Free Fatty Acid Receptor 2

– A G protein coupled receptor with unique signaling properties in neutrophils

Simon Lind

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

Gothenburg, Sweden, 2022

UNIVERSITY OF
GOTHENBURG

Cover illustration by Simon Lind

Free Fatty Acid Receptor 2 – A G protein coupled receptor with unique signaling properties in neutrophils

simon.lind@gu.se

ISBN 978-91-8009-855-7 (PRINT)
ISBN 978-91-8009-856-4 (PDF)
http://hdl.handle.net/2077/71517
Printed in Borås, Sweden 2022
Printed by Stema Specialtryck

“Whatever it is you´re seeking won´t come in the form you’re expecting”
Haruki Murakami

Abstract

The overall aim of the PhD thesis was to determine the role of neutrophil pattern recognition receptors in the initiation and resolution of inflammatory processes. Neutrophil pattern recognition receptors such as the formyl peptide receptors (FPRs) and the short chain free fatty acid receptors (FFARs) belong to the family of G protein coupled receptors (GPCR). The FPRs recognize danger peptides having a formylated methionyl group in the N-terminus, peptides generated not only by bacteria but also during synthesis of proteins encoded for by the mitochondrial DNA. FFARs recognize metabolites generated during fiber fermentation by gut microbes. The thesis work has been focused on the coupling between ligand recognition by these receptors and the down-stream intracellular signals transduced. The work started with studies on the FPRs, receptors that have been implicated to trigger both pro- and anti-inflammatory responses. However, the work on one of the FFARs (FFA2R) expanded rapidly to become the mainstage. Receptor recognition of orthosteric ligands and allosteric modulators and the subsequent novel receptor down-stream signaling induced by newly developed tool compounds have been studied. The neutrophil pattern recognition receptors are regarded as promising therapeutic targets for the treatment of diseases in which the inflammatory response needs to be properly controlled. The results presented in the thesis have increase our knowledge about the regulatory roles not only of the FFARs but also of the FPRs in inflammation. Hopefully, this knowledge will be of help in the efforts to develop pharmaceutical drugs that can be used to modulate inflammatory processes in direct and controlled ways.

Keywords
Neutrophil, G protein coupled receptors, FFA2R, signal transduction, allosteric modulation, reactive oxygen species, biased signaling

Populärvetenskaplig sammanfattning

Vårt immunförsvar är mycket viktigt och utan det skulle vi inte klara hotet från alla mikroorganismer som finns i vår omgivning. Immunsystemet är komplicerat och uppbyggt av många olika celler och lösliga ämnen som tillsammans har till uppgift att stå emot/försvara oss mot attacker från bakterier, virus, svampar och andra parasiter. En av det naturliga immunförsvarets celler som finns i stort antal i blodet är den vita blodkropp som kallas neutrofil granulocyt. Neutrofilen är en av våra så kallade fagocyter (ätarceller), som har som sin främsta uppgift att snabbt leta reda på farliga organismer (patogener) och döda dessa genom att först ”äta upp dem” och sedan utsätta mikroberna för olika avdödningssystem där produktionen så kallade fria syreradikaler är en viktig del. Neutrofilens avdödningssystem kan liknas vid ett tveeggat svärd eftersom det som dödar oönskade mikrober också kan skada våra egna celler och vävnader i samband med att immunsystemet aktiveras. Detta är något som händer vid många så kallade inflammatoriska sjukdomar. För att kunna utveckla läkemedel som kan minska risken för vävnadsskada i samband med sjukdom behöver vi öka våra kunskaper om hur inflammationsreaktionen regleras.

För att lokalisera var det finns något främmande (typ farliga mikrober) har neutrofilen förmåga att känna igen ett brett spektrum av olika typer av signalmolekyler som produceras av mikroberna och/eller våra egna varningssystem. De signalmolekyler som reglerar neutrofilernas olika funktioner, känner våra försvarsceller igen med hjälp av så kallade receptorer som sitter på cellytan och "läser av" omgivningen. Många av dessa igenkänningsstrukturer tillhör en grupp receptor som kallas G protein kopplade, eftersom de förmedlar informationen från signalmolekylen på ytan till cellens olika funktioner på samma sätt, nämligen genom att aktivera ett protein (G proteinet) på insidan av det cellmembran där receptorn sitter. Neutrofilens funktionella svar ser olika ut beroende på såväl vilken signalmolekyl (en så kallade agonist) det är som sätter i gång cellen och vilken receptor som känner igen signalmolekylen. Det visades för ungefär 20 år sedan att det finns receptorer som känner igen korta fettsyror som produceras av våra tarmbakterier. En av dessa receptorer som kallas FFA2R (förkortning av engelskans Free Fatty Acid Receptor 2) uttrycks av neutrofiler och har föreslagits vara viktig för reglering av många olika funktioner där inflammationsreaktionen

är en. Denna receptor har varit fokus i det arbete som presenteras i avhandlingen.

Många G protein kopplad receptor kan binda andra molekyler än den så kallade agonisten och dessa binder då till ett annat ställe (allosteriskt) på receptorn än det som agonisten binder. Dessa andra molekyler aktiverar inte de receptorer de binder till på egen hand, men förändrar (modulerar) receptorns funktion när signalmolekylen binder. Målsättningen med mitt avhandlingsarbete har varit att öka våra kunskaper om hur olika typer av molekyler som FFA2R känner igen, påverkar neutrofilers funktion. Delarbetena har handlat om allosteriska modulatorer som gör receptorn mer effektiv eller aktiverar receptorn på nya sätt. Allosteriska modulatorer kan under vissa omständigheter aktivera FFA2R och de kan också ändra receptorns struktur så att agonister binder bättre eller att de signaler som receptors skickar i väg blir starkare. Jag har också visat att FFA2R, när den receptorn är allosteriskt modulerad, kan aktiveras av signaler som kommer från cellens insida, och detta sker utan att det bundit någon agonist till FFA2R. Detta är ett nytt sätt på vilket en G protein kopplad receptor kan aktiveras genom att två olika receptorer ”pratar med varandra”. Genom att undersöka hur olika allosteriska FFA2R modulatorer fungerar har jag i mitt arbete kunnat visa att receptorn har två distinkt olika allosteriska bindningsställen och att tillsammans kommer två modulatorer (som var för sig inte aktiverar receptorn) som har olika bindningsställen, att aktivera receptorn. Denna mekanism för aktivering av en G protein kopplad receptor är unik då receptorn aktiveras utan att någon agonist behövs, och att en ligand som normal minskar receptor aktivitet (en så kallad antagonist) i stället ökar aktiviteten. Resultaten som presenteras i avhandlingen har ökat vår kunskap om hur FFA2R fungerar och förhoppningsvis kan denna kunskap bidra till att öka förståelsen för hur neutrofilers funktioner regleras och vara till hjälp i arbetet med att utveckla farmakologiskt aktiva molekyler som kan användas för att begränsa de skador som kan orsakas av en inflammationsreaktion.

List of papers

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

I. Functional selective ATP receptor signaling controlled by the free fatty acid receptor 2 through a novel allosteric modulation mechanism.

Simon Lind, André Holdfeldt, Jonas Mårtensson, Martina Sundqvist, Lena Björkman, Huamei Forsman, Claes Dahlgren. Published in: FASEB J. 2019; 33: 6887-6903

II. Interdependent allosteric free fatty acid receptor 2 modulators synergistically induce functional selective activation and desensitization in neutrophils.

Simon Lind, André Holdfeldt, Jonas Mårtensson, Martina Sundqvist, Terry P Kenakin, Lena Björkman, Huamei Forsman, Claes Dahlgren. Published in: Biochim Biophys Acta Mol Cell Res. 2020; 1867: 118689.

III. Multiple ligand recognition sites in free fatty acid receptor 2 (FFAR2) direct distinct neutrophil activation patterns

Simon Lind, André Holdfeldt, Jonas Mårtensson, Kenneth L. Granberg, Huamei Forsman, Claes Dahlgren. Published in: Biochemical Pharmacology 2021; 193: 114762

IV. Allosteric Receptor Modulation uncovers an FFAR2 antagonist as a positive orthosteric modulator/agonist in disguise.

Simon Lind, Dagny Olofsson Hoffman, Huamei Forsman, Claes Dahlgren. Published in: Cellular Signalling 2022; 90: 110208

List of papers by Simon Lind not included in the thesis

The papers listed below were also published during my PhD studies, however, these will not be the main focus in the thesis. Nevertheless, these will be referred to in the thesis by their alphabetic character.

Original papers

A. Mitocryptides from Human Mitochondrial DNA-Encoded Proteins Activate Neutrophil Formyl Peptide Receptors: Receptor Preference and Signaling Properties

Michael Gabl, Martina Sundqvist, Andre Holdfeldt, **Simon Lind**, Jonas Mårtensson, Karin Christenson, Takayuki Marutani, Claes Dahlgren, Hidehito Mukai, Huamei Forsman. Published in: The Journal of Immunology. 2018; 200: 3269

B. Identification of Residues Critical for FPR2 Activation by the Cryptic Peptide Mitocryptide-2 Originating from the Mitochondrial DNA−Encoded Cytochrome b

Simon Lind, Michael Gabl, André Holdfeldt, Jonas Mårtensson, Martina Sundqvist, Kodai Nishino, Claes Dahlgren, Hidehito Mukai and Huamei Forsman. Published in: The Journal of Immunology. 2019; 202: 2710

C. Functional and signaling characterization of the neutrophil FPR2 selective agonist Act-389949

Simon Lind, Martina Sundqvist, Rikard Holmdahl, Claes Dahlgren, Huamei Forsman,1, Peter Olofsson. Published in: Biochemical Pharmacology. 2019; 166; 163

D. Functional selective FPR1 signaling in favor of an activation of the neutrophil superoxide generating NOX2-complex

Simon Lind, Claes Dahlgren, Rikard Holmdahl, Peter Olofsson, Huamei Forsman. Published in: Journal of Leukocyte Biology. 2020; 109: 1105

E. The PAR4-derived pepducin P4Pal $_{10}$ lacks effect on neutrophil GPCRs that couple to Gαq for signaling but distinctly modulates function of the Gαi-coupled FPR2 and FFAR2

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

F. Structural Determinants in the *Staphylococcus aureus*-Derived Phenol-Soluble Modulin α2 Peptide Required for Neutrophil Formyl Peptide Receptor Activation

Moa Viklund, Johanna Fredriksson, André Holdfeldt, **Simon Lind**, Henrik Franzyk, Claes Dahlgren, Martina Sundqvist, Huamei Forsman. Published in: The Journal of Immunology. 2022; 208: 1632

Review

G. Neutrophil Signaling That Challenges Dogmata of G Protein-Coupled Receptor Regulated Functions

Claes Dahlgren, André Holdfeldt, **Simon Lind**, Jonas Mårtensson, Michael Gabl, Lena Björkman, Martina Sundqvist, and Huamei Forsman. Published in: ACS Pharmacology & Translational Science. 2020; 3: 204

Content

Abbreviations

5-HT 5-hydroxytryptamine receptors	LPS Lipopolysaccharides
ADP Adenosine diphosphate	mtDNA mitochondrial DNA
AT1R Angiotensin II receptor type 1	NADPH-oxidase Nicotinamide adenine dinucleotide phosphate oxidase
ATP Adenosine triphosphate	NAM Negative allosteric modulator
C5aR Component 5a receptor	NET Neutrophil extracellular trap
Ca^{2+} Calcium ion	NK Natural killer
CGD chronic granulomatous disease	O_2^- Superoxide anion
DAG Diacylglycerol	PAF Platelet activating factor
DAMP Danger associated molecular pattern	PAFR Platelet-activating factor receptor
DC Dendritic cell	PAM Positive allosteric modulator
ERK Extracellular signal-regulated kinase	PAMP Pathogen associated molecular pattern
FFAR Free fatty acid receptor	PIP2 Phosphatidylinositol 4, 5-bisphosphate
FPR Formyl peptide receptor	PIP3 Phosphatidylinositol (3, 4, 5)-trisphosphate
GDP Guanosine diphosphate	PKC Protein kinase C
GLP-1 glucagon-like peptide 1	PLC Phospholipase C
GM-CSF Granulocyte colony-stimulating factor	PMN Polymorphonuclear leukocyte
GPCRs G-protein coupled receptors	PRR Pattern recognition receptor
GRK G-protein coupled receptor kinase	PYY gut hormones peptide YY
GTP Guanosine triphosphate	ROS Reactive oxygen species
HDAC histone deacetylases	SCFA Short chain fatty acid
IL Interleukin	SOD superoxide dismutase
ILR Interleukin receptor	TEER transepithelial electrical resistance
IP3 Inositol trisphosphate 3	TNFR Tumor necrosis factor receptor
	TREG regulatory T cells

1. The Innate immune system

1.1 Overview

The innate immune system constitutes the first line of defense against foreign material (immunogens). Innate meaning an inborn natural ability to defend the host against harmful material entering our bodies including microbial pathogens. The innate immune system has the ability to discriminate between self and non-self, a very important function based on an inborn ability to recognize molecular patterns (1). One important part of the recognition system is constituted by a group of cell receptors known as pattern recognition receptors (PRRs) (2). These receptors are proteins that have the ability to sense molecules that are commonly found in potential pathogenic microbes (PAMPs; Pathogen-Associated Molecular Patterns) and/or released by damaged host cells/tissues (DAMPs; Damage-Associated Molecular Patterns) (3). The innate immune response is very rapidly mobilized and the killing/degradation/tissue resolving "weaponry" potently prevent, impede, or decelerate infection. However, despite the lack of the adaptivity characterizing the other major part of our immune system, the innate immune system efficiently protects the host against a broad spectrum of pathogens and tissue destructing insults/causes.

1.2 Inflammation

The skin and mucosal surfaces are important parts of the physical barriers of our innate immune system. In normal healthy conditions (see **Fig 1**) the skin is an almost impervious organ (4). A part of the natural ability of the skin to protect the host against infections, is attributed by the low pH and lack of nutrients for microbial pathogens. The pH level is regulated by lactic acid as well as fatty acids secretion from sebaceous glands. Despite this, wounds are regularly formed throughout life in these barrier-organs as a result of injury. Independent of the localization of the injury this will represent a threat to the host. Different repair programs are quickly mobilized to restore the integrity, and one part of this repair program involves the accumulation of innate immune cells such as monocytes/macrophages, granulocytes, and dendritic cells, at the injured site ((5), Paper G). Neutrophils are the first cells to respond and be recruited to an infected/damaged tissue and their arrival is an early sign of the initiation of an inflammatory reaction. This is the basic process by which body tissues respond to injury.

The classical signs of inflammation (calor, heat; dolor, pain; rubor, redness; tumor, swelling, and functio laesa; loss of function) were described already 2000 years ago (**Fig 1**). The reaction is initiated by the production/release of chemoattractants originating from invading microbes, the activated complement system (part of the humoral innate immune system) and/or the injured tissue (6). These inflammatory mediators activate tissue resident macrophages and recruit both neutrophils and monocytes from the blood stream to the site of injury. The large number of inflammatory mediators formed locally account together with the cells recruited to the inflammatory site, for the changes in temperature, redness, swelling, pain and functional loss, but in the best-case scenario, they also contribute to the resolution/healing of the inflammatory reaction once the cause to the reaction has been eliminated (4, 7).

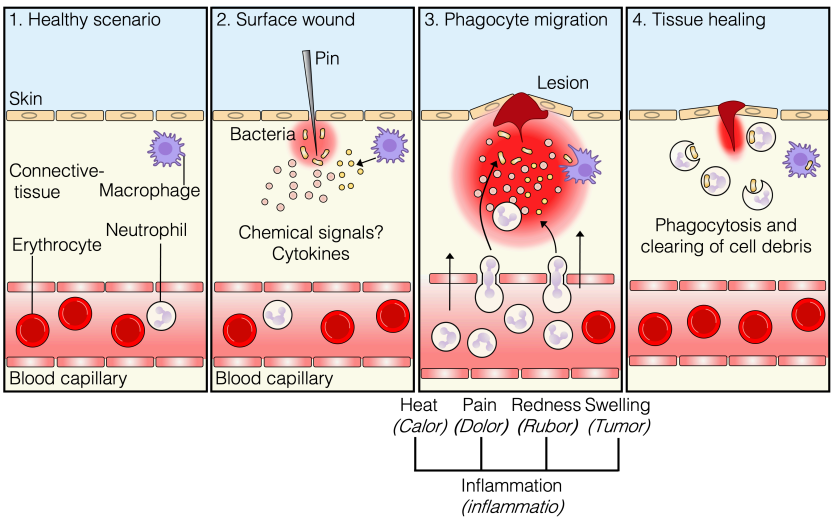

Figure 1: The five classic signs of inflammation
*Intact skin can protect us from potentially harmful microbes (**1**). A wound change, however, the balance (**2**) and when such an injury is recognized by tissue macrophages and/or mast cells, inflammatory mediators such as cytokines and chemokines will be released, and an inflammatory response is initiated. The inflammatory response (**3**) increases blood flow to the injured area, and increased vascular permeability allows fluid, proteins, neutrophils, and other immune cells to enter the tissue. These events result in the swelling (Tumor) and reddening (Rubor) of the injured site, and the increased blood flow to the injured area gives rise to a local increase in temperature (Calor). Inflammation is also associated with pain (Dolor) due to that these events stimulate nerve pain receptors in the tissue and loss of function (function laesa). The last phase of inflammation should be resolution (**4**). This process is controlled by several factors and involves many cell functions of which phagocytosis of bacteria and clearing of cell debris are crucial events.*

1.3 Inflammatory cells

The cellular branch of the innate immune system consists of a large number of tissue resident cells as well as cells present in peripheral blood. Tissue macrophages in skin and mucosa originate from blood monocytes recruited to the tissues where they continuously guard the specific tissues. As mentioned, macrophages are also able to produce pro-inflammatory cytokines such as the tumor necrosis factor alpha (TNFα) and interferon gamma, important links that participate also in the triggering of an adaptive immune response (8). Resident liver macrophages (Kupffer cells) are key players in liver homeostasis where they have the capacity to rapidly clear the blood from microbes and infected/apoptotic cells (9). Monocytes/macrophages are, together with neutrophil granulocytes, professional phagocytes, immune cells that have as their main functions to engulf (phagocytose), kill (if a microbe is engulfed) and digest foreign material. Neutrophil granulocytes are rapidly recruited from the blood circulation to inflammatory sites through a process called diapedesis (trans endothelial extravasation) (10).

2.The Neutrophil

The polymorphonuclear leukocytes (PMNs), also known as granulocytes, comprise of neutrophils, eosinophils and basophils, of which neutrophils are the most abundant white cell (leukocyte; around 60%) in human peripheral blood (5, 10). The naming stems from the facts that their nucleus has a multilobulated shape, and their cytoplasm contains a large number of storage organelles (granules). Focusing on the neutrophil, the shape of the nucleus has been suggested to reduce the steric hindrance when these cells migrate through the endothelium into an inflamed tissue (11). The granules store preformed receptors, the killing arsenal and proteolytic enzymes, and the presence of storage organelles facilitates the ability for a very rapid mobilization of the defense system (12). Mature neutrophils are produced from stem cells in the bone marrow (at least 10^{11} cells are produced daily), where they remain for 4-6 days, forming a reservoir of mature neutrophils that can rapidly be released into circulation in response to an infection or injury. The number of circulating neutrophils (13, 14), increases several folds when the host immune system is challenged (10, 15). This shows their importance in host defense, and this is also evident when the number of circulating neutrophils is substantially reduced (neutropenia) due to a genetic disorder or as an effect of pharmacological treatment. Such conditions are plagued by persistent, severe, and often life-threatening infections (15, 16).

Circulating neutrophils are considered to have a relatively short life span of 1-2 days (17). When chemotactically recruited to an inflammatory site (see **Fig 2**) they may survive for an additional 1-2 days. Chemoattractants, such as interleukin 8 (IL-8) and platelet activating factor (PAF), may also prime the neutrophils in their response to other inflammatory mediators, a function shared with microbial compounds such lipopolysaccharides (LPS) from the cell wall of Gram negative bacteria (18). Neutrophils combat pathogens by several different mechanisms. One of these is through internalization (phagocytosis) of a particulate prey (microbe or cell debris), during which an intracellular vesicle (the phagosome) enclosing the prey is formed. The phagosome interior is then made hostile to the engulfed material, and this is achieved through fusion with granules containing anti-microbial proteins and hydrolytic enzymes. The granules fusing with the phagosome (forming a phagolysosome) also contains the membrane components of an electron transporting enzyme system (the NADPH-oxidase) (19). The NADPH oxidase is an enzyme system that transports electrons and catalyzes the reduction of molecular oxygen (primarily to superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2)) to generate reactive oxygen species (ROS). The reduced oxygen species will be further metabolized to generate other reactive oxygen metabolites with anti-

microbial effects such as hypochlorous acid. Another mechanism to kill microbial pathogens used by neutrophils is through the formation of neutrophil extracellular traps (NETs) (20). Such NETs are formed through an active form of cell death where the neutrophil membranes surrounding the cell as well as the nucleus are disintegrated, allowing chromatin, decorated with antimicrobial granule proteins, to be released and these net-like structures can bind/trap both Gram-positive and Gram-negative bacteria. The classical formation of NETs is dependent on ROS production by the NADPH oxidase (21). While the ROS and hydrolytic enzymes released from neutrophils constitute essential parts of the first line of immune defense, these systems are double-edged swords. If not properly controlled, severe damage to surrounding tissue may be inflicted.

The process leading to a resolution of an inflammatory response is complex and involves many different steps. This includes an initiation involving the inflammatory cells, a special type of programmed cell death (apoptosis), and generation/release of host-derived pro-resolving mediators (22). Morphologically, neutrophil apoptosis is characterized by a loss of cytoplasmic granules, a rounding of the nucleus, and condensation of the nuclear heterochromatin, and the process is regarded non-inflammatory. Irrespectively if the process is initiated by phagocytosis (a process also known as phagocytosis-induced cell death (23)) or some other means, apoptotic neutrophils are subsequently ingested by macrophages, providing the means to resolve the inflammatory response without releasing cytotoxic molecules that would otherwise damage host tissues (24).

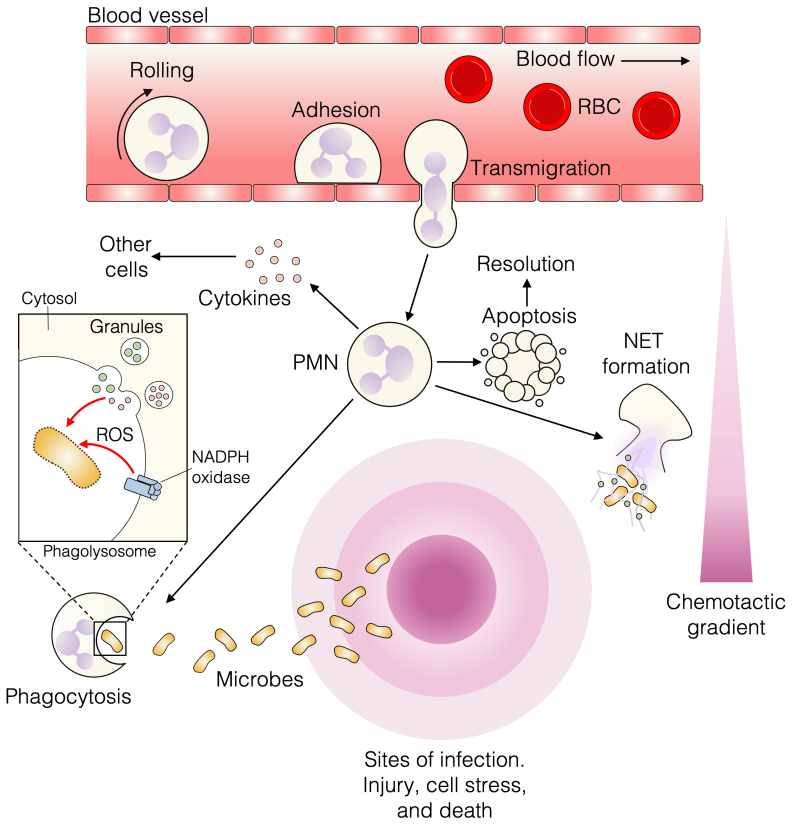

Figure 2. Tissue recruitment of neutrophil
Blood neutrophils are recruited to sites of infections/tissue destruction by a process called chemotaxis. The cells detect and migrate in response to a concentration gradient of chemoattractants generated by the host and/or the invading microbes. Neutrophils kill and clear the tissue through phagocytosis, release of preformed antimicrobial proteins and hydrolytic enzymes from granule stores, and release of microbe-trapping fibers of DNA (NETs). Neutrophils also release signal molecules including cytokines, to alert other part of the immune system. If these systems are not properly controlled the surrounding host tissues can be damaged. Neutrophil apoptosis (programmed cell death) is part of the resolution phase and a process important to reduce the risk for the surrounding tissues.

2.1 Formation and maturation of neutrophils

Neutrophils are derived from pluripotent stem cells, and they mature in the bone marrow and once matured, the cells are released into the bloodstream (25). The maturation process from myeloid progenitor cell can be divided into six different developmental stages. The myeloblast stage is followed in sequence by the

promyelocyte, myelocyte, metamyelocyte, band cell, and finally a mature neutrophil stage. The maturation process in the bone marrow takes around 13 days and once fully differentiated, the neutrophil that is no longer dividing reaches a fairly short-lived end-stage (26). The longevity of neutrophils in circulation has, however, been highly debated (17, 27). The general view is that neutrophils in circulation have a life span of around one day, but this time period may be extended if the neutrophils are subjected to inflammatory stimuli. Under healthy conditions, neutrophils remain in circulation for a relatively short time before they migrate and die in a controlled manner in the bone marrow or are cleared in the liver by Kupffer cells (9).

2.2 Neutrophil granules

As previously mentioned, neutrophils in circulation are non-dividing cells and the ability to synthesize new proteins is fairly limited (28). Despite this, neutrophils are equipped with the proteins needed for killing microbes and to clear infections, and these proteins are present in preformed storage granules (29). These sub-cellular structures are membrane enclosed vesicles filled with antimicrobial and hydrolytic peptides/proteins (see **Fig 3**). There are four distinct types of granules/vesicles formed during neutrophil maturation, and the first granule type formed, the primary or azurophil granules, are enriched in myeloperoxidase (MPO), an enzyme that contributes to microbial killing in the phagolysosome (discussed in more detail below) by increasing the toxic potential of H_2O_2 that is formed by the NADPH-oxidase (30). The azurophil granules also contain a large number of other proteins/peptides of importance for killing and degradation of microbes and tissue components (see **Fig 3**) (31). Later in the maturation process, two fairly similar granule types are formed, the specific granules enriched in vitamin B_{12} binding protein, and the more easily mobilized gelatinase granules (32). The membrane of both these granules contains a number of adhesion proteins and receptors together with a b-type cytochrome (cytochrome b_{558}), the membrane component of the oxygen radical producing NADPH oxidase (33). The specific granules also contain lactoferrin, an iron-binding antimicrobial protein, and as indicated by their name, the gelatinase granules also contain this matrix degrading enzyme, required for neutrophil migration from the blood stream (34). The synthesis of the proteins, enriched and stored in the different granule types and packed in the Golgi, takes place in the endoplasmic reticulum and is turned on and off during maturation in a process referred to as targeting/sorting-by-timing (35). The fourth granule/vesicle type (the secretory vesicles), formed very late in the maturation process, originates from the plasma membrane, and is formed through an

endocytosis process (36). These vesicles contain plasma proteins, and in addition, the membrane of this very easily mobilized organelle contains receptors that are mobilized to the cell surface during neutrophil activation.

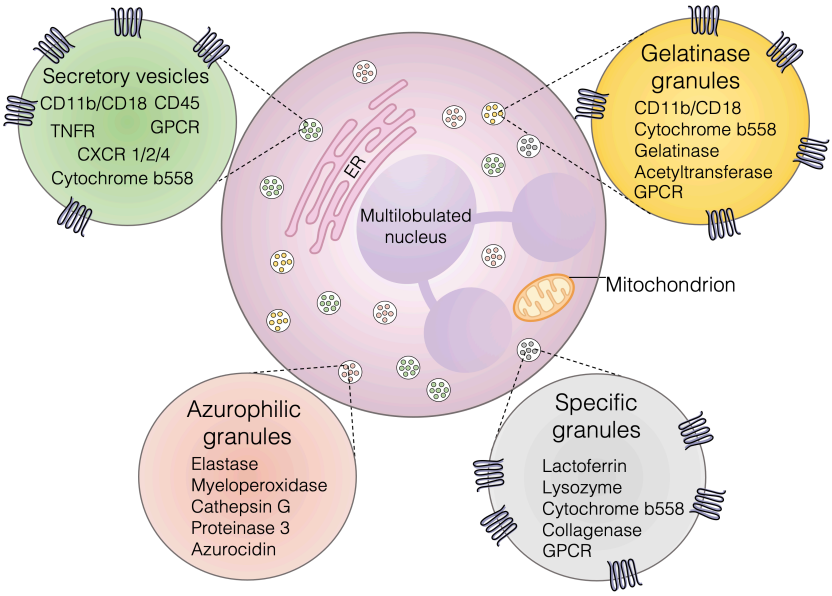

Figure 3 The content in the different neutrophil granules are important for the functions of the cells *A simplified overview showing the four distinct mobilizable neutrophil granules/vesicles and some of the marker proteins (examples of both membrane-bound and matrix proteins are given) stored in the different granules/vesicles.*

2.3 Phagocytosis

At the site of infection, neutrophils recognize, bind, and ingest invading microorganisms by a process known as phagocytosis (37, 38). The process is initiated when the microbe/particle binds to the phagocyte plasma membrane. This binding leads to activation of signaling pathways, which in turn leads to changes in the actin cytoskeleton (39). Long membrane protrusions surrounding the microbe/particle to be phagocytosed are formed through the polymerization of actin. At the same time, small vesicles migrate towards the cell membrane to make it easier for the cell membrane to enclose the particle to be phagocytosed (40). Once the phagocytic vesicle, the phagosome (see **Fig 4**), has been ligated off from the cell membrane, the layer of actin filaments is removed (41). After this, a phagolysosome

can be formed when granules are mobilized and fused with the phagosome. The granule content emptied into the phagolysosome will be part of the mechanisms that kill the engulfed microorganism. The superoxide producing NADPH-oxidase, assembled, and activated in the phagolysosomal membrane is also part of the killing machinery (42). Neutrophils have the capacity to recognize microbial surface components such as LPS and peptidoglycans which can initiate phagocytosis, however, the efficiency of elimination and clearance of microbes often require and/or is further enhanced by so called opsonins (complement components and/or antibody) bound to the surface of the microbe (43). These opsonins are recognized by neutrophil receptors for the complement component C3b (CR3) and the Fc-part of opsonizing antibodies (FcRII).

2.4 Production of reactive oxygen species

It is clear that to efficiently handle invading microbes and tissue debris, phagocytosing neutrophils are equipped with a diverse killing/clearance arsenal. In addition to the discussed anti-bacterial products stored in the different granule populations, the massive generation of ROS by the membrane-localized NADPH-oxidase is an important system designed (at least in part) to kill microorganism. The increased susceptibility for severe microbial infections in a group of patients suffering from chronic granulomatous disease (CGD), demonstrates the importance of the system (44). CGD is a disease caused by a mutation in one of the subunits of the NADPH oxidase complex, leading to a diminished superoxide production due to an absence or reduced function of the mutated subunit (45, 46).

The neutrophil NADPH-oxidase is a multicomponent enzyme complex that reduces molecular oxygen to superoxide anions (O_2^-), using electrons from NADPH (47, 48). In resting cells, the oxidase subunits reside in the cytosol ($p47^{phox}$, $p67^{phox}$, $p40^{phox}$) as well as in membrane compartments (the b cytochrome in plasma membrane and granule membranes). The membrane spanning component is a flavohemeprotein composed of two subunits, $gp91^{phox}$ and $p22^{phox}$ (49). During cell activation, the cytosolic oxidase components translocate to the b cytochrome containing membranes together with a GTP-binding protein, to form the assembled electron transporting oxidase complex. The phagosome become enriched in the b_{558} cytochrome through granule fusion specifically thought to come from granules belonging to the specific granule subtype (step 0 in **Fig 4**).

Glucose-6-phosphate dehydrogenase, an enzyme in the pentose phosphate metabolic pathway, is required for the production of the electron donor (e^-)

nicotinamide adenine dinucleotide phosphate (NADPH), essential for function of the oxidase system (50) (Fig 4, step 1). The O_2^- generated (Step 2) are rapidly dismutated (spontaneously or by the enzyme superoxide dismutase (SOD)) to hydrogen peroxide (H_2O_2) (Step 3). These two reduced oxygen metabolites are weakly microbicidal, but myeloperoxidase (MPO), an abundant haem-containing protein that resides in neutrophil primary (azurophilic) granules, potently increase the killing capacity. When delivered to a phagolysosome, MPO catalyzes a reaction involving halogen ions and H_2O_2 to produce potent microbicidal compounds such as HOCl (hypochlorous acid) and chloramines (19) (Step 4) that together with other reactive oxygen species constitute the oxygen dependent part of the mechanisms used to kill pathogens (Step 5). Mice deficient in MPO are much more susceptible to some micro-organisms compared with wild-type animals (51), but conversely, humans with an MPO deficiency, a relatively common genetic defect (incidence of 1 in 2000–4000), typically do not have an increased susceptibility to infections (52). Thus, it has been proposed that O_2^- and H_2O_2 or secondarily derived ROS, such as OH•, may be sufficient to compensate for the lack of the MPO–halide system under certain conditions (53). It raises the question about the precise role of ROS in microbial killing, a question still debated (47, 53-56). The widespread enzyme catalase, that is part of the antioxidant defense system of most cells, further processes H_2O_2 to H_2O and O_2 (Step 6).

2.5 Importance of extracellular ROS and regulation of inflammation

The first functions of neutrophil produced ROS to be revealed was the microbicidal activities, particularly related to the large quantities formed in the phagolysosome (57). However, the neutrophil NADPH-oxidase/NOX also produce and secrete ROS extracellularly. Such a release may occur by accident, if the pathogen/antigen is too large to be phagocytosed as is the case with hyphae of from the fungus *Aspergillus fulmigatus* (58). ROS produced by the NOX can possibly also act as signaling molecules that modify the redox state of proteins or lipids (59). As some ROS, especially H_2O_2, are membrane permeable, and this makes them suitable as paracrine signaling molecules which can affect the cells and the milieu around them, especially in an inflammatory setting (60).

ROS are often regarded as a pro-inflammatory mediator as well as being directly or indirectly involved in inflammatory tissue damage: however, new data suggest that ROS may have anti-inflammatory effects and facilitate resolution of inflammatory reactions/processes (56, 61, 62). Different animal models of inflammatory

diseases clearly illustrate the anti-inflammatory effects of ROS. In a rat model of arthritis, CGD animals that lacked one of the cytosolic components, develop a more severe disease (63). This was also shown to be true in a mouse model of gout (64). A molecular mechanism suggested for the development of a more severe disease involves the inability of CGD neutrophils to form NETs (64); the hypothesis presented was that NETs absorb and block the functions of pro-inflammatory cytokines and by that a local inflammation will be reduced (65).

2.6 Methods to determine neutrophil production of reactive oxygen species

Many techniques have been developed over the years to measure the cellular production of different ROS, and techniques based on detection of colorimetric changes as well as fluorescence or chemiluminescence have been used. There are some basic criteria that should be met and used to evaluate the method of choice. Ideally a method should target a specific oxygen metabolite, be sensitive and if possible, not interfere with the function of the cells. Preferentially, the method should also be easy to set up/perform, and it should be possible to determine the localization of the ROS produced. There is, however, no method that fulfils all these criteria and different measurement techniques have different drawbacks. It is clear that the NADPH oxidase can be assembled in the plasma membrane (leading to a release of ROS) or in intracellular compartments (phagosomes and/or granules in which the ROS are retained). A method that can measure both intra and extracellular ROS that comes from phagocytes that has been activated with different stimuli would be ideal. There are several dyes that after being excited by ROS release energy in the form of light (chemiluminescence). One of these dyes luminol/isoluminol; see **Fig 4**) is redox-sensitive and emits blue luminescence(47). Luminol/isoluminol is oxidized by oxygen radicals into the resonance-stabilized dianion, which is oxidized by oxygen species into the dicarboxylate ion, accompanied by the loss of molecular nitrogen, N_2. The molecule is transferred to an instable excited (higher energy/higher orbit) state that emits light when it returns to its ground state (see **Fig 4**) that is energetically more favorable than the excited state (66). Luminol is (in contrast to isoluminol) a membrane permeable dye, and when applied to neutrophils the light production is dependent on the enzyme MPO and both intra- and extracellular ROS production contributes to the activity measured (67). A change in the position of an amino group in the phthalate ring of luminol affects the properties of the molecule. The modified molecule (isoluminol) does no longer traverse the neutrophil plasma membrane but is excited and emits light in the same way as luminol (68). This means that isoluminol

can be used to specifically measure neutrophil superoxide production/release extracellularly. The shortcoming of using either luminol or isoluminol to measure ROS production is that the precise amounts of O_2^- (nmoles/min/cell) cannot be directly determined, but the relative ROS production can be followed continuously in real time (69).

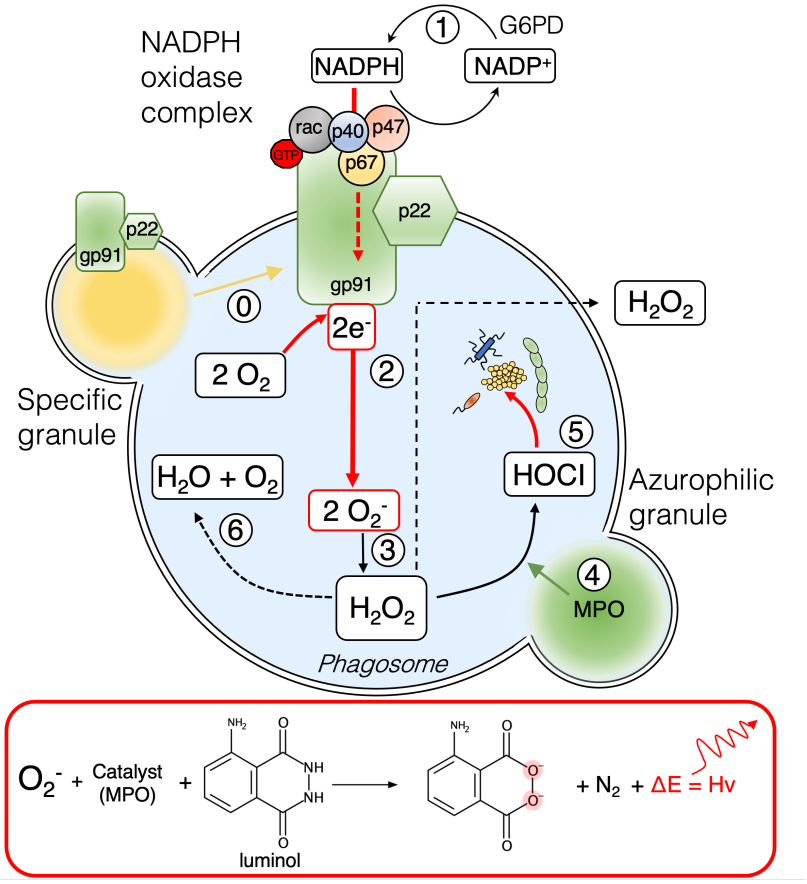

Figure 4 Production of reactive oxygen species

By transporting electrons from NADPH, the phagosomal membrane depolarizes as superoxide is produced in the phagosome. Superoxide is processed to a whole spectrum of reactive oxygen species that are highly reactive and toxic to microbes. See text for full details in the steps indicated by numbers in the figure. To measure radicals in the phagosome a dye such as luminol can be used.

3. Free fatty acids metabolism

The human gut is colonized by a staggering number of microorganisms and it is estimated that 90% of "our cells" are microbes (70). The microbes mainly help us to digest food products and regulate gut homeostasis. One major product of the metabolic digestion of carbohydrates is short chain fatty acids (SCFAs) generated during anaerobic bacterial fermentation of fiber diets. In a day, roughly 600 mmoles of SCFA are produced in the gut (71) and the most abundant SCFAs are acetate (C2), propionate (C3) and butyrate (C4). SCFAs are build up by a carboxylic acid moiety and a short hydrocarbon chain (72).

The SCFAs are primarily used as an energy source and the metabolic pathway is shown in **Fig 6**. The SCFAs can be absorbed by colonocytes utilizing different pathways. The process is either passive through diffusion or active through symporters (73). The absorption of SCFA can also occur through an exchange with bicarbonate (HCO_3^-) via a still an unknown exchanger. Colonocytes then use SCFAs as energy substrates in the Krebs cycle and generate energy in the form of ATP. This is important since SCFAs provide ~10% of our daily caloric requirements (74). The non-metabolized SCFAs are transported into the portal circulation where the concentrations may be fairly high (30 to 260 μM concentrations for propionate/butyrate and acetate, respectively) (75). When reaching the liver they will be an energy source for hepatocytes and also be used for the biosynthesis of glucose, cholesterol, and longer fatty acids (76). Hence, a smaller portion of the SCFAs reach the systemic circulation and peripheral tissues (acetate 36%, propionate 9% and butyrate 2%, respectively).

The finding that a group of orphan GPCRs recognized SCFAs opened a new research field. The GPCRs were shown to be highly expressed in immune cells (77) and were later termed free fatty acid receptors (FFARs). New investigations described a link between gut microbiota and inflammation (78), and this suggests that SCFAs are more important than what was originally thought.

4. Neutrophil G protein coupled receptors

Inflammatory mediators that often (but not always) are chemoattractants have important functions for the immune system to localize sites of tissue injury or an infection. These mediators are generated and released either by intruding microbes or from host cells or tissues (13, 79). One of the important non-chemotactic inflammatory mediators is Tumor Necrosis Factor α (TNFα), a pro-inflammatory chemokine produced by activated macrophages, T lymphocytes, and natural killer (NK) cells (80). This mediator is recognized by TNF receptors (TNFRs) expressed in most tissues (8). Basically, TNFRs are divided into TNFR-1, which binds soluble and membrane-bound TNF, and TNFR-2, which only binds membrane-bound TNF. Both receptors are expressed in neutrophils and require a specific adaptor protein for the downstream signaling that in neutrophils trigger exocytosis/granule mobilization but no rise in intracellular Ca^{2+} (81). The signaling pathway involving a rise in Ca^{2+} is typical for the responses triggered during activation of many neutrophil GPCRs (Paper G). TNFα induces a mobilization of the secretory vesicles that fuse with the plasma membrane and by that, the receptors stored in these vesicles are integrated into the plasma membrane. The ability of neutrophils to mobilize granule constituents is not static. Accordingly, the ability to mobilize granules is augmented in neutrophils treated with bacterial LPS as well as several cytokines, including not only TNFα, but also granulocyte/macrophage-colony-stimulating factor (GM-CSF), and IL-1β. Some cytokines may also enhance the neutrophil response when the cells are activated by chemoattractants (82-85). This process is termed priming. Naïve neutrophils exhibit limited antimicrobial effects; the cells must be reinstructed (primed) by microbial or endogenous agents to exert maximal phagocyte functionality, as measured by bacterial uptake and activation of the respiratory burst oxidase ((4, 5), Paper G). Priming is thus a key event whereby neutrophil responsiveness to an activating stimulus is markedly augmented by prior exposure to a priming agent. The biochemical events that mediate cytokine dependent priming of neutrophils are unfortunately poorly understood (86). Activation of the neutrophil NADPH-oxidase, by agents such as the formylated peptide fMLF (see below), is accompanied by a number of crucial signaling events, including an increase in the concentration of free calcium ions in the cytoplasm and activation and translocation of protein kinase C (PKC) to the cell membrane.

Following the identification in 1975 of powerful neutrophil chemotactic peptides such as fMLF (formyl-methionyl-leucyl-phenylalanine) generated by bacteria

(87), the number of such chemoattractants has increased significantly and now includes metabolites such as platelet activating factor (PAF), the complement component C5a (C5a) and several different interleukins (ILs), such as IL8 (18). These inflammatory mediators are recognized by distinct receptors expressed by the neutrophil (88, 89). These chemoattractant receptors belong to a large family of receptors that couple to heterotrimeric guanosine triphosphate-binding proteins (G proteins) (90). About thousand different G protein coupled receptors (GPCRs) are encoded for by the human genome and around 50% of these recognize odor molecules and are thus the basis for our ability to identify unpleasant as well as pleasant smells (91). Whereas around 100 of the human GPCRs lack known ligands (by definition these are orphan receptors) the diversity of ligands for the deorphanized is very large (92). Several GPCRs have been highly associated with immune reactivity of neutrophils and the ligands recognized by these receptors are also diverse but at the same time highly specific. This makes GPCRs highly appealing as drug targets, and it is estimated that around 40 percent of all approved drugs on the market target GPCRs (93).

All GPCRs have in common that they comprise of a single 342-360 amino acid long protein that spans the cell membrane seven times (**Fig 5**) (94). The ligand recognition receptor domain (orthosteric site) involves the N-terminus, the extracellular loops and some transmembrane parts reachable from the outside of the cell plasma membrane. In turn, the signaling mediated by such receptors is thought to be transduced through the three intracellular loops as well as the cytosolic C-terminal tail. Once an activating ligand (agonist) binds to the orthosteric receptor site, a heterotrimeric G protein is activated on the cytosolic side of the membrane in which the receptor is expressed. The trimeric G protein is made up of an α-subunit and a heterodimeric β/γ complex. Recognition of a chemoattractant induces multiple receptor-down-stream effector functions in neutrophils such as chemotactic migration, secretion of pro-inflammatory mediators and production of ROS (16, 69, 88, 95), but as mentioned, neutrophils store some of the GPCRs in mobilizable secretory organelles, and in order for these receptors to be functionally active, they have to be recruited to and be present on the neutrophil surface.

Neutrophils express many different GPCRs and a couple of these are described in more detail below.

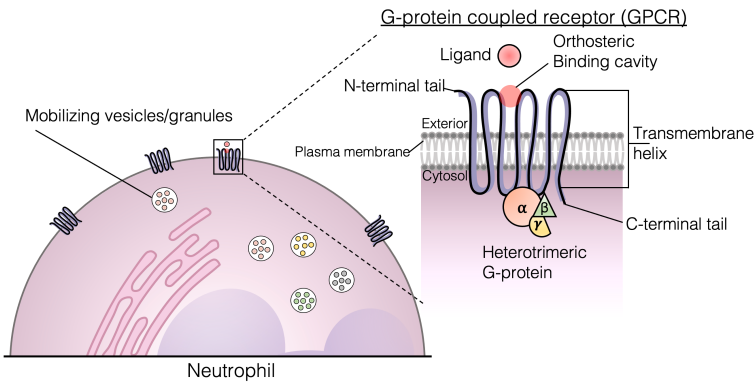

Figure 5 Overview and localization of G-protein coupled receptors
A generalized schematic description of a GPCR. These receptors are expressed in the neutrophil plasma membrane and/or a mobilizable granule/vesicle membrane. The receptor protein passes through the cell membrane seven times with the N terminal exposed on the cell surface or in the granule matrix and the C terminal tail faces the cytosol. The G-protein interacts with the cytosolic parts of the receptors. Conventional ligands (agonist/antagonist) bind to the orthosteric binding site present on outside of the plasma membrane and the intracellular signal is initiated by the heterotrimeric G protein on the cytosolic side of the membrane. Granule/vesicle-localized GPCRs can be mobilized to the cell surface through a secretion process.

4.1 Formyl peptide receptors

Synthetic N-formylated peptides were shown almost 50 years ago to be chemoattractants for neutrophils and it was then postulated that specific surface receptors recognized a molecular pattern common for prokaryotic microbes. The rational being, that protein synthesis in prokaryotic cells starts with a formylated methionine (fMet) (87). The concept rapidly gained support when highly potent chemotactic fMet-containing peptide were identified in culture supernatants of growing *E. coli* bacteria (96), and the pathogen associated molecular pattern (PAMP) recognition concept was rapidly found applicable to the group of receptors named formyl peptide receptors (FPRs; (88)). These findings opened for further receptor related research which led to the cloning of the gene for FPR1, one of the FPRs (97, 98), and since then this has become the most studied neutrophil GPCR. Shortly after the cloning of FPR1, two originally orphan receptors (now known as FPR2 and FPR3) with large sequence homologies to FPR1 were identified. The three human receptors in the FPR family are grouped together on chromosome 19q.13.3, and the encoding proteins probably share similar biological functions (99). Whereas a large number of formylated and non-formylated ligands have been shown to be recognized by FPR2, no potent and selective agonists for FPR3 have yet been described.

Formylated peptides are produced not only by bacteria, but also by mitochondria that are of prokaryotic origin. The mitochondrial DNA (mtDNA) encodes 13 proteins that belong to the respiratory chain that generates the bulk of cellular ATP via oxidative phosphorylation (100), and the encoding genes are transcribed and translated in a microbial like manner. Accordingly, the initiating amino acid in the mtDNA-encoded proteins is a formylated methionine, and these proteins (or N-terminal peptides thereof) will be recognized by neutrophil FPRs as a tissue damage molecular pattern (DAMP) (Paper A, Paper B, (96, 101-104). The release of mtDNA and formylated proteins/peptides from injured/destructed tissues leads to exposure of otherwise concealed/hidden or cryptic endogenous DAMPs, which include the mitochondrial cryptic peptides (mitocryptides; MCTs) (105-108). From an evolutionary point of view, one can assume that the receptors that recognize peptides starting with a formylated methionine have developed both to combat infections and to resolve an aseptic inflammation in a traumatized tissue. Indeed, mitochondrial-derived DAMPs have been shown to activate blood neutrophils (Paper B) and, hence, when produced and released in large amounts during severe injury, induce a sepsis-like state (109).

Pathogenic *Staphylococcus aureus* bacteria produce a special type of non-chemotactic N-formylated peptides recognized by FPR2, and a recent study in which a pharmacological as well as a genetic approach was used, gained increased molecular insights into the pattern of signaling as well as into the receptor preference for formyl peptides. The data presented show that not only the N-terminal part of a peptide that fits into the so called orthosteric binding site/pocket is of importance for recognition and the signals induced (Paper F).
Despite the large number of FPR agonists described to have anti-inflammatory effects in animal disease models, very few have entered clinical trials. Act-389949, a small compound agonist, is an exception and when tested it was found to be safe and well tolerated in healthy humans (110). The receptor preference and down-stream signaling characteristics induced by this first-in-class compound has been determined, and the data obtained show that FPR2 is the preferred receptor, and the signals and functional repertoires down-stream of the receptor is the same as for many earlier described FPR2 agonists (Paper C). The fact that Act-389949 was shown to be resistant to oxidation by MPO-H_2O_2-halide derived oxidants, suggests that it may serve as tool-compound for further studies about the regulatory roles of FPR2 in inflammation. Another small compound (RE-04-001) recognized preferentially by FPR1, has been shown to be a potent functional selective agonist that activates neutrophils (Paper D) that may also serve as tool-compound for future studies of the regulatory roles of FPR1 in inflammation.

4.2 The purinergic receptors

In addition to its function as an energy source in a large number of physiological processes taking place in actively metabolizing cells/tissues, ATP is also a DAMP that may be actively secreted and/or passively released from damaged cells/tissues (111). Thus, ATP affects inflammatory processes and purinergic receptors sense both ATP and ADP. Out of the not so well studied neutrophil purinergic receptors, the $P2Y_2$ receptor is the best characterized and this GPCR binds ATP with high affinity. It is said that $P2Y_2R$ couples mainly to $G\alpha_q$ in neutrophils, but the receptor has the capacity to also interact with $G\alpha_i$ and $G\alpha_{12/13}$ (112-114). The ATP activated $P2Y_2R$ triggers a rise in the concentration of intracellular calcium ($[Ca^{2+}]_i$) but in neutrophils this emptying of Ca^{2+} from intracellular stores is achieved without any activation of superoxide generating NADPH oxidase (115). The pathway downstream of the ATP-activated receptor in naïve cells, is blocked through an inhibitory mechanism involving the actin cytoskeleton (88, 115). Hence, the ROS generating oxidase is activated by the ATP/P2Y2R ligand/receptor in neutrophils in which the actin cytoskeleton has been disrupted.

ATP is not a chemotactic ligand in itself, yet if ATP occupies $P2Y_2R$ the fMLF-mediated chemotaxis is enhanced (116), and it has been suggested that such an effect may be of physiological importance and mediated either by ATP in the environment or by ATP secreted from neutrophils at the leading edge of the migrating cells (117). The presence of ATP can enhance the generation of superoxide through an activation achieved through a novel receptor cross-talk/transactivation mechanism by which signals generated by $P2Y_2R$ turn another GPCR into a signaling state (Paper I, (118))

4.3 Free fatty acid receptors focusing on the neutrophil FFA2R

The SCFAs constitute not only an energy source and the basis for synthesis of more complex molecules (119), but the identification of receptors that recognized these molecules suggested that these metabolites constitute a molecular pattern of microbial origin that can act as signaling molecules (120, 121). SCFAs can interact with and activate at least four distinct GPCRs originally known as GPR41, GPR43, GPR109a (HCA2) and olfactory receptor Olfr78. The SCFA-receptors initially termed GPR43 and GPR41 are now known as free fatty acid receptor 2 and 3 (FFA2R and FFA3R), respectively (122). These receptors are expressed in tissues

that are connected to metabolic regulation such as adipose tissue, pancreatic β-cells and intestinal enteroendocrine cells (123), but at least FFA2R is also highly expressed in different immune cells including neutrophils (124). Accordingly, the basic functions of SCFAs have been slightly changed and now include not only the importance in gut energy homeostasis and metabolic regulation but also a role in immune reactivity (77, 125). Until recently, the lack of potent and selective ligands for the FFARs limited the likelihoods to advancement of our knowledge about the pathophysiological roles of the receptors, but now several selective synthetic ligands have been identified, and this has opened up for an increased understanding about the regulatory role of the FFARs and their ligands in inflammation and immune function ((126-130) Paper I-IV). The selective ligands for the FFARs include not only activating and inhibiting ligands (agonist and antagonists) but also a new type of receptor specific ligands that selective modulate receptor function. These modulators interact with allosteric receptor sites rather than with the orthosteric site, and they modulate receptor functions positively (increase the activity induced by an orthosteric agonist) or negatively (inhibit the activity induced by an orthosteric agonist), respectively (Paper I-IV).

Several neutrophil functions are regulated by SCFAs. Results obtained in *in vitro* as well as in *in vivo* studies suggest that SCFAs are neutrophil chemoattractants (78, 131-133). The direct involvement of one of the receptors that recognize SCFAs, FFA2R, in neutrophil recruitment to inflammatory sites has been shown in a bowel disease model in mouse, and in accordance with a role for FFA2R, receptor knockout animals exhibited diminished inflammation, suggesting that this receptor is involved in the recruitment process (133). This suggestion has gained support from the finding that neutrophil recruitment and the inflammatory activity in such a colitis model, was reduced in wild-type mice fed with SCFAs (131). Regarding the ability for SCFAs to affect neutrophil production of reactive oxygen species and the ability to phagocytose a prey (Paper I-III (131, 134)), conflicting inhibitory as well as enhancing effects have been reported. Furthermore, SCFAs have also been shown to inhibit neutrophil production of the pro-inflammatory cytokine TNFα (135).

The lack of potent and selective FFA2R ligands has made it difficult to properly define the regulatory roles/functions of the neutrophil SCFA receptors. Recent advances in the field have opened new possibilities as potent and selective agonists, antagonists as well as allosteric modulators for FFA2R have been described. The small compounds CATPB (136) and Cmp1 (81, 137) are ligands that fulfil the requirements of being both potent and receptor selective. Cmp1 is an orthosteric agonist that has been shown to activate the neutrophil FFA2R, and the receptor

down-stream signaling cascade involves a pertussis toxin-sensitive G-protein and the PLC-PIP2-IP_3 route to induce a release of Ca^{2+} from intracellular storage organelles, a response induced also by the neutrophil FPR1 (138). CATPB was identified as an FFA2R antagonists, and accordingly it inhibits the response induced not only by SCFA but also by Cmp1. Results obtained dealing with the regulatory mechanisms for activation of FFA2R and termination of the response, disclose both similarities and differences to the mechanisms that regulate the FPRs. It is clear that although Cmp1 induces a rise in intracellular Ca^{2+} in neutrophils, this response is not accompanied by any mobilization of CR3 to the cell surface or cleavage of L-selectin from the cell surface (115). GLPG0974 (130), another selective FFA2R antagonist was recently tested in a clinical trial to evaluate its safety and efficacy in treating patients with ulcerative colitis. This antagonist has also been thoroughly investigated in neutrophils and it potently inhibited FFA2R dependent responses (Paper I, Paper IV), but its effects following binding is fairly complex (Pater IV; see also 7.3 below).

4.4 SCFA and FFAR in health and disease

Gut microbiota and secondary metabolites generated during microbial growth participate in the regulation of several organ systems including the immune system, and SCFAs are one of the key regulatory metabolites identified (76, 124, 131). The microbiota is said to affect immune cell functions such as the ability to produce soluble effector molecules such as cytokines, antibodies, antimicrobial peptides, and several other compounds vital for the host defense (139-141). Predictably if a loss of balance occurs between the host immune system and the components generated by the microbiota, the result could lead not only to infections but also to the development of inflammatory diseases. Notably, this has been suggested to be of importance in periodontitis, rheumatoid arthritis, diabetes, and inflammatory bowel disease. (142-146).

SCFAs were recently shown to affect several cellular processes regulated by signals generated down-stream of activated FFARs (depicted in **Fig 6** and discussed below), as well as gene expression, cell differentiation (147), proliferation (148), apoptosis (149), inhibition of histone deacetylases (HDACs) (150), and stimulation of histone acetyltransferase activity (150).

It has been shown that SCFAs can interact with FFA2R and FFA3R expressed by colonocytes and by that affect the plasma glucose levels regulated by gut hormones such as the peptide YY (PYY) (151) and glucagon-like peptide 1 (GLP-1)

(119, 152). PYY is known as an indulgence hormone, but it also boosts the insulin action on glucose disposal in muscle and adipose tissue. PYY and GLP-1 is said to also influence learning, memory, and mood (119, 153). The direct link between FFARs and hormone production is clearly illustrated by the fact that the colonic PYY production is reduced in FFA2R and FFA3R knockout animals and this affects the whole-body glucose tolerance.

The increased susceptibility to dextran sulfate sodium-induced colitis in FFA2R mice knockouts, suggests that this receptor is of importance also for gut homeostasis (131). This is also supported by the fact FFA2R agonists interacting with their receptor on intestinal epithelial cells, activate the NLRP3 inflammasome and induce the production of IL-18 in these cells (154). This response is said to be critical for the ability to maintain epithelial integrity and intestinal homeostasis.

SCFAs may also regulate (possibly through binding of FFA2R) intestinal barrier function (155). This is accomplished by inducing intestinal epithelial cell-secretion of mucin and antimicrobial peptides, as well as by affecting the function of the tight junctions and maintaining the transepithelial electrical resistance (TEER) (156, 157). A problem to maintain the status of the intestinal barrier could give rise to a "leaky gut" that allows gut bacteria and toxins to pass the intestinal wall (158).

SCFAs also play an important role in inducing neutrophil migration to inflammatory sites and enhance their phagocytic capability (78). Additionally, SCFAs can inhibit or induce production of proinflammatory cytokines (e.g., IL-6, IL-1β and TNFα) and the outcome is probably dependent on the concentration of SCFAs and the cell type targeted (159-162).

Additionally, SCFA can bind FFA2R on regulatory T (Treg) cells to induce the production of the anti-inflammatory cytokine interleukin-10 (IL-10) (163-166). Tregs treated with SCFA promote IL10 production and $FFA2R^{-/-}$ Tregs were not affected by SCFA. IL-10 has shown to be important for the development of an inflammatory response without inflicting self-tissue damage (167). Loss of IL10 production due to mutations in IL10 or its receptor is also linked to problematic enterocolitis in infants (168)

SCFA have also been shown to affect the ability of dendritic cells (DCs) to promote $CD4^{+}$ T cells to become Tregs (169). Moreover, SCFAs has also been seen to inhibit carcinogenesis through promoting apoptosis and suppressing proliferation of tumor cells which is also indicated mediated through FFA2R (170, 171). Furthermore, a *Staphylococcus aureus* induced sepsis study in mice showed that

acetate administration not only primed neutrophils but also rescued wild-type mice and substantially reduced bacterial numbers in peripheral organs (172).

Taken together, these data show the importance of SCFAs in human health and when it comes to inflammation, show a complex system were the balanced between the gut microbiota derived SCFA are deeply connected to inflammation. Supplementation of dietary fibre may be an effective strategy for future treatment of inflammatory and immune-related diseases, such as rheumatoid arthritis (173-175).

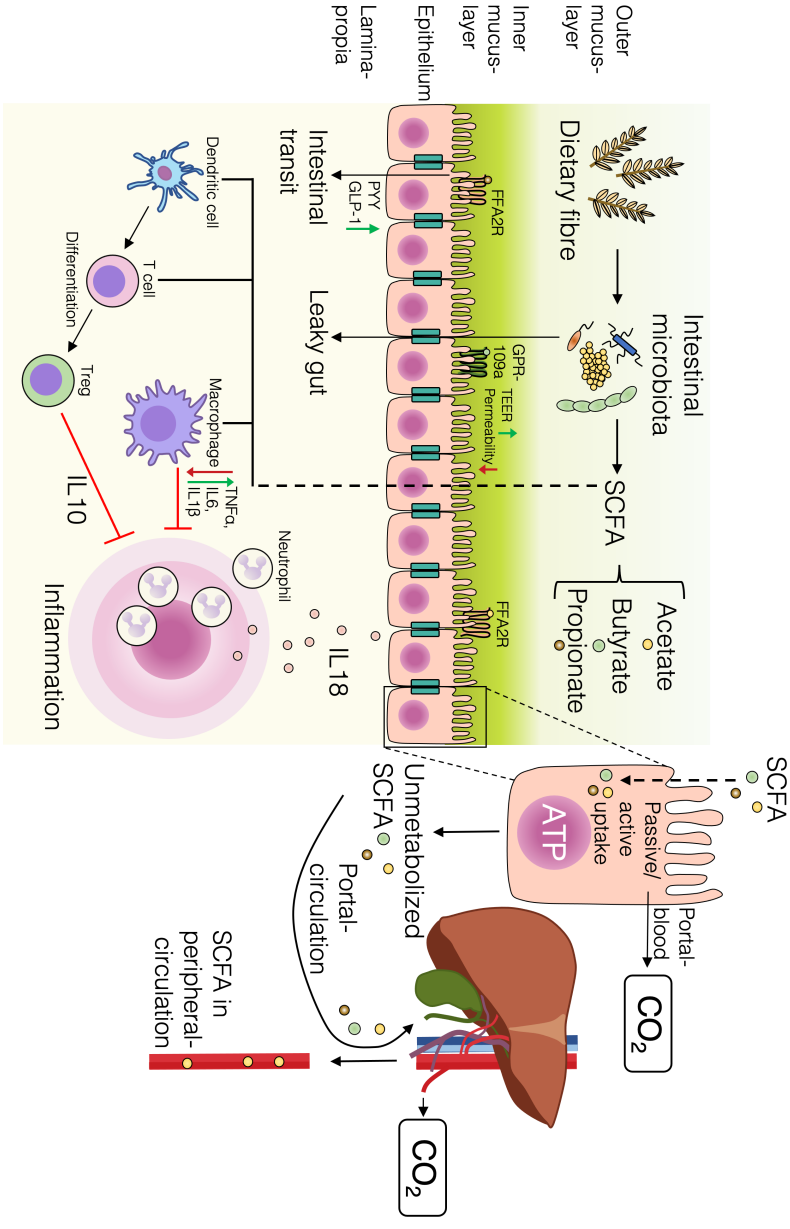

Figure 6 Metabolism of SCFA and its effect on health and disease. *Overview picture depicting the SCFA, a commensal bacterial fermentation product that is both used an energy source but also have connection in health and immunity.*

5. Characteristics of GPCR signaling

Orthosteric GPCR ligands bind to a receptor pocket reached from the outside of the cells that express the receptor (176, 177). Binding of activating orthosteric agonists induces conformational changes in the receptor (178). The conformational/structural changes involve the cytosolic parts of the receptor that interact with the receptor down-stream heterotrimeric G protein (94). The Gα and Gβγ parts of the activated G protein dissociate, and the subunits initiate downstream signaling cascades. The Gα subunit has an intrinsic GTPase activity, which regulates the association/dissociation of the subunits and by that the level of signaling. Receptor activation (**Fig 7**) promotes an exchange of the bound guanosine diphosphate (GDP) that is replaced by guanosine triphosphate (GTP) and, this exchange leads to the dissociation of the two G protein subunits (179). Once dissociated both subunits have the capacity to interact with cytosolic as well as membrane bound/associated proteins that further transduce signaling.

5.1 An early signaling cascade generated by activated GPCRs

G protein transduced intracellular signaling induced by activated GPCRs commonly include a rise in the intracellular concentration of free calcium ions ($[Ca^{2+}]_i$) (180, 181) (see **Fig 7**). The signaling cascade leading to a rise in $[Ca^{2+}]_i$ is initiated by an activation of a phospholipase (PLCβ) (182). This activation is mediated by one of the G protein subunits (the Gα subunit of $G\alpha_q$ containing G proteins and the Gβγ dimer of $G\alpha_i$ containing G proteins) and the activated PLCβ hydrolyzes phosphatidylinositol bisphosphate (PIP2) that is cleaved into inositol triphosphate (IP3) and diacylglycerol (DAG). The IP3 formed, regulates calcium channels present in the membrane of calcium storing organelles (i.e., the endoplasmic reticulum) and an opening of these channels results in a release of Ca^{2+} from the stores to the cytoplasm and an elevation of the level of the $[Ca^{2+}]_i$ (183) . A further rise in $[Ca^{2+}]_i$ is commonly induced through an opening of plasma membrane localized Ca^{2+}channels. The opening of these channels is regulated by the filling state of the storage organelles. These store operated Ca^{2+} channels (SOCs) open when the stores are emptied. In a positive feedback loop the membrane anchored DAG formed by the activated PLCβ recruits protein kinase C (PKC) to the membrane and activates this enzyme in a Ca^{2+} regulated process (184). Downstream signals

of activated PKC lead to activation of many proteins and also an activation of the NADPH-oxidase that produces O_2^- (185).

Out of the four main G protein subtypes, the $G\alpha_i$ and $G\alpha_q$ are considered the most important for neutrophil function. Of the two other main subtypes ($G\alpha_s$ and $G_{12/13}$), $G\alpha_s$ couples to GPCRs such as the 5-HT receptor (186) (serotonin receptor) and $G_{12/13}$ has been shown/suggested to have roles in the regulation of the actin cytoskeleton (187). It should be noticed that there are also several isoforms of the two parts of $G\beta\gamma$ heterodimer (188). Due to the fairly high sequence homology between the different subunits, it is hard to determine the precise identity of the G proteins involved in signaling in primary cells (189). Bacterial toxins have long been the only available tools to determine the signaling role of the different G proteins. A toxin from *Bordetella pertussis* has long been used to determine the involvement of $G\alpha_i$ (190), however, the specificity of the pertussis toxin in neutrophils has been questioned. Pertussis toxin inhibits signaling in neutrophils also by GPCRs that couple to $G\alpha_q$ containing G proteins, questioning the exact mechanism by which this toxin interacts with the G proteins (191). A toxin from *Vibrio cholera* is a tool compound that can be used to determine $G\alpha_s$ involvement (192). Recently selective $G\alpha_q$ inhibitors have been described (193, 194). Data obtained using these inhibitors have revealed that some neutrophil GPCRs use a $G\alpha_q$-pathway to initiate a rise in $[Ca^{2+}]_i$ (Paper G).

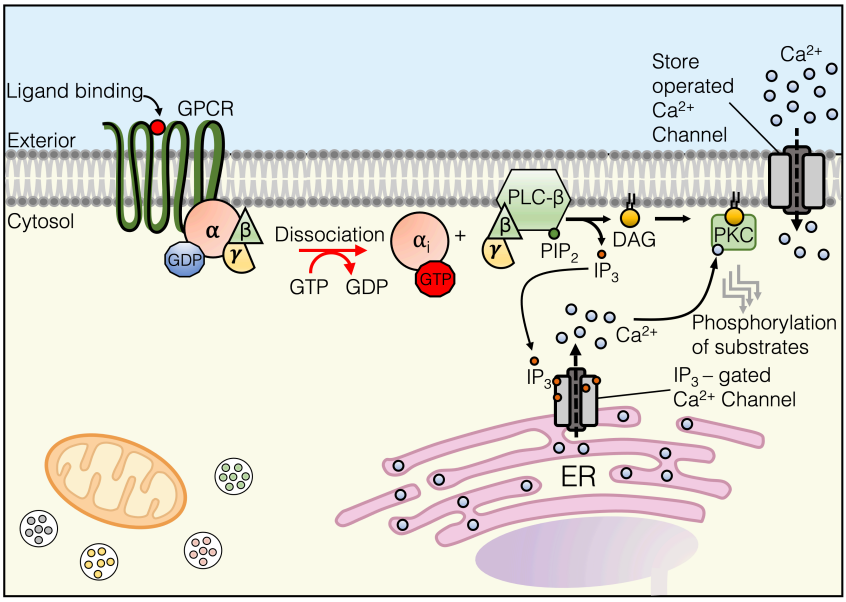

Figure 7 Schematic description of the signaling pathway that leads to increased intracellular concentration calcium ions in the cytosol initiated by activated GPCRs. *See 5.1 for a description of the early signaling cascade pathway generated by activating GPCRs. Once the agonist binds to the orthosteric site of the GPCR the trimeric G protein is activated, and the subunits dissociate. The βγ heterodimer triggers a PLC dependent release of calcium ions from storage organelles (mostly likely from endoplasmic reticulum (ER)) and a second phase of increase is initiated when store operated Ca^{2+} channels in the cell membrane are opened. This opening is regulated by a depletion of calcium ions in the ER.*

5.2 Allosteric receptor modulators

The change in function of a protein, mediated by a compound that binds to a site in the protein not identical to the active site, is defined as allosterism or an allosteric behavior (195). Allosteric effects are in most cases linked to some type of structural/conformational change of the protein, and the change is induced by the allosteric effector. Originally, this phenomenon was described as an important mechanism by which different allosteric effector components regulate enzyme activity (195, 196), and the new model replaced the so called “lock and key model” in which enzymes were regarded as rigid protein structures (197). The idea that enzymes are flexible protein structures that have the capacity to change conformation has become vital for our understanding not only of how enzyme activity is regulated but also for the regulation of ion-channels and signaling properties of GPCRs (198).

According to the conformation selection model, a protein stabilizes into different states when ligands bind. Given that GPCRs are complex macromolecules that can take several different tertiary forms and in any given time, a GPCR can adopt a collection of different conformations of similar free energy (199, 200). Binding of a ligand to its receptor initiates a conformational change, and once a high affinity binding is established the new conformation is stabilized.

However, it is not always clear if, once a ligand binds to a receptor, the adopted structure is unique and can only be adopted once the ligand is bound to the receptor (201). Another possibility is that the receptor has the ability to adopt the unique conformation also without the ligand, and there are time and energy rational for this in a biological setting (202, 203).

The development of receptor selective allosteric modulators as drug candidates has become an alternative to meet the challenges in the development of small orthosteric ligands (204). The allosteric receptor modulation concept states, that the response elicited by the endogenous agonist/antagonist (see **Fig 8** for visual reference) is affected by a non-activating but modulating receptor binding ligand. Such an allosteric modulator can positively (increase) or negatively (decrease) affect the receptor activities, or more importantly, change signaling to a unique functional selective pathway. Such receptor ligands are termed biased allosteric modulators (205).

Binding of an agonist to the orthosteric binding pocket reached from the outside of a cell, will change receptor parts on the cytosolic side of the cell membrane, a structural change that triggers an activation of the G protein. An agonist induced cellular response mediated by a GPCR, thus, clearly involve an allosteric conformational change. Also binding of non-activating allosteric GPCR modulators induces conformational changes of the recognizing receptor, changes that affect the behavior of the receptor. Allostery may be described as a process of cooperativity between the binding sites for the modulator and orthosteric ligands; binding of a molecule to one site in the GPCR modify the binding/signaling characteristic of a ligand that binds to another site in the same receptor (206). The physical separation of the two binding sites is one of the important features when defining allosterism. The effects of a modulator may also depend on the co-binding ligand, and this probe dependency is also an important feature of allostery (207). Another important feature of allosteric modulators is the saturation of their effect (208). This means that the allosteric effect reaches a maximum when the binding to the allosteric receptor site is saturated.

Typically, it is hard to predict the effects of an allosteric modulator. Binding of an allosteric modulator could alter the affinity and/or efficacy when the receptor is activated or the allosteric modulator may by itself generate a response (209). This means that an allosteric GPCR modulator can display one or more of the pharmacological effects mentioned above (see **Fig 8** for visual representation). To reiterate, affinity modulation will lead to a conformational change that affects the orthosteric binding pocket. This can change the association or dissociation rate (or both) for an orthosteric ligand. Efficacy modulation will affect signaling properties achieved through a modulation of the cytosolic part of the receptor (also called intrinsic efficacy) (210).

Additionally, there are no restrictions as to the combinations of effects that an allosteric modulator can have (195). That means that affinity may be increased or decreased, and this is true also for the efficacy. Allosteric efficacy is still subjected to the normal limitations for the agonists. If we consider a dose response curve for an agonist induced activity, an increase in affinity will cause a left shift of the curve and a decreased affinity will cause a right shift. If the agonist is a partial agonist an increase in the maximal signaling output is also a possible outcome. Likewise, a decrease in efficacy results in a right shift of the dose response curve for a full agonist if the receptor reserve is large and to a lower maximum if the receptor reserve is small. For a partial agonist, which by definition has no receptor reserve, a decrease in efficacy will reduce the maximal response. Taking in all considerations of allosteric modulation effects would lead to numerous possible combinations however in a practical sense there are three phenotypes of activity. If binding of an allosteric modulator negatively affect affinity and/or efficacy of an orthosteric ligand, this will result in negative allosteric modulation and these ligands are termed negative allosteric modulators (NAMs). If there are instead positive effects of the affinity and/or efficacy of an orthosteric ligand the allosteric modulator is called positive allosteric modulator (PAM). There are also unique phenotypes that, if the affinity is positive and efficacy is negative, are referred to as PAMs with antagonistic properties (211). It should also be stated that the theory of probe dependence states that different probes (agonist) may be affected in different ways by the same allosteric modulator (212). This means that on agonist can be negatively or positively affected by a structural change whereas another agonist is not affected at all. These allosteric modulation models are, however, challenging to fit to experimental data (213) but different classes of allosteric modulators are now being utilized in therapeutic settings.

Some 15 years ago a new class of allosteric GPCRs modulators were described. These lipopeptides (pepducins), containing a peptide chain coupled to a fatty acid, were suggested to regulate GPCR activity through a novel mechanism. A peptide sequence identical to that in one of the intracellular domains in a GPCR was suggested to selectively interact with this receptor on the cytoplasmic side of the membrane and the result will be an activation or an inhibition of receptor signaling (214). This is obviously a regulatory mechanism vastly different from that of conventional GPCR-ligands that mediate their activation (agonist) or inhibition (antagonist) of receptor function through a direct interaction with the orthosteric binding sites exposed on the cell surface. In agreement with the allosteric GPCR modulation concept, pepducin are expected to modulate functions solely of receptors that contains a sequence of amino acids that is identical to the peptide sequence present in one of the cytosol receptor domains. However, data obtained in studies of the FPRs call the concept in question (Paper G).

An exception regarding the proposed GPCR selectivity for pepducin, was described for a pepducin ($P4Pal_{10}$), containing a 10 amino acid long peptide originating from the protease-activated receptor 4. This pepducin was suggested to inhibit signaling by many receptors having in common that they coupled to a G protein containing a $G\alpha_q$-subunit (215). Yet in neutrophils, $P4Pal_{10}$ has been shown to be without effect on signaling by the ATP and PAF receptors, two receptors that couple to a $G\alpha_q$-containing G protein. In contrast, however, the response induced by FPR2 agonists is inhibited and more importantly, this pepducin activates FFA2R in neutrophils (Paper E).

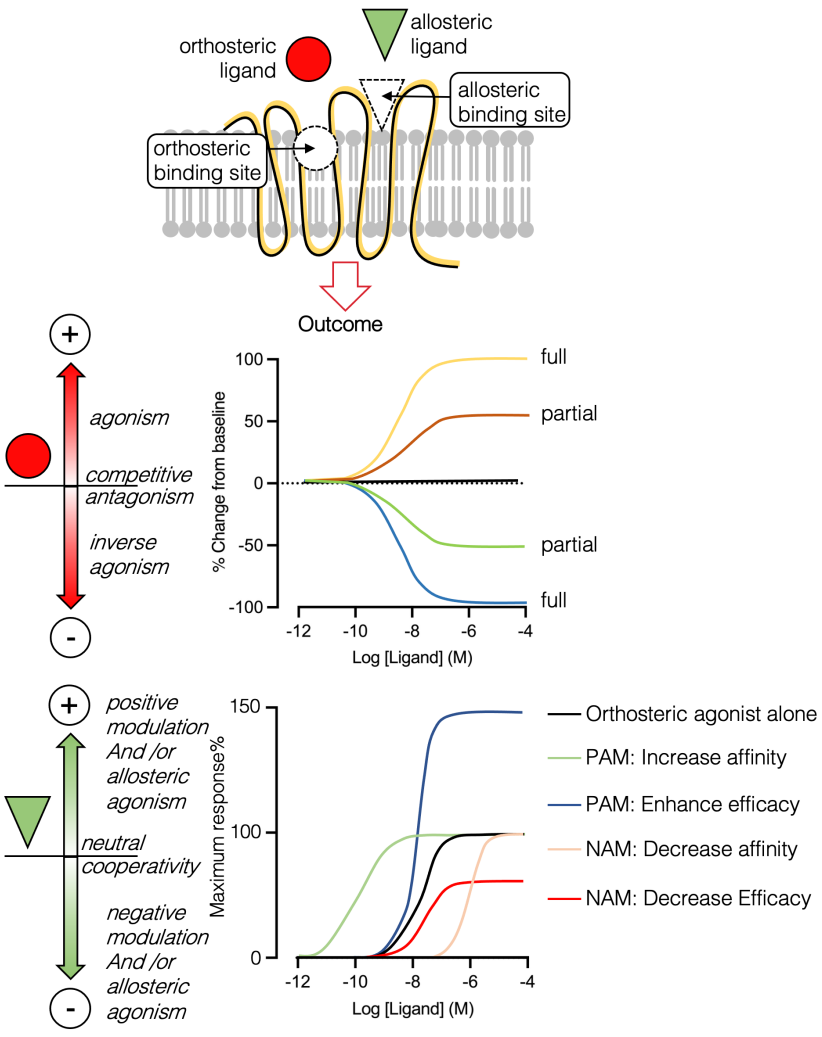

Figure 8 Mechanism of action and phenotypic profiles for allosteric modulators. *GPCRs have an orthosteric binding site that interacts with the endogenous ligand for the receptor and that is where all orthosteric ligands interact. Distinct from the orthosteric binding pocket is the allosteric site where allosteric modulators bind. The old allosterism models tells us that the allosteric modulator can only affect the intracellular signal pathway outcome only if the orthosteric site is also engaged. Middle part of the figure summaries the log dose–effect curves for full agonist, partial agonist, antagonist, partial inverse agonist and full inverse agonists in percentage change from baseline. Lower part of the figure represents the log dose-effect curves effects in the presence of different allosteric modulators. Black color represents the profile for the agonist dose curve alone and the different allosteric modulation effects on the agonist is presented in different colors as indicated in the figure. The allosteric modulators can either or both change the binding and functional response of the agonist.*

5.3 Biased signaling/functional selectivity

GPCRs are allosteric proteins that can engage many different structural conformations and signaling states (216, 217). This means that depending on the ligand that binds to a GPCR, the receptor has the ability to selectively engage one or several different receptor down-stream signaling pathways and cell functions (see **Fig 9**). The description of this so-called biased receptor signaling phenomenon directly linked to functional selective responses by GPCRs, became the starting point for a deeper understanding of the complexity of the signaling events and the importance of the ligands for regulation the receptor activities (218). Biased ligands that selectively activate (agonists) or inhibit (antagonists and reversed agonists) different signaling pathways also became appealing when searching for drug candidates. A novel ligand that affects a subset of functions of a single receptor, could minimize cross reactivity and thereby optimizing the therapeutic effect (176). The concept of biased signaling/functional selectivity is also valid for neutrophils. This is illustrated for example by the effects of different types of FPR2 specific agonist (i.e., lipopeptides, pepducins and formylated peptides) that have in common that they trigger both a rise in $[Ca^{2+}]_i$ and an activation of the superoxide generating system in neutrophils, whereas they lack the ability to recruit β-arrestin and induce chemotaxis. The FPR1 specific agonist RE-04-001 (Paper D) has the same biased signaling and functional characteristics, favoring ROS production, ERK1/2 phosphorylation and a rise of $[Ca^{2+}]_i$ over β-arrestin recruitment and chemotaxis. Similar to an angiotensin 1 receptor (AT_1R) specific agonist (219), the pro-resolving FPR2 specific lipids LipoxinA4 and Resolvin D1 have been shown be biased β-arrestin recruiting agonist (220). We have however, not been able to confirm these results (unpublished data).

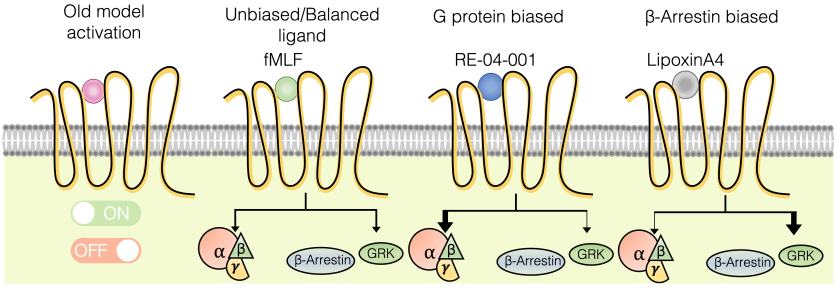

Figure 9 Representation of functional selectivity for GPCRs. *The old model of GPCR activation was simply that receptor signaling was either on or off but the introduction of the biased signaling concept was a paradigm shift. Depending on the ligand and the receptor conformation induced signaling will either be balanced (no signal preference/functional selectivity) or biased (one or the other signaling pathways is preferred.*

6. Allosteric modulation and biased signaling of FFA2R

The allosteric receptor modulation concept, stating that the response elicited by an orthosteric agonists can be modulated by specific non-activating receptor ligands that bind to an alternative (allosteric) site, has been shown to be valid also for FFA2R. Two compounds, Cmp58 and AZ1729, were identified using screening protocols to search for FFA2R ligands. The two compounds lack the carboxylic acid entity shown to be of prime importance for the ability of orthosteric FFA2R agonists to activate the receptor (221). Accordingly, Cmp58 (222) as well as AZ1729 (129) were shown to be allosteric FFA2R modulators that positively modulated the response induced by FFA2R agonists. Recent data also show that an allosteric modulator may affect not only the response induced by a ligand recognized by the orthosteric binding site, but also signaling induced by a second allosteric modulator that engage another allosteric receptor site (Paper II and below). The allosterically modulated FFA2R may also be activated by a transactivation/receptor cross talk mechanism without the involvement of any orthosteric FFA2R agonist (Paper I and below). The identification and characterization of new allosteric modulators opens not only for possibilities to use unique way to regulate function of the targeted GPCRs but also increases the therapeutic potential and improve the possibilities to develop new drugs. Accordingly, better tools to identify and characterize allosteric modulators will increase the possibilities to find highly selective allosteric GPCR modulators.

6.1 Biased FFA2R signaling achieved without involvement of any orthosteric agonist

A novel receptor activation mechanism was described (Paper II**)** when showing that FFA2R can be activated without the involvement of any orthosteric agonist. As previously mentioned, the number of ligands for FFA2R increases steadily, and the data obtained using the new research tools show that this receptor has a more central regulatory role in immunological reactivity than previously thought (123). The identification/description of new allosteric modulators that can be used to regulate receptor functions induced by orthosteric agonists has attracted many researchers (223) and one of the allosteric modulators recently identified is AZ1729 (129), an allosteric modulator for FFA2R developed by AstraZeneca. We show (Paper II) that in neutrophils this allosteric FFA2R modulator displays

properties very similar to those of Cmp58. These similarities include both the positive modulating effects on the response induced by orthosteric FFA2R agonists and also on the ability to transfer FFA2R to an activated state through the receptor-cross-talk mechanism involving also the agonist/receptor pair ATP/$P2Y_2R$ (see Paper I). In addition, AZ1729 also turns the PAM Cmp58 into a potent activating allosteric agonist. The interdependent neutrophil activation by the two PAMs is evident when they were added to the neutrophils together as well as in sequence. When added in sequence the order is reciprocal. This means that Cmp58 turns AZ1729 into a potent activating allosteric agonist, a character typical for the relation between a modulator and an agonist. The activation of the allosterically modulated FFA2R by the transactivating signals generated by $P2Y_2R$ was, however, not reciprocal (Paper I and below).

Furthermore, no orthosteric agonist is required for activation by the two PAMs, and signaling down-stream of the activated FFA2R, partly differs from that induced when an orthosteric agonist participates. The receptor down-stream signals generated by FFA2R activated by the PAMs, bypasses the PLC-PIP_2-IP_3 pathway leading to a rise in intracellular Ca^{2+}. This type of biased signaling is not seen when orthosteric agonists participate in the activation of FFA2R. When the response induced by the two PAMs is terminated, FFA2R is desensitized and this non-responding state of FFA2R is functional selective. Despite the fact that the response induced in Cm58/Az1729 activated/desensitized neutrophils by an orthosteric FFA2R agonist is lowered when measured as superoxide production, the corresponding response is potentiated when determined as the transient rise in $[Ca^{2+}]_i$. Based on the data presented and the fact that Cmp58 as well as AZ1729 lack the carboxylic acid structure shown to be of vital importance for the ability of orthosteric agonists to activate FFA2R (129), we concluded that FFA2R must have two different allosteric binding sites structurally separated from the orthosteric binding site.

6.2 Using a small compound library to gain further insight into allosteric activation

The hypothesis/model presented in Paper II has been further tested. According to the model, FFA2R has two different binding sites that recognize Cmp58 and AZ1729, respectively, and these allosteric sites are structurally/physically separated from the orthosteric binding site. The three binding site model gains full support from the data presented in Paper III.

To gain further insights into modulation and signaling properties of FFA2R, the activities induced by a set of new small molecules with some structural similarity to earlier described FFA2R ligands were investigated. By definition, non-activating ligands that turn propionate (an orthosteric FFA2R specific agonist) into an activating agonist, classify as positive allosteric FFA2R modulators (PAM). Accordingly, several of the compounds also classified as allosteric FFA2R modulators.
To determine the precise allosteric site used by the different allosteric modulators, neutrophils were sensitized with each respective compound and the cells were then activated with either Cmp58 or AZ1729 and the neutrophils production of O_2^- was determined. According to the binding/activation model with two different allosteric sites in FFA2R, AZ1729 should not activate neutrophils sensitized with allosteric modulators that bind to the AZ1729 site, whereas Cmp58 should. Eight of allosteric modulators were active when combined with Cmp58, showing that these compounds are recognized by the AZ1729 site and should be classified as allosteric modulators that are functionally "AZ1729-like" allosteric FFA2R modulators. Two compounds classified as being "Cmp58-like" meaning that they activated the cells when combined with AZ1729 and interact with the same receptor site as Cmp58.

To further determine the credibility of our model with two different allosteric binding sites, the response induced was determined when the new "AZ1729 like" and "Cmp58 like" allosteric FFA2R modulators were combined. The results obtained gave full support to the model for activation in which FFA2R has, in addition to the orthosteric binding site, two sites that recognize allosteric modulators. The novel patterns for activation of neutrophils and the biased receptor down-stream signaling mediated by cross-sensitizing allosteric FFA2R modulators, represent a new mechanism for the control of receptor functions.

7. Termination of signaling and receptor desensitization

Similar to a double-edged sword, neutrophils constitute a highly effective first line of defense against most invading pathogens, but they also have the potential to cause substantial tissue damage. Accordingly, appropriate mechanisms for termination of receptor signals are important. Neutrophil receptors belonging to the family of GPCRs exert proinflammatory responses that must be terminated to allow the signals required to resolve the inflammatory reaction. Accordingly, activated receptors become homologous desensitization by time, and by that they are nonresponsive to the activating agonist and to other agonists recognized by the same receptor. Desensitized receptors may, however, be reactivated/transactivated by signals generated by other receptors, a topic that will be addressed below.

The desensitization process is for many receptors linked to kinase mediated phosphorylation of the cytosolic domains of the receptor that participate in G-protein binding and/or recruitment of β-arrestin (224).

β-arrestins are multipurpose adapter proteins having the ability to interact and form complexes with most GPCRs. This receptor-arrestin interaction/recruitment is part of the dynamic receptor activation process initiated when the activated receptors have been phosphorylated by G protein coupled receptor kinases (GRKs) (225). For many GPCRs, β-arrestin has been reported to be the main regulator of homologous desensitization and sequestration of the desensitized receptors. The receptor-bound β-arrestin uncouple and block G protein binding to the receptor. This uncoupling is achieved through steric hindrance of G protein interaction and the functional outcome is a homologous receptor desensitization. The bound β-arrestin also targets GPCRs to clathrin coated pits for endocytosis (226). A visual representation of the role of β-arrestin in the desensitization process is shown (**Fig 10**). In short, the waning of GPCR signaling in the continued presence of agonist is accomplished by a coordinated series of events that are typically considered as three distinct processes: receptor desensitization, sequestration, and downregulation.

Moreover, the recruited β-arrestin proteins might mediate novel GPCR signals down-stream of the activated/"desensitized" receptor. As recently shown/suggested the receptor recruited β-arrestin interacts directly with several different proteins involved in the transduction of signals, such as Src family kinases and components of the ERK1/2 and JNK3 MAP kinase cascades (227). These proteins,

when recruited by β-arrestin coupled to a GPCR, can confer distinct enzymatic receptor dependent activities that generate signals that are important for the regulation of cell functions.

The role of β-arrestins in the processing of neutrophil GPCRs is, however, more complex. Signaling induced by FPR1 and FPR2 specific agonists includes a rapid and transiently activation of the ERK pathway, suggested to be downstream of β-arrestin. The ERK pathway is, however, also activated by FPR agonist that lack the ability to recruit β-arrestin (Paper D). These results suggest that the ERK pathway is activated downstream of the G protein rather than downstream of β-arrestin. Another possibility is that activation the ERK pathway occurs independent of a G protein as well as of β-arrestin. Signaling induced by FPR agonist that lack the ability to recruit β-arrestin is still rapidly terminated and the receptors are homologously desensitized (Paper D, (228). These results clearly show that in neutrophils, there is no direct link between β-arrestin recruitment and homologous desensitization. The direct link between β-arrestin and activation of the ERK pathway is also missing in neutrophils. However there seem to be a clear link between β-arrestin signaling and chemotaxis. Recently published data show that β-arrestin is required for cell migration mediated through FPR1 in neutrophil-like HL-60 cells and that FPR1/FPR2 agonists that do not induce β-arrestin recruitment also lack chemotactic activity (228-230).

Since neutrophil signaling/responses are fairly rapidly terminated also when the activating agonist is unable to recruit β-arrestin this suggests that neutrophils have alternative mechanisms for the termination of signaling and receptor desensitization (228, 230) (88, 231, 232). The actin cytoskeleton is known for its importance for active shape changes in cells and for cell motility/migration (233). Yet it is evident that the cytoskeleton is involved also in other functions such as changes in receptor affinity, apoptosis, and receptor desensitization. Accordingly, actin on the cytosolic side of the plasma membrane (in its polymerized form and together with different actin binding proteins linked to the cytoskeleton) has been shown to interact/associate with activated receptors and by that be a part of the process that terminates FPR signaling (88, 234). This interaction/binding physically separates the G protein from the activated receptor and by that, signaling is terminated and the receptors becomes desensitized. The functional effect is reciprocal, meaning that that receptor signaling both regulates cytoskeleton dependent functions and is regulated by the cytoskeleton. The role of the actin cytoskeleton in GPCR signaling is apparent from experiment involving drugs that inhibit the actin polymerization and disturb the integrity of the cytoskeleton. (230, 235) Paper C)

There are also other mechanisms by which receptors could be desensitized. Heterologous receptor desensitization is one such mechanism, that is part of a neutrophil receptor hierarchy. This is illustrated by the fact that activated FPRs desensitizes also CXCR1/CXCR2, the receptors for IL8 (PAPER D). This desensitization of CXCRs is achieved without involvement of IL8 and has been shown to be part of a receptor hierarchy by which neutrophils are able to prioritize end-target chemoattractants over those regarded as intermediate (232).

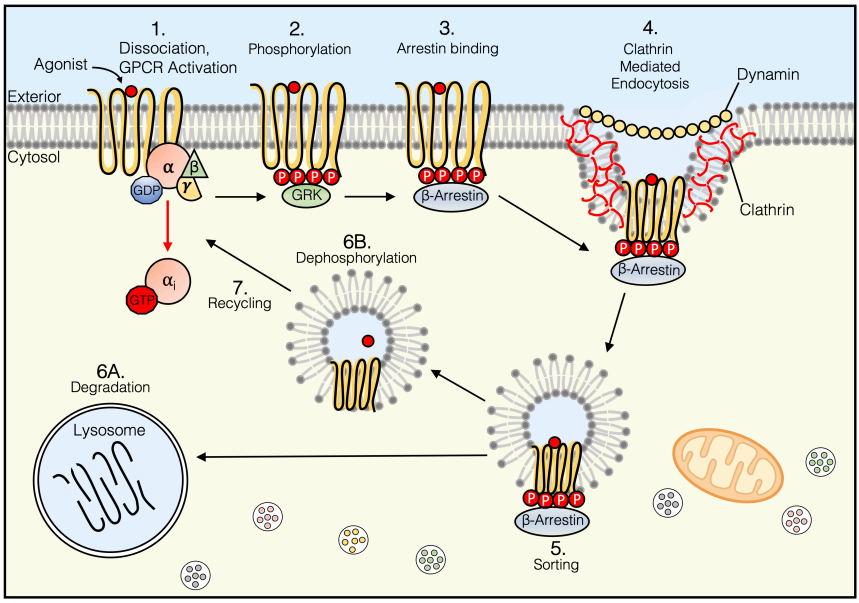

Figure 10 Role of β-arrestins in homologous GPCR desensitization. *Once an agonist (red dot) has induced an intracellular signaling event starting with the activation and dissociation of the heterotrimeric G-protein* ***(1)*** *G-protein-coupled receptor kinases (GRKs) initiate the homologous desensitization process. This is initiated by a phosphorylation of activated receptors* ***(2)****. β-arrestin then binds the phosphorylated receptors* ***(3)****. This binding leads to a termination of signaling, and this is achieved through an uncoupling and hindrance of the trimeric G-protein to interact with the receptor. The β-arrestin also act as an adapter protein that mediates binding to components of the clathrin endocytic machinery including clathrin and dynamin. The sequestration and receptor dependent endocytosis is performed via clathrin-coated pits* ***(4)****. Once internalized, the receptor-containing endosomes are sorted* ***(5)****. The receptor will either be degraded in the lysosome* ***(6A)*** *or be dephosphorylated to be free of bound β-arrestin* ***(6B)*** *and be recycled back to the plasma membrane* ***(7)****.*

7.1 Receptor cross-talk in neutrophils

There are a couple of novel mechanisms by which some neutrophil GPCRs can be activated and activate other receptors, respectively. One such mechanism was disclosed when it was shown that desensitized FPRs were reactivated by signals generated by the ATP receptor $P2Y_2R$ and the receptor for PAF (platelet activating factor) ((118) Paper D). The FPR reactivating signals generated by the ATP and PAF receptors are formed downstream of the G protein coupled to $P2Y_2R$/PAFR, suggesting that the FPR reactivating signal interact with the cytosolic parts of the FPRs. Accordingly, the response induced by ATP in neutrophils with desensitized FPRs is inhibited not only by FPR specific antagonist but also by a $P2Y_2R$ antagonist and by specific inhibitors of $G\alpha_q$ containing G proteins coupled to $P2Y_2R$. Moreover, Calyculin A inhibits the cross-talk reactivation of desensitized FPR1 when induced by the PAFR, implying a regulatory role of the serine/threonine phosphatases inhibited by Calyculin A (118). In contrast to the direct response induced by FPR agonists, the reactivation of desensitized FPRs is functional selective. This is illustrated by the fact that $P2Y_2R$ activates the ROS generating NADPH-oxidase down-stream of the reactivated FPRs, but this is achieved without any FPR dependent rise in $[Ca^{2+}]_i$ (236).

7.2 Transactivation/receptor cross-talk of allosteric modulated FFA2R – a novel GPCR activation mechanism.

The generally accepted concept of how allosteric modulators work is challenged by the data presented in Paper I; the challenged concept states that when a PAM binds the allosteric site, it transfers the receptor to a state in which the affinity of the orthosteric agonist is increased or/and the ability to activate the receptor, i.e. the efficacy, is increased (122, 123). This means that, a receptor specific PAM should affect solely the response induced by orthosteric agonists that are specific for the same (modulated) receptor. In Paper I, we show that allosteric modulation of FFA2R, turns not only specific FFA2R agonists into potent activators of the neutrophil NADPH oxidase, but the response induced also by ATP, a mitochondrial produced danger molecule, is greatly increased. The receptor for ATP is $P2Y_2R$, and the increased response is achieved through a novel receptor cross-talk mechanism between this receptor and the allosterically modulated FFA2R. The allosteric FFA2R modulator Cmp58 turned ATP, an otherwise inert NADPH-oxidase activating agonist, into a potent activator of the superoxide generating

oxidase system. A specific orthosteric FFA2R agonist could not replace the allosteric FFA2R modulator in the receptor cross talk with $P2Y_2R$. The $P2Y_2R$ induced activation of FFA2R is disclosed by the effects of a selection of inhibitors/antagonists. Accordingly, the ATP induced response in neutrophils with their FFA2Rs allosterically modulated is inhibited both by a $P2Y_2R$ specific antagonist and by an inhibition of the $G\alpha_q$ containing G protein coupled to P2Y2R. This was also true irrespectively if Cmp58 or AZ1729 was used to modulate FFA2R. More important, the response induced by ATP was fully inhibited also by antagonist specific for FFA2R. The FFA2R dependent generation/secretion of O_2^- induced by signals generated down-stream of $P2Y_2R$, was achieved without the participation of any orthosteric agonist for FFA2R. The FFA2R activating signals are generated down-stream of the G protein coupled to P2Y2R. Furthermore, the ATP-induced signal leading to a transient rise in intracellular Ca^{2+} was not changed by the allosteric FFA2R modulator, suggesting a functional selective (biased) signaling shift mediated by Cmp58 (Paper I).

Classical allosteric modulation is characterized by a reciprocity (237), which means that that the order by which the ligands are added can be reversed. This mean that the non-activating PAM increases the response induced by the orthosteric agonist but in addition, a non-activating concentration of the orthosteric agonist turns the PAM to an activating ligand. In contrast, however, for receptor transactivation, the order by which ATP and the allosteric FFA2R modulator is added to the cells, cannot be reversed.

The mechanism for the receptor transactivation of the allosterically modulated FFA2R, by signals generated by $P2Y_2R$ has noticeable similarities with the receptor cross-talk/transactivation of desensitized FPRs. This is illustrated for example by the facts, i) that responses are inhibited by the respective antagonists, CATPB for the allosterically modulated FFA2R and PBP10 for the desensitized FPR2 and, ii) the response dependent on FFA2R and FPR2, respectively, is, when mediated by the $G\alpha_q$ coupled ATP receptor, abolished by an inhibitor specific for this G protein. The desensitized FPRs as well as the allosterically modulated FFA2Rs are obviously both activated by signals generated down-stream of the $G\alpha_q$ containing G protein coupled to $P2Y_2R$ and these activate the receptors from the cytosolic side of the plasma membrane, (Paper I, II, IV). It should also be noticed that $P2Y_2R$ is not the only receptor able to transactivate allosterically modulated FFA2R. Signals that transactivate FFA2R are generated also by the FPRs (Paper C, D) and the receptor for platelet activating factor (to be published).

In summary, the data presented clearly show that the model for receptor selectivity coupled to the allosteric modulation concept must be abandoned; it is clear from the data presented that an allosteric modulator may affect not only the response induced by agonists that interacts with the same receptor.

7.3 The interaction pattern for FFA2R ligands is even more complex than originally anticipated

Despite the fact that the binding characteristic of the two FFA2R specific antagonists GLPG0974 and CATPB, differ at the molecular level, they both have been shown to be recognized specifically by the orthosteric binding site in FFA2R (221, 238). The affinity for FFA2R is also comparable or even indistinguishable for the two antagonists (221, 239)

The effects of the FFA2R specific antagonists on neutrophil FFA2R-activation has been investigated (Paper IV) when the activation was induced by the two PAMs/co-allosteric agonists Cmp58 and AZ1729. It is clear from the data presented that the two antagonists inhibit the interaction of the PAMs with their respective allosteric FFA2R site. This was determined by the inhibition of the novel receptor transactivation induced by ATP in the presence of either of the two PAMs. This inhibition was used to determine the allosteric site-over-lap of the two antagonists.

As previously revealed, no neutrophil activation was induced by AZ1729 or Cmp58 alone, but together they activate neutrophils and the biased activation pattern earlier described was evident. The superoxide generating NADPH-oxidase was activated without any concomitant transient rise in $[Ca^{2+}]_i$. When the concentration of the two co-activating PAMs was reduced, CATPB inhibited also this response, however, no such effect was obtained with GLPG0974. On the contrary, GLPG0974 acted as a positive modulator that increased the potency but not the efficacy of the response. At the signaling level, GLPG0974 altered signaling downstream of FFA2R activated by the co-agonistic PAMs. The biased signaling pattern was changed by GLPG0974 and the activation of the NADPH-oxidase induced by the two co-agonistic PAMs was in the presence of GLPG0974 accompanied by a rise in $[Ca^{2+}]_i$. This effect was selectively reciprocal – i.e., the non-activating GLPG0974 triggered a rise in $[Ca^{2+}]_2$, in neutrophils first activated/sensitized with the two PAMs, but no corresponding activation of the oxidase was induced by GLPG0974. In addition, the response by GLPG0974 was inhibited by propionate but also by the other antagonist CATPB, suggesting that the response

is inhibited both when the receptor is desensitized following an activation by an orthosteric agonist and in the presence of a competing FFA2R antagonist. In summary, the results obtained with GLPG0974, an FFA2R ligand presented as an orthosteric antagonist, clearly show that in addition to be an antagonist, this ligand displays also agonistic as well as positive FFA2R modulating effects on the FFA2R mediated response.

8. Future Perspective

Neutrophils have for long been recognized as a leukocyte population of fundamental importance for protection against infections. Out of the many GPCRs expressed by neutrophils, FFA2R, one of the short chain free fatty acid receptors, has been suggested to be an important target for controlling inflammatory reactivity. The agonistic SCFAs have the ability to affect regulatory mechanisms of importance for the immune system and their presence/absence has in model systems been shown to be associated with a vast number of inflammatory disease conditions. Allosteric receptor modulators have during recent years been adopted as innovative tool compounds. They are able to target GPCRs with precision, and the identification of defined receptor sites that recognize allosteric FFA2R modulators has opened new opportunities to target and potentially treat diseases involving these receptors. In addition, the fact that the receptor has multiple binding sites for allosteric mediators extends the possibilities of how the complex GPCR pharmacology can be allosterically modulated, and potentially fine-tuned by ligands that specifically target these allosteric receptor sites. The two distinct allosteric sites on FFA2R discovered and discussed in this thesis show just how multifaceted these signaling events can be. The allosteric receptor modulation area of research has great future opportunities, however, with opportunities there are also challenges.

One of the initial benefits regarded important for both amplifying and inhibiting allosteric receptor modulators was that they opened for the ability to affect the functional outcome to endogenous receptor ligands. This could be achieved without any effects of the receptor signaling profile. That is, allosteric ligands rarely signal on their own which could be an advantage in a physiological setting; they, thus, maintain the physiological tone, timing, and location of the natural receptor stimulus. Another advantage distinguishing the research today on allosteric targeting from the classical approach using orthosteric ligands for drug development is the promotion of more complex pharmacology including functional selective/biased signaling. Accordingly, the allosterically modulated FFA2R has the ability to adopt conformations that trigger distinct biased signaling pathways. This is clearly illustrated by the selective desensitization of the propionate response when this orthosteric agonist was combined with an allosteric FFA2R modulator. More important, when two non-activating allosteric FFA2R modulators (Cmp58 and AZ1729) are combined they activate the oxidase activity without any involvement of the classical GPCR pathway, a transient rise in levels of free intracellular calcium ions. Activation of distinct signaling pathways could be exploited as it is known that certain signaling events but not others are associated with distinct physiological outcomes (240), and future work has to determine the precise

mechanisms for how the different signaling pathways can be regulated. A new class of bitopic GPCR ligands has also been described (241), that are made up of an orthosteric ligand coupled to an allosteric one. There are also other ways of engaging both an allosteric- and the orthosteric receptor site. A new concept of functional modulation was described for the allosteric FFA2R agonist 4-CMTB which activates and engages two distinct FFA2 sites namely the orthosteric site and one of the allosteric sites in a step wise mechanism (242, 243), and future constructs may also combine two allosteric receptor modulators that are recognized by distinct different allosteric sites.

It is also clear from the data presented in the thesis that the generally accepted models have to be reconsidered, for how desensitized GPCRs are processed and for the precise receptor specificity of allosteric GPCR modulators. The model for how activated GPCRs is processed states, that the receptor by time after activation is transferred to a non-signaling state that lacks the ability to return to a signaling state first after endocytic uptake and recirculation to the plasma membrane. However, a novel receptor cross-talk process can reactivate desensitized receptors, a reactivation mechanism initially studied with desensitized neutrophil FPRs. Regarding receptor selectivity, the generally accepted model for the selectivity of allosteric GPCR modulator states that such a modulator affects the activities induced solely by orthosteric agonists that are specifically recognized by the receptor that also recognizes the allosteric modulator. The fact that the FFA2R PAMs studied, modulates the neutrophil response also to agonists recognized by other neutrophil GPCRs, clearly show the allosteric modulatory effect is not restricted to agonist recognized by the modulated receptor. These findings show that an additional layer of complexity must be included in the models for modulation and receptor activation. Future work could hopefully define the precise transactivating signals generated by one GPCR and activate another GPCR family member, and also determine if this type of modulation/activation mechanisms are unique for the neutrophil pattern recognition receptors.

In spite of the aforesaid advantages of targeting allosteric modulation in drug development, also the discovery and development of allosteric GPCR drugs have major challenges. One screening obstacle is obvious. When performing high-throughput screening (HTS) of large compound libraries in functional assay systems to identify allosteric modulators, the associated use of the right co-ligand is required (244). The background to this is the allosteric term probe dependency, which dictates that the allosteric modulator affects the binding/signaling properties differently depending which orthosteric ligands are used. This must be taken into account in any screening campaign and the natural ligand is an apparent option

since this is the endogenous orthosteric ligand in the biological setting. However, you may also want to include a ligand with unique activating properties. Another difficulty using structure-based approaches to identify allosteric ligands is associated with the limited amount of knowledge available on physiologically relevant structural information of the GPCRs (245). Future advances in this field especially in the description of structural flexibility, will have to show whether this leads to useful information for the design of GPCR ligands, especially for the structurally less conserved domains of allosteric sites (196).

When more structural information for the neutrophil pattern recognition receptors such as the FFARs have been generated, this knowledge will hopefully make it possible to link structure and structural changes, to signaling and functional outcome in more detail. Optimistically this will provide conformational insights into activation induced both by conventional and biased signaling ligands when these interact with naïve receptors as well as with those that are allosterically modulated. If achieved, we get one step closer of understanding the mechanism of desensitization and how a desensitized receptor can be reactivated. Such knowledge will be of great importance for the development of new treatments as well as of preventive strategies for inflammatory or autoimmune diseases. Even though GPCRs are a highly valuable class of receptors for drug discovery that already have been in focus for many years in screening for and characterization of drug candidates, the allosteric receptor modulation concept has become an attractive approach that can provide innovative mechanisms of action for GPCRs. This has broadened the innovative level for a given target, but more important, the tools available for future work in medicinal cell physiology/biochemistry/molecular biology have increased very much. Applying the allosteric modulation concept will most probably be included as novel and viable approaches for future discoveries related to the role of pattern recognition receptors in innate immunity and regulation of inflammation.

Acknowledgement

When I first came to the Phagocyte research group, I thought I came here for a one-year master project. That project turned out to become over five years and has been a life changing journey for me in more ways than one.

This journey would not be possible without my main supervisor **Claes Dahlgren**. I have told both you and everyone that I met during this experience that I would never have done a PhD if you were not my supervisor. You been incredibly patient with me and kept me grounded so that I have had time to grow both as a person and a researcher. You are also my inspiration in research, and I love all the scientific discussions we have had during my time. I have been blessed with a supervisor that always have the door open for advice which has been essential for the accomplishment that I have made during my PhD. I want to thank you from the bottom of my heart that I have been able to have this experience with you Claes and this journey will always have a very special place in my heart.

I also want to thank my co supervisor **Huamei**. You have an overflowing energy and excitement for research that is highly contagious. You have been an excellent leader in the group which has opened for amazing collaborations and experiences that I will never forget.

I also want to thank all my old and new colleagues at the lab **André**, **Mike**, **Jonas**, **Yanling, Lena and Martina**. Thanks for a wonderful time together and I have learned a lot from you.

A special thanks I want to give to **Linda**. Without you the lab would fall apart, and I have always enjoyed working together with you in the lab!

I also want to thank other amazing people that I have meet during my PhD such as **Ebba**, **Caroline**, **Alessandro**, **Christina**, **Jauquline**, **Bea**, **Agnes**, **Felix**, **Karin**, **Johan**, **Jonas**, **Jonathan**, **Timothy**, **Anders**, **Zhicheng**, **Vignesh**, **Moa**, **Majd** and **so many more,** thanks for the great time!

Thanks to external collaborators that made my PhD time more comprehensive, **Henrik**, **Kenneth**, **Michel**, **Shane**, **Tudor** and **Trond**.

Thanks to all students that I have had the pleasure to supervise during my PhD. Teaching you has been rewarding.

Thanks to all other members of the department of Rheumatology I had the privilege to get to know.

I also want to thank my family and friends that always have been supportive of me. I especially want to thank my brother, **Sebastian**. You have been my rock in times of worry, and I know I can always count on you.

Thank you, **William** for being an awesome friend and classmate. I would never have done a PhD if you did not encourage me to do so.

Lastly, I want to thank my fiancé, **Lucy**. Had it not been for this journey I would probably never have met you, which to say the least, would have lifechanging effects. I have grown as a researcher during our years together and having you along the way has made every bit enjoyable. Your determination to complete your goals in life had made me work harder but also enjoy the process of learning along the way. Thank you for not only for making me happy but finally in my life giving me a place that I can truly call home.

References

1. Janeway CA, Medzhitov R. Innate Immune Recognition. Annual Review of Immunology. 2002;20(1):197-216.
2. Weber F. Innate Immunity: Introduction. Encyclopedia of Virology. 2008:111-7.
3. Mogensen TH. Pathogen recognition and inflammatory signaling in innate immune defenses. Clin Microbiol Rev. 2009;22(2):240-73.
4. Nguyen AV, Soulika AM. The Dynamics of the Skin's Immune System. International journal of molecular sciences. 2019;20(8):1811.
5. Rosales C. Neutrophil: A Cell with Many Roles in Inflammation or Several Cell Types? Frontiers in Physiology. 2018;9.
6. Pasparakis M, Haase I, Nestle FO. Mechanisms regulating skin immunity and inflammation. Nature Reviews Immunology. 2014;14(5):289-301.
7. Ansar W, Ghosh S. Inflammation and Inflammatory Diseases, Markers, and Mediators: Role of CRP in Some Inflammatory Diseases. Biology of C Reactive Protein in Health and Disease. 2016:67-107.
8. Kany S, Vollrath JT, Relja B. Cytokines in Inflammatory Disease. International journal of molecular sciences. 2019;20(23):6008.
9. Wen Y, Lambrecht J, Ju C, Tacke F. Hepatic macrophages in liver homeostasis and diseases-diversity, plasticity and therapeutic opportunities. Cellular & Molecular Immunology. 2021;18(1):45-56.
10. Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. Nature reviews Immunology. 2013;13(3):159-75.
11. Salvermoser M, Begandt D, Alon R, Walzog B. Nuclear Deformation During Neutrophil Migration at Sites of Inflammation. Frontiers in immunology. 2018;9:2680-.
12. Faurschou M, Borregaard N. Neutrophil granules and secretory vesicles in inflammation. Microbes and Infection. 2003;5(14):1317-27.
13. Segal AW. How Neutrophils Kill Microbes. Annual review of immunology. 2005;23:197-223.
14. Kruger P, Saffarzadeh M, Weber ANR, Rieber N, Radsak M, von Bernuth H, et al. Neutrophils: Between Host Defence, Immune Modulation, and Tissue Injury. PLOS Pathogens. 2015;11(3):e1004651.
15. Wright HL, Moots RJ, Bucknall RC, Edwards SW. Neutrophil function in inflammation and inflammatory diseases. Rheumatology (Oxford, England). 2010;49(9):1618-31.
16. Sundqvist M. Neutrophil function in health and disease. Department of Rheumatology and Inflammation Research: University of Gothenburg; 2013.
17. Hidalgo A, Chilvers ER, Summers C, Koenderman L. The Neutrophil Life Cycle. Trends Immunol. 2019;40(7):584-97.
18. Metzemaekers M, Gouwy M, Proost P. Neutrophil chemoattractant receptors in health and disease: double-edged swords. Cell Mol Immunol. 2020;17(5):433-50.

19. Hurst JK. What really happens in the neutrophil phagosome? Free radical biology & medicine. 2012;53(3):508-20.
20. Bjornsdottir H, Welin A, Michaelsson E, Osla V, Berg S, Christenson K, et al. Neutrophil NET formation is regulated from the inside by myeloperoxidase-processed reactive oxygen species. Free radical biology & medicine. 2015;89:1024-35.
21. Fuchs TA, Abed U, Goosmann C, Hurwitz R, Schulze I, Wahn V, et al. Novel cell death program leads to neutrophil extracellular traps. J Cell Biol. 2007;176(2):231-41.
22. Kennedy AD, DeLeo FR. Neutrophil apoptosis and the resolution of infection. Immunologic Research. 2009;43(1):25-61.
23. Kobayashi SD, Malachowa N, DeLeo FR. Influence of Microbes on Neutrophil Life and Death. Frontiers in cellular and infection microbiology. 2017;7:159-.
24. Bratton DL, Henson PM. Neutrophil clearance: when the party is over, clean-up begins. Trends in immunology. 2011;32(8):350-7.
25. Lehman HK, Segal BH. The role of neutrophils in host defense and disease. Journal of Allergy and Clinical Immunology. 2020;145(6):1535-44.
26. Summers C, Rankin SM, Condliffe AM, Singh N, Peters AM, Chilvers ER. Neutrophil kinetics in health and disease. Trends in immunology. 2010;31(8):318-24.
27. Simon SI, Kim M-H. A day (or 5) in a neutrophil's life. Blood. 2010;116(4):511-2.
28. Fox S, Leitch AE, Duffin R, Haslett C, Rossi AG. Neutrophil apoptosis: relevance to the innate immune response and inflammatory disease. Journal of innate immunity. 2010;2(3):216-27.
29. Sheshachalam A, Srivastava N, Mitchell T, Lacy P, Eitzen G. Granule Protein Processing and Regulated Secretion in Neutrophils. Frontiers in Immunology. 2014;5.
30. Cassatella MA, Östberg NK, Tamassia N, Soehnlein O. Biological Roles of Neutrophil-Derived Granule Proteins and Cytokines. Trends in Immunology. 2019;40(7):648-64.
31. John E. Bennett RDMJB. Mandell, Douglas, and Bennetts principles and practice of infectious diseases: Eighth edition. Philadelphia, PA : Elsevier/Saunders, [2015]; 2015.
32. Kjeldsen L, Bainton DF, Sengel⊘v H, Borregaard N. Structural and Functional Heterogeneity Among Peroxidase-Negative Granules in Human Neutrophils: Identification of a Distinct Gelatinase-Containing Granule Subset by Combined Immunocytochemistry and Subcellular Fractionation. Blood. 1993;82(10):3183-91.
33. Mollinedo F, Schneider DL. Subcellular localization of cytochrome b and ubiquinone in a tertiary granule of resting human neutrophils and evidence for a proton pump ATPase. The Journal of biological chemistry. 1984;259(11):7143-50.

34. Breton-Gorius J, Mason DY, Buriot D, Vilde JL, Griscelli C. Lactoferrin deficiency as a consequence of a lack of specific granules in neutrophils from a patient with recurrent infections. Detection by immunoperoxidase staining for lactoferrin and cytochemical electron microscopy. Am J Pathol. 1980;99(2):413-28.
35. Rørvig S, Østergaard O, Heegaard NH, Borregaard N. Proteome profiling of human neutrophil granule subsets, secretory vesicles, and cell membrane: correlation with transcriptome profiling of neutrophil precursors. J Leukoc Biol. 2013;94(4):711-21.
36. Uriarte SM, Powell DW, Luerman GC, Merchant ML, Cummins TD, Jog NR, et al. Comparison of proteins expressed on secretory vesicle membranes and plasma membranes of human neutrophils. J Immunol. 2008;180(8):5575-81.
37. Lee WL, Harrison RE, Grinstein S. Phagocytosis by neutrophils. Microbes Infect. 2003;5(14):1299-306.
38. Kobayashi S, Voyich J, Burlak C, DeLeo F. Neutrophils in the innate immune response. Archivum immunologiae et therapiae experimentalis. 2004;53:505-17.
39. DiNubile MJ, Huang S. High concentrations of phosphatidylinositol-4,5-bisphosphate may promote actin filament growth by three potential mechanisms: Inhibiting capping by neutrophil lysates, severing actin filaments and removing capping protein-β2 from barbed ends1This work was supported by Cooper Faculty Grant SPF 2821-94.1. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research. 1997;1358(3):261-78.
40. Lee WL, Harrison RE, Grinstein S. Phagocytosis by neutrophils. Microbes and Infection. 2003;5(14):1299-306.
41. Jaumouillé V, Waterman CM. Physical Constraints and Forces Involved in Phagocytosis. Frontiers in Immunology. 2020;11.
42. Nguyen GT, Green ER, Mecsas J. Neutrophils to the ROScue: Mechanisms of NADPH Oxidase Activation and Bacterial Resistance. Frontiers in cellular and infection microbiology. 2017;7:373-.
43. Katzenmeyer KN, Szott LM, Bryers JD. Artificial opsonin enhances bacterial phagocytosis, oxidative burst and chemokine production by human neutrophils. Pathogens and Disease. 2017;75(6).
44. Roos D. Chronic granulomatous disease. Br Med Bull. 2016;118(1):50-63.
45. Rada B, Leto T. Oxidative innate immune defenses by Nox/Duox Family NADPH oxidases. Contributions to microbiology. 2008;15:164-87.
46. Patiño PJ, Perez JE, Lopez JA, Condino-Neto A, Grumach AS, Botero JH, et al. Molecular analysis of chronic granulomatous disease caused by defects in gp91-phox. Hum Mutat. 1999;13(1):29-37.
47. Dahlgren C, Björnsdottir H, Sundqvist M, Christenson K, Bylund J. Measurement of Respiratory Burst Products, Released or Retained, During Activation of Professional Phagocytes. Methods Mol Biol. 2020;2087:301-24.
48. Dahlgren C, Karlsson A, Bylund J. Intracellular Neutrophil Oxidants: From Laboratory Curiosity to Clinical Reality. The Journal of Immunology. 2019;202(11):3127-34.

49. Quinn Mark T, Ammons Mary Cloud B, DeLeo Frank R. The expanding role of NADPH oxidases in health and disease: no longer just agents of death and destruction. Clinical Science. 2006;111(1):1-20.
50. Tiwari M. Glucose 6 phosphatase dehydrogenase (G6PD) and neurodegenerative disorders: Mapping diagnostic and therapeutic opportunities. Genes Dis. 2017;4(4):196-203.
51. Brennan ML, Anderson MM, Shih DM, Qu XD, Wang X, Mehta AC, et al. Increased atherosclerosis in myeloperoxidase-deficient mice. J Clin Invest. 2001;107(4):419-30.
52. Aratani Y, Kura F, Watanabe H, Akagawa H, Takano Y, Suzuki K, et al. Differential Host Susceptibility to Pulmonary Infections with Bacteria and Fungi in Mice Deficient in Myeloperoxidase. The Journal of Infectious Diseases. 2000;182(4):1276-9.
53. Klebanoff SJ, Kettle AJ, Rosen H, Winterbourn CC, Nauseef WM. Myeloperoxidase: a front-line defender against phagocytosed microorganisms. Journal of leukocyte biology. 2013;93(2):185-98.
54. Mittal M, Siddiqui MR, Tran K, Reddy SP, Malik AB. Reactive oxygen species in inflammation and tissue injury. Antioxidants & redox signaling. 2014;20(7):1126-67.
55. Liou G-Y, Storz P. Reactive oxygen species in cancer. Free radical research. 2010;44(5):10.3109/10715761003667554.
56. Sareila O, Kelkka T, Pizzolla A, Hultqvist M, Holmdahl R. NOX2 Complex–Derived ROS as Immune Regulators. Antioxidants & Redox Signaling. 2010;15(8):2197-208.
57. Bylund J, Campsall PA, Ma RC, Conway B-AD, Speert DP. Burkholderia cenocepacia Induces Neutrophil Necrosis in Chronic Granulomatous Disease. The Journal of Immunology. 2005;174(6):3562.
58. Balloy V, Chignard M. The innate immune response to Aspergillus fumigatus. Microbes Infect. 2009;11(12):919-27.
59. Finkel T. Signal transduction by reactive oxygen species. J Cell Biol. 2011;194(1):7-15.
60. D'Autréaux B, Toledano MB. ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. Nature Reviews Molecular Cell Biology. 2007;8(10):813-24.
61. Holmdahl R, Sareila O, Olsson LM, Bäckdahl L, Wing K. Ncf1 polymorphism reveals oxidative regulation of autoimmune chronic inflammation. Immunol Rev. 2016;269(1):228-47.
62. Chelombitko MA. Role of Reactive Oxygen Species in Inflammation: A Minireview. Moscow University Biological Sciences Bulletin. 2018;73(4):199-202.
63. Hultqvist M, Olofsson P, Holmberg J, Bäckström BT, Tordsson J, Holmdahl R. Enhanced autoimmunity, arthritis, and encephalomyelitis in mice with a reduced oxidative burst due to a mutation in the Ncf1 gene. Proceedings of the National Academy of Sciences of the United States of America. 2004;101(34):12646-51.

64. Schauer C, Janko C, Munoz LE, Zhao Y, Kienhöfer D, Frey B, et al. Aggregated neutrophil extracellular traps limit inflammation by degrading cytokines and chemokines. Nat Med. 2014;20(5):511-7.
65. Jaeschke H. Reactive oxygen and mechanisms of inflammatory liver injury: Present concepts. J Gastroenterol Hepatol. 2011;26 Suppl 1:173-9.
66. Karabchevsky A, Mosayyebi A, Kavokin AV. Tuning the chemiluminescence of a luminol flow using plasmonic nanoparticles. Light: Science & Applications. 2016;5(11):e16164-e.
67. Gross S, Gammon ST, Moss BL, Rauch D, Harding J, Heinecke JW, et al. Bioluminescence imaging of myeloperoxidase activity in vivo. Nat Med. 2009;15(4):455-61.
68. Lundqvist H, Dahlgren C. Isoluminol-enhanced chemiluminescence: a sensitive method to study the release of superoxide anion from human neutrophils. Free radical biology & medicine. 1996;20(6):785-92.
69. Dahlgren C, Karlsson A. Respiratory burst in human neutrophils. J Immunol Methods. 1999;232(1-2):3-14.
70. O'Hara AM, Shanahan F. The gut flora as a forgotten organ. EMBO Rep. 2006;7(7):688-93.
71. Dalile B, Van Oudenhove L, Vervliet B, Verbeke K. The role of short-chain fatty acids in microbiota-gut-brain communication. Nat Rev Gastroenterol Hepatol. 2019;16(8):461-78.
72. Parada Venegas D, De la Fuente MK, Landskron G, González MJ, Quera R, Dijkstra G, et al. Short Chain Fatty Acids (SCFAs)-Mediated Gut Epithelial and Immune Regulation and Its Relevance for Inflammatory Bowel Diseases. Frontiers in Immunology. 2019;10.
73. Stumpff F. A look at the smelly side of physiology: transport of short chain fatty acids. Pflugers Arch. 2018;470(4):571-98.
74. Bergman EN. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiol Rev. 1990;70(2):567-90.
75. Bloemen JG, Venema K, van de Poll MC, Olde Damink SW, Buurman WA, Dejong CH. Short chain fatty acids exchange across the gut and liver in humans measured at surgery. Clinical Nutrition. 2009;28(6):657-61.
76. Silva YP, Bernardi A, Frozza RL. The Role of Short-Chain Fatty Acids From Gut Microbiota in Gut-Brain Communication. Frontiers in endocrinology. 2020;11:25-.
77. Kimura I, Inoue D, Hirano K, Tsujimoto G. The SCFA Receptor GPR43 and Energy Metabolism. Frontiers in endocrinology. 2014;5:85.
78. Vinolo MAR, Rodrigues HG, Nachbar RT, Curi R. Regulation of inflammation by short chain fatty acids. Nutrients. 2011;3(10):858-76.
79. Kumar V, Sharma A. Neutrophils: Cinderella of innate immune system. International Immunopharmacology. 2010;10(11):1325-34.
80. Grivennikov SI, Tumanov AV, Liepinsh DJ, Kruglov AA, Marakusha BI, Shakhov AN, et al. Distinct and Nonredundant In Vivo Functions of TNF Produced by T Cells and Macrophages/Neutrophils: Protective and Deleterious Effects. Immunity. 2005;22(1):93-104.

81. Björkman L, Mårtensson J, Winther M, Gabl M, Holdfeldt A, Uhrbom M, et al. The Neutrophil Response Induced by an Agonist for Free Fatty Acid Receptor 2 (GPR43) Is Primed by Tumor Necrosis Factor Alpha and by Receptor Uncoupling from the Cytoskeleton but Attenuated by Tissue Recruitment. Molecular and Cellular Biology. 2016;36(20):2583-95.
82. Condliffe AM, Kitchen E, Chilvers ER. Neutrophil priming: pathophysiological consequences and underlying mechanisms. Clin Sci (Lond). 1998;94(5):461-71.
83. Miralda I, Uriarte SM, McLeish KR. Multiple Phenotypic Changes Define Neutrophil Priming. Frontiers in Cellular and Infection Microbiology. 2017;7.
84. Potera RM, Jensen MJ, Hilkin BM, South GK, Hook JS, Gross EA, et al. Neutrophil azurophilic granule exocytosis is primed by TNF-α and partially regulated by NADPH oxidase. Innate Immunity. 2016;22(8):635-46.
85. Almkvist J, Faldt J, Dahlgren C, Leffler H, Karlsson A. Lipopolysaccharide-induced gelatinase granule mobilization primes neutrophils for activation by galectin-3 and formylmethionyl-Leu-Phe. Infect Immun. 2001;69(2):832-7.
86. Thelen M, Rosen A, Nairn AC, Aderem A. Tumor necrosis factor alpha modifies agonist-dependent responses in human neutrophils by inducing the synthesis and myristoylation of a specific protein kinase C substrate. Proceedings of the National Academy of Sciences. 1990;87(15):5603.
87. Schiffmann E, Corcoran BA, Wahl SM. N-formylmethionyl peptides as chemoattractants for leucocytes. Proc Natl Acad Sci U S A. 1975;72(3):1059-62.
88. Dahlgren C, Gabl M, Holdfeldt A, Winther M, Forsman H. Basic characteristics of the neutrophil receptors that recognize formylated peptides, a danger-associated molecular pattern generated by bacteria and mitochondria. Biochem Pharmacol. 2016;114:22-39.
89. Skovbakke S. Modulation of Innate Immunity by Host Defense Petidomimetics. Graduate School of Health and Medical Science University of Coppenhagen: Graduate School of Health and Medical Science University of Coppenhagen; 2016.
90. Migeotte I, Communi D, Parmentier M. Formyl peptide receptors: A promiscuous subfamily of G protein-coupled receptors controlling immune responses. Cytokine Growth Factor Rev. 2006;17(6):501-19.
91. Di Pizio A, Behrens M, Krautwurst D. Beyond the Flavour: The Potential Druggability of Chemosensory G Protein-Coupled Receptors. International journal of molecular sciences. 2019;20(6):1402.
92. Fachi JL, Sécca C, Rodrigues PB, Mato FCPd, Di Luccia B, Felipe JdS, et al. Acetate coordinates neutrophil and ILC3 responses against C. difficile through FFAR2. Journal of Experimental Medicine. 2019;217(3).
93. Sriram K, Insel PA. G Protein-Coupled Receptors as Targets for Approved Drugs: How Many Targets and How Many Drugs? Molecular pharmacology. 2018;93(4):251-8.
94. Rosenbaum DM, Rasmussen SG, Kobilka BK. The structure and function of G-protein-coupled receptors. Nature. 2009;459(7245):356-63.

95. Bylund J, Samuelsson M, Collins LV, Karlsson A. NADPH-oxidase activation in murine neutrophils via formyl peptide receptors. Experimental cell research. 2003;282(2):70-7.
96. Marasco WA, Phan SH, Krutzsch H, Showell HJ, Feltner DE, Nairn R, et al. Purification and identification of formyl-methionyl-leucyl-phenylalanine as the major peptide neutrophil chemotactic factor produced by Escherichia coli. J Biol Chem. 1984;259(9):5430-9.
97. Boulay F, Tardif M, Brouchon L, Vignais P. The human N-formylpeptide receptor. Characterization of two cDNA isolates and evidence for a new subfamily of G-protein-coupled receptors. Biochemistry. 1990;29(50):11123-33.
98. Boulay F, Tardif M, Brouchon L, Vignais P. Synthesis and use of a novel N-formyl peptide derivative to isolate a human N-formyl peptide receptor cDNA. Biochem Biophys Res Commun. 1990;168(3):1103-9.
99. Bao L, Gerard NP, Eddy RL, Jr., Shows TB, Gerard C. Mapping of genes for the human C5a receptor (C5AR), human FMLP receptor (FPR), and two FMLP receptor homologue orphan receptors (FPRH1, FPRH2) to chromosome 19. Genomics. 1992;13(2):437-40.
100. Anderson S, Bankier AT, Barrell BG, de Bruijn MHL, Coulson AR, Drouin J, et al. Sequence and organization of the human mitochondrial genome. Nature. 1981;290:457.
101. Kretschmer D, Gleske AK, Rautenberg M, Wang R, Koberle M, Bohn E, et al. Human formyl peptide receptor 2 senses highly pathogenic Staphylococcus aureus. Cell host & microbe. 2010;7(6):463-73.
102. Rabiet M-J, Huet E, Boulay F. Human mitochondria-derived N-formylated peptides are novel agonists equally active on FPR and FPRL1, while Listeria monocytogenes-derived peptides preferentially activate FPR. European Journal of Immunology. 2005;35(8):2486-95.
103. Rot A, Henderson LE, Sowder R, Leonard EJ. Staphylococcus aureus tetrapeptide with high chemotactic potency and efficacy for human leukocytes. Journal of leukocyte biology. 1989;45(2):114-20.
104. Gabl M, Sundqvist M, Holdfeldt A, Lind S, Martensson J, Christenson K, et al. Mitocryptides from Human Mitochondrial DNA-Encoded Proteins Activate Neutrophil Formyl Peptide Receptors: Receptor Preference and Signaling Properties. J Immunol. 2018.
105. Marutani T, Hattori T, Tsutsumi K, Koike Y, Harada A, Noguchi K, et al. Mitochondrial protein-derived cryptides: Are endogenous N-formylated peptides including mitocryptide-2 components of mitochondrial damage-associated molecular patterns? Biopolymers. 2016;106(4):580-7.
106. McDonald B, Pittman K, Menezes GB, Hirota SA, Slaba I, Waterhouse CC, et al. Intravascular danger signals guide neutrophils to sites of sterile inflammation. Science. 2010;330(6002):362-6.
107. Monlun M, Hyernard C, Blanco P, Lartigue L, Faustin B. Mitochondria as Molecular Platforms Integrating Multiple Innate Immune Signalings. J Mol Biol. 2017;429(1):1-13.
108. Jin HS, Suh H-W, Kim S-J, Jo E-K. Mitochondrial Control of Innate Immunity and Inflammation. Immune Network. 2017;17(2):77-88.

109. Zhang Q, Raoof M, Chen Y, Sumi Y, Sursal T, Junger W, et al. Circulating mitochondrial DAMPs cause inflammatory responses to injury. Nature. 2010;464(7285):104-7.
110. Stalder AK, Lott D, Strasser DS, Cruz HG, Krause A, Groenen PM, et al. Biomarker-guided clinical development of the first-in-class anti-inflammatory FPR2/ALX agonist ACT-389949. British journal of clinical pharmacology. 2017;83(3):476-86.
111. Pittman K, Kubes P. Damage-associated molecular patterns control neutrophil recruitment. Journal of innate immunity. 2013;5(4):315-23.
112. Bagchi S, Liao Z, Gonzalez FA, Chorna NE, Seye CI, Weisman GA, et al. The P2Y2 nucleotide receptor interacts with alphav integrins to activate Go and induce cell migration. The Journal of biological chemistry. 2005;280(47):39050-7.
113. Liao Z, Seye CI, Weisman GA, Erb L. The P2Y2 nucleotide receptor requires interaction with alpha v integrins to access and activate G12. J Cell Sci. 2007;120(Pt 9):1654-62.
114. Taboubi S, Milanini J, Delamarre E, Parat F, Garrouste F, Pommier G, et al. G alpha(q/11)-coupled P2Y2 nucleotide receptor inhibits human keratinocyte spreading and migration. Faseb j. 2007;21(14):4047-58.
115. Gabl M, Winther M, Welin A, Karlsson A, Oprea T, Bylund J, et al. P2Y2 receptor signaling in neutrophils is regulated from inside by a novel cytoskeleton-dependent mechanism. Exp Cell Res. 2015;336(2):242-52.
116. Junger WG. Immune cell regulation by autocrine purinergic signalling. Nat Rev Immunol. 2011;11(3):201-12.
117. Chen Y, Corriden R, Inoue Y, Yip L, Hashiguchi N, Zinkernagel A, et al. ATP Release Guides Neutrophil Chemotaxis via P2Y2 and A3 Receptors. Science. 2006;314(5806):1792.
118. Gabl M, Winther M, Skovbakke SL, Bylund J, Dahlgren C, Forsman H. 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.
119. den Besten G, van Eunen K, Groen AK, Venema K, Reijngoud DJ, Bakker BM. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. J Lipid Res. 2013;54(9):2325-40.
120. Brown AJ, Goldsworthy SM, Barnes AA, Eilert MM, Tcheang L, Daniels D, et al. The Orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids. J Biol Chem. 2003;278(13):11312-9.
121. Le Poul E, Loison C, Struyf S, Springael JY, Lannoy V, Decobecq ME, et al. Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell activation. The Journal of biological chemistry. 2003;278(28):25481-9.
122. Stoddart LA, Smith NJ, Milligan G. International Union of Pharmacology. LXXI. Free fatty acid receptors FFA1, -2, and -3: pharmacology and pathophysiological functions. Pharmacol Rev. 2008;60(4):405-17.

123. Bolognini D, Tobin AB, Milligan G, Moss CE. The Pharmacology and Function of Receptors for Short-Chain Fatty Acids. Mol Pharmacol. 2016;89(3):388-98.
124. Alvarez-Curto E, Milligan G. Metabolism meets immunity: The role of free fatty acid receptors in the immune system. Biochem Pharmacol. 2016;114:3-13.
125. Mohammad S. Role of Free Fatty Acid Receptor 2 (FFAR2) in the Regulation of Metabolic Homeostasis. Curr Drug Targets. 2015;16(7):771-5.
126. Milligan G, Shimpukade B, Ulven T, Hudson BD. Complex Pharmacology of Free Fatty Acid Receptors. Chem Rev. 2017;117(1):67-110.
127. Schmidt J, Smith NJ, Christiansen E, Tikhonova IG, Grundmann M, Hudson BD, et al. Selective orthosteric free fatty acid receptor 2 (FFA2) agonists: identification of the structural and chemical requirements for selective activation of FFA2 versus FFA3. The Journal of biological chemistry. 2011;286(12):10628-40.
128. Hansen AH, Sergeev E, Bolognini D, Sprenger RR, Ekberg JH, Ejsing CS, et al. Discovery of a Potent Thiazolidine Free Fatty Acid Receptor 2 Agonist with Favorable Pharmacokinetic Properties. Journal of medicinal chemistry. 2018;61(21):9534-50.
129. Bolognini D, Moss CE, Nilsson K, Petersson AU, Donnelly I, Sergeev E, et al. A Novel Allosteric Activator of Free Fatty Acid 2 Receptor Displays Unique G(i)-functional Bias. The Journal of biological chemistry. 2016;291(36):18915-31.
130. Namour F, Galien R, Van Kaem T, Van der Aa A, Vanhoutte F, Beetens J, et al. Safety, pharmacokinetics and pharmacodynamics of GLPG0974, a potent and selective FFA2 antagonist, in healthy male subjects. British journal of clinical pharmacology. 2016;82(1):139-48.
131. Maslowski KM, Vieira AT, Ng A, Kranich J, Sierro F, Yu D, et al. Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. Nature. 2009;461(7268):1282-6.
132. Rodrigues HG, Takeo Sato F, Curi R, Vinolo MAR. Fatty acids as modulators of neutrophil recruitment, function and survival. European Journal of Pharmacology. 2016;785:50-8.
133. Sina C, Gavrilova O, Forster M, Till A, Derer S, Hildebrand F, et al. G protein-coupled receptor 43 is essential for neutrophil recruitment during intestinal inflammation. J Immunol. 2009;183(11):7514-22.
134. Vinolo MA, Hatanaka E, Lambertucci RH, Newsholme P, Curi R. Effects of short chain fatty acids on effector mechanisms of neutrophils. Cell Biochem Funct. 2009;27(1):48-55.
135. Vinolo MA, Rodrigues HG, Hatanaka E, Sato FT, Sampaio SC, Curi R. Suppressive effect of short-chain fatty acids on production of proinflammatory mediators by neutrophils. J Nutr Biochem. 2011;22(9):849-55.
136. Hudson BD, Tikhonova IG, Pandey SK, Ulven T, Milligan G. Extracellular ionic locks determine variation in constitutive activity and ligand potency between species orthologs of the free fatty acid receptors FFA2 and FFA3. The Journal of biological chemistry. 2012;287(49):41195-209.

137. Hudson BD, Due-Hansen ME, Christiansen E, Hansen AM, Mackenzie AE, Murdoch H, et al. Defining the molecular basis for the first potent and selective orthosteric agonists of the FFA2 free fatty acid receptor. The Journal of biological chemistry. 2013;288(24):17296-312.
138. Björkman L, Mårtensson J, Winther M, Gabl M, Holdfeldt A, Uhrbom M, et al. The Neutrophil Response Induced by an Agonist for Free Fatty Acid Receptor 2 (GPR43) Is Primed by Tumor Necrosis Factor Alpha and by Receptor Uncoupling from the Cytoskeleton but Attenuated by Tissue Recruitment. Mol Cell Biol. 2016;36(20):2583-95.
139. Kamada N, Seo SU, Chen GY, Núñez G. Role of the gut microbiota in immunity and inflammatory disease. Nat Rev Immunol. 2013;13(5):321-35.
140. Gomez de Agüero M, Ganal-Vonarburg SC, Fuhrer T, Rupp S, Uchimura Y, Li H, et al. The maternal microbiota drives early postnatal innate immune development. Science. 2016;351(6279):1296-302.
141. Koch MA, Reiner GL, Lugo KA, Kreuk LS, Stanbery AG, Ansaldo E, et al. Maternal IgG and IgA Antibodies Dampen Mucosal T Helper Cell Responses in Early Life. Cell. 2016;165(4):827-41.
142. Hand TW, Vujkovic-Cvijin I, Ridaura VK, Belkaid Y. Linking the Microbiota, Chronic Disease, and the Immune System. Trends Endocrinol Metab. 2016;27(12):831-43.
143. Maeda Y, Takeda K. Host–microbiota interactions in rheumatoid arthritis. Experimental & Molecular Medicine. 2019;51(12):1-6.
144. Lourenço TGB, Spencer SJ, Alm EJ, Colombo APV. Defining the gut microbiota in individuals with periodontal diseases: an exploratory study. J Oral Microbiol. 2018;10(1):1487741-.
145. Amabebe E, Anumba DOC. Female Gut and Genital Tract Microbiota-Induced Crosstalk and Differential Effects of Short-Chain Fatty Acids on Immune Sequelae. Frontiers in Immunology. 2020;11.
146. Scheithauer TPM, Rampanelli E, Nieuwdorp M, Vallance BA, Verchere CB, van Raalte DH, et al. Gut Microbiota as a Trigger for Metabolic Inflammation in Obesity and Type 2 Diabetes. Frontiers in immunology. 2020;11:571731-.
147. Park JH, Kotani T, Konno T, Setiawan J, Kitamura Y, Imada S, et al. Promotion of Intestinal Epithelial Cell Turnover by Commensal Bacteria: Role of Short-Chain Fatty Acids. PloS one. 2016;11(5):e0156334.
148. Kaiko GE, Ryu SH, Koues OI, Collins PL, Solnica-Krezel L, Pearce EJ, et al. The Colonic Crypt Protects Stem Cells from Microbiota-Derived Metabolites. Cell. 2016;165(7):1708-20.
149. Aoyama M, Kotani J, Usami M. Butyrate and propionate induced activated or non-activated neutrophil apoptosis via HDAC inhibitor activity but without activating GPR-41/GPR-43 pathways. Nutrition. 2010;26(6):653-61.
150. Lin MY, de Zoete MR, van Putten JPM, Strijbis K. Redirection of Epithelial Immune Responses by Short-Chain Fatty Acids through Inhibition of Histone Deacetylases. Frontiers in Immunology. 2015;6.

151. Brooks L, Viardot A, Tsakmaki A, Stolarczyk E, Howard JK, Cani PD, et al. Fermentable carbohydrate stimulates FFAR2-dependent colonic PYY cell expansion to increase satiety. Mol Metab. 2017;6(1):48-60.
152. Tolhurst G, Heffron H, Lam YS, Parker HE, Habib AM, Diakogiannaki E, et al. Short-chain fatty acids stimulate glucagon-like peptide-1 secretion via the G-protein-coupled receptor FFAR2. Diabetes. 2012;61(2):364-71.
153. den Besten G, Lange K, Havinga R, van Dijk TH, Gerding A, van Eunen K, et al. Gut-derived short-chain fatty acids are vividly assimilated into host carbohydrates and lipids. Am J Physiol Gastrointest Liver Physiol. 2013;305(12):G900-10.
154. Macia L, Tan J, Vieira AT, Leach K, Stanley D, Luong S, et al. Metabolite-sensing receptors GPR43 and GPR109A facilitate dietary fibre-induced gut homeostasis through regulation of the inflammasome. Nat Commun. 2015;6:6734.
155. Hiippala K, Jouhten H, Ronkainen A, Hartikainen A, Kainulainen V, Jalanka J, et al. The Potential of Gut Commensals in Reinforcing Intestinal Barrier Function and Alleviating Inflammation. Nutrients. 2018;10(8).
156. Śliżewska K, Markowiak-Kopeć P, Śliżewska W. The Role of Probiotics in Cancer Prevention. Cancers (Basel). 2020;13(1):20.
157. Pérez-Reytor D, Puebla C, Karahanian E, García K. Use of Short-Chain Fatty Acids for the Recovery of the Intestinal Epithelial Barrier Affected by Bacterial Toxins. Frontiers in physiology. 2021;12:650313-.
158. Maes M, Kubera M, Leunis JC. The gut-brain barrier in major depression: intestinal mucosal dysfunction with an increased translocation of LPS from gram negative enterobacteria (leaky gut) plays a role in the inflammatory pathophysiology of depression. Neuro Endocrinol Lett. 2008;29(1):117-24.
159. Nakajima A, Nakatani A, Hasegawa S, Irie J, Ozawa K, Tsujimoto G, et al. The short chain fatty acid receptor GPR43 regulates inflammatory signals in adipose tissue M2-type macrophages. PloS one. 2017;12(7):e0179696.
160. He J, Zhang P, Shen L, Niu L, Tan Y, Chen L, et al. Short-Chain Fatty Acids and Their Association with Signalling Pathways in Inflammation, Glucose and Lipid Metabolism. International journal of molecular sciences. 2020;21(17):6356.
161. Ohira H, Fujioka Y, Katagiri C, Mamoto R, Aoyama-Ishikawa M, Amako K, et al. Butyrate attenuates inflammation and lipolysis generated by the interaction of adipocytes and macrophages. J Atheroscler Thromb. 2013;20(5):425-42.
162. Mirmonsef P, Zariffard MR, Gilbert D, Makinde H, Landay AL, Spear GT. Short-Chain Fatty Acids Induce Pro-Inflammatory Cytokine Production Alone and in Combination with Toll-Like Receptor Ligands. American Journal of Reproductive Immunology. 2012;67(5):391-400.
163. Park J, Kim M, Kang SG, Jannasch AH, Cooper B, Patterson J, et al. Short-chain fatty acids induce both effector and regulatory T cells by suppression of histone deacetylases and regulation of the mTOR-S6K pathway. Mucosal Immunol. 2015;8(1):80-93.

164. Sun M, Wu W, Liu Z, Cong Y. Microbiota metabolite short chain fatty acids, GPCR, and inflammatory bowel diseases. J Gastroenterol. 2017;52(1):1-8.
165. Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly-Y M, et al. The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. Science (New York, NY). 2013;341(6145):569-73.
166. Sun M, Wu W, Chen L, Yang W, Huang X, Ma C, et al. Microbiota-derived short-chain fatty acids promote Th1 cell IL-10 production to maintain intestinal homeostasis. Nat Commun. 2018;9(1):3555.
167. Metzger DW, Salmon SL, Kirimanjeswara G. Differing effects of interleukin-10 on cutaneous and pulmonary Francisella tularensis live vaccine strain infection. Infect Immun. 2013;81(6):2022-7.
168. Glocker EO, Kotlarz D, Klein C, Shah N, Grimbacher B. IL-10 and IL-10 receptor defects in humans. Ann N Y Acad Sci. 2011;1246:102-7.
169. Kaisar MMM, Pelgrom LR, van der Ham AJ, Yazdanbakhsh M, Everts B. Butyrate Conditions Human Dendritic Cells to Prime Type 1 Regulatory T Cells via both Histone Deacetylase Inhibition and G Protein-Coupled Receptor 109A Signaling. Frontiers in immunology. 2017;8:1429-.
170. Chen J, Zhao K-N, Vitetta L. Effects of Intestinal Microbial⁻Elaborated Butyrate on Oncogenic Signaling Pathways. Nutrients. 2019;11(5):1026.
171. Bindels LB, Porporato P, Dewulf EM, Verrax J, Neyrinck AM, Martin JC, et al. Gut microbiota-derived propionate reduces cancer cell proliferation in the liver. British Journal of Cancer. 2012;107(8):1337-44.
172. Schlatterer K, Beck C, Schoppmeier U, Peschel A, Kretschmer D. Acetate sensing by GPR43 alarms neutrophils and protects from severe sepsis. Communications Biology. 2021;4(1):928.
173. Dürholz K, Hofmann J, Iljazovic A, Häger J, Lucas S, Sarter K, et al. Dietary Short-Term Fiber Interventions in Arthritis Patients Increase Systemic SCFA Levels and Regulate Inflammation. Nutrients. 2020;12(10).
174. Singh RK, Chang H-W, Yan D, Lee KM, Ucmak D, Wong K, et al. Influence of diet on the gut microbiome and implications for human health. J Transl Med. 2017;15(1):73-.
175. Macia L, Tan J, Vieira AT, Leach K, Stanley D, Luong S, et al. Metabolite-sensing receptors GPR43 and GPR109A facilitate dietary fibre-induced gut homeostasis through regulation of the inflammasome. Nat Commun. 2015;6(1):6734.
176. Yang D, Zhou Q, Labroska V, Qin S, Darbalaei S, Wu Y, et al. G protein-coupled receptors: structure- and function-based drug discovery. Signal Transduction and Targeted Therapy. 2021;6(1):7.
177. Chan HCS, Li Y, Dahoun T, Vogel H, Yuan S. New Binding Sites, New Opportunities for GPCR Drug Discovery. Trends in Biochemical Sciences. 2019;44(4):312-30.
178. Manglik A, Kim TH, Masureel M, Altenbach C, Yang Z, Hilger D, et al. Structural Insights into the Dynamic Process of β2-Adrenergic Receptor Signaling. Cell. 2015;161(5):1101-11.
179. Hilger D, Masureel M, Kobilka BK. Structure and dynamics of GPCR signaling complexes. Nature Structural & Molecular Biology. 2018;25(1):4-12.

180. Predescu D-V, Crețoiu SM, Crețoiu D, Pavelescu LA, Suciu N, Radu BM, et al. G Protein-Coupled Receptors (GPCRs)-Mediated Calcium Signaling in Ovarian Cancer: Focus on GPCRs activated by Neurotransmitters and Inflammation-Associated Molecules. International journal of molecular sciences. 2019;20(22):5568.
181. Clapham DE. Calcium Signaling. Cell. 2007;131(6):1047-58.
182. Futosi K, Fodor S, Mocsai A. Neutrophil cell surface receptors and their intracellular signal transduction pathways. Int Immunopharmacol. 2013;17(3):638-50.
183. Mikoshiba K. IP3 receptor/Ca2+ channel: from discovery to new signaling concepts. Journal of Neurochemistry. 2007;102(5):1426-46.
184. Schaefer M. Protein Kinase C. In: Enna SJ, Bylund DB, editors. xPharm: The Comprehensive Pharmacology Reference. New York: Elsevier; 2007. p. 1-4.
185. Bertram A, Ley K. Protein kinase C isoforms in neutrophil adhesion and activation. Arch Immunol Ther Exp (Warsz). 2011;59(2):79-87.
186. Kvachnina E, Liu G, Dityatev A, Renner U, Dumuis A, Richter DW, et al. 5-HT7 receptor is coupled to G alpha subunits of heterotrimeric G12-protein to regulate gene transcription and neuronal morphology. J Neurosci. 2005;25(34):7821-30.
187. Dhanasekaran N, Dermott JM. Signaling by the G12 class of G proteins. Cellular Signalling. 1996;8(4):235-45.
188. Dupré DJ, Robitaille M, Rebois RV, Hébert TE. The role of Gbetagamma subunits in the organization, assembly, and function of GPCR signaling complexes. Annu Rev Pharmacol Toxicol. 2009;49:31-56.
189. Milligan G, Kostenis E. Heterotrimeric G-proteins: a short history. British journal of pharmacology. 2006;147 Suppl 1(Suppl 1):S46-S55.
190. Cubillos S, Norgauer J, Lehmann K. Toxins-useful biochemical tools for leukocyte research. Toxins (Basel). 2010;2(4):428-52.
191. Holdfeldt A, Dahlstrand Rudin A, Gabl M, Rajabkhani Z, König GM, Kostenis E, et al. Reactivation of Gαi-coupled formyl peptide receptors is inhibited by Gαq-selective inhibitors when induced by signals generated by the platelet-activating factor receptor. J Leukoc Biol. 2017;102(3):871-80.
192. Bharati K, Ganguly NK. Cholera toxin: a paradigm of a multifunctional protein. Indian J Med Res. 2011;133(2):179-87.
193. Uemura T, Kawasaki T, Taniguchi M, Moritani Y, Hayashi K, Saito T, et al. Biological properties of a specific Galpha q/11 inhibitor, YM-254890, on platelet functions and thrombus formation under high-shear stress. Br J Pharmacol. 2006;148(1):61-9.
194. Schlegel JG, Tahoun M, Seidinger A, Voss JH, Kuschak M, Kehraus S, et al. Macrocyclic Gq Protein Inhibitors FR900359 and/or YM-254890-Fit for Translation? ACS Pharmacol Transl Sci. 2021;4(2):888-97.
195. Christopoulos A, Kenakin T. G protein-coupled receptor allosterism and complexing. Pharmacol Rev. 2002;54(2):323-74.
196. Kenakin TP. Chapter 5 - Allosteric Drug Effects. In: Kenakin TP, editor. Pharmacology in Drug Discovery and Development (Second Edition): Academic Press; 2017. p. 101-29.

197. Koshland Jr. DE. The Key–Lock Theory and the Induced Fit Theory. Angewandte Chemie International Edition in English. 1995;33(23-24):2375-8.
198. Preininger AM, Meiler J, Hamm HE. Conformational flexibility and structural dynamics in GPCR-mediated G protein activation: a perspective. J Mol Biol. 2013;425(13):2288-98.
199. Niesen MJ, Bhattacharya S, Vaidehi N. The role of conformational ensembles in ligand recognition in G-protein coupled receptors. J Am Chem Soc. 2011;133(33):13197-204.
200. Kenakin T, Miller LJ. Seven transmembrane receptors as shapeshifting proteins: the impact of allosteric modulation and functional selectivity on new drug discovery. Pharmacol Rev. 2010;62(2):265-304.
201. Pargellis C, Tong L, Churchill L, Cirillo PF, Gilmore T, Graham AG, et al. Inhibition of p38 MAP kinase by utilizing a novel allosteric binding site. Nature Structural Biology. 2002;9(4):268-72.
202. Nussinov R, Ma B, Tsai C-J. Multiple conformational selection and induced fit events take place in allosteric propagation. Biophysical Chemistry. 2014;186:22-30.
203. Kenakin T. New concepts in pharmacological efficacy at 7TM receptors: IUPHAR review 2. Br J Pharmacol. 2013;168(3):554-75.
204. Nussinov R, Tsai C-J. The different ways through which specificity works in orthosteric and allosteric drugs. Curr Pharm Des. 2012;18(9):1311-6.
205. Gentry PR, Sexton PM, Christopoulos A. Novel Allosteric Modulators of G Protein-coupled Receptors. The Journal of biological chemistry. 2015;290(32):19478-88.
206. Prilla S, Schrobang J, Ellis J, Höltje H-D, Mohr K. Allosteric Interactions with Muscarinic Acetylcholine Receptors: Complex Role of the Conserved Tryptophan M₂⁴²²Trp in a Critical Cluster of Amino Acids for Baseline Affinity, Subtype Selectivity, and Cooperativity. Molecular Pharmacology. 2006;70(1):181-93.
207. Valant C, Felder CC, Sexton PM, Christopoulos A. Probe dependence in the allosteric modulation of a G protein-coupled receptor: implications for detection and validation of allosteric ligand effects. Mol Pharmacol. 2012;81(1):41-52.
208. Kenakin TP. Chapter 11 - “Hit” to Drug: Lead Optimization. In: Kenakin TP, editor. A Pharmacology Primer (Third Edition). New York: Academic Press; 2009. p. 239-72.
209. Jakubík J, Randáková A, Chetverikov N, El-Fakahany EE, Doležal V. The operational model of allosteric modulation of pharmacological agonism. Scientific Reports. 2020;10(1):14421.
210. Johnstone S, Albert JS. Pharmacological property optimization for allosteric ligands: A medicinal chemistry perspective. Bioorganic & Medicinal Chemistry Letters. 2017;27(11):2239-58.
211. Kenakin T, Strachan RT. PAM-Antagonists: A Better Way to Block Pathological Receptor Signaling? Trends Pharmacol Sci. 2018;39(8):748-65.
212. Kenakin T. Allosteric theory: taking therapeutic advantage of the malleable nature of GPCRs. Curr Neuropharmacol. 2007;5(3):149-56.

213. Klein MT, Vinson PN, Niswender CM. Chapter One - Approaches for Probing Allosteric Interactions at 7 Transmembrane Spanning Receptors. In: Kenakin T, editor. Progress in Molecular Biology and Translational Science. 115: Academic Press; 2013. p. 1-59.
214. O'Callaghan K, Kuliopulos A, Covic L. Turning receptors on and off with intracellular pepducins: new insights into G-protein-coupled receptor drug development. The Journal of biological chemistry. 2012;287(16):12787-96.
215. Covic L, Misra M, Badar J, Singh C, Kuliopulos A. Pepducin-based intervention of thrombin-receptor signaling and systemic platelet activation. Nat Med. 2002;8(10):1161-5.
216. Chang SD, Bruchas MR. Functional Selectivity at GPCRs: New Opportunities in Psychiatric Drug Discovery. Neuropsychopharmacology. 2014;39(1):248-9.
217. Urban JD, Clarke WP, von Zastrow M, Nichols DE, Kobilka B, Weinstein H, et al. Functional selectivity and classical concepts of quantitative pharmacology. J Pharmacol Exp Ther. 2007;320(1):1-13.
218. Seyedabadi M, Ghahremani MH, Albert PR. Biased signaling of G protein coupled receptors (GPCRs): Molecular determinants of GPCR/transducer selectivity and therapeutic potential. Pharmacol Ther. 2019;200:148-78.
219. Teixeira LB, Parreiras-E-Silva LT, Bruder-Nascimento T, Duarte DA, Simões SC, Costa RM, et al. Ang-(1-7) is an endogenous β-arrestin-biased agonist of the AT(1) receptor with protective action in cardiac hypertrophy. Scientific reports. 2017;7(1):11903-.
220. Schebb NH, Kühn H, Kahnt AS, Rund KM, O'Donnell VB, Flamand N, et al. Formation, Signaling and Occurrence of Specialized Pro-Resolving Lipid Mediators—What is the Evidence so far? Frontiers in pharmacology. 2022;13.
221. Sergeev E, Hansen AH, Pandey SK, MacKenzie AE, Hudson BD, Ulven T, et al. Non-equivalence of Key Positively Charged Residues of the Free Fatty Acid 2 Receptor in the Recognition and Function of Agonist Versus Antagonist Ligands. The Journal of biological chemistry. 2016;291(1):303-17.
222. Wang Y, Jiao X, Kayser F, Liu J, Wang Z, Wanska M, et al. The first synthetic agonists of FFA2: Discovery and SAR of phenylacetamides as allosteric modulators. Bioorganic & Medicinal Chemistry Letters. 2010;20(2):493-8.
223. Felder CC. Chapter One - GPCR drug discovery-moving beyond the orthosteric to the allosteric domain. In: Witkin JM, editor. Advances in Pharmacology. 86: Academic Press; 2019. p. 1-20.
224. Lorenz W, Inglese J, Palczewski K, Onorato JJ, Caron MG, Lefkowitz RJ. The receptor kinase family: primary structure of rhodopsin kinase reveals similarities to the beta-adrenergic receptor kinase. Proceedings of the National Academy of Sciences of the United States of America. 1991;88(19):8715-9.
225. Smith JS, Rajagopal S. The β-Arrestins: Multifunctional Regulators of G Protein-coupled Receptors. The Journal of biological chemistry. 2016;291(17):8969-77.

226. Luttrell LM, Lefkowitz RJ. The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. J Cell Sci. 2002;115(Pt 3):455-65.
227. Gurevich VV, Gurevich EV. GPCR Signaling Regulation: The Role of GRKs and Arrestins. Frontiers in pharmacology. 2019;10:125-.
228. Gabl M, Holdfeldt A, Sundqvist M, Lomei J, Dahlgren C, Forsman H. FPR2 signaling without beta-arrestin recruitment alters the functional repertoire of neutrophils. Biochem Pharmacol. 2017;145:114-22.
229. Gera N, Swanson KD, Jin T. β-Arrestin 1-dependent regulation of Rap2 is required for fMLP-stimulated chemotaxis in neutrophil-like HL-60 cells. Journal of Leukocyte Biology. 2017;101(1):239-51.
230. Lind S, Dahlgren C, Holmdahl R, Olofsson P, Forsman H. Functional selective FPR1 signaling in favor of an activation of the neutrophil superoxide generating NOX2 complex. J Leukoc Biol. 2021;109(6):1105-20.
231. Pitcher JA, Freedman NJ, Lefkowitz RJ. G protein-coupled receptor kinases. Annu Rev Biochem. 1998;67:653-92.
232. Kelly E, Bailey CP, Henderson G. Agonist-selective mechanisms of GPCR desensitization. British Journal of Pharmacology. 2008;153(Suppl 1):S379-S88.
233. Torres M, Coates TD. Function of the cytoskeleton in human neutrophils and methods for evaluation. Journal of immunological methods. 1999;232(1):89-109.
234. Dahlgren C, Holdfeldt A, Lind S, Mårtensson J, Gabl M, Björkman L, et al. Neutrophil Signaling That Challenges Dogmata of G Protein-Coupled Receptor Regulated Functions. ACS Pharmacol Transl Sci. 2020;3(2):203-20.
235. Lind S, Sundqvist M, Holmdahl R, Dahlgren C, Forsman H, Olofsson P. Functional and signaling characterization of the neutrophil FPR2 selective agonist Act-389949. Biochem Pharmacol. 2019;166:163-73.
236. Onnheim K, Christenson K, Gabl M, Burbiel JC, Muller CE, Oprea TI, et al. A novel receptor cross-talk between the ATP receptor P2Y2 and formyl peptide receptors reactivates desensitized neutrophils to produce superoxide. Exp Cell Res. 2014;323(1):209-17.
237. Kenakin TP. Ligand Detection in the Allosteric World. Journal of Biomolecular Screening. 2010;15(2):119-30.
238. Pizzonero M, Dupont S, Babel M, Beaumont S, Bienvenu N, Blanqué R, et al. Discovery and Optimization of an Azetidine Chemical Series As a Free Fatty Acid Receptor 2 (FFA2) Antagonist: From Hit to Clinic. Journal of medicinal chemistry. 2014;57(23):10044-57.
239. Milligan G, Barki N, Tobin AB. Chemogenetic Approaches to Explore the Functions of Free Fatty Acid Receptor 2. Trends in Pharmacological Sciences. 2021;42(3):191-202.
240. Bosier B, Hermans E. Chapter 8 - Promises of Biased Signaling in the Development of Improved Therapeutics. In: Arey BJ, editor. Biased Signaling in Physiology, Pharmacology and Therapeutics. San Diego: Academic Press; 2014. p. 251-92.

241. Lane R, Sexton P, Christopoulos A. Bridging the gap: Bitopic ligands of G-protein-coupled receptors. Trends in pharmacological sciences. 2012;34.
242. Grundmann M, Tikhonova IG, Hudson BD, Smith NJ, Mohr K, Ulven T, et al. A Molecular Mechanism for Sequential Activation of a G Protein-Coupled Receptor. Cell Chem Biol. 2016;23(3):392-403.
243. Grundmann M, Kostenis E. Temporal Bias: Time-Encoded Dynamic GPCR Signaling. Trends in Pharmacological Sciences. 2017;38(12):1110-24.
244. Bertekap RL, Burford NT, Li Z, Alt A. High-Throughput Screening for Allosteric Modulators of GPCRs. In: Filizola M, editor. G Protein-Coupled Receptors in Drug Discovery: Methods and Protocols. New York, NY: Springer New York; 2015. p. 223-40.
245. Burger WAC, Sexton PM, Christopoulos A, Thal DM. Toward an understanding of the structural basis of allostery in muscarinic acetylcholine receptors. J Gen Physiol. 2018;150(10):1360-72.