), other downstream mitogen-activated protein kinases (MAPKs) and the release of calcium from intracellular stores (Figure 3). Fc receptor-mediated responses include activation of transcription factors and cytokine release, cytoskeleton remodeling, phagocytosis and cytotoxicity [59].

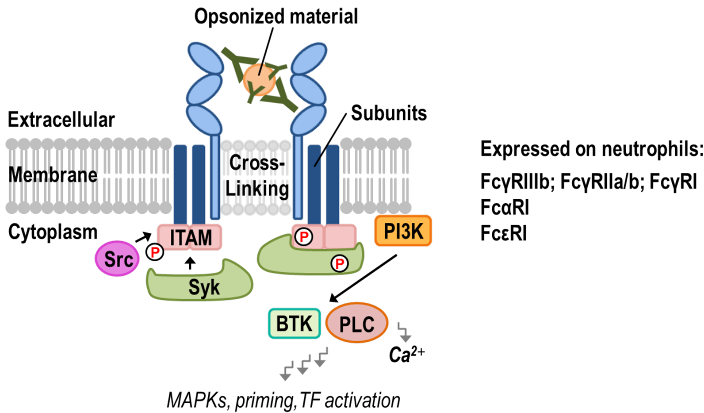


Figure 3) Schematic of an FcγRI and characteristic signaling events.
Fc receptors are predominantly expressed by cells of the immune system and bind to the constant region of antibodies. Activation by a respective ligand promotes receptor cross-linking and initiates various downstream signaling cascades that stimulate immune cells for antimicrobial and cytotoxic activity.

Cytokine Receptors

Cytokine receptors are activated by molecules that are typically released by immune cells (chemokines, interferons, interleukins and tumor necrosis factors) to mediate particular responses during an infection or inflammation. They are all similar in function but so-called type I receptors are characterized by a conserved amino acid motif (WSXWS) in their extracellular domain which is lacking in type II receptors. G-CSF and GM-CSF (granulocyte and granulocyte/monocyte/monocyte colony-stimulating factor, respectively) are ligands for neutrophil cytokine receptors that are important during neutrophil development but they also prime mature neutrophils for antimicrobial activities [60]. Neutrophils have been shown to express also several cytokine receptors for interleukins. Of these receptors, IL4R, IL6R, IL13R and IL15R regulate immunomodulatory and proinflammatory functions such as cell adhesion and cytoskeletal rearrangements, priming and neutrophil cytokine release [61-64]. In the presence of additional stimuli IL2R and IL12R can induce co-stimulatory signals, gene transcription and IL8 production [65, 66]. IL5R has been found to be expressed

on neutrophils from sepsis patients [67] and the IL10R is stored in specific granules and generates anti-inflammatory signals upon membrane expression and activation [68]. Interferons are important for the immune response towards viral infections and interact either with type I interferon alpha/beta receptors (IFNAR) or type II interferon gamma receptors (IFNGR), which may prime neutrophils for receptor expression and some modest protein synthesis, increased phagocytosis and an anti-apoptotic phenotype [69, 70]. Also IL1R and IL18R are important neutrophil cytokine receptors that induce proinflammatory responses but their signaling pathways are more similar to toll-like receptors (see below). Neutrophil activation with IL1 delays neutrophil apoptosis [71] and IL18 enhances phagocytic burst activity, degranulation and cytokine release [72]. Typical for type I and type II cytokine receptor signaling is the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway (Figure 4). Ligand binding to receptor homo-, heterodimers or oligomers increases receptor-associated JAK activity which in turn phosphorylates tyrosine residues on the receptor. This promotes STAT proteins to be recruited to the receptor site. Phosphorylated STAT proteins dimerize and translocate to the nucleus to induce gene transcription. In addition, cytokine receptor activation can also trigger Ras-Raf-MAPK pathway-mediated gene transcription and PI3K activation [73].

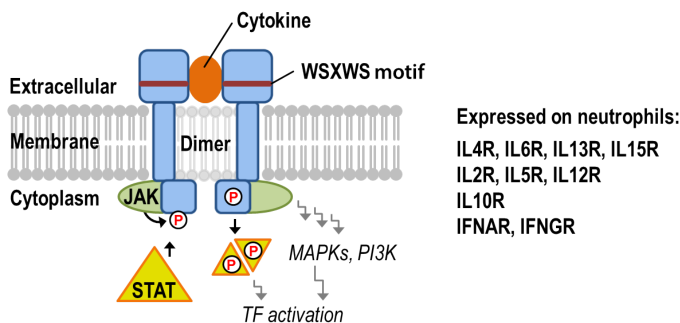


Figure 4) Schematic of a type I cytokine receptor and characteristic signaling events.
Cytokine receptors are activated by host-derived molecules during an in inflammatory situation or viral infection. Cytokines are produced by a broad range of cell types including leukocytes and lymphocytes. Most cytokines act as proinflammatory signaling molecule for immune cells, while some exhibit a resolving anti-inflammatory profile.

TNF Receptors

Receptors of the tumor necrosis factor receptor superfamily (TNFR) bind cytokines that can mediate apoptotic cell death, of which TNFα and its receptors are best characterized. TNFRs are divided into TNFR-1, which contains a so-called death domain and binds soluble and membrane-bound TNF, and TNFR-2, which only binds membrane-bound TNF and does not have a death domain. Both receptors are expressed on neutrophils. An activated TNFR-1 forms homotrimers and can bind two protein complexes in the intracellular domain, depending on the inflammatory environment surrounding the neutrophil. Complex 1 mediates antiapoptotic signals through activation of NFκB protein and c-Jun N-terminal kinase (JNK) and is formed by association of the receptor with multiple proteins, namely TNFR-1-associated death domain protein (TRADD), TNFR-associated factors 2 and 3 (TRAF2/3), receptor interacting protein (RIP) and cellular inhibitor of apoptosis proteins 1 and 2 (cIAP1/2). This promotes upregulation of the c-FLIP protein which interferes with the pro-apoptotic signals form complex 2. Complex 2 is formed after the receptor and complex 1 receive posttranslational modifications that causes their dissociation in the cytosol and let TRADD associate instead with the death-inducing signaling complex (DISC), consisting of Fas-associated death domain (FADD) protein and pro-caspases 8 and 10. This initiates caspase-mediated neutrophil apoptosis [74, 75] (Figure 5). Another prominent member of the TNFR superfamily is the first apoptotic signal (FAS) receptor that also evokes neutrophil apoptosis by caspase signaling but is suggested to involve a mitochondria-dependent pathway, whereas the TNFR-1 pathway depends more on the presence of intracellular oxygen radicals [76]. Furthermore, neutrophils have been shown to functionally express pro-apoptotic TNF-related apoptosis-inducing ligand (TRAIL) receptor 2 and 3 [77], as well as receptor activator of NFκB (RANK) in patients with persistent bacterial infections [78]. Other TNF receptor-mediated proinflammatory signals on neutrophils include priming for NADPH-oxidase activity, degranulation and membrane receptor upregulation [79].

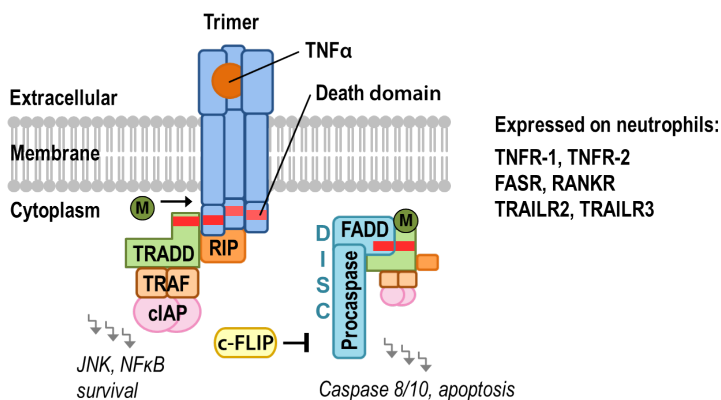


Figure 5) Schematic of a TNFR-1 and characteristic signaling events.
TNF receptors are cytokine receptors which predominantly induce proapoptotic signals to mediate controlled cell death. In connection to inflammatory situations TNFR ligands can act as proinflammatory stimuli for cells of the immune system and may cause prolonged cell survival.

Toll-like Receptors

Toll-like receptors (TLR) recognize conserved microbial patterns and are expressed predominantly by tissue-resident sentinel cells. Their ligands include lipopolysaccharides (LPS), lipopeptides, proteins, double-stranded viral RNA and DNA motifs. Although formally cytokines receptors, IL1Rs and IL18R are similar to TLRs in signal transduction and together they form the IL1R/TLR receptor superfamily. There are 13 identified mammalian TLRs (TLR1-13) but in humans TLR11 is a non-functional pseudogene and TLR12 and 13 are lacking [80]. Human neutrophils express TLR1, 2, 4, 5, 6, 8, 9, 10 but not TLR3 and 7, yet the expression levels of TLR2, 5 and 9 vary depending on the presence of additional neutrophil stimuli [81-83]. TLR activation can trigger L-selectin shedding, priming for oxygen radical production, phagocytosis, production and release of the neutrophil chemoattractant IL8 and influences chemotactic migration [83, 84]. The extracellular domains of TLRs are defined by leucine-rich repeats (LRR) and a horseshoe-like shape. Upon ligand binding, TLR monomers form homo- or heterodimers which recruit adaptor proteins to specific regions of their intracellular domains. Interaction occurs through Toll-IL-1-resistence (TIR) domains present on receptors and adaptors, which consist either of myeloid differentiation primary-response protein 88 (MYD88) and MYD88-adaptor-like protein (MAL), or of TIR domain-containing adaptor-inducing IFNβ (TRIF) and TRIF-related adaptor molecule (TRAM). The MYD88 pathway is used by all

TLRs and activates PI3K, interleukin-1 receptor-associated kinases (IRAKs), TNF receptor-associated factor 6 (TRAF6) and TGFβ-activated kinase 1 (TAK1) to mediate MAPK- and IKK-γ dependent activation of transcription factors (Figure 6). In contrast, TLR3 on endosomes exclusively engages the TRIF pathway which activates NF-κB transcription independently of MYD88 and promotes translocation of interferon regulatory factors (IRFs) to the nucleus in response to viral infections. Only TLR4 can address both of these pathways [85].

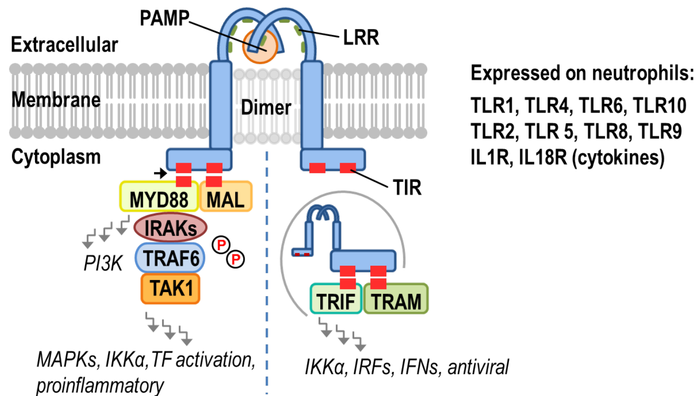


Figure 6) Schematic of the two signaling cascades initiated by toll-like receptors.
TLRs are important pattern recognition receptors of the innate immune system that recognize conserved microbial structures. Activation of TLRs induces various proinflammatory cellular responses and the production of cytokines, and stimulates the adaptive immunity. Most TLRs use the MYD88 pathway, only TLR3 and 4 utilize the TRIF-pathway.

G-Protein Coupled Receptors (GPCRs)

GPCRs represent the largest family of cell surface receptors with about 950 genes predicted in the human genome. About 500 are related to smell and taste and around 350 receptors are suggested to bind to endogenous ligands [86]. GPCRs are implemented in many biological processes including immune reactivity and their ligands are numerous and diverse, ranging from ions and photons to hormones, cytokines, small molecules, and peptides/proteins. Being integrated into the cell membrane makes it particularly difficult to obtain structural information at high resolution for this group of receptors. In the year 2000, bovine rhodopsin (photoreceptor) was the first GPCR to be crystalized [87] and to date over 800 sequence-based comparative structural predictions exist [88] along

with about 130 crystal structures for more than 30 receptors with and without ligand and/or protein interactions [89]. Vertebrate and invertebrate GPCRs have been grouped together by sequence homology into six classes, and in 2003 the human GPCRs were divided into five groups based on a phylogeny [90, 91]:

Table 1) GRAFS classification system of human GPCRs

Receptor family	Family members	Characteristics
Glutamate	22	primarily neurotransmission
Rhodopsin	701/ non-olfactory: 284	high sequence similarities and amino acid motifs
Adhesion	33	extracellular adhesion domain
Frizzled/Taste2	24	cell polarity and development
Secretin	15	bind large peptides/peptide hormones
unclassified	23	atypical (loop) domains

The rhodopsin family is by far the largest group and consists predominantly of receptors related to the sense of smell (olfactory). Of those that are non-olfactory, more than 50% are still classified as orphan receptors, meaning they have not yet been linked to an endogenous ligand [92]. Most well-known GPCRs belong to the rhodopsin group, and amongst them can be mentioned the adrenergic receptors, opioid receptors, dopamine receptors, histamine receptors as well as the formyl peptide receptors, chemokine receptors and the IL8 receptor, etc. Also known as seven-transmembrane receptors (7TMR), GPCRs comprise an N-terminal extracellular tail, seven transmembrane-spanning α-helices which are connected by three extra- and intracellular loops and a C-terminal intracellular tail (Figure 7). To be able to mediate cellular responses from ligand binding to their extracellular/transmembrane binding cavity, GPCRs require coupling to a heterotrimeric G-protein (consisting of α-subunit and β/γ complex) at the cytosolic side of the membrane.

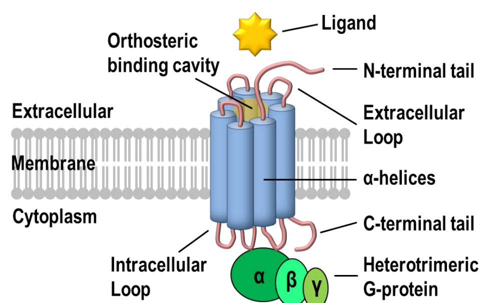


Figure 7) Schematic of a GPCR. GPCRs consist of an extracellular N-terminal tail, seven membrane-spanning domains that are connected via extra- and intracellular loops, and an intracellular C-terminal tail. Agonist binding at the extracellular domain induces dissociation of the heterotrimeric G-protein from the receptor in the cytoplasm into α subunit and β/γ complex, which initiate various signaling events.

Neutrophil GPCRs

Overview of GPCRs expressed by Neutrophils

Neutrophils are highly motile and the first immune cells that are recruited to sites of inflammation and infection. Their segmented nucleus is suggested to be of advantage for exudation from the blood stream through the endothelial wall towards an afflicted tissue. Key characteristics of neutrophils are chemotactic migration, phagocytosis, ability to release cytotoxic/microbicidal substances and proteolytic enzymes, and their competence to generate oxygen radicals. GPCRs, either readily expressed on the cell surface or contained within granules to be mobilized in response to an inflammatory environment, are implemented in all of these processes. The neutrophil GPCRs include the platelet activating factor receptor (PAFR), the complement component 5a receptor (C5aR), the leukotriene B4 receptor (LTB4R), the formyl peptide receptors (FPR1 and FPR2), and the interleukin 8 receptor (IL8R) [93], but also the purinergic receptor $P2Y_2R$ (receptor for extracellular ATP) [33] as well as different subtypes of adenosine receptors [94]. Neutrophils further express receptors that recognize fatty acids of different length, i.e. short chain fatty acid receptor GPR43/FFAR2 [95-97] and medium chain fatty acid receptor GPR84 [98], which both exhibit proinflammatory activation profiles. Chemokine receptor 4 (CXCR4) regulates neutrophil release from the bone marrow [99], and the $G\alpha_s$-coupled histamine H_2-receptor has inhibitory effects on FPR activation [100, 101]. Similar to the histamine receptor, also the β2-adrenergic receptor inhibits FPR signaling, reduces chemotactic migration, adhesion and superoxide production, suggestively by adenylyl cyclase activation and cAMP production [102-104]. The following table shows a summary of characterized GPCRs expressed by human neutrophils (Table 2). Neutrophil GPCRs differ in their expression levels, recognize endogenous and/or foreign ligands, execute distinctive functions, they may interact with different G-proteins and some are able to communicate with other receptors in the presence of multiple stimuli. All these factors play important roles in the fine-tuning of neutrophil responses to resolve an inflammatory situation accordingly, as well as they demonstrate the complexity of GPCR signaling *in vivo*.

Table 2) Selected characterized GPCRs expressed by human neutrophils.

Name	Agonists	Primary implications	References
FPR1	f-pep; pep; s.m.	Priming; chemotaxis; ROS	[105-107]
FPR2/ALX	f-pep; pep; s.m.; pdc.	Priming; chemotaxis; ROS	[108, 109]
PAFR	PAF	Priming; chemotaxis; ROS	[110]
C5aR	C5a	Priming; chemotaxis; ROS	[111]
CXCR1/2	IL8; other CXCLs	Priming; chemotaxis; ROS	[112]
LTB_4R	LTB_4	Adhesion; chemotaxis	[113]
$P2Y_2R$	ATP; UTP; variants	Migration; ROS	[114, 115]
CXCR4	SDF-1 (CXCL12)	Homeostasis	[99]
H_2R	Histamine; s.m.	Inhibitory on chemotaxis, ROS	[100, 101]
GPR43/FFA2R	Acetate; s.c.FAs	Regulatory on inflammation	[95-97]

Abbreviations: f-pep formylated peptide; s.m. small molecule; pdc pepducin; FA fatty acid; ROS reactive oxygen species

In addition to the receptors mentioned above, there are also less profound reports on other GPCRs expressed by human neutrophils. Some have investigated the cannabinoid receptor CB_2R and related receptor GPR55 in relation to superoxide production and chemotactic migration [116-118]. GPCR68 is suggested to antagonize superoxide production [119], the arachidonic acid metabolite receptor OXE1R and related receptor GPCR R527 are proposed to induce chemotactic activity [120, 121] and prostaglandin receptors EP_2 and DP inhibit neutrophil functions [122]. Purinergic receptor $P2Y_{11}R$ was shown to mediate cell survival [123, 124] and $P2Y_{14}R$, receptor for UDP-glucose was reported to be functionally expressed [125]. In regard to chemokine receptors it was suggested that CCR6 expression is dependent on cytokine stimulation [126] and CCR7 is expressed heterogeneously [127], whereas CCRL2 was upregulated in synovial fluid neutrophils of rheumatoid arthritis patients. Although a large body of neutrophil receptor research already exists to date, gene expression analysis implies the existence of additional receptors of different types, waiting to be identified and characterized [128].

The Formyl Peptide Receptors (FPRs)

A difference in the protein synthesis machinery between eukaryotic and prokaryotic cells is one of the profound mechanisms by which microbes generate chemoattractants for leukocyte. Although the first amino acid of new proteins is always a methionine both in eukaryotes and in prokaryotes, only bacteria and mitochondria, which are suggested to originate from endosymbiosis with prokaryotic cells, possess a formylated initiator tRNA (fMet-$tRNA_i^{Met}$) that adds a

formyl group to the amino-terminal part of the translated proteins [129]. In 1975 N-formylated peptides were discovered to be strong chemoattractants for leukocytes [130] and two years later the “formyl peptide receptor” (FPR1) was identified via binding of a radio-labeled variant of the formylated tripeptide fMet-Leu-Phe (fMLF) [131]. FPR1 was successfully cloned in 1990 [132, 133] and shortly after two FPR1 homologs, all together located in a cluster on chromosome 19, were described. FPR2 (former FPRL1), which shares 69% amino acid sequence similarity with FPR1, is also expressed on neutrophils and was initially regarded as a low affinity receptor for formylated peptides. FPR3 (former FPRL2) has 56% similarity to FPR1 and is expressed in monocytes and dendritic cells but not neutrophils [134-137].
Based on the observation that pertussis toxin, an exotoxin produced by the gram-negative bacterium *Bordetella pertussis* which causes ADP ribosylation of $G\alpha_i$ subunits and thereby prevents proper receptor interaction, inhibits FPR signaling, FPRs have been suggested to couple to the Gαi class of G-proteins [138, 139] (see below, signaling downstream of GPCRs).
Despite being classified as immune receptors and primarily expressed in leukocytes, FPRs are also found in other tissues, i.e. in endo- and epithelial cells, Kupffer cells (macrophages) of the liver, in lung tissue, cells of the nervous system and skeletal muscles [140-142], yet their functions in these tissues are largely unknown. FPRs are found on mammalian leukocytes including primates, dog, horse, cow, rat and mouse, but their respective genes expanded differently after the divergence of rodents, as exemplified by the murine genome where the FPRs expanded to eight homologs [143]. Six mouse FPRs have been cloned, of which, Fpr1, Fpr-rs1 and Fpr-rs5 are suggested to be the orthologs of human FPR1, 2 and 3 according to sequence similarities. FPR2 arose from FPR1 through gene duplication prior to divergence of mouse and man and its subsequent replication lead to today’s gene diversity [144]. This discrepancy and the lack of clear orthology between human and mouse orthologs is an important factor to consider when investigating FPRs across species, as ligands that work in one species may have a different or no affinity for the respective receptor in another species [145-147], a phenomenon clearly illustrated by the activity of FPR-derived lipopeptide ligands (pepducins, see below) [148, 149].

The Platelet Activating Factor Receptor (PAFR)

This GPCR binds the endogenous proinflammatory mediator platelet activating factor, also known as l-O-alkyl-2-acetyl-sn-glycero-3-phospho-choline (AGEPC) or in short PAF. This agonist is a soluble phospholipid that initially was identified in rabbits and was shown to be released from activated basophils and to cause platelet aggregation [150]. PAF can, however, be produced also by other cell types including monocytes, neutrophils, endothelial cells and even platelets itself [151-153]. Cells can synthesize PAF *de novo* but the more common mechanism is remodeling of ether-linked phospholipid membrane components. Arachidonic acid-containing glycerophosphocholins (GPC) are processed by phospholipase A_2 which generates the precursor lyso-PAF. Acetyl coenzyme A and lyso-PAF acetyl transferase then generate the active form of PAF from the precursor. In reverse, the enzyme PAF-acetyl hydrolase (PAF-AH) can convert PAF back to lyso-PAF [154-156]. The remodeling route and release is thought to be of importance for inflammatory responses, whereas *de novo* synthesis is suggested to be implemented in preserving homeostasis. Besides platelets, primarily neutrophils, monocytes and eosinophils express the PAF receptor but it can also be found in lung tissue and Kupffer cells of the liver [157]. The PAF receptor was shown to couple to $G\alpha_i$ and $G\alpha_q$ which can promote similar as well as G-protein subtype-specific downstream signals [158] (see below). PAF is a strong chemoattractant for neutrophils and mediates cell migration similar to the FPR1 agonist fMLF [159]. Contrary to FPRs, on human neutrophils PAF receptors are expressed solely on the cell membrane and absent in intracellular stores. Their activation also induces calcium release from intracellular stores, L-selectin shedding, degranulation and oxygen radical production [160]. In addition, PAFR can reactivate desensitized FPRs for superoxide production through receptor cross-talk signaling ([161] Paper I and IV).

The Purinergic Receptors

The nucleotide adenosine triphosphate (ATP) is substrate for intracellular energy transfer through conversion into adenosine diphosphate (ADP) or adenosine monophosphate (AMP) via hydrolytic phosphate cleavage; it further serves as substrate for various kinases and has a second messenger function when cata-

lyzed to cAMP by the enzyme adenylyl cyclase. But ATP that is actively secreted or released from damaged cells/tissue can also function as a danger signal (DAMP) during an infection/inflammation. Purinergic receptors are ubiquitous membrane receptors that take part in many biological processes and bind nucleotides and nucleosides. They are divided into three categories, i.e. the P2X ligand-gated ion channels and two classes of GPCRs, namely four P1 receptors which sense adenosine, and eight P2Y receptors which sense ADP, ATP, UTP, UDP and UDP-glucose [162, 163]. $P2Y_{14}R$ with affinity for UDP-glucose was defined as functionally expressed on neutrophils and suggested to have modulatory effects on cAMP levels and FPR activation by fMLF but its functional role is not yet precisely defined [125]. $P2Y_{11}R$ was shown to mediate antiapoptotic effects on neutrophils in the presence of ATP or β-nicotinamide adenine dinucleotide (NAD+) [123, 124] and $P2Y_6R$ is assumed to promote neutrophil IL8 production when activated with UTP [164]. The $P2Y_2$ receptor is the best characterized purinergic receptor expressed by neutrophils and has affinity for both ATP and UTP. $P2Y_2R$ typically couples to $G\alpha_q$ but is also able to interact with $G\alpha_i$ and $G\alpha_{12/13}$ [165-168]. Although not a chemoattractant receptor itself, $P2Y_2R$ enhances fMLF-mediated chemotaxis through ATP sensing in the extracellular milieu or secreted from neutrophils at the leading edge [33, 169]. In neutrophils, $P2Y_2R$ activation with high ATP concentrations induces the release of calcium from intracellular stores through a pertussis toxin-sensitive $G\alpha_i$ signaling pathway and mediates extracellular signal-regulated kinase (ERK) and MAP kinase activation [170]. Contrary to FPRs, $P2Y_2R$ stimulation does not trigger NADPH-oxidase-mediated superoxide production, as this pathway is blocked in naïve cells through an inhibitory mechanism involving the actin cytoskeleton. Accordingly, $P2Y_2R$-mediated oxygen radical production requires precedent disruption of filamentous actin (Paper II). The presence of ATP can enhance the generation of superoxide induced through activation of other neutrophil GPCRs and, similar to the PAF receptor, the $P2Y_2R$ has also been shown to reactivate desensitized FPRs for respiratory burst activity by a novel receptor cross-talk mechanism [114, 171].

Signaling downstream of GPCRs

Heterotrimeric G-Proteins

The human genome encodes for 32 G-protein α/β/γ subunit proteins [172] and the α type subunits are categorized in four classes, i.e. $G\alpha_s$, $G\alpha_q$, $G\alpha_i$ and $G\alpha_{12/13}$, which initiate distinct, as well as overlapping signaling cascades. All Gα subunits possess an intrinsic GTPase activity which keeps signaling of unbound receptors at a very low or zero level [173]. In the absence of an agonist, the β/γ subunits form a single inactive complex together with the Gα subunit but ligand binding to the 7TM receptor induces conformational changes that promote exchange of G-protein-bound GDP (guanosine diphosphate) with GTP (guanosine triphosphate), which leads to the dissociation and activation of the G_α subunit. As the human genome encodes for multiple copies of all G-protein subunits, they can be found in varying combinations. Activated GPCRs mediate signals through second messenger cascades and subsequently get desensitized by physical separation of receptor and G-protein. The predominant mechanism therefore is recruitment and binding of arrestin proteins which additionally mediate the receptor internalization process [174]. Endocytosed GPCRs are either degraded or they can be recycled back to the cell surface and regain their function.

$G\alpha_{12/13}$ Signaling Characteristics

$G\alpha_{12/13}$ signaling mechanisms are the least characterized amongst all G-proteins. This group consists of two members, α12 and α13, and receptors that have affinity for $G\alpha_{12/13}$ have been shown to also bind to other Gα subtypes [175]. Activated and dissociated $G\alpha_{12/13}$ proteins inactivate themselves at a relatively slow pace via hydrolyzation of their bound GTP. This can lead to prolonged signaling and therefore $G\alpha_{12/13}$ proteins are controlled by guanosine nucleotide exchange factors (RhoGEF) that are recruited from the cytosol and directly interact with the G-protein subunits. RhoGEFs are not only GTPase-activating proteins (GAP) but also mediate downstream signaling cascades by activation of Ras homolog gene family member A (RhoA) and its release from RhoGDI (guanine nucleo-

tide dissociation inhibitor). RhoA is thereby enabled to activate Rho-associated protein kinase (ROCK). ROCK inhibits myosin light chain phosphatase (MLCP) which leads to cell contraction and signaling through cytoskeletal proteins, activates serum response factor (SRF-) mediated gene transcription and further phosphorylates multiple other substrates (Figure 8A). These include c-Jun N-terminal kinase (JNK) and focal adhesion kinase (FAK) which is implemented in cell adhesion and movement [176]. Studies on knock-out mice have suggested that murine neutrophils require $G\alpha_{12/13}$ signaling for polarization, adhesion and migration [177]. In a neutrophil-like human promyelocytic leukemia (HL-60) cell line $G\alpha_{12/13}$ was shown to be involved in the formation of the trailing edge during fMLF-mediated polarization and migration [178, 179].

$G\alpha_s$ Signaling Characteristics

Stimulation of a $G\alpha_s$-protein coupled receptor is characterized by activation of the membrane-bound adenylyl cyclase enzyme which catalyzes conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). cAMP is a second messenger that binds co-called EPACs (exchange proteins directly activated by cAMP) which then activate regulatory Ras-like small GTPases (Rap) [180]. cAMP is also an activator of protein kinase A (PKA) that phosphorylates many downstream targets, such as MAPKs and it also activates cAMP response element-binding proteins (CREB) which induce gene transcription [181]. $G\alpha_s$-protein activity is regulated by phosphodiesterases (PDE) that convert cAMP to AMP. The cAMP pathway executes many cellular functions ranging from insulin secretion, neuronal and cardiovascular regulation to pro- and anti-inflammatory signaling [180] (Figure 8A). In neutrophils increased levels of cAMP can negatively affect oxygen radical production and decrease chemotactic migration [182, 183].

$G\alpha_i$ Signaling Characteristics

Signaling by the $G\alpha_i$ subunit (also known as $G\alpha_{i/o}$) is characterized by an inhibitory effect on adenylyl cyclase activity and thereby downregulates the levels of cAMP. $G\alpha_i$ can directly bind to Src which mediates activation of the transcription factor STAT3 (signal transducer and activator of transcription 3) [184]. $G\alpha_i$

promotes activation of multiple transcription factors by extracellular signal-regulated kinase (ERK) via the Ras-Raf-MAPK pathway and also activates PLC, which regulates the release of calcium from intracellular stores and thereby PKC activation [185] (Figure 8A). The neutrophil chemoattractant receptors FPR1 and FPR2 are prominent examples for $G\alpha_i$-coupling GPCRs.

$G\alpha_q$ Signaling Characteristics

$G\alpha_q$ signaling is characterized by activation of PLC, which hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP2) to diacylglycerol (DAG) and inositol trisphosphate (IP_3). DAG then activates PKC and IP_3 mediates calcium release from intracellular stores by binding to IP_3 receptors on the endoplasmic reticulum. PKC phosphorylates multiple downstream proteins and can regulate the activation of transcription factors (Figure 8A). $G\alpha_q$ signaling also leads to activation of MAPK and is generally suggested to overlap with $G\alpha_{12/13}$-related signaling pathways [186].

The Gβ/γ Subunit

The Gβ/γ complex is a heterodimeric protein that is bound to the Gα subunit under resting conditions (i.e. when a GPCR is in its non-signaling state). Gβ/γ prevents Gα activation by regulating its high affinity for GDP and mediates GTP exchange at the Gα subunit upon GPCR activation. The Gβ/γ complex is also involved in GPCR signaling, although through direct protein-protein interactions, as it misses the Gα subunit's catalytic center. Plenty of proteins, cytosolic and membrane-bound, can serve as interaction partners, including MAP kinases, ion channels, adenylyl cyclase, etc. [187]. Gβ/γ recruits and activates PI3K to processes PIP_2 to PIP_3 (phosphatidylinosi-tol-4,5-trisphosphate), which has been shown to play a regulatory role in actin reorganization, chemotaxis and motility [188]. Protein kinase B (Akt), which has many regulatory roles, can bind $PIP_{2/3}$ and mediates antiapoptotic down-stream signals through NFκB activation (Figure 8B).

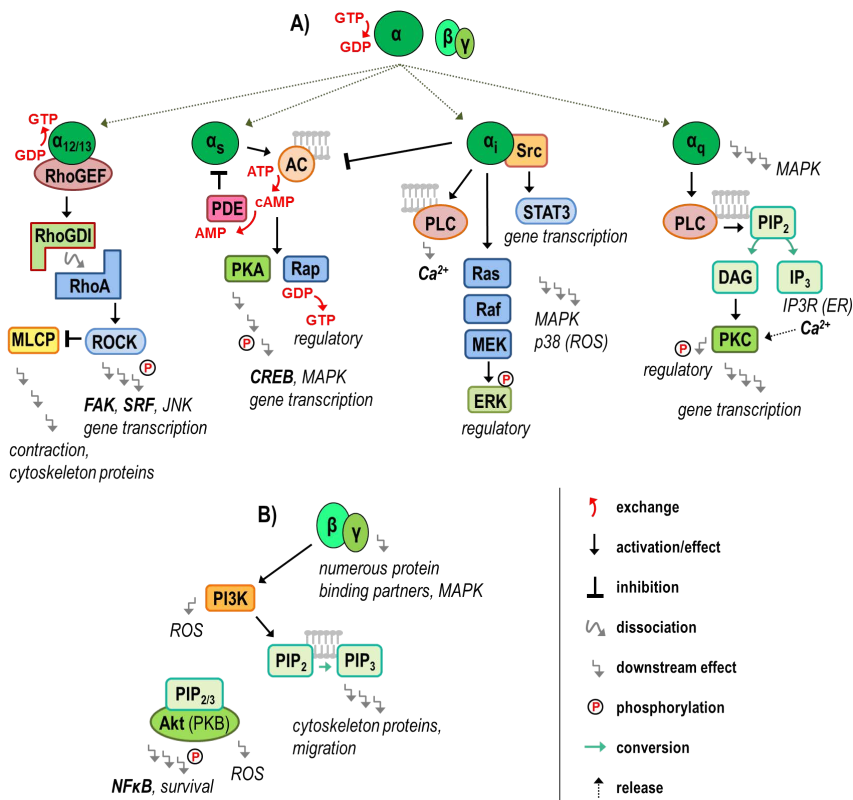


Figure 8) Signaling characteristics of G-protein subunits.
A) Agonist binding to a GPCR induces the exchange of GDP with GTP bound in the α-subunit of the G-protein and subsequent activation and dissociation from the heterotrimeric protein complex. Each type of Gα-subunit is characterized by class-specific downstream signaling events. Gq typically activates phospholipase C (PLC), Gs activates the adenylyl cyclase (AC) and cAMP-dependent pathways, whereas Gi inhibits adenylate cyclase activity and G12/13 interacts with Rho guanine nucleotide exchange factors (RhoGEFs) and is mainly associated with proliferation and motility. Certain signaling pathways and mediator molecules (e.g. kinases) can be activated by multiple Gα-subunits and also cell-type specific differences in relation to G-protein signaling exist. **B)** The Gβ/γ complex functions as a negative regulator for Gα-proteins but GPCR activation also leads to Gβ/γ complex-mediated responses which do not directly depend on the type of coupling Gα subunit.

G-Protein Signaling and Lessons from Neutrophils

The Signaling Cascade:

It is noteworthy that the signaling pathways triggered by the different G-protein subtypes can overlap and that cellular responses mediated by GPCRs depend highly on the cell type and individual receptor, as well as on factors like the type of activating ligand and its concentration, co-stimulatory influences and the state of a cell. The release of calcium (Ca^{2+}) from intracellular stores into the cytoplasm is one of the earliest events of receptor activation. The starting point for this signaling pathway is the activation of phospholipase C (PLCβ) downstream of the activated receptor and this enzyme hydrolyzes phosphatidylinositol bisphosphate (PIP_2) into inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 then promotes a transient Ca^{2+} release from intracellular stores (in many cells the endoplasmic reticulum (ER)) and these ions activate protein kinase C (PKC) together with DAG. Depletion of intracellular Ca^{2+} from stores opens store-operated Ca^{2+} channels (SOCs) in the plasma membrane. Ca^{2+} also activates cytosolic phospholipase A2 (cPLA2) to liberate arachidonic acid (AA) from membrane phospholipids. The $p47^{phox}$ subunit of the cytosolic component of the superoxide generating NADPH-oxidase is phosphorylated by kinases, a modification that enables translocation from the cytosol and interaction with cytochrome b_{558} in the membrane. Arachidonic acid binding to Rho GDP dissociation inhibitor (RhoGDI) causes release of Rac2 and subsequent activation through GDP-GTP exchange by guanine nucleotide exchange factors (GEF). Binding of Rac to $p67^{phox}$ completes assembly and activation of the NADPH-oxidase complex [189, 190]. The Gβ/γ subunit and PI3K have been shown to play roles in neutrophil and HL-60 cell-line migration and fMLF-mediated neutrophil superoxide production [191, 192]. Phosphatidylinositol-3-phosphate (PI3P), the product of $G_{\beta/\gamma}$ subunit-activated PI3 kinase, can bind to $p40^{phox}$ and thereby augments NADPH-oxidase activity in phagosomes [192, 193] (Figure 9). Besides PLC also other kinases including Akt, p38 MAPK and ERK have been suggested to have roles in NADPH-oxidase activation [194-197] but as of yet, not all regulatory aspects have been identified. FPRs can, however, induce a transient rise in intracellular Ca^{2+} that is not necessarily accompanied by an activation of the NADPH-oxidase and, in similar manner, FPR-mediated NADPH-oxidase activa-

tion can occur without any rise in intracellular Ca^{2+} (see below, regulation of GPCRs in neutrophils).

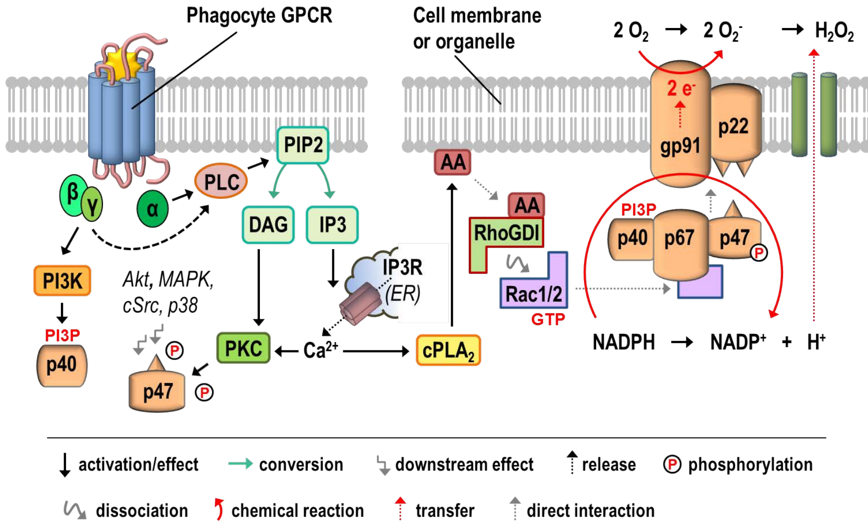


Figure 9) GPCR-mediated activation of the phagocyte NADPH-oxidase.
The release of calcium from intracellular stores is one of the earliest events of neutrophil GPCR activation. FPRs mediated the release of Ca^{2+} already in the presence of low agonist concentrations. Activation of neutrophil GPCRs with higher agonist concentrations may lead to NADPH-oxidase-mediated generation of oxygen radicals. Signaling events downstream of activated receptors/G-proteins induce recruitment of the cytosolic heterotrimeric protein complex of the NADPH-oxidase enzyme, consisting of $p40^{phox}$, $p67^{phox}$ and $p47^{phox}$, to its membrane-bound component cytochrome b_{558} ($gp91^{phox}$ and $p22^{phox}$). The assembled NADPH-oxidase enzyme additionally requires the presence of a small GTPase to mediate the transport of two electrons from the substrate NADPH across the plasma membrane or the membrane of an organelle or phagosome to reduce two oxygen molecules into two superoxide anions.

The transient Ca^{2+} Response:

The release of Ca^{2+} from intracellular stores into the cytoplasm has primarily been regarded as a signaling hallmark of $G\alpha_q$-coupled receptors, whereas $G\alpha_i$-coupled receptors regulate signaling of the cAMP-generating enzyme adenylate cyclase. In neutrophils activation of a GPCR typically triggers a transient increase in cytosolic calcium, irrespectively if the coupling is through the $G\alpha_i$ or the $G\alpha_q$ subtype of the G-protein. Accordingly, an activation of $G\alpha_i$-coupled receptors, such as the neutrophil FPRs and $P2Y_2R$ is accompanied by alterations in the level of intracellular Ca^{2+}, and this is true also for the $G\alpha_q$-coupled PAFR (Paper I – IV, [160]) The transient rise in Ca^{2+} is not substantially changed when extracellular Ca^{2+} is chelated, suggesting that the primary source is an inositol

trisphosphate (IP_3-) triggered ion release from intracellular storage organelles. Also this observation is valid for both the PAFR ($G\alpha_q$-linked) and the FPRs ($G\alpha_i$-linked). It should be noticed that the hydrolysis of PIP_2 to generate IP_3 is suggested to be mediated directly by the $G\alpha_q$ subunit but possibly the same signaling pathway is triggered by the $\beta\gamma$ complex of $G\alpha_i$-linked FPRs [198].

Even though less is known about FPR2 signaling than about FPR1 signaling, it is very likely that they use similar pathways based on the fact that the two receptors are structurally related in their signaling domains, and they mediate comparable cellular responses. A fundamental difference has, however, been described in signaling profiles between the two receptors when it was shown that one of the receptors (FPR2) triggers a unique Ca^{2+} influx. The opening of plasma membrane channels was suggested to occur without the involvement of intracellular storage organelles [199]. This has later been disproven and the basic signaling scheme downstream of FPR2 follows the same route as FPR1 [200]. More importantly, different signal transduction pathways can be activated, depending on the precise conformational change in the receptor induced by the bound agonist (see the section about biased signaling below). This phenomenon was initially described for two agonists that bind to the same receptor, but with respect to the FPRs only one agonist is required. Stimulation of neutrophil FPRs with an agonist in high concentration induces a temporary rise in intracellular Ca^{2+} (as described above) and activates the superoxide-generating NADPH oxidase. But superoxide produced through a reactivation process of desensitized FPRs triggered either by cytoskeletal disruption or by a receptor cross-talk mechanism occurs without any FPR-related transient rise in intracellular Ca^{2+} [114, 161, 201]. It should also be mentioned that a conformational change in the ATP receptor $P2Y_2R$ induced by agonist binding leads to G-protein-mediated signaling that results in a rise in intracellular Ca^{2+} but not in an activation of the NADPH-oxidase ([114], Paper II and III).

Inhibitors of G-Proteins:

The large sequence homologies between the different G-proteins make it hard to conclusively determine the identity of the precise subtype of G-protein involved in signaling. For long, bacterial toxins have been the tools available to identify the involvement of $G\alpha_i$ and $G\alpha_s$ (sensitive to the *Bordetella pertussis* and *Vibrio cholerae* toxins, respectively). Since recently, selective and efficient inhibitors of

$G\alpha_q$ are available [202, 203] which lack effect on FPR-mediated activities in naïve neutrophils, whereas the same functions mediated by the PAFR are inhibited [198]. This stands in direct contrast to the general belief that cellular responses triggered by neutrophil chemoattractant receptors (including the PAFR) are inhibited by the pertussis toxin effect on $G\alpha_i$ [204], although the precise mechanism regarding inhibition of GPCRs that are characterized as coupling to different subtypes than $G\alpha_i$ (e.g. PAFR) is not known. Recently, it has been shown that $G\alpha_q$-dependent activation signals generated by the PAFR can also activate the $G\alpha_i$-coupled FPRs through a receptor cross-talk that represents a novel pathway for GPCR regulation. In that regard, PAFR-mediated reactivation of desensitized FPRs is in complete dependence of functional $G\alpha_q$ proteins and does not rely on $G\alpha_i$-mediated signals [198]. In conclusion, identification of small inhibitors that are selective for the respective G-protein subtypes would be advantageous for a more detailed characterization of the pathways involved in neutrophil GPCR signaling.

Arrestin Proteins

Arrestin Translocation and Functions

Arrestin has initially been described as a mechanism for homologous desensitization of rhodopsin [205]. Arrestins play important roles in the termination of G-protein-dependent signaling of ligand-activated receptors, as well as they mediate signals themselves. The four known arrestin isotypes differ somewhat in function and expression pattern; arrestin 1 (S-antigen or visual arrestin) [206] and arrestin 4 (cone arrestin) [207] are mainly restricted to the retina and the regulation of photoreceptors, whereas arrestin 2 (β-arrestin 1) and arrestin 3 (β-arrestin 2) are ubiquitously expressed [208, 209]. Arrestins not only terminate G-protein-mediated signaling and promote receptor endocytosis but they are also capable of G-protein-independent signal transduction of activated receptors. Phosphorylation of ligand-bound GPCRs by G protein-coupled receptor kinases (GRK) increases binding affinity for β-arrestin and thereby causes a sterical separation of the receptor from its G-protein. Recruited β-arrestin can directly bind to adaptor protein 2 (AP-2) and the heavy chain of clathrin [210] which initiates the endocytic process via receptor clustering and clathrin-coated pit formation. The GTPase dynamin (Dyn) then executes the clipping of clathrin-coated pits from the plasma membrane to complete the endocytic process [174, 211]. Depending on the strength of the receptor-arrestin interaction, internalized GPCRs are either subsequently degraded, or recycled if the binding is rather transient [212].

Receptor-bound arrestins can also recruit and directly interact with various signaling proteins including Src kinases and ERK1/2 which are involved in the regulation of arrestin and GRK function, desensitization, exo- and endocytosis [213-216]. Arrestins also scaffold stress-related JNK3 kinase [217] and regulatory ubiquitin E3 ligase [218] (Figure 10). Much is still unknown about arrestin-mediated signaling but their interaction with GPCRs and other proteins is suggested to depend on distinct conformational changes in the receptor as well as in the arrestin protein itself [219, 220].

Studies on a basophilic leukemia cell line linked arrestin to IL8-dependent degranulation involving Src activation [213] and human embryonic kidney (HEK) cells have been shown to perform cytoskeleton rearrangements to stimulation with fMLF in dependency of arrestin [221]. Arrestin can serve as a scaffold for cofilin, an actin-chopping protein that is in complex with the phosphatase chronophin and with the actin-binding kinase LIM [222]. This complex regulates assembly of filamentous actin at the leading edge during cell migration [223, 224].

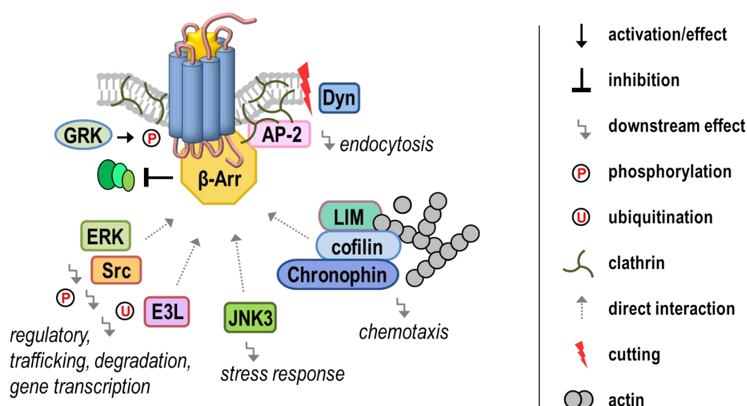


Figure 10) Arrestin-mediated effects of GPCR activation.
The predominant mechanism for GPCR desensitization and receptor-mediated endocytosis is recruitment and binding of arrestin proteins which physically interfere with G-protein coupling and promote the uptake of desensitized GPCRs in clathrin coated vesicles. Arrestins may also serve as scaffolds for various signaling proteins and these arrestin-dependent signaling pathways can be triggered independently of G-protein activation.

Structural Requirements for G-Protein and Arrestin Binding

High resolution crystal structures are critical to understand the structural requirements of particular GPCR-mediated responses. Ideally, such data should allow comparison of receptors bound to balanced and biased agonists and their coupling to either G-protein or arrestin. Although multiple crystal structures for several receptors exist, information on receptor complexes is still limited. In the year 2011 the β2-adrenergic receptor in complex with its G-protein was successfully crystalized and revealed that an outward shift of transmembrane domains

TM5 and TM6 (which confine intracellular loop ICL3) and a small change in ICL2, caused by an inward shift of TM7/helix 8, precede G-protein coupling. TM5, TM6 and ICL3 all interact with the C-terminal domain of the Gα subunit and ICL2 forms contact with a cleft within the protein that undergoes conformational changes during the coupling process [225]. A large body of evidence suggests the existence of common fundamental structural similarities in relation to receptor activation and signaling, despite variations in GPCR architecture and amino acid composition. Some receptors, including CCR5, lack the characteristic regulatory E/DRY of rhodopsin class GPCRs, yet they display comparable profiles of activation-mediated conformational alterations [226]. Accordingly, also a high resolution crystal structure of a G-protein-bound adenosine A_{2A} receptor from the year 2016 revealed a large degree of similarity to the structure of the crystallized β2-adrenergic receptor [227].

Arrestins contain an N-domain-related part and a C-domain-related part in their overall configuration, which form a so-called N-C lock in their inactive state. This lock requires receptor phosphorylation prior to activation and binding, whereby the phosphorylated C-terminal tail and ICL3 interact with a positively charged N-terminal cleft of the arrestin protein and trigger a conformational change. This enables the arrestin for full interaction with the receptor and consequential blocking of the G-protein binding site [228]. The crystal structure of arrestin-bound rhodopsin from the year 2015 and data from computational modeling supports the theory of a biphasic mechanism of arrestin binding [229, 230]. The initial contact is formed between TM7/helix 8 and a loop domain of the arrestin, followed by interactions with TM5/TM6/ICL3 and TM3/ICL2. Phosphorylation of the C-terminal tail of the receptor provides charge interaction sites for the arrestin that are suggested to be required for recruitment and interaction with TM7/helix 8. Arrestin-bound rhodopsin displays a less significant outward shift of TM3 and ICL3 as compared to its G-protein-bound form. Nuclear magnetic resonance labeling in the β2-adrenergic receptor in complex with various ligands showed that TM6 and TM7 adopt two major conformational states and agonist binding primarily alters TM6 whereas arrestin-biased ligands predominantly alter TM7 [231].

Another requirement for arrestin-biased ligands is the ability to mediate receptor phosphorylation. Different types of β2-adrenergic receptor agonists can induce distinct phosphorylation patterns and arrestin conformations through activation

of different G-protein coupled receptor kinases (GRKs) [232], which is in accordance with reports on other GPCRs demonstrating that GRK2 and GRK3 primarily mediate arrestin recruitment and internalization, whereas GRK5 and GRK6 are necessary for β-arrestin-dependent ERK signaling [233-235]. Contrariwise, absence of μ-opioid receptor phosphorylation by morphine, as compared to etorphine, was linked to its inability to promote arrestin recruitment and receptor internalization [236].

Arrestin and Lessons from Neutrophils

In many cells β-arrestins have the role of adaptor molecules which, when recruited to GPCRs, not only inhibit the receptors to bind the signaling G-protein, but the signals generated by arrestin-bound GPCRs are also shifted to components regulated by the ERK signaling cascade that secondarily mediate receptor internalization. The role of β-arrestins in FPR signaling is, however, more complex. When expressed in different cell lines the agonist-occupied FPRs rapidly and transiently activate ERK, and this activation is most probably coupled to G-protein activation. Moreover internalization of FPR2 has been shown to require a co-expression of arrestin, but the endocytic uptake of FPR1 does not [237].
Termination of the G-protein-mediated signaling activities can be achieved through a physical separation of the agonist-occupied receptor from the G-protein in the plane of the plasma membrane. It is clear that β-arrestin binding, as well as the associated consequential homologous receptor desensitization is one possible mechanism therefore. But also other receptor-binding molecules may constitute the basis for physical separation of the G-protein from the activated receptor, for example actin, in its polymerized form [238]. In FPRs, the window of active signaling that follows agonist binding is fairly rapidly ceased through an actin cytoskeleton-dependent mechanism, as disruption of filamentous actin affects FPR desensitization and modulates receptor signaling (for details see chapter: Regulation of GPCRs in Neutrophils). Moreover, the patterns for signal termination and desensitization are very similar for agonists able to recruit arrestin and for those that do not (Paper IV). Although activated FPRs are desensitized by an arrestin-independent mechanism, it has been demonstrated that knockdown of β-arrestin in HL-60 cells results in reduced migration towards the bacterial chemoattractant fMLF [239]. In similar manner, FPR2-mediated

chemotaxis is impaired in primary neutrophils when exposed to an agonist that activates this receptor but does not induce recruitment of β-arrestin (Paper IV). This suggests not only a link between arrestin recruitment and chemotactic migration but also demonstrates that G-protein-dependent and arrestin-dependent signaling (and their physiological consequences) are not necessarily linked in neutrophil GPCRs.

GPCR Ligands

Orthosteric Ligands

Conventional extracellular ligands are incapable of traversing the plasma membrane and bind to GPCRs specifically at their designated binding pocket region which is typically formed by the extracellular/transmembrane domains and accessible from outside of the receptor-expressing membrane. The binding pocket for an endogenous ligand, which originates from within an organism, is defined as the natural, or orthosteric binding site. An exogenous agonist is a receptor-specific ligand that originates from an external source; drugs or microbial metabolites being prominent examples. Exogenous ligands often bind the orthosteric binding site of the targeted receptor. Any agonist that induces a maximal cellular response is classified as a full agonist and agonists with comparably lower efficacy at saturating concentrations are classified as partial agonists, respectively (Paper I). Inverse agonists are inhibitors that not only dampen agonist-induced receptor activity but also reduce the basal activity of a constitutively active (non-occupied) GPCR. Dual agonists are ligands that bind and activate two distinct receptors, however, not necessarily with similar affinity or potency. Contrary to inverse agonists, neutral antagonists do not alone cause a measurable receptor-mediated effect or a cellular response but simply occupy a receptor binding site and thereby prevent interaction with a receptor-specific agonist.

Allosteric Modulators and Agonists

An allosteric modulator is a molecule that interacts with a receptor at a location distinct from the orthosteric binding site and thereby modulates the effects of a conventional agonist. In the absence of an agonist, allosteric modulators are inert and they do not compete for receptor binding with natural ligands or conventional antagonists. A negative allosteric modulator (NAM) stabilizes the inactive (or low signaling) conformation of a receptor, whereas a positive allosteric modulator (NAM) promotes ligand binding or receptor activation by an agonist [240]. Allosteric modulators are not solely synthetic compounds and small molecules

but also exist endogenously in form of ions and peptides. Some prominent GPCRs that are characterized for being influenced by endo- and/or exogenous allosteric modulators include 5-HT serotonin receptors, adenosine and adrenergic receptors, various chemokine receptors and opioid receptors, muscarinic acetylcholine receptors (mAChRs), dopamine and $GABA_B$ receptor, etc. [241, 242]. Ligands that bind a GPCR distinct from the orthosteric site and display receptor-activating properties in the absence of a conventional agonist are classified as allosteric activators and the mediated effects of such molecules can reflect the properties of the different types of orthosteric GPCR ligands [243].

Pepducins: Activation/Inhibition through a novel Mechanism?

Binding of conventional orthosteric ligands induces conformational changes in parts of the respective receptor. Transmembrane region TM3, TM6 and TM7 have been found to play critical roles in this process, which consequently also causes alterations in the conformation of one or more intracellular loops [244, 245]. Receptor mutagenesis and cross-linking experiments (i.e. covalent binding of interacting proteins or peptides by forming chemical bonds between amino acids) showed that all intracellular loops ICL1, ICL2 and ICL3 can establish contact with the G-protein but TM3/ICL3 is suggested to be the main contact site [246-250].

A new concept for GPCR regulation was introduced with the first pepducins designed in the year 2002, based on findings showing that i) peptides with amino acid sequences from the ICL3 of the β_2-adrenergic receptor, as well as ii) the wasp peptide toxin mastoparan, with structural similarities to an intracellular GPCR loop, were able to activate G-proteins [251, 252], and that iii) lipidated peptides derived from an integrin subunit glycoprotein GpIIb penetrate the plasma membrane of platelets and specifically mediate their aggregation and activation [253]. Pepducins contain a fatty acid (typically a palmitic acid) that is linked to a peptide with an amino acid sequence identical to the whole or a part of one of the intracellular loops of a GPCR. The first pepducins, with peptide sequences derived from the ICL3s of the protease-activated receptors PAR1 and PAR2 and of the melanocortin 4 receptor MC4R, affected the functions of their cognate GPCRs when expressed in platelets and thereby created the starting point for the

concept of pepducin-mediated receptor modulation [254, 255]. The physiochemical properties of pepducins, i.e. charge and especially their hydrophobicity, allow for anchoring of the molecules to the cell membrane, as their lipid part sticks into the membrane phospholipids. The peptide part of the pepducin may then reach the area of the membrane facing the cytoplasm though some type of flipping mechanism. Based on similarities between pepducins and natural post-translational modifications (e.g. palmitoylation) used to anchor a large number of proteins (including G-protein subunits) to the inner leaflet of the plasma membrane [256, 257], also pepducins should prefer this localization. The peptide part that mimics the intracellular receptor loop of identical amino acid sequence is suggested to alter the interaction between the GPCR and the coupling G-protein. Consequently, pepducins then either promote an activation of down-stream signaling without receptor activation by a conventional agonist at the orthosteric binding site, or they cause an inhibition of receptor signaling induced by a conventional agonist [255]. Based on the presumed mechanism for receptor interaction, pepducins are regarded as allosteric ligands (Figure 11). Therefore, their mode of action, should fulfill certain criteria: i) they require a fatty acid to mediate their functions, ii) they are specific for the receptor containing an identical amino acid sequence, iii) if the mediated effect is receptor activation, this should not be affected by conventional receptor antagonists and iv) there should not be any competition with orthosteric ligands for receptor binding.

Established concept of pepducin-receptor interaction

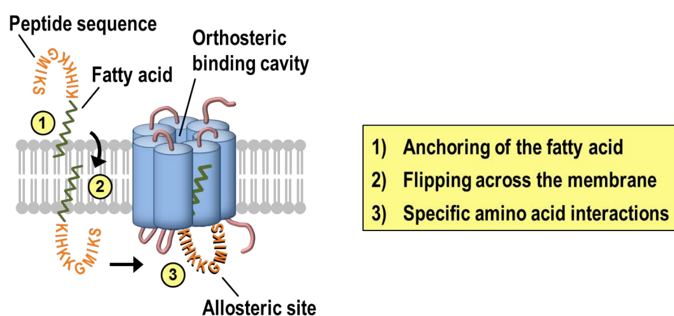


Figure 11) Established model of pepducin-mediated allosteric modulation of a GPCR.
The fatty acid part of the pepducin enables anchoring to the phospholipids of plasma membrane and its typical hydrophobic nature promotes a flipping of the peptide part into the cytosolic domain of the cell. With high specificity the peptide part then allosterically modulates or activates the targeted GPCR on basis of amino acid sequence identity to one of the intracellular loops of the receptor.

Both the primal PAR1-based activating pepducin P1pal-19 and the truncated version thereof, P1pal-13, fulfill these criteria, although P1pal-19 also fully activates the closely related receptor PAR2 (determined by the ability to induce calcium mobilization, platelet aggregation and activation of PLC-β). It is also worth mentioning that P1pal-12, one amino acid shorter than P1pal-13, is a PAR1-specific antagonist instead. Further, PAR2-derived P2pal-21 and MC4R-derived MC4pal-14 both activated their cognate receptors specifically but the truncated PAR4 antagonist P4pal-10 gained some degree of affinity for PAR1 [254, 255, 258]. Such off-target effects are suggested to occur due to the high degree of similarity in the intracellular domains of these receptors.

Since the introduction of the concept of lipopeptide-based GPCR modulation in the year 2002, activating and inhibitory pepducins have been identified for several receptors. To list some, intracellular loop ICL1- and ICL3-derived pepducins of CXCR1/2 (x1/2LCA-i1 and x1/2pal-i3) inhibit intracellular calcium mobilization and human neutrophil chemotaxis, induced by agonists that interact with the orthosteric binding site of the respective receptor, and this was also shown for CXCR4-derived x4pal-i1 and x4pal-i3. Interestingly, x1/2pal-i3 cross-inhibited CXCR4-mediated neutrophil chemotaxis, but in overexpressing HEK cells this occurred solely if CXCR1/2 was co-expressed, indicating a receptor dimerization-dependent mode of action [259]. ATI2341, a receptor-activating CXCR4 pepducin identified through a screening approach in receptor-overexpressing HEK cells [260, 261], was shown, when characterized in detail, to be a biased allosteric agonist for CXCR4. As discovered by bioluminescence resonance energy transfer (BRET) assay in HEK cells, ATI2341 interaction with the receptor mediated coupling in favor for $G\alpha_i$, whereas the natural ligand SDF1 promotes engagement of $G\alpha_i$, $G\alpha_{13}$ and arrestin [262]. In a similar approach, the pepducin ICL1–9 of the β_2-adrenergic receptor was identified as a biased ligand in human osteosarcoma (U2S) cells, as it promotes a receptor conformation favoring β-arrestin signaling over G-protein signaling [263]. The ICL2 pepducin KRX-725 derived from the sphingosine-1-phosphate receptor 3 $S1P_3$ has been suggested to have pro-angiogenic effects in human cell lines [264], an LGR7 relaxin receptor pepducin 619-629-Lys(Palm) was found to be inhibitory on adenylyl cyclase stimulation in rat tissue [265], and three inhibitory pepducins SMOi1-1, SMOi2-1, SMOi3-1 that were synthesized from the intracellular loops

of smoothened, a GPCR-like receptor involved in the hedgehog pathway, have been characterized on human cancer cell lines [266].

Also palmitoylated peptides based on proteins other than GPCRs have been designed and demonstrated to have physiological effects. Palpeptides derived from $G\alpha_q$, $G\alpha_o$ modulate GPCR-regulated neuronal potassium channel functions but this is achieved through direct interference with G-protein coupling [267]. In rat primary sensory neurons the pepducin TRP-p5, directed against a protein interaction sequence of the cation channel vanilloid receptor 1 (TrpV1), was suggested to interfere with the conformational change required for opening [268]. The myristoylated peptide mSRI is based on the $G\alpha_{13}$ subunit switch region for RhoGEF interaction and could inhibit PAR1-mediated RhoA activation in human platelets and inhibited $G\alpha_{13}$ interaction with integrin $a_{IIb}b_3$ in murine platelets [269, 270]. In an overexpressing insect cell line the short palmitoylated peptide sequence IL3-8, derived from the third intracellular loop of the β2-adrenergic receptor, was proposed to interact directly with the $G\alpha_s$ subunit in a receptor-independent manner [271].

Pepducins and Lessons from Neutrophils

According to the model, the peptide part of a pepducin determines the receptor specificity and consequently whether the targeted receptor is activated or inhibited. The molecular basis for interaction is the amino acid identity between the pepducin and the respective receptor from which the sequence originates. It is not easy to understand the basic mechanism for how the peptide part of a pepducin translocates to the inner leaflet of the membrane and how two identical peptide sequences interact and by that either inhibit or activate receptor signaling. Irrespective of this, the results obtained with pepducins and neutrophils are in many aspects not consistent with these restrictions ([109, 149, 272], Paper I and III). This is, for example, illustrated by the fact that pepducins that activate FPR2 are sensitive to conventional antagonists and that peptide agonists specific for FPR2 compete for receptor binding [109]. Moreover a chimeric FPR in which the cytosolic tail of FPR1 is replaced with the tail of FPR2 gains affinity for a pepducin with an amino acid sequence that represents FPR2 rather than FPR1 [273]. The functional activities induced by third intracellular loop pepducins with amino acid sequences that originate from the human and mouse

FPRs/Fprs all target one of the receptors (FPR2/Fpr2), but not necessarily the one they are derived from [149]. Taken together, a large amount of neutrophil-related results question the validity of the pepducin concept, not only in relation to receptor selectivity but also regarding the mechanistic concept for how they activate, inhibit or modulate receptor function.

FPR Ligands – Formylated Peptides and beyond

To date, there are no crystal structures available for any of the FPRs and all structural analyses and data on ligand binding properties are based on computational modeling, docking studies, receptor chimera constructs, receptor mutagenesis and structure–activity relationship (SAR) predictions. Far more ligands and of greater diversity are known for FPR2 than for FPR1 and no selective high affinity agonist has yet been characterized for FPR3. Although FPR1 and FPR2 share 70% amino acid sequence identity, substantial differences in their binding sites have been suggested. Receptor point mutation studies with different formyl peptide ligands identified multiple charged amino acids in the extracellular domain and transmembrane regions TM2 and TM7 of FPR1 that were suggested to be critically required for agonist binding [274, 275]. FPR2 lacks some of these key amino acids but others have been shown to be of importance instead and they create affinity for somewhat longer formylated peptides with preference for a positively charged C-terminus [276]. Further, FPR2 is predicted to have a deeper binding pocket than FPR1 which holds three hydrophobic clusters within the protein, of importance for non-peptide ligand binding [277]. FPR2 generally seems to tend to bind larger molecules, supported by the observations that truncated versions of PSM (phenol soluble modulin) molecules, i.e. *S. aureus* α-helical peptides with affinity for FPR2, gain affinity for FPR1 and hybrid molecules created from the FPR1-specific fMIFL peptide and PSMα2 (fused at the C-terminal side) switch receptor preference depending on length – shorter peptides prefer FPR1, medium-sized are dual FPR1/FPR2 agonists, whereas longer peptides prefer FPR2 [273, 278].

Formylated Peptides derived from Microbes and Mitochondria

After the discovery that FPR1 is activated by N-formylated peptides, such structures have been isolated from various bacterial cultures and human mitochon-

dria. These organelles most likely evolved from prokaryotic cells and, in similar manner, they express a formylated initiator tRNA. Cells of the immune system are able to recognize formylated peptide structures either as a sign of microbial infection, or as a consequence of damaged host tissue. fMLF is an *E. coli* derived formylated tripeptide and the primal classical ligand for FPR1 [279]; *S. aureus* produces cytotoxic phenol-soluble modulin peptides (PSMα2, PSMα3) with affinity for FPR2 as well as the peptide fMIFL, a potent agonist for FPR1 [280, 281]. *L. monocytogenes*-derived formylated peptides display high affinity for murine Fprs and primarily bind human FPR1, whereas shorter mitochondrial peptide sequences show affinities for both FPR1 and FPR2 and longer mitochondrial amino acid structures (e.g. mitocryptide-2) bind FPR2 [282-284]. Although not formylated, the *Helicobacter pylori* peptide Hp (2-20) was shown to activate FPR2 and FPR3 in monocytes [285], and also viral (HIV-1) envelope proteins are suggested to bind to FPRs [286, 287].

Non-formylated Agonists

Besides pathogenic agonists, also endogenous peptides and lipid structures have been claimed to be ligands for FPRs. The antimicrobial peptide LL37, a cleavage product of the neutrophil granule protein cathelicidin binds FPR2 and activates neutrophils [288]. Various amyloidogenic proteins (i.e. proteins with the capacity to form aggregates), including amyloid-beta peptide ($A\beta_{42}$), humanin and acute phase protein serum amyloid A (SAA, the first identified endogenous ligand) are suggested to bind to FPR2 [289-291]. A critical fact about SAA is that for experimental purposes usually a recombinant form is used – a hybrid of SAA1 and SAA2 – so the true physiological effects of naturally occurring SAA can be debated [292, 293]. The antimicrobial/proinflammatory neutrophil granule protein cathepsin G was reported to be able to cause FPR1-dependent migration but does not induce an intracellular calcium transient [294]. Lipoxins (derived from arachidonic acid), resolvins (derived from fatty acids) and annexins (phospholipid-binding proteins) are regarded as anti-inflammatory mediators that form during and infection/inflammation and some reports suggest these molecules mediate their effects through FPRs [140, 295-297]. Cleaved annexin 1 peptide fragments can have activating and inhibitory functions [298], but *in vitro*, lipoxin A4 and resolvin D1 fail to induce any response that can be as-

signed to formyl peptide receptors (e.g. calcium mobilization or oxygen radical production), labelling them as somewhat controversial FPR agonists [299, 300].
Numerous synthetic FPR agonists have been identified in high throughput screening approaches with random peptide libraries and small molecules. Of these, the hexapeptide WKYMVm (D-chiral methionine) is a potent dual agonist for both FPR1 and FPR2 [106]. WKYMVM (L-chiral methionine) is a selective agonist for FPR2 in neutrophils with weak affinity for FPR3 in monocytes [301]. Another peptide agonist that was identified via screening is the 13-mer MMK-1 which activates FPR2 [302]. The quinazolinone-derived compound Quin-C1 was the first small molecule discovered to bind formyl peptide receptors. Quin-C1 has affinity for FPR2 and selectively induces a calcium response and chemotactic migration in neutrophils but it does not trigger oxygen radical production [303]. And the pyrazolone-derived small molecule compound 43 was initially identified as a FPR2 agonist but ultimately turned out to be a dual agonist with preference for FPR1 [304, 305].

FPR Antagonists

Because microbes naturally attract leukocytes, they have developed strategies to avoid immune-detection. *S. aureus* is known to produce “chemotaxis inhibitory proteins” (CHIPs) and their N-terminal residues show antagonistic effects on FPR1, as well as they synthesize “FPR1-like inhibitory proteins” targeting FPR2 [306, 307]. Cyclosporin H is a fungal toxin (*Tolypocladium inflatum*) and a natural antagonist selective for neutrophil FPR1 [308, 309]. In addition, cyclosporins also have medical implications as immunosuppressants. Exchange of the N-terminal formyl group of the fMLF agonist with a tert-butyloxycarbonyl group or a carbamate analog group created antagonists with high affinity for FPR1, i.e. Boc-1, Boc-2, respectively [309, 310]. A peptide library screening approach identified the hexapeptide WRWWWW as a selective FPR2 antagonist [311]. The membrane-permeable decapeptide inhibitor PBP10, derived from an amino acid sequence of the cytoskeleton protein gelsolin that is linked to an N-terminal rhodamine, is to date the most potent established neutrophil FPR2 inhibitor [312]. In human monocytes, PBP10 has been shown to have some inhibitory off-target effects towards *Herpes simplex* virus glycoprotein gG-2p20-mediated oxygen radical production through an unknown receptor distinct from FPR2 [313].

FPR-modulating Pepducins and Peptidomimetics

Most of our knowledge about pepducins is based on recombinant systems in which they are suggested to mediate their effects through an allosteric binding mechanism on intracellular parts of the receptor (as opposed to orthosteric binding to the extracellular cavity). But much less is known about their mode of action in primary cells. Lately, this novel class of cell-penetrating lipopeptides has been shown to interact with formyl peptide receptors on human neutrophils. Neutrophils express FPR1 and FPR2 that differ only in two amino acids in their third intracellular loop domains. As proposed by the pepducin dogma, the FPR2-ICL3-derived full-length palmitoylated 16-mer $F2Pal_{16}$ specifically activates neutrophils through its cognate receptor for intracellular calcium mobilization and oxygen radical production. C-terminal truncations of the $F2Pal_{16}$ pepducin showed that 12-amino acid long $F2Pal_{12}$ and 10-amino acid long $F2Pal_{10}$ had even greater efficacy than the full-length peptide and still retained their FPR2 specificity. On the contrary, no activating or inhibitory effects on FPR1 were seen by its respective ICL3 pepducin $F1Pal_{16}$. Surprisingly, in receptor overexpressing HL-60 cells substitutions in the third intracellular loop of FPR2 with amino acids of FPR1 did not affect binding and activity of any of the FPR2-derived pepducins. Further, point mutations in the peptide sequences indicated positive charges to be beneficial for lipopeptide activity [109]. $F1Pal_{16}$, initially shown to be inactive in FPR1, turned out to be an inhibitory ligand that is also highly specific for FPR2 [314].

These off-target effects could be explained by the sequence similarities between FPR1 and FPR2, as similar observations were made for pepducins derived from the PAR receptors, where optimal peptide length, charge and particular amino acids seemed to factor into pepducin activity [254, 255, 258, 315]. Yet, to date there is still no FPR1-derived pepducin with specificity for FPR1. Other phenomena that do not correlate with the pepducin concept are that FPR palpeptides compete for receptor binding with extracellular agonists in primary neutrophils and their activity is influenced by the presence of extracellular agonists. This argues against allosteric receptor modulation by pepducins in these cells [109, 314]. Neutrophils express CXCR4 predominantly during maturation in the bone marrow but receptor expression is diminished in fully developed peripheral blood neutrophils before it returns again for the clearing process of aged cells

[99]. Although the CXCR4 pepducin ATI2341 was demonstrated to bind to its cognate receptor in recombinant cross-linking experiments [261], in primary human neutrophils the ATI2341-mediated oxygen radical production was specifically sensitive to FPR2 antagonists and to desensitization by the FPR2 pepducin $F2Pal_{10}$ [272]. Similar results were obtained with palpeptides created from the intracellular loops of the ATP receptor $P2Y_2R$ (Paper III). In short, $P2Y_2Pal_{IC2}$ and $P2Y_2Pal_{IC3}$ activated neutrophil intracellular calcium release and superoxide production and these responses were all sensitive to FPR2 antagonists and agonists (conventional and pepducins), but not to ATP or a $P2Y_2R$-specific inhibitor. Further, $P2Y_2Pal_{IC2}$ competed for FPR2 binding and primed neutrophils for $P2Y_2R$-mediated radical production by ATP stimulation, which resembles an FPR-dependent receptor cross-talk mechanism [114] (Paper I).

All tested pepducins with effects on primary human neutrophils, i.e. FPR2-derived $F2Pal_{16}$ and $F2Pal_{10}$, FPR1-derived $F1Pal_{16}$, $P2Y_2R$-derived $P2Y_2PalIC2$ and $P2Y_2PalIC3$, as well as CXCR4-derived ATI-2341, involve/require FPR2 to trigger or modulate a functional response. These data, in combination with the data on competitive binding at the orthosteric receptor site are in strong contradiction to the proposed allosteric binding model for pepducins.

To date, some pepducins are under investigation for therapeutic and clinical implications in the treatment of cardiovascular diseases, asthma, bone marrow transplantation, inflammation, sepsis and cancer [260, 271, 315-320] and in the year 2016 the pepducin PZ-128, an inhibitor of protease-activated receptor 1 (PAR1) successfully passed phase I trial as a safe and highly reversible anti-platelet agent that prevents blood clots in patients with coronary artery disease [321]. Despite the fact that pepducin-based allosteric receptor modulation has been conclusively demonstrated, data on primary cells, as well as *in vivo* data, is lacking. In contrast to the proposed model of allosteric modulation by pepducins, the data presented in this thesis, based on experiments on isolated human neutrophils, suggests that FPR2 has pattern recognition properties for lipidated peptides and questions the pepducin dogma in reference to these cells (Paper I, III and IV). Clearly, more research is required to fully understand the mechanisms underlying receptor-pepducin interaction and to exploit potential perks of this novel class of lipopeptide ligands.

Another type of GPCR ligands that has recently been introduced as modulators of FPR signaling are peptidomimetics, i.e. small proteolytically stable molecules

designed to mimic proteins. Such molecules are either based on modifications of normal amino acids, or they are synthesized from amino acid/peptide-like molecules. Short activating fMLF-based peptidomimetics were identified as selective for FPR1 [322] but longer structures bind FPR2 and can exhibit activating and inhibitory properties. F2M2 is a lipidated α-peptide/β-peptoid hybrid (Lau-((S)-Aoc)-(Lys- βNphe)$_6$-NH_2) with structural similarities to pepducin molecules that activates human neutrophils through interaction with FPR2. The structurally related palmitoylated peptidomimetic Pam-(Lys-βNSpe)$_6$-NH_2 inhibits FPR2 activation with high efficiency and specificity [323, 324].

The notion that all the FPR2-specific pepducins and peptidomimetics mentioned above have the molecular profile of lipidated peptides, despite sharing or lacking amino acid sequence homology with one of the intracellular loops of FPR2, further support the idea that this neutrophil GPCR might be a pattern recognition receptor able to sense medium-sized lipopeptide structures – possibly from the extracellular side of the cell membrane. This theory would be in agreement with existing data on FPR binding pockets, suggesting that FPR1 holds a small extracellular ligand binding cavity and tends to be restricted to interaction with shorter molecules (besides having no affinity for pepducins), whereas FPR2 with its comparatively versatile binding pocket can interact with more ligands of greater diversity [273, 277, 278, 325].

During the past decades of research, many molecules have been claimed to be agonists or antagonists for the human formyl peptide receptors 1 and 2. A list of selected characterized FPR ligands is given below (Table 3).

Table 3) Selected neutrophil-activating FPR ligands and inhibitors.

FPR activators	Origin	Affinity	Reference
f-MLF	*E. coli*	FPR1	[279]
f-MIFL	*S. aureus*	FPR1	[280, 283]
PSMα2	*S. aureus*	FPR2	[281]
PSMα3	*S. aureus*	FPR2	[281]
f-MIVIL	*L. monocytogenes*	FPR1 > FPR2	[282, 283]
f-MIVTLF	*L. monocytogenes*	FPR1 > FPR2	[282]
f-MIGWI(I)	*L. monocytogenes*	FPR1 / FPR1 > FPR2	[282]
Hp (2-20)	*H. pylori*	FPR2 > FPR3 (Mo)	[285]
Mitocryptide-2 (15aa)	Mitochondria	FPR2	[284]
f-MFADRW (MCT-2)	Mitochondria	FPR1, FPR2	[282]
f-MMYALF	Mitochondria	FPR1, FPR2	[282]
f-MLKLIV	Mitochondria	FPR1, FPR2	[282]
LL37	Endogenous	FPR2	[288]
WKYMVm	Peptide library	FPR1 > FPR2	[106]
WKYMVM	Peptide library	FPR2 > FPR3 (Mo)	[301]
MMK-1	Peptide library	FPR2	[302]
Quin-C1	Small molecule screening	FPR2	[303]
Comp43	Small molecule screening	FPR1 > FPR2	[304, 305]
$F2Pal_{16}$	FPR2-derived pepducin	FPR2	[109]
$F2Pal_{10}$	FPR2-derived pepducin	FPR2	[109]
$P2Y_2PalIC2$	$P2Y_2R$-derived pepducin	FPR2	(Paper III)
$P2Y_2PalIC3$	$P2Y_2R$-derived pepducin	FPR2	(Paper III)
ATI-2341	CXCR4-derived pepducin	FPR2	[272]
F2M2	Peptidomimetic	FPR2	[324]
FPR inhibitors	**Origin**	**Affinity**	**Reference**
$F1Pal_{16}$	FPR1-derived pepducin	FPR2	[314]
CHIPs	*S. aureus*	FPR1	[306]
FLIPr	*S. aureus*	FPR2	[307]
CysH	*T. inflatum*	FPR1	[308, 309]
Boc-1, Boc-2	f-MLF N-terminal modification	FPR1	[309, 310]
WRW^4	Peptide library	FPR2	[311]
PBP10	Derived from gelsolin protein	FPR2	[312]
Pam-$(Lys\text{-}\beta NSpe)_6$-$NH_2$	Peptidomimetic	FPR2	[146]

Abbreviations: (Mo) monocytes

Regulation of GPCRs in Neutrophils

Homologous and Heterologous Desensitization

FPRs, but also other neutrophil GPCRs, are well-known triggers of NADPH-oxidase-mediated oxygen radical production and to avoid unspecific tissue damage during inflammation their responsiveness to extended or repeated agonist exposure is limited through receptor desensitization. The basic GPCR signaling scheme hereby includes i) ligand binding to the receptor and change in conformation, which leads to ii) activation of the associated G-protein and downstream signaling cascades, as well as iii) consequential phosphorylation and initiation of the signal termination process through a separation of the receptor from the G-protein.

In contrast to homologous desensitization, when agonist-occupied receptors turn insensitive to further stimulation, heterologous desensitization refers to a process where subsequent to activation of one GPCR the inability to respond is transferred to another unrelated GPCR. This desensitization is induced in the absence of the receptor-specific agonist. Receptor phosphorylation is a key event in desensitization and G-protein coupled receptor kinases (GRKs) are predominantly responsible for phosphorylation during homologous desensitization subsequent to receptor activation [326]. Heterologous desensitization instead involves phosphorylation by second messenger-dependent kinases, such as protein kinase A and C [327, 328], as well as by other signaling- and regulatory molecules including RGSs (regulators of G-protein signaling) [329, 330]. At a site of inflammation, neutrophils are exposed to numerous different stimuli and in order to execute specific and appropriate functions, their responses have to be firmly regulated. It has been shown that in relation to heterologous desensitization there is a hierarchy between the neutrophil chemoattractant receptors and in this context FPR1 and C5aR can be classified as end-target chemoattractant receptors, as their activation can overrule other (intermediate) receptors, such as CXCR1/2 and LTB4R [331, 332]. As a consequence, neutrophils always migrate towards

end-target chemoattractants, even when facing opposing chemotactic gradients. It has been suggested that end-target-directed migration relies on p38 MAP kinase activity, whereas intermediate chemoattractant receptors depend on PI3 kinase and Akt [333]. Heterologous desensitization of neutrophil GPCRs also occurs through activation of $G\alpha_s$-coupled histamine H_2-receptors and β-adrenergic receptors. These GPCRs inhibit $G\alpha_i$-dependent FPR-mediated functions through activation of adenylyl cyclase and the generation of cAMP [100, 101, 103].

The Actin Cytoskeleton as an endogenous GPCR Modulator

Desensitization has long been regarded as an irreversible state, and for most activated GPCRs arrestins interfere with G-protein coupling and promote internalization and receptor degradation or recycling. But for FPRs, the cytoskeleton is accountable for desensitization and can directly interact with the receptors even at suboptimal (low) temperatures [238, 334, 335]. Consistent with these findings, it was shown in overexpressing cell lines that desensitization and internalization of FPRs and C5aR required C-terminal phosphorylation, but these processes occurred independently of arrestin binding (the precise mechanism is still unknown) [336, 337]. Although not required for their desensitization/internalization, β-arrestin have been shown to bind to FPRs [338] and in an arrestin knock out cell line, internalized receptors were unable to recycle back to the cell membrane in the absence of β-arrestin. Similar results were obtained by arrestin mutagenesis experiments [339, 340]. Further, β-arrestin is suggested to be required for FPR-dependent adhesion and chemotaxis in HL-60 cells [239].
The importance of the cytoskeleton for regulation of FPR signaling is also reflected by the effects of actin-interfering drugs. Cytochalasin B binds to barbed ends of microfilaments and impairs assembly of filamentous actin, and latrunculin A binds to globular actin monomers and thereby prevents their integration during F-actin formation, which results in disruption of the actin cytoskeleton network during ongoing actin turnover processes [341]. Treatment of neutrophils with cytochalasin B or latrunculin A leads to primed and prolonged FPR-mediated oxygen radical production. Further, FPR-desensitized neutrophils can be reactivated for respiratory burst activity by cytoskeleton disintegration. As

opposed to direct receptor activation, reactivation signals for radical production are not accompanied by (or dependent on) the release of intracellular calcium [201] (Figure 12). Similar to neutrophils, also HL-60 cells that lack intracellular storage pools of receptors can be reactivated, indicating that mobilization of novel receptors to the cell surface is neither required nor the reason for this effect [201]. Alike FPRs, also desensitized neutrophil C5aR can be reactivated, but recently characterized GPR43/FFA2R, the IL8R or PAFR cannot [96, 342] (Figure 12). This is in agreement with the findings in receptor-overexpressing cells where PAFR desensitization and internalization was shown to depend on β-arrestin interaction [343, 344].

In naïve human neutrophils stimulation of the ATP receptor $P2Y_2R$ induces a transient release of calcium from intracellular stores but not generation of oxygen radicals. A $P2Y_2R$-mediated respiratory burst response requires pre-treatment with latrunculin A or cytochalasin B, as the signaling route to activate the NADPH-oxidase is selectively blocked by the cytoskeleton. In contrast, $P2Y_2R$ desensitization is unaffected by the integrity of the actin cytoskeleton and cannot be reversed through its disruption (Figure 12, Paper II). The same regulatory mechanism is valid also for FFAR2-mediated NADPH-oxidase activity [96] (Figure 12). In line with these observations, studies on overexpressing HEK-293 cells have shown that all human P2Y receptors are desensitized and internalized through interaction with β-arrestins [345]. The physiological role of the actin cytoskeleton in preventing $P2Y_2R$ to mediate NADPH-oxidase activity is currently not known. ATP is present in any given cell and tissue to provide chemical energy, and in the cytosol it can reach concentrations in the millimolar range [346]. Due to these factors, the cytoskeleton might serve as a failsafe mechanism for neutrophils to prevent unnecessary damage to injured tissue through $P2Y_2R$-induced radical production. Hypothetically, besides ATP other yet unknown endogenous $P2Y_2R$ ligands might exist that can unlock the cytoskeleton-dependent NADPH-oxidase signaling route in naïve neutrophils.

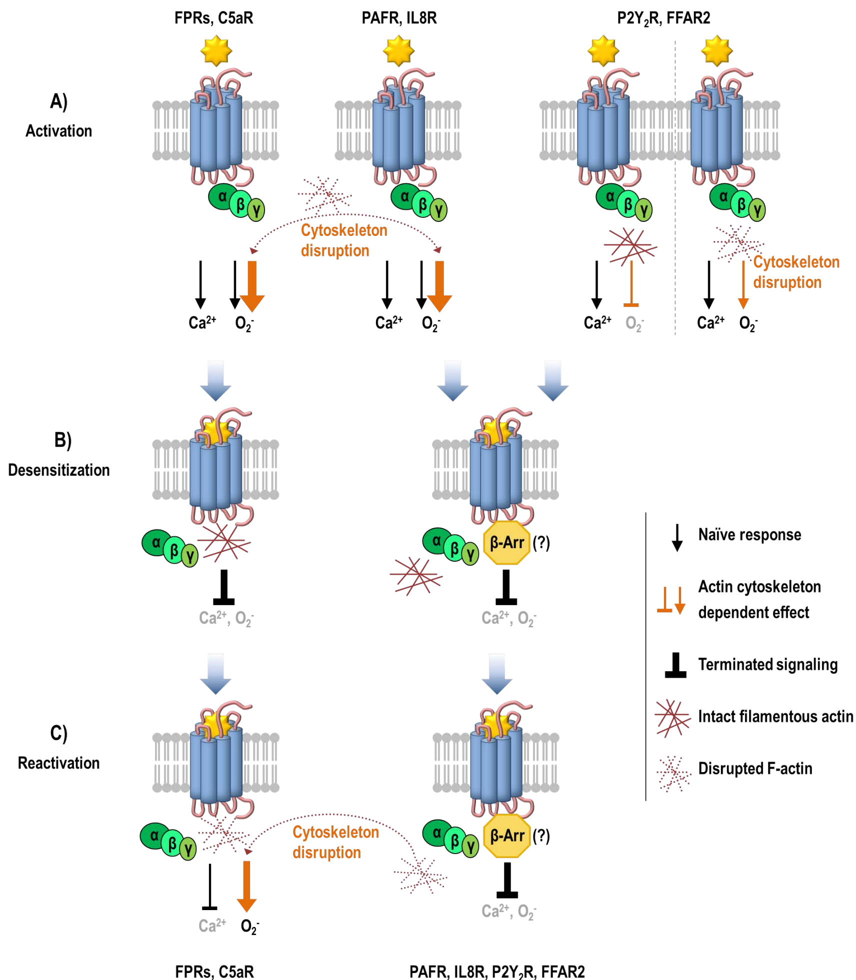


Figure 12) Regulation of neutrophil GPCR signaling by the actin cytoskeleton and β-arrestin.
A) Some neutrophil GPCRs can be primed to induce increased levels of oxygen radical production by disruption of the actin cytoskeleton. Other receptors are incapable of triggering respiratory burst activity in naïve cells and require initial disintegration of filamentous actin to access this signaling route. **B)** Two distinct mechanisms regulate the desensitization of activated neutrophil GPCRs. One pathway utilizes the actin cytoskeleton to physically separate certain receptors from their respective G-proteins, whereas other GPCRs are desensitized in an actin cytoskeleton-independent manner, either via arrestin binding or by another unidentified mechanism. **C)** Receptors that are desensitized through direct interaction with the actin cytoskeleton (interfering with G-protein coupling) can be reactivated for oxygen radical production through disruption of filamentous actin structures. This secondary respiratory burst activity is not accompanied by the release of calcium from intracellular stores. Receptors that depend on alternative mechanisms of desensitization cannot be transferred back to a state of active signaling.

Receptor Cross-Talk

A novel form of receptor cross-talk has recently been described in which FPRs can be reactivated to induce neutrophil oxygen radical production [114, 161]. Homologously desensitized FPRs do not respond to a second dose of the same agonist or of another agonist that binds to the same receptor. However, such cells still respond to PAF or ATP and activation of their receptors (PAFR and P2Y2, respectively) results in a primed respiratory burst response, as compared to naïve neutrophils that have not been desensitized to FPRs. In contrast, PAFR- and $P2Y_2R$-dependent calcium signaling is not affected by FPR desensitization (Figure 13). More important than the priming effect on respiratory burst activity itself is the underlying mechanism therefore. The basis for this effect is re-engagement of the FPR-induced NADPH-oxidase signaling route through a heterologous receptor-activation process. Inhibitory effects of receptor-specific antagonists clearly demonstrate a reversal of FPR desensitization through secondary receptors. The pepducin agonist $F2Pal_{10}$ exhibits superior potential over conventional agonists, including the hexapeptide WKYMVM, in receptor cross-talk between the FPRs and PAFR or $P2Y_2R$ (Paper I).

The signaling route that leads to reactivation of desensitized FPRs appears to be restricted to certain GPCRs and does not occur between FPR homologs. FPR-desensitization prevents CXCR1/2-mediated NADPH-oxidase activity in neutrophils via heterologous desensitization when stimulated with IL8. The phosphatase inhibitor Calyculin A was shown to inhibit cross-talk between FPR1 and PAFR, implying a regulatory role of serine/threonine phosphatases [161] and a recent study using novel $G\alpha_q$ subunit inhibitors demonstrated that the reactivation signal for desensitized FPR2 ($G\alpha_i$) is in complete dependency of PAFR-coupling $G\alpha_q$ proteins [198]. As mentioned before, FPRs are not only important chemokine receptors that guide migration during bacterial infections; they are also potent inducers of cytotoxic oxygen radical production. Therefore, a reactivating receptor cross-talk signaling pathway could be a mechanism by which desensitized FPRs are reused in a situation of severe infection to enhance pathogen clearance. The reason why the FPR2 pepducin $F2Pal_{10}$ has superior potency in cross-talk signaling could be connected to its proposed mode of allosteric receptor modulation or, independently thereof, its receptor-binding properties might induce a conformation that favors reactivation over activation.

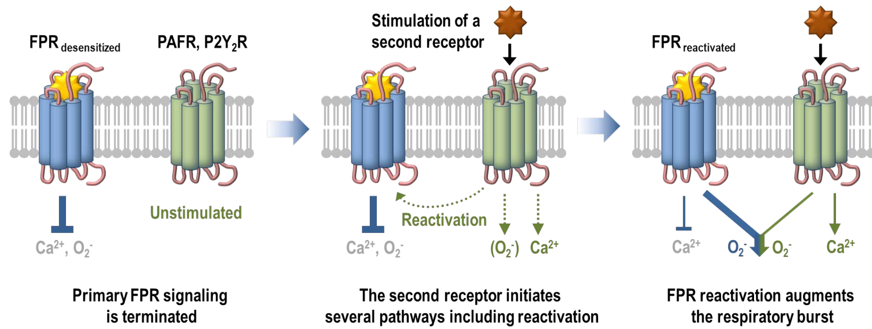


Figure 13) Receptor cross-talk.

Homologously desensitized FPRs are non-responsive to further stimulation with any agonist that is specific for these receptors, as they cannot induce another transient rise in intracellular calcium or activate the NADPH-oxidase for generation of reactive oxygen species. In neutrophils, subsequent stimulation of certain unrelated GPCRs (e.g. PAFR or $P2Y_2R$) can induce an FPR-reactivating receptor cross-talk signaling pathway. Thereby, initially desensitized FPRs are transferred back to a state of active signaling. This cross-talk-dependent FPR reactivation results in an augmented net respiratory burst activity induced by stimulation of the second/unrelated receptor. In contrast, the amount of calcium that is released from the intracellular stores through activation of the second/unrelated receptor is not affected by prior FPR desensitization.

Functional Selectivity

"Functional selectivity" or "biased signaling" describes the ability of GPCRs or GPCR ligands to stabilize distinct receptor conformations which promote activation of certain signaling events over others. This can result in varying efficacies for the different receptor-mediated responses as well as in complete avoidance of particular pathways, whereas signaling by "balanced agonists" does not favor any pathways over others. In recent years, the field of functional selectivity research gained a lot of interest as a novel strategy for GPCR-related drug development with the aim to stimulate only beneficial receptor responses while avoiding unwanted side effects.
Already in the year 1956 it was shown that the relation between receptor and agonist is not linear. Different ligands have varying capacities to induce a receptor-mediated response and the activity of an agonist was defined as a product of its affinity and efficacy [347]. The corresponding classical linear two state model suggested that receptors exist in an equilibrium of two affinity states, namely R for inactive and $R*$ for active (Figure 14A). Ligand binding and causal receptor activation would shift this equilibrium towards the $R*$ state, which was also in agreement with the concept of full and partial agonism. Accordingly, inverse agonists would shift the receptor equilibrium towards the inactive R state. Over time, this two-state model was expanded to various ternary complex models that introduced additional factors, like G-protein coupling and basal activity [348]. Based on the discovery that GPCRs can adopt multiple conformations of active signaling, the concept of functional selectivity was first proposed in 1995 under the term "agonist trafficking" and resulted from experimental data on overexpressing cells showing that agonists can induce distinct binding patterns in receptors with affinities for multiple G-proteins [349]. In the early 2000's, biased ligands and biased signaling have been described in recombinant expression systems for several GPCRs, including for the angiotensin II receptor 1 (AT_1R), the β2-adrenergic receptor, the V2 vasopressin receptor (regulating water retention), the δ-opioid receptor and for the serotonin 5-HT_{2C} receptor. It was shown that these GPCRs were capable of MAPK activation and β-arrestin recruitment in a G-protein-independent manner and exhibited reversed efficacies for distinct

signaling pathways [350-353]. At the same time, arrestins have been recognized as multifunctional proteins that, in addition to receptor desensitization, also mediate GPCR internalization and can act as scaffolds for adaptor molecules and signal transduction proteins [354]. Since then, the number of identified biased ligands is steadily increasing and today functional selectivity most often discriminates between G-protein-dependent and arrestin-dependent signaling (Figure 14B). But the observed differences do not necessarily have to be absolute and can instead relate to differences in efficacy for distinct signaling pathways. Accordingly, it has been demonstrated that CCR2, CCR5 and CCR7 are capable of coupling to multiple isoforms of $G\alpha_{i/o}$ proteins and their respective receptor ligands exhibited varying potency profiles in different assay systems [355]. The human genome encodes for about 20 chemokine receptors which are predominantly expressed by cells of the immune system and recognize about 40 chemokine ligands [356]. Consequently, some agonists can interact with several receptors and contrariwise some receptors can interact with several agonists. Based on these promiscuous binding patterns, functional selective responses for multiple combinations of receptor-chemokine-interaction have been identified and divided into three forms: i) ligand bias, occurring for different agonists acting on a single receptor, ii) receptor bias, where a single agonist induces distinct responses when binding to different receptors and iii) tissue bias, where the receptor-mediated response to a ligand is dependent on the cell type [357, 358]. In addition to endogenous chemokine receptor ligands with functional selective profiles, also synthetic molecules with biased activity have been identified for several receptors [262, 359, 360]. Current knowledge implies that biased agonism as a concept is generally applicable to chemokine receptors, and possibly serves as a fine-tuning mechanism in response to their ligands during inflammatory situations.

The assumption that receptors do not just exist in a dichotomy state of being either active or inactive, but that they instead can adopt various conformations is not only the basis for functional selectivity, it further extends this concept to a multi-state model of receptor signaling. The multi-state model suggests that any given ligand stabilizes a unique receptor conformation and the number of possible conformations is only limited by the number of ligands, which differ from each other in their efficacies/properties to promote receptor interaction with

available G-proteins, G-protein subtypes, and arrestins [361, 362]. Consequently, every agonist induces unique receptor-mediated responses (Figure 14C).
To date, medication exhibiting biased signaling properties on GPCRs is already commercially available and more biased ligands are subject of clinical studies [363]. For example, patients with acute heart failure benefit from β-arrestin biased medication for the angiotensin receptor (AT_1R) to reduce blood pressure, while increasing cardiac contractility and preserving renal function [364]. Blood pressure-lowering medication for the adrenergic receptor (β-blockers) with β-arrestin biased properties is suggested to have additional positive effects in the treatment of cardiovascular diseases [365] and G-protein biased medication for opioid receptors relieves pain but causes less respiratory depression and side effects [366, 367].

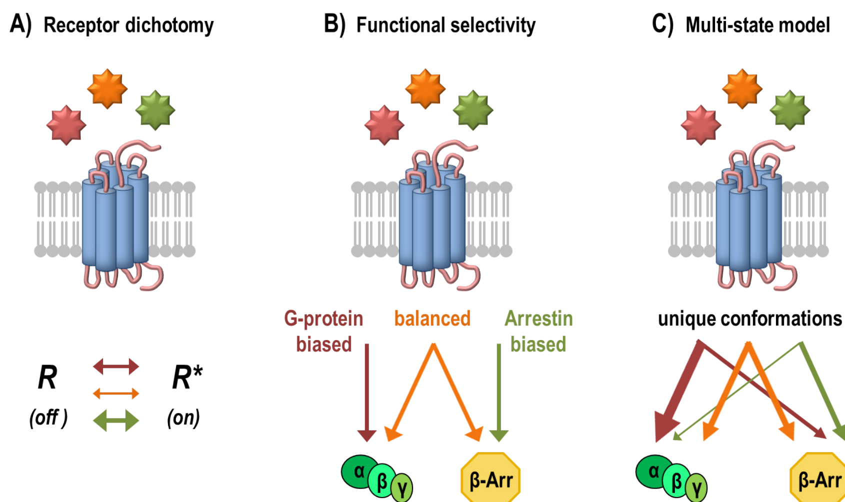


Figure 14) Models of agonist-dependent GPCR signaling.
A) The classic (but outdated) model of receptor dichotomy suggests that GPCRs can engage two distinct conformational states, namely signaling (on, R*) and non-signaling (off, R). Presence of an agonist, either full, or partial or inverse, shifts the proportion of signaling and non-signaling receptors in a respective direction. **B)** The concept of functional selectivity typically distinguishes between G-protein- and arrestin-dependent signaling which occurs downstream of receptor activation. According to this model, GPCR agonists can further be classified as being either balanced, if they mediate signaling through both pathways, or biased, if receptor activation favors one pathway (i.e. G-protein- or arrestin-dependent) over another. **C)** The multi-state model suggests that every agonist for any given GPCR is unique in its properties to induce G-protein- and/or arrestin-dependent signaling and consequently exhibits individual affinities and efficacies for their corresponding pathways.

Signaling Bias in Human Neutrophils

Today, functional selectivity is a generally accepted model to describe GPCR signaling mechanisms and the concept is applicable to basically all receptors. Yet, the lion's share of knowledge within this field of research is based on recombinant systems with highly overexpressed receptors. Much less is known about biased signaling and their functional outcomes in primary cells. In that perspective, the neutrophil is an ideal cell type, as large numbers are easily obtainable. Already before the concept of biased signaling was even established, agonists of the $G\alpha_s$-coupled histamine H_2 receptor have been shown to have varying pharmacological profiles for inhibition of receptor-mediated oxygen radical production and stimulation of cAMP production [100, 101, 368]. A recent study, suggested the existence of ligand-specific conformations induced by H_2 receptor ligands in human neutrophils [369]. In similar manner, this was demonstrated for the adenosine A_2 receptor and the β2-adrenergic receptor, as their inhibitory effects on other receptors were not directly correlating to respective cAMP levels [370, 371]. Functional selectivity was later postulated for β2-AR agonists in human neutrophils [372]. Neutrophils express all four subtypes of adenosine receptors. A_1 receptor and A_3 receptor promote neutrophil chemotaxis and phagocytosis at low agonist concentrations, whereas receptor A_{2A} and A_{2B} inhibit neutrophil functions, such as respiratory burst activity, at increasing concentrations [94]. In reference to chemokine receptors and their ligands this can be seen as a form of receptor bias, mediated by a single agonist. The CXCR4-selective pepducin ATI-2341 was classified as a receptor-specific allosteric activator with biased signaling properties in overexpressing cell lines suggested to mediate coupling to $G\alpha_i$ subunits but not to $G\alpha_s$ or arrestin [262]. In primary human neutrophils, ATI-2341 seems to induce cellular responses through FPR2 instead. It desensitizes cells for the FPR2-derived pepducin $F2Pal_{10}$ and its mediated effects are sensitive to FPR2-selective inhibitors [272]. Despite the observed off-target effects of ATI-2341 in neutrophils, no data is exists for signaling (bias) of the pepducin through its associated receptor CXCR4. In reference to ATI-2341 activity in recombinant systems, the CXCR4 pepducin thereby classifies as a tissue biased agonist. The ATP receptor $P2Y_2R$ exhibits a novel form of signaling bias in human neutrophils that involves only a single agonist and GPCR. Superoxide production through ATP stimulation is modulated by the state of actin cytoskele-

ton, whereas other signaling routs operate independently thereof (Paper II). FPR2 has binding affinity for numerous and diverse ligands and the non-peptide molecule Quin-C1 was characterized as a selective biased ligand which stimulates calcium mobilization but not superoxide production [303]. And an apolipoprotein-derived (lipid-binding protein) peptide was shown to mobilize calcium release in neutrophils and to mediate migration through FPR2, but failed to induce respiratory burst activity [373]. Recently, the small molecule dual FPR1/FPR2 agonist Compd17b was identified as being negatively biased for intracellular calcium signaling in comparison to dual agonist Compound 43, while retaining similar patterns for phosphorylation of ERK1/2 and Akt. These results rely on recombinant systems and data on primary cells is currently not available. The pepducin $F2Pal_{10}$ is a partial agonist for FPR2 and shares many similarities with the conventional hexapeptide agonist WKYMVM when mediating functional responses in neutrophils, like the release of intracellular calcium, priming/degranulation and superoxide production. Despite a generally lower potency and efficacy of $F2Pal_{10}$ when compared to the hexapeptide agonist WKYMVM, in relation to cross-talk mediated superoxide production the pepducin turns into a full (and the most potent) agonist for FPR2 reactivation. This biased signaling profile for the two pathways that mediate oxygen radical production in neutrophils, i.e. receptor activation and reactivation, classify this pepducin as a functional selective FPR2 ligand (Paper I). Further, in cells overexpressing FPR2, the $F2Pal_{10}$ pepducin lacks the ability to recruit β-arrestin, and in contrast to WKYMVM, $F2Pal_{10}$ does not induce FPR2-mediated chemotactic migration of neutrophils (Paper IV). Although the direct link between these two phenomena remains to be established, β-arrestin is known to be involved in cell motility. A recent study on neutrophil-like HL-60 cells showed arrestin-dependency for FPR1-mediated migration [239].

In reference to the general concept of functional selectivity, it is important to consider that signaling bias does not necessarily exclude certain pathways, as it may also relate to profound differences in agonist efficacies for distinct signaling cascades. Not much is known yet about the physiological consequences of FPR modulation and functional selective activation but in a future perspective this area of research could prove invaluable for the development of therapeutic strategies to treat FPR-linked malignancies.

Concluding Remarks

Analysis on drug-target interactions from 2011 showed that 60% of nearly 1000 approved drugs were targeting receptors of which ca. 200 were GPCRs [374]. From a pharmacological point of view, allosteric modulator drugs can hold advantages over drugs that bind to orthosteric sites, as they should not compete with natural ligands for binding and their influence on a given receptor should only be effective in the presence of the natural ligand, potentially reducing the risk of unwanted side effects. Formyl peptide receptors have been subject to research for several decades and are primarily known as leukocyte chemoattractant receptors that are linked to inflammation and microbial infections. But a multitude of functions and ligands have been characterized over the years and various animal and disease models suggest that FPRs are involved in a variety of human afflictions, including angio- and tumorigenesis, obesity and diabetes, HIV infection, Alzheimer's disease, cardiovascular conditions and autoimmunity [375, 376]. Enduring inflammation can lead to destruction of host tissue, for example through FPR-mediated oxygen radical production, or indirectly through proinflammatory signals that are generated within the affected tissue. Systemic dampening of immune responses with receptor inhibitors, for example treatment of rheumatic patients with TNFα blockers, can alleviate the symptoms of a disease but may also simultaneously increase the risk of novel infections. Accordingly, re-balancing the immune system instead in its inflammatory and resolving signals by use of allosteric modulators (pepducins?) could be of advantage.

Homologous desensitization of GPCRs as a consequence to agonist stimulation is usually seen as a point of no return but a recently described cross-talk mechanism leading to FPR reactivation in human neutrophils has challenged this dogma. Yet, on a molecular level the exact signaling pathways involved in this receptor cross-talk are still oblivious. Identification of critical signaling events will improve our understanding of neutrophil physiology in relation to *in vivo* inflammatory situations, when cells are exposed to multiple stimuli.

The concept of functional selectivity, defined by biased activation of GPCR signaling pathways, created a novel strategy for the development of more specific and effective drugs, as therapeutic treatments often require the modulation of

only a particular receptor-mediated effect. Consequently, biased ligands will exert fewer adverse effects than conventional receptor agonists or inhibitors. FPRs are potent inducers of oxygen radical production and their dysregulation can cause unspecific destruction of host tissue. In reference to the FPR2-derived pepducin F2Pal$_{10,}$ a partial agonist with bias for receptor cross-talk that does not induce neutrophil chemotactic migration, drugs with respective functional selective properties could be of use to modulate immune responses under inflammatory condition. Pharmaceuticals of this type could dampen neutrophil recruitment to sites of inflammation and would allow excessive oxygen radical production solely in the presence of multiple stimuli for different receptors.

Various therapeutic approaches already profit from allosteric receptor modulation and biased ligands and respective advances in the field of leukocyte biology could constitute the basis for prospective treatments of receptor/neutrophil-linked inflammatory disorders and diseases.

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