# Activation of formyl-peptide receptors by *Staphylococcus aureus* and consequences for inflammation

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## Abstract

*Staphylococcus aureus* is a major human pathogen causing a variety of diseases such as local skin infections, sepsis, and endocarditis. Phagocytes of the innate immune system are the first line of defense against invading pathogens. They express a variety of pattern recognition receptors (PRRs), which ensure the recognition of microbe-associated molecular patterns (MAMPs). One class of PRRs are the formyl-peptide receptors (FPRs). They belong to the family of seven-transmembrane G protein-coupled receptors (GPCRs), which are involved in host defense against bacterial infections and in the clearance of damaged host cells. Human neutrophils express two of the FPRs – the FPR1 and FPR2 – which exhibit a large overall amino acid sequence similarity. FPR1 generally recognizes short peptides initiated with N-formyl-methionine, which are cleavage products of bacterial and mitochondrial proteins. In contrast, FPR2 prefers longer peptides with α-helical, amphipathic properties. The most prominent bacterial FPR2 ligands are the phenol-soluble modulins (PSMs) of staphylococci.

FPRs are well conserved among mammals and outside the mammalian clade, such as in fish. Since it has been shown that FPRs from different species often recognize distinct ligands, we investigated if the mouse FPR2 (mFpr2) could be activated by *S. aureus* PSMs as the human FPR2. By using a *S. aureus* peritonitis model we could show that early recruitment of leukocytes to the infection site is FPR2 and PSM-mediated. Furthermore, we observed that coactivation of both FPRs leads to a synergistic release of the chemokine interleukin (IL)-8 from neutrophils. Moreover, our data suggest a crucial role for the FPRs during phagocytosis and killing of *S. aureus*. Stimulation of FPRs by their cognate ligands significantly enhanced the engulfment of Gram-positive and Gram-negative bacteria. The increased phagocytosis is mediated by an enhanced expression of the complement receptors 1 and 3, as well as the FCγ receptor I. Interestingly, the consequence of the augmented phagocytosis is also a synergistic release of the chemokine IL-8 and a time- and ratio-dependent shift of bacterial killing. *In vivo*, although lack of mFpr2 and PSMs results in less phagocytosis of *S. aureus*, a better killing of PSM-releasing *S. aureus* could be observed in mFpr2$^{-/-}$ mice. Thus, the following study provides deep insights into the functional role of FPRs in phagocytes during *S. aureus* infection.

## Zusammenfassung

*Staphylococcus aureus* ist ein humanes Pathogen, welches eine Vielzahl von Krankheiten verursacht, wie zum Beispiel lokale Hautinfektionen, Sepsis und Endokarditis. Phagozyten des angeborenen Immunsystems stellen die erste Verteidigungslinie gegen eindringende Pathogene dar. Sie exprimieren eine Vielzahl von „Muster-Erkennungs-Rezeptoren“ (PRRs), welche die Erkennung von “mikrobiell-assoziierten molekularen Mustern“ (MAMPs) sicherstellen. Eine Klasse der PRRs sind die Formylpeptid-Rezeptoren (FPRs). Sie gehören zur Familie der sieben-transmembran G-Protein-gekoppelten Rezeptoren (GPCRs), welche an der Wirtsabwehr gegen bakterielle Infektionen und der Beseitigung von geschädigten Wirtszellen beteiligt sind. Humane neutrophile Granulozyten exprimieren zwei der FPRs – FPR1 und FPR2 – welche eine große Ähnlichkeit in der Aminosäuresequenz zeigen. FPR1 erkennt kurze Peptide, die mit einem N-Formyl-Methionin beginnen. Diese sind Spaltprodukte bakterieller und mitochondrialer Proteine. Im Gegensatz dazu bevorzugt der FPR2 längere Peptide mit α-helikalen, amphipathischen Eigenschaften. Die bekanntesten bakteriellen FPR2-Liganden sind die Phenol-löslichen Moduline (PSMs) der Staphylokokken.

FPRs sind hoch konservierte Rezeptoren bei Säugetieren, aber auch außerhalb der Säugetiergruppe, wie zum Beispiel bei Fischen. Da allerdings bekannt ist, dass FPRs verschiedener Spezies oft unterschiedliche Liganden erkennen, war ein Ziel dieser Arbeit, herauszufinden, ob der FPR2 von Mäusen (mFpr2) im gleichen Maße durch PSMs von *S. aureus* aktiviert werden kann, wie der humane FPR2. Wir konnten in einem Peritonitismodell zeigen, dass für die frühe Rekrutierung von Leukozyten zum Infektionsort der FPR2 und die PSMs essentiell sind. Weiterhin konnte gezeigt werden, dass die simultane Stimulation beider FPRs auf neutrophilen Granulozyten zu einer synergistischen Freisetzung des Chemokins Interleukin (IL)-8 führt. *In vitro* Daten zeigen außerdem, dass die Stimulierung der FPRs durch bakterielle Liganden die Phagozytose von gram-positiven und gram-negativen Bakterien signifikant verstärkt. Weiterhin konnte gezeigt werden, dass diese verstärkte Aufnahme durch eine erhöhte Expression der Komplementrezeptoren 1 und 3, sowie des FCγ Rezeptors I vermittelt wird. Die Konsequenz der gesteigerten Phagozytose ist eine synergistische Freisetzung des Chemokins IL-8 und eine Zeit- und Verhältnis-

abhängige Verschiebung des Abtötens der Bakterien. Obwohl das Fehlen von mFpr2 und PSMs *in vivo* in weniger Phagozytose von *S. aureus* resultierte, konnte ein besseres Abtöten von PSM-freisetzendem *S. aureus* in mFpr2$^{-/-}$ Mäusen beobachtet werden. Zusammenfassend, die folgende Studie liefert neue Aspekte der funktionalen Rolle der FPRs in Phagozyten während einer *S. aureus* Infektion.

# CHAPTER 1

## General introduction

## Detection of invading pathogens by pattern recognition receptors

The innate immune system discriminates between self and foreign by the recognition of microbe-associated molecular patterns (MAMPs) sensed by pattern recognition receptors (PRRs). These receptors include membrane-bound receptors that scan the extracellular milieu and endosomal compartments (Toll-like receptors – TLRs; C-type lectin receptors – CLRs), cytoplasmic nucleic acid-sensing receptors (absent in melanoma 2 (AIM2), which senses DNA, or retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5), which sense RNA) and the intracellular nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (figure 1) [1]. This receptor repertoire provides a specific immune response to a certain class of pathogens (Gram-positive and -negative bacteria, protozoa, viruses, and fungi) and to the location of a recognized pathogen (within the cytoplasm, internal compartments or outside of the cell). For *Salmonella typhimurium* it was shown that extracellular detection of the pathogen by TLRs leads to proinflammatory cytokine production and phagocytic antimicrobial pathways whereas the detection of intracellular bacteria leads to NLR-dependent programmed, proinflammatory cell death [2, 3].

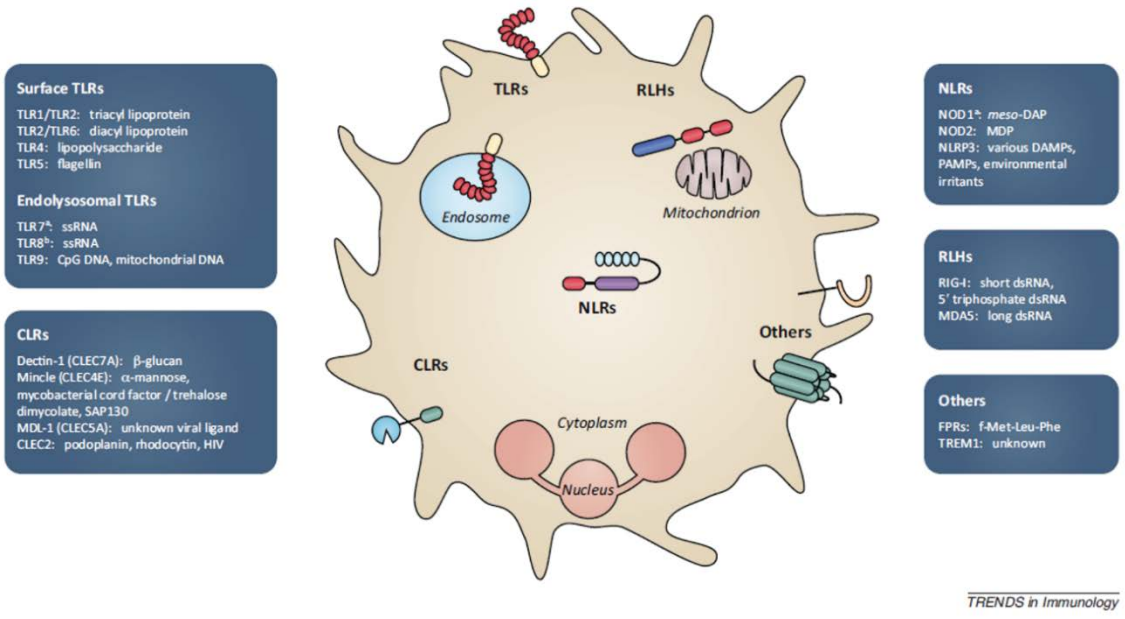


**Figure 1: Families of pattern recognition receptors expressed by neutrophils and their ligands [1].** Abbreviations: CLEC, C-type lectin domain family; CLR, C-type lectin receptor; DAMP, danger-associated molecular pattern; meso-DAP, meso-diaminopimelic acid; FPR, formyl peptide receptor; MDL, myeloid DAP12-associating lectin; NLR, nucleotide-binding oligomerization domain (NOD)-like receptor; NLRC, NLR with a CARD domain; NLRP, NLR with a pyrin domain; PAMP, pathogen-associated molecular pattern; RLH, retinoic acid-inducible gene (RIG)-like helicase; SAP, spliceosome-associated protein; TLR, Toll-like receptor; TREM, triggering receptor expressed on myeloid cells.a Mouse but not human neutrophils.b Human but not mouse neutrophils.

## Formyl-peptide receptors – understudied but important PRRs

Most PRR studies focus on the role of TLRs during infection but another class of PRRs – the formyl-peptide receptors (FPRs) – has been largely neglected. FPRs belong to the family of seven-transmembrane G protein-coupled receptors (GPCRs), which are involved in host defense against bacterial infections and in the clearance of damaged cells [4]. FPRs are well conserved among mammals and they were also found outside the mammalian clade, such as in fish. The number of expressed FPRs differ between species. For example, humans express three different FPRs (FPR1, FPR2, and FPR3), whereas mice express seven receptors (Fpr1, Fpr2, Fpr3, Fpr-rs3, Fpr-rs4, Fpr-rs6, and Fpr-rs7) [4, 5]. In humans the receptors are mainly expressed in immune cells, like neutrophils, macrophages, monocytes, dendritic cells, and T cells but also in astrocytes, microglia, hepatocytes, keratinocytes, and epithelial cells. Human neutrophils express only two of the FPRs – the FPR1 and FPR2 – which exhibit a large overall amino acid sequence similarity [6, 7]. These receptors show a higher degree of identity in the cytosolic parts and a lower in the extracellular domains suggesting that the two receptors differ more in ligand binding than in intracellular signaling. Several endogenous and exogenous ligands have been described for human FPR1 and FPR2.

FPR1 recognizes peptides initiated with N-formyl-methionine, which are cleavage products of bacterial and mitochondrial proteins. The most potent FPR1 ligand is the peptide N-formyl-methionyl-leucyl-phenylalanine (fMLF), which was first found in *Escherichia coli* [8]. Upon binding to FPR1 on the surface of neutrophils, fMLF is internalized within approximately 30 seconds resulting in a variety of cellular responses, like chemotaxis, production of reactive oxygen species (ROS), degranulation, and cytokine expression [4, 9, 10]. Several studies have shown the role of fMLF in the pathogenesis of multiple inflammatory diseases such as colitis [11, 12], pouchitis, ulcerative colitis and Crohn´s disease [13, 14], and juvenile peridotitis [15]. It was also reported that inhalation or injection of fMLF can cause rapid neutropenia and bronchial inflammation in human and other mammals [16, 17].

FPR2 is a low affinity receptor for many of the potent FPR1 agonists. Whereas, FPR1 recognizes mainly short N-formylated peptides, FPR2 prefers longer peptides with α-helical, amphipathic properties [18]. The most prominent bacterial FPR2 ligands are the *S. aureus* derived phenol soluble modulins (PSMs). These peptides share an α-

helical, amphipathic structure but they can be divided by their amino acid length into two subgroups: the α-type PSMs – PSMα1-4 and δ-toxin – consisting of 20-26 amino acids and the β-type PSMs – PSMβ1 and PSMβ2 – consisting of 40-44 amino acids [19]. Additionally, *S. epidermidis [20]*, *S. lugdunensis* [21], *S. haemolyticus* [22], and many other staphylococci [23] produce PSMs or PSM-like peptides. At micromolar concentrations PSMs are potent toxins, which disrupt the cytoplasmic membrane of eukaryotic host cells [24] and in some cases competing bacteria [25] receptor independently. At nanomolar concentrations PSMs are very potent FPR2 agonists and induce chemotaxis, calcium influx into the cytosol, chemokine release, and degranulation in human and mouse neutrophils [24, 26]. As staphylococcal virulence correlates with PSM levels, FPR2 may allow neutrophils to distinguish between highly-pathogenic, low-pathogenic, and non-pathogenic staphylococci, thereby enabling the innate immune system to adjust immune responses appropriately [23]. *S. aureus* produces two inhibitors of FPR2 – FPR-like 1 inhibitory protein (FLIPr) and FLIPr-like – showing the importance of FPR2 for the host during *S. aureus* infection [27, 28]. The significance of PSMs as virulence determinants in pathogenesis was already shown for skin and soft tissue infections [29], biofilm-associated infections [30], osteomyelitis [31], and atopic dermatitis [32].

Besides pathogen-derived ligands many host-derived non-formylated peptide agonists for FPRs have been described. These are generally peptides and proteins associated with human diseases and inflammation showing a preference for FPR2 [33]. Several endogenous FPR2 ligands are involved in neurodegenerative diseases, such as the amyloidogenic peptides serum amyloid A (SAA), the prion fragment PrP (106-126), the 42-amino acid form of Aβ amyloid peptide ($A\beta_{42}$), and humanin indicating a role of FPR2 in amyloidosis, prion disease, and Alzheimer´s disease, respectively [34]. In addition the FPRs play also a role in HIV infection, diabetes, and cancer [35].

## Initiation of inflammation – recruitment of neutrophils to the infection site

Inflammation is a tightly regulated process induced by invading pathogens or tissue injury. The main function of inflammation is to eradicate pathogens and to remove

damaged tissue followed by restoring tissue homeostasis. The effective elimination of pathogens and cell debris is achieved by professional phagocytes – neutrophils, monocytes, and macrophages – of the innate immune system [36, 37]. Neutrophils are typically the first leukocytes to be recruited to the infection site. The localization of neutrophils to the site of inflammation is crucial for clearance of infection and a marked decrease in neutrophil numbers in the blood leads to severe immunodeficiency in humans [38, 39].

Microbial invasion and/or tissue damage leads to necrotic cell death. This results in the release of cytoplasmic and nuclear components [40], which contain damage-associated molecular patterns (DAMPs). Together with MAMPs, such as bacterial and fungal cell wall components, and viral nucleic acids [41] they are recognized by PRRs expressed by leukocytes. These alarm signals activate tissue-resident sentinel cells, like macrophages and mast cells to release proinflammatory mediators, such as interleukin (IL)-1β and tumor necrosis factor α (TNFα), and chemoattractants (table 1) [37]. These mediators initiate the so called neutrophil recruitment cascade, which consists of the following steps: 1) tethering of the neutrophil to the activated endothelium, 2) rolling of the neutrophil along the endothelium, 3) adhesion of the neutrophil to the endothelium, 4) crawling of the neutrophil along the endothelium, and 5) transmigration of the neutrophil into the tissue (figure 2) [42].

**Table 1: Major human neutrophil-active chemoattractants and their receptors expressed by neutrophils [43].**

| Chemoattractant | Receptor |
|---|---|
| **Chemokines** | |
| CXCL1 (GROα) | CXCR2 |
| CXCL2 (GROβ) | CXCR2 |
| CXCL3 (GROγ) | CXCR2 |
| CXCL5 (ENA-78) | CXCR2 |
| CXCL6 (GCP-2) | CXCR1/CXCR2 |
| CXCL7 (NAP-2) | CXCR1/CXCR2 |
| CXCL8 (IL-8) | CXCR1/CXCR2 |
| CXCL12 (SDF-1α) | CXCR4/CXCR7 |
| CCL3 (MIP-1α) | CCR1/CCR5 |
| CCL5 (RANTES) | CCR1/CCR3/CCR5 |
| CCL7 (MCP-3) | CCR1/CCR2/CCR3 |

| **Peptides/Anaphylotoxins** | |
|---|---|
| N-formylated peptides (e.g. fMLF) | FPR1 |
| LL37 | FPR2 |
| Phenol soluble modulins (PSMs) | FPR2 |
| C3a | C3aR |
| C5a | C5aR |
| | |
| **Lipids** | |
| Leukotriene $B_4$ ($LTB_4$) | BLT1 |
| Platelet activating factor (PAF) | PAFR |

Emigrated neutrophils have to follow a chemotactic gradient to reach the site of infection. It has recently been shown that temporal and spatial cascades of chemoattractants are necessary to guide neutrophils from the blood into the tissue and to the inflammatory site. The signals of chemoattractants are transmitted through GPCRs at the cell surface of neutrophils (table 1) leading to directed cell migration. Lipid mediators, like leukotriene $B_4$ ($LTB_4$) seem to initiate neutrophil recruitment, probably due to their rapid synthesis. $LTB_4$-stimulated leukocytes produce cytokines, such as IL-6, IL-1β, and TNFα, which induce the release of chemokines, mainly CXCR2 and CCR1 ligands. Chemokines act later in chemoattractant cascades, as they are transcriptionally regulated, and at longer distances. [44]. Besides lipid mediators and chemokines, the so called intermediate chemoattractants, end-target chemoattractants, like formylated peptides of bacteria or complement component C5a guide neutrophils to the inflammatory site. The classification of chemoattractants is based on their intracellular signaling hierarchy. Neutrophils prefer end-target chemoattractants over intermediate chemoattractants, which ensures a directed migration to the infection site (figure 2) [45]. The differentiation between intermediate and end-target chemoattractants is achieved by distinct signaling molecules inside neutrophils and the availability of discrete chemotactic receptors on the cell surface. Intermediate chemoattractants signal primarily via the PI3K/Akt pathway, whereas end-target chemoattractants function mainly by stimulating p38 MAPK and thereby inhibiting the PI3K/Akt pathway [46]. Receptors for intermediate chemoattractants are constitutively localized at the cell surface, whereas the majority of receptors for end-target chemoattractants are stored in intracellular vesicles. These receptors are localized to the cell surface upon activation of neutrophils, leading to delayed and

possibly more robust response to end-target chemoattractants [47]. Differential desensitization mechanisms described for both chemoattractant groups might also contribute to the distinction between these groups. Binding of the intermediate chemokine IL-8 to its receptor leads to homologous desensitization. Internalization of CXCR1/CXCR2 prevents subsequent activation with the same chemokine without affecting the signaling of other chemotactic receptors. In contrast, activation of FPR1 by its end-target ligand fMLF results in heterologous desensitization. Signaling by both end-target and intermediate chemoattractants is blocked [48].

Thus, different mechanisms, such as the lipid-cytokine-chemokine cascade and the discrimination between intermediate and end-target chemoattractants are involved in the temporal and spatial precise migration of neutrophils to the infection site.

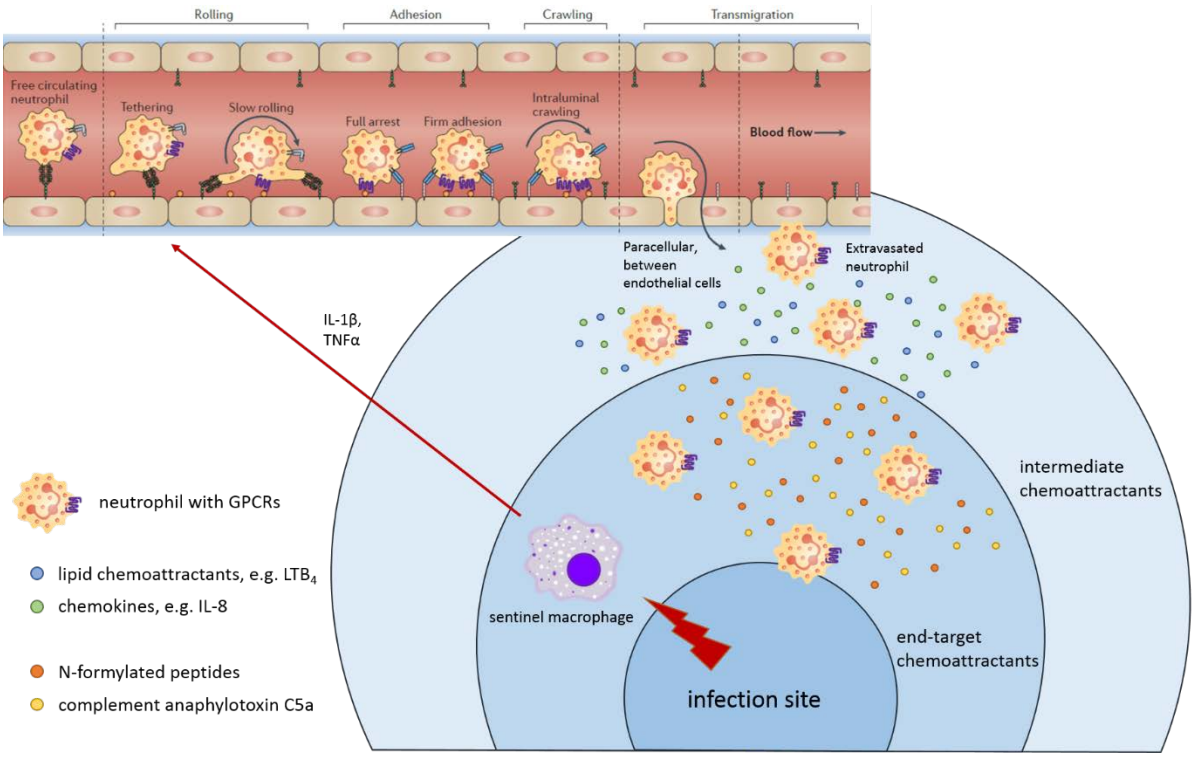


**Figure 2: Recruitment of neutrophils to the infection site** (adapted from Kolaczkowska and Kubes, 2011 [45]). Sentinel macrophages are activated by tissue injury and release IL-1β and TNFα to activate endothelial cells. This leads to initiation of the neutrophil recruitment cascade, extravasation of neutrophils from the blood stream into the tissue, and guided migration by intermediate and end-target chemoattractants to the infection site.

## Fighting invading pathogens – phagocytosis and killing of bacteria by neutrophils

Once at the infection site, neutrophils fulfill a set of antimicrobial functions. They release cytokines and chemokines to activate and recruit further immune cells, release reactive oxygen species (ROS) and antimicrobial peptides (AMPs) to kill extracellular pathogens, and they phagocytose pathogens to kill them intracellularly.

Phagocytosis is a process by which other cells, cell fragments, and microorganisms are engulfed by professional phagocytes of the innate immune system. Surface structures of pathogens are opsonized by IgG antibodies and complement factor C3b, which are recognized by FCγ receptors (FCγR) and complement receptors (CR) on the surface of phagocytes, respectively. Peripheral blood neutrophils express FCγRII (CD32) and FCγRIIIB (CD16). During systemic infections and sepsis they also express FCγRI (CD64). Complement receptors expressed by neutrophils are CR1 (CD35) and CR3 (CD11b/CD18) [49]. Recognition of opsonized pathogens by these phagocytic receptors initiates the vigorous extension of pseudopods that surround and ultimately entrap the pathogen [50]. The formed phagosome undergoes “phagosome maturation”, a series of remodeling events, to acquire the cellular machinery necessary for killing of pathogens. In macrophages this process shows parallels with endocytosis. The newly formed phagosome fuses with early, then late endosomes, before finally fusing with lysosomes to yield a highly acidic phagolysosome [51]. Phagosome maturation in neutrophils is completely different and results in a compartment with neutral pH [52]. The phagosome fuses with secretory vesicles and granules in a process called degranulation to gain its antimicrobial function. Neutrophils contain four different types of granules: primary (or azurophilic) granules, secondary granules, tertiary granules, and secretory vesicles. These organelles contain a powerful arsenal of microbicidal peptides and proteolytic enzymes, as well as numerous membrane-bound proteins that contribute to pathogen elimination (figure 3) [53]. Fusion of granules with the phagosome is controlled by intracellular calcium levels. The individual secretory compartments have different calcium thresholds for secretion, with secretory vesicles having the lowest and azurophilic granules the highest threshold [54]. Azurophilic granules enrich the phagosome with numerous AMPs, including α-defensins, cathepsins, proteinase-3, elastase, and azurocidin [55]. Secondary granules are thought to be the main source

of phagosomal NADPH-dependent oxidase [56]. This membrane-bound complex generates high levels of superoxide, which is termed “respiratory burst”. Superoxide is short-lived and rapidly dismutates to hydrogen peroxide and other secondary reactive products, which are effective microbicidal compounds [57]. Patients with chronic granulomatous disease (CGD) have a defect in NADPH oxidase, leading to recurrent bacterial and fungal infections, especially caused by *S. aureus* [58].

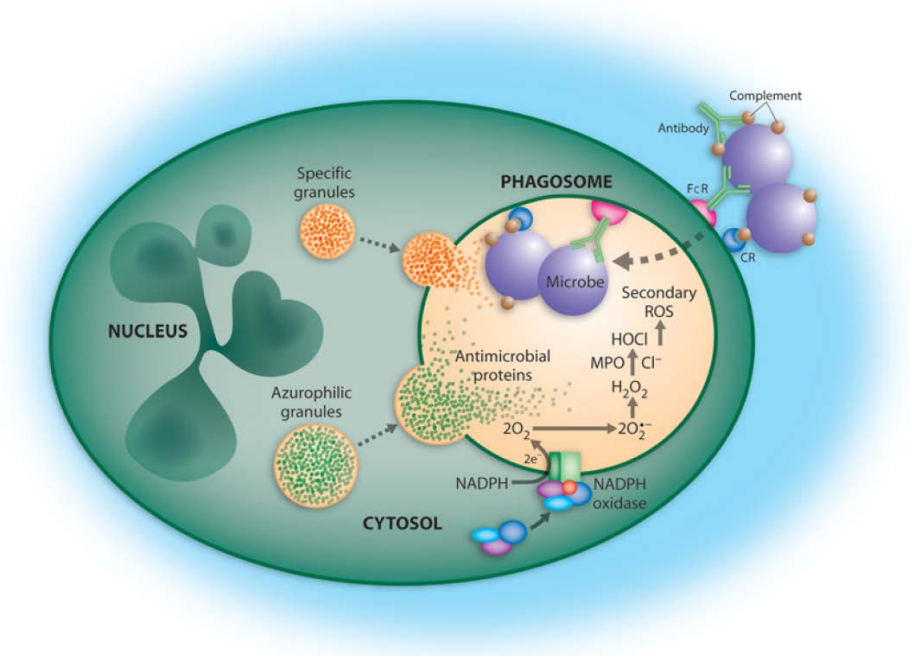


**Figure 3: Phagocytosis of pathogens and subsequent killing within the mature phagosome [57].** Complement and antibody opsonized pathogens are recognized by complement receptor (CR) and FCγ receptor (FCγR), respectively, leading to uptake of bacteria. Several granules fuse with the phagosome to provide AMPs and NADPH oxidase for bacterial killing.

Several pathogens have evolved strategies to circumvent phagocytosis and/or phagosomal killing. *S. aureus* exhibits many characteristics of anti-neutrophil pathogens. Although this bacterium has developed many ways to block the uptake by neutrophils, it is rapidly phagocytosed and phagocytosis is significantly greater than that of *Klebsiella pneumoniae*, group A streptococcus, or *Streptococcus pneumoniae [59]*. Therefore, the role of *S. aureus* “antiphagocytic” molecules in the pathogenesis of human infections remains unclear. However, ~15-50 % of the initial ingested inoculum of *S. aureus* survive within the neutrophil phagosome and cause lysis of neutrophils and, in turn, escape and disseminate [60]. It was recently shown that cytolytic PSMs of *S. aureus* are responsible for phagosomal escape into the cytoplasm, whereas the bicomponent leucocidins LukAB and PVL are responsible for

lysis of the cytoplasmic membrane [61]. Thereby, *S. aureus* is hypothesized to be an intracellular pathogen and uses neutrophils as Trojan horses to disseminate [62, 63].

## References


1. Thomas, C.J. and K. Schroder, *Pattern recognition receptor function in neutrophils.* Trends Immunol, 2013. **34**(7): p. 317-28.
2. Miao, E.A., et al., *TLR5 and Ipaf: dual sensors of bacterial flagellin in the innate immune system.* Semin Immunopathol, 2007. **29**(3): p. 275-88.
3. Miao, E.A., et al., *Caspase-1-induced pyroptosis is an innate immune effector mechanism against intracellular bacteria.* Nat Immunol, 2010. **11**(12): p. 1136-42.
4. Ye, R.D., et al., *International Union of Basic and Clinical Pharmacology. LXXIII. Nomenclature for the formyl peptide receptor (FPR) family.* Pharmacol Rev, 2009. **61**(2): p. 119-61.
5. Gao, J.L., et al., *Differential expansion of the N-formylpeptide receptor gene cluster in human and mouse.* Genomics, 1998. **51**(2): p. 270-6.
6. Dahlgren, C., et al., *Basic characteristics of the neutrophil receptors that recognize formylated peptides, a danger-associated molecular pattern generated by bacteria and mitochondria.* Biochem Pharmacol, 2016. **114**: p. 22-39.
7. Dorward, D.A., et al., *The role of formylated peptides and formyl peptide receptor 1 in governing neutrophil function during acute inflammation.* Am J Pathol, 2015. **185**(5): p. 1172-84.
8. Marasco, W.A., 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): p. 5430-9.
9. Liu, M., et al., *Formylpeptide receptors are critical for rapid neutrophil mobilization in host defense against Listeria monocytogenes.* Sci Rep, 2012. **2**: p. 786.
10. Prossnitz, E.R. and R.D. Ye, *The N-formyl peptide receptor: a model for the study of chemoattractant receptor structure and function.* Pharmacol Ther, 1997. **74**(1): p. 73-102.
11. Chester, J.F., et al., *Acute colitis produced by chemotactic peptides in rats and mice.* Am J Pathol, 1985. **121**(2): p. 284-90.
12. LeDuc, L.E. and C.C. Nast, *Chemotactic peptide-induced acute colitis in rabbits.* Gastroenterology, 1990. **98**(4): p. 929-35.
13. Nast, C.C. and L.E. LeDuc, *Chemotactic peptides. Mechanisms, functions, and possible role in inflammatory bowel disease.* Dig Dis Sci, 1988. **33**(3 Suppl): p. 50S-57S.
14. Anton, P.A., S.R. Targan, and F. Shanahan, *Increased neutrophil receptors for and response to the proinflammatory bacterial peptide formyl-methionyl-leucyl-phenylalanine in Crohn's disease.* Gastroenterology, 1989. **97**(1): p. 20-8.
15. Perez, H.D., et al., *Defective polymorphonuclear leukocyte formyl peptide receptor(s) in juvenile periodontitis.* J Clin Invest, 1991. **87**(3): p. 971-6.
16. Peters, M.J., et al., *Haematological effects of inhalation of N-formyl-methionyl-leucyl-phenylalanine in man.* Thorax, 1992. **47**(4): p. 284-7.
17. Jonsson, M., et al., *Hemodynamic and metabolic effects of intravenous formyl-methionyl-leucyl-phenylalanine (FMLP) in rabbits.* In Vivo, 1997. **11**(2): p. 133-9.
18. Kretschmer, D., et al., *Peptide length and folding state govern the capacity of staphylococcal beta-type phenol-soluble modulins to activate human formyl-peptide receptors 1 or 2.* J Leukoc Biol, 2015. **97**(4): p. 689-97.
19. Peschel, A. and M. Otto, *Phenol-soluble modulins and staphylococcal infection.* Nat Rev Microbiol, 2013. **11**(10): p. 667-73.

20. Vuong, C., et al., *Regulated expression of pathogen-associated molecular pattern molecules in Staphylococcus epidermidis: quorum-sensing determines pro-inflammatory capacity and production of phenol-soluble modulins.* Cell Microbiol, 2004. **6**(8): p. 753-9.
21. Donvito, B., et al., *Synergistic hemolytic activity of Staphylococcus lugdunensis is mediated by three peptides encoded by a non-agr genetic locus.* Infect Immun, 1997. **65**(1): p. 95-100.
22. Frenette, M., et al., *Chemical and biological characterization of a gonococcal growth inhibitor produced by Staphylococcus haemolyticus isolated from urogenital flora.* Infect Immun, 1984. **46**(2): p. 340-5.
23. Rautenberg, M., et al., *Neutrophil responses to staphylococcal pathogens and commensals via the formyl peptide receptor 2 relates to phenol-soluble modulin release and virulence.* FASEB J, 2011. **25**(4): p. 1254-63.
24. Kretschmer, D., et al., *Human formyl peptide receptor 2 senses highly pathogenic Staphylococcus aureus.* Cell Host Microbe, 2010. **7**(6): p. 463-73.
25. Gonzalez, D.J., et al., *Novel phenol-soluble modulin derivatives in community-associated methicillin-resistant Staphylococcus aureus identified through imaging mass spectrometry.* J Biol Chem, 2012. **287**(17): p. 13889-98.
26. Weiss, E., et al., *Formyl-peptide receptor 2 governs leukocyte influx in local Staphylococcus aureus infections.* FASEB J, 2017.
27. Prat, C., et al., *A new staphylococcal anti-inflammatory protein that antagonizes the formyl peptide receptor-like 1.* J Immunol, 2006. **177**(11): p. 8017-26.
28. Prat, C., et al., *A homolog of formyl peptide receptor-like 1 (FPRL1) inhibitor from Staphylococcus aureus (FPRL1 inhibitory protein) that inhibits FPRL1 and FPR.* J Immunol, 2009. **183**(10): p. 6569-78.
29. Wang, R., et al., *Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA.* Nat Med, 2007. **13**(12): p. 1510-4.
30. Wang, R., et al., *Staphylococcus epidermidis surfactant peptides promote biofilm maturation and dissemination of biofilm-associated infection in mice.* J Clin Invest, 2011. **121**(1): p. 238-48.
31. Cassat, J.E., et al., *A secreted bacterial protease tailors the Staphylococcus aureus virulence repertoire to modulate bone remodeling during osteomyelitis.* Cell Host Microbe, 2013. **13**(6): p. 759-72.
32. Nakamura, Y., et al., *Staphylococcus delta-toxin induces allergic skin disease by activating mast cells.* Nature, 2013. **503**(7476): p. 397-401.
33. He, H.Q. and R.D. Ye, *The Formyl Peptide Receptors: Diversity of Ligands and Mechanism for Recognition.* Molecules, 2017. **22**(3).
34. Panaro, M.A., et al., *Biological role of the N-formyl peptide receptors.* Immunopharmacol Immunotoxicol, 2006. **28**(1): p. 103-27.
35. Li, Y. and D. Ye, *Molecular biology for formyl peptide receptors in human diseases.* J Mol Med (Berl), 2013. **91**(7): p. 781-9.
36. Silva, M.T., *Neutrophils and macrophages work in concert as inducers and effectors of adaptive immunity against extracellular and intracellular microbial pathogens.* J Leukoc Biol, 2010. **87**(5): p. 805-13.
37. Soehnlein, O. and L. Lindbom, *Phagocyte partnership during the onset and resolution of inflammation.* Nat Rev Immunol, 2010. **10**(6): p. 427-39.
38. Dotta, L., L. Tassone, and R. Badolato, *Clinical and genetic features of Warts, Hypogammaglobulinemia, Infections and Myelokathexis (WHIM) syndrome.* Curr Mol Med, 2011. **11**(4): p. 317-25.
39. Zeidler, C., et al., *Clinical implications of ELA2-, HAX1-, and G-CSF-receptor (CSF3R) mutations in severe congenital neutropenia.* Br J Haematol, 2009. **144**(4): p. 459-67.
40. Kono, H. and K.L. Rock, *How dying cells alert the immune system to danger.* Nat Rev Immunol, 2008. **8**(4): p. 279-89.
41. Iwasaki, A. and R. Medzhitov, *Control of adaptive immunity by the innate immune system.* Nat Immunol, 2015. **16**(4): p. 343-53.

42. Mayadas, T.N., X. Cullere, and C.A. Lowell, *The multifaceted functions of neutrophils.* Annu Rev Pathol, 2014. **9**: p. 181-218.
43. Sadik, C.D., N.D. Kim, and A.D. Luster, *Neutrophils cascading their way to inflammation.* Trends Immunol, 2011. **32**(10): p. 452-60.
44. Sadik, C.D. and A.D. Luster, *Lipid-cytokine-chemokine cascades orchestrate leukocyte recruitment in inflammation.* J Leukoc Biol, 2012. **91**(2): p. 207-15.
45. Kolaczkowska, E. and P. Kubes, *Neutrophil recruitment and function in health and inflammation.* Nat Rev Immunol, 2013. **13**(3): p. 159-75.
46. Heit, B., et al., *An intracellular signaling hierarchy determines direction of migration in opposing chemotactic gradients.* J Cell Biol, 2002. **159**(1): p. 91-102.
47. Andreasson, E., K. Onnheim, and H. Forsman, *The subcellular localization of the receptor for platelet-activating factor in neutrophils affects signaling and activation characteristics.* Clin Dev Immunol, 2013. **2013**: p. 456407.
48. Fu, H., et al., *The mechanism for activation of the neutrophil NADPH-oxidase by the peptides formyl-Met-Leu-Phe and Trp-Lys-Tyr-Met-Val-Met differs from that for interleukin-8.* Immunology, 2004. **112**(2): p. 201-10.
49. van Kessel, K.P., J. Bestebroer, and J.A. van Strijp, *Neutrophil-Mediated Phagocytosis of Staphylococcus aureus.* Front Immunol, 2014. **5**: p. 467.
50. Greenberg, S. and S. Grinstein, *Phagocytosis and innate immunity.* Curr Opin Immunol, 2002. **14**(1): p. 136-45.
51. Vieira, O.V., R.J. Botelho, and S. Grinstein, *Phagosome maturation: aging gracefully.* Biochem J, 2002. **366**(Pt 3): p. 689-704.
52. Jankowski, A., C.C. Scott, and S. Grinstein, *Determinants of the phagosomal pH in neutrophils.* J Biol Chem, 2002. **277**(8): p. 6059-66.
53. Lee, W.L., R.E. Harrison, and S. Grinstein, *Phagocytosis by neutrophils.* Microbes Infect, 2003. **5**(14): p. 1299-306.
54. Sengelov, H., L. Kjeldsen, and N. Borregaard, *Control of exocytosis in early neutrophil activation.* J Immunol, 1993. **150**(4): p. 1535-43.
55. Faurschou, M. and N. Borregaard, *Neutrophil granules and secretory vesicles in inflammation.* Microbes Infect, 2003. **5**(14): p. 1317-27.
56. Borregaard, N. and J.B. Cowland, *Granules of the human neutrophilic polymorphonuclear leukocyte.* Blood, 1997. **89**(10): p. 3503-21.
57. Quinn, M.T., M.C. Ammons, and F.R. Deleo, *The expanding role of NADPH oxidases in health and disease: no longer just agents of death and destruction.* Clin Sci (Lond), 2006. **111**(1): p. 1-20.
58. Lekstrom-Himes, J.A. and J.I. Gallin, *Immunodeficiency diseases caused by defects in phagocytes.* N Engl J Med, 2000. **343**(23): p. 1703-14.
59. Rogers, D.E. and R. Tompsett, *The survival of staphylococci within human leukocytes.* J Exp Med, 1952. **95**(2): p. 209-30.
60. Voyich, J.M., et al., *Insights into mechanisms used by Staphylococcus aureus to avoid destruction by human neutrophils.* J Immunol, 2005. **175**(6): p. 3907-19.
61. Munzenmayer, L., et al., *Influence of Sae-regulated and Agr-regulated factors on the escape of Staphylococcus aureus from human macrophages.* Cell Microbiol, 2016. **18**(8): p. 1172-83.
62. Lehar, S.M., et al., *Novel antibody-antibiotic conjugate eliminates intracellular S. aureus.* Nature, 2015. **527**(7578): p. 323-8.
63. Thwaites, G.E. and V. Gant, *Are bloodstream leukocytes Trojan Horses for the metastasis of Staphylococcus aureus?* Nat Rev Microbiol, 2011. **9**(3): p. 215-22.

# CHAPTER 2

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## Formyl-Peptide Receptors in Infection, Inflammation, and Cancer

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## Abstract

Formyl-peptide receptors (FPRs) recognize bacterial and mitochondrial formylated peptides as well as endogenous non-formylated peptides and even lipids. FPRs are expressed on various host cell types but most strongly on neutrophils and macrophages. After the discovery of FPRs on leukocytes, it was assumed that these receptors predominantly govern a proinflammatory response resulting in chemotaxis, degranulation, and oxidative burst during infection. However, it is clear that the activation of FPRs has more complex consequences and can also promote the resolution of inflammation. Recent studies have highlighted associations between FPR function and inflammatory conditions, including inflammatory disorders, cancer, and infection. In this review we discuss these recent findings.

## Highlights

Formyl-peptide receptors (FPRs) belong to the family of seven-transmembrane G protein-coupled receptors, which are involved in host defense against infections and in clearance of damaged host cells.

FPR1 is activated by short N-formylated peptides; these are cleavage products of bacterial and mitochondrial proteins. FPR2 is activated by endogenous non-formylated ligands as well as by longer amphipathic, $\alpha$-helical bacterial peptides such as staphylococcal phenol-soluble modulins.

Endogenous FPR2 ligands can increase host survival during bacterial infections by reducing bacterial loads and by preventing excessive inflammatory responses.

FPRs are critical for effective healing of sterile wounds, as they can mediate the first wave of neutrophil infiltration.

FPR expression is associated with tumor progression in various cancer types. However, chemotherapy-induced tumor regression can also require FPR1 expression.

## Formyl-Peptide Receptors and Their Ligands in Health and Disease

The innate immune system discriminates between self and foreign cells or substances through **pattern recognition receptors** (**PRRs**; see Glossary), which recognize **pathogen-associated molecular patterns (PAMPs)**. The **Toll-like receptors (TLRs)** are the best studied PRRs, whereas formyl-peptide receptors (FPRs), also considered PRRs, have not been studied in nearly as much depth. FPRs belong to the family of seven-transmembrane G protein-coupled receptors (GPCRs), many of which are involved in host defense against bacterial infections and clearance of damaged cells [1]. FPRs are well conserved among mammals [1]. The human FPRs (FPR1, FPR2, and FPR3) are primarily regarded as myeloid cell receptors, as they are mainly expressed in neutrophils (except FPR3) and monocytes [2]. In addition to myeloid cells, FPR1 is also expressed in astrocytes, microglia, hepatocytes, and immature dendritic cells, whereas FPR2 is expressed in astrocytoma cells, epithelial cells, hepatocytes, microvascular endothelial cells, and neuroblastoma cells [2]. FPR1 generally recognizes short peptides of approximately 3–5 amino acids that start with N-formylmethionine [3]; these peptides are cleavage products of bacterial and mitochondrial proteins. One of the most potent ligands for human FPR1 is the *Escherichia coli*-derived peptide N-formylmethionyl-leucyl-phenylalanine (fMLF) [1]. By contrast, FPR2 is a low-affinity receptor for many of the potent FPR1 agonists and is activated by longer peptides with a-helical, amphipathic properties [4]. The most prominent bacterial FPR2 ligands are the staphylococcal-derived **phenol-soluble modulins (PSMs)** [5]. In addition to staphylococci, many other bacteria and viruses produce FPR2 ligands, including *Listeria monocytogenes* [6], **Enterococcus faecium** [7], *Helicobacter pylori* [8], *Streptococcus pneumoniae* [9,10], and human immunodeficiency virus (HIV) [11–15] (Table 1). In addition, many host-derived peptide and lipid FPR agonists (Table 1) have been shown to be associated with inflammation and have demonstrated a preference for human and mouse FPR2 [2]. For example, administration of **resolvin D1 (RvD1)** and **lipoxin $A_4$** (**$LXA_4$**; both FPR2 ligands) has been shown to resolve inflammation in a murine model of Alzheimer's disease [16]. FPR3 ligands hardly overlap with those of FPR1 or FPR2, and the overall function of the FPR3 receptor remains unclear (Box 1). Activation of FPRs in myeloid cells results in dissociation of heterotrimeric G proteins coupled to FPRs into $\alpha$ and $\beta\gamma$ subunits [17]. Multiple signaling pathways activated by the dissociated G proteins are involved in a variety of antimicrobial cellular

responses, such as the migration of these cells to peritoneal infection sites, phagocytosis, the release of reactive oxygen species and antimicrobial peptides (AMPs), such as LL-37, as well as the expression of chemokines, such as interleukin (IL)-8 (Figure 1). These have been shown using *in vivo* mouse models, as well as primary mouse and human leukocytes [5,6,18,19].

**Table 1: Representative Pathogen- and Host-derived FPR ligands and their Selectivity.**

| Ligand | Origin / Description | Selectivity | Refs |
|---|---|---|---|
| ***Pathogen-derived ligands*** | | | |
| fMLF | *Escherichia coli* | FPR1 | [1] |
| fMIFL | *Staphylococcus aureus* | FPR1 | [106] |
| fMIVIL | *Listeria monocytogenes* | FPR1 | [106] |
| T20 (DP178) | HIV gp41 | FPR1 | [14] |
| PSMs | *S. aureus* | FPR2 | [5] |
| Hp (2-20) | *Helicobacter pylori* | FPR2 | [8] |
| T21 (DP107) | HIV gp41 | FPR2 | [13] |
| F peptide | HIV gp120 | FPR2 | [11] |
| V3 peptide | HIV gp120 | FPR2 | [12] |
| | | | |
| ***Host-derived ligands*** | | | |
| Cathepsin G | Host defense | FPR1 | [1] |
| Annexin A1 | Wound healing | FPR1; FPR2 | [104] |
| Serum amyloid A | Inflammation; Acute Phase Protein | FPR2 | [1] |
| Lipoxin $A_4$ | Inflammation | FPR2 | [1] |
| Resolvin D1 | Inflammation | FPR2 | [107] |
| LL-37 (mouse CRAMP) | Host defense | FPR2 | [108] |
| $A\beta_{42}$ | Alzheimer's disease | FPR2 | [1] |

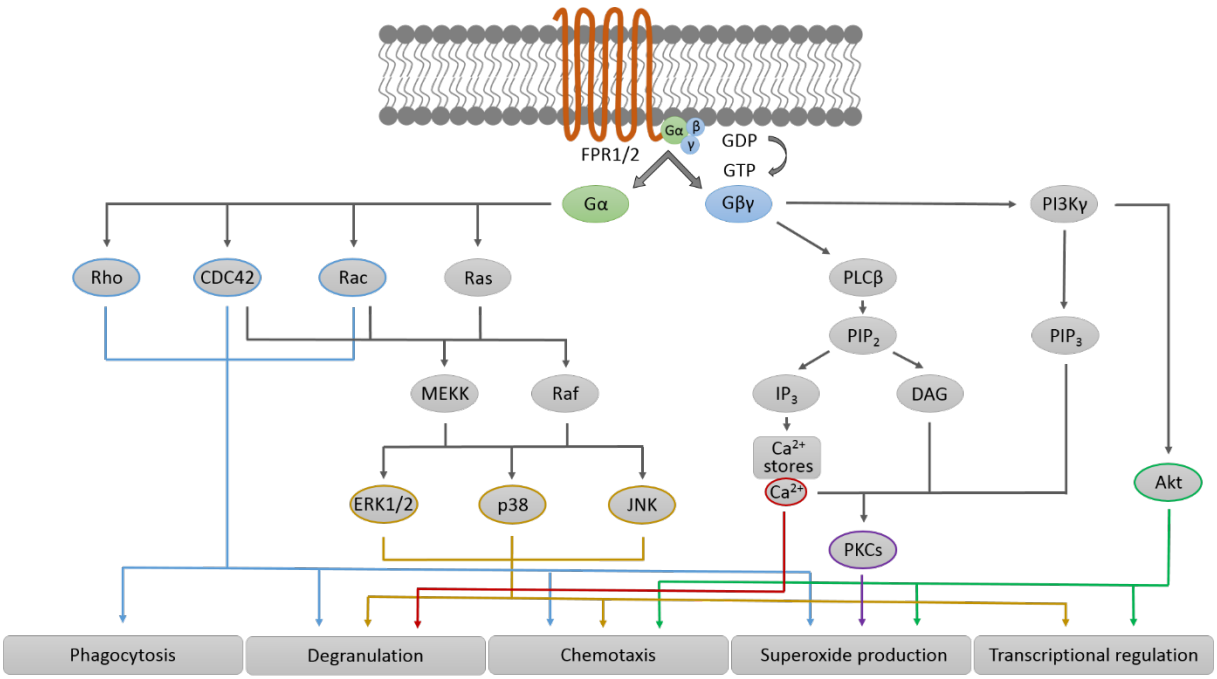


**Figure 1. Signal Transduction Pathways of Formyl-Peptide Receptors (FPRs).** Human FPR1 and FPR2 share a 69% amino acid sequence identity and are thought to signal via the same pathways [1]. Activation of FPRs results in conversion of guanosine diphosphate to guanosine triphosphate, which leads to dissociation of heterotrimeric G proteins coupled to FPRs into α and βγ subunits [17]. The βγ subunit activates phospholipase Cβ (PLCβ) leading to release of calcium from intracellular stores and activation of protein kinases C (PKCs), which are necessary for degranulation and superoxide production, respectively [17,109]. Phosphoinositide-3-kinase γ (PI3Kγ) is also activated by the βγ subunit, which results in activation of PKCs and protein kinase B (Akt). Akt is essential for chemotaxis, superoxide production, and transcriptional regulation [17]. GTPases of the Ras superfamily (Ras, Rho, CDC42, and Rac) are activated by the α G protein subunit (Gα) leading to activation of the mitogen-activated protein kinase (MAPK) pathways, extracellular-regulated protein kinase 1/2 (ERK1/2), p38, and JUN-N-terminal protein kinase (JNK) [110]. The Ras GTPases lead directly to cytoskeletal reorganization [111], necessary for phagocytosis, degranulation, and chemotaxis [17]. In addition, the NADPH oxidase complex is formed and thereby reactive oxygen species (ROS) are released. Activation of the MAPKs results in degranulation, chemotaxis, and transcriptional regulation [17]. DAG, diacylglycerol; IP3, inositol 1,4,5-trisphosphate; MEKK, MAP kinase kinase kinase; PIP, phosphatidylinositol 4,5-bisphosphate.

In recent years, many studies have demonstrated that the release of endogenous FPR ligands can influence severe diseases associated with inflammation, including **systemic inflammatory response syndrome (SIRS)** [20,21] and cancers, such as

glioblastoma as well as gastric and colorectal cancer [22–24]. Indeed, tumoral FPRs frequently serve to support cancer proliferation and metastasis [22–24]. Moreover, heightened FPR expression in tumor cells often correlates with poor prognosis relative to low FPR1 expressing tumors [25]. However, this is only one side of the coin. In addition, effective chemotherapy-induced antitumor responses against breast cancer have been reported to require functional FPR1 expression, since breast cancer patients who bear loss-of-function alleles in FPR1 exhibit reduced survival after treatment with adjuvant chemotherapy [26,27]. As all bacteria (and many pathogens) release FPR ligands and some even FPR inhibitors [such as the chemotaxis-inhibitory protein of *Staphylococcus aureus* (CHIPS) and FPR2-inhibitory protein (FLIPr); Box 2], it is therefore crucial to clarify the role of FPRs in infection as well as in inflammatory processes. Here, we review recent findings concerning the role of FPRs in disease. We provide an overview on the consequences of FPR expression and activation in the context of bacterial and viral infections as well as during inflammation and cancer. Since FPRs are involved in such diverse processes, to our knowledge, we try to connect for the first time the role of FPRs in these various scientific fields. It will be necessary to further interrelate these research topics in the future to better understand the mechanistic roles of these pathways in health and disease.

## Sensing Invading Bacterial Pathogens via FPRs

### Pneumonia and Meningitis

Lower respiratory tract infections in humans are the leading infection-related cause of death worldwide and are mainly caused by *S. pneumoniae* [28]. For pneumococcal pneumonia in mice, an increased expression of several chemokines, such as MIP-2, together with formylated peptides has been shown to be essential in the recruitment of neutrophils into the lung and subsequent effective bacterial clearance [29]. An acute coinfection pneumonia model in mice [coinfection of *S. pneumoniae* and influenza A virus (IAV)] resulted in markedly increased pneumococcal lung load and neutrophilic inflammation [30]. Moreover, expression of mouse Fpr2 (mFpr2; a homolog of human FPR2) has been found to be significantly enhanced in the lungs of coinfected mice, along with the concentrations of **acute phase protein serum amyloid A (SAA)** – an inflammatory agonist of mFpr2 – in both the lung and serum

(Table 1) [30]. SAA induces a proinflammatory macrophage phenotype in monocytes of patients with chronic obstructive pulmonary disease (COPD) [31], reported to stimulate acute neutrophilic lung inflammation in an mFpr2-dependent manner in mice with COPD [32]. SAA can also oppose the organ protective and pro-resolving functions of the lipid agonists RvD1 and $LXA_4$ (Table 1), facilitating the resolution of inflammation via mFpr2 in the absence of SAA (Figure 2A), during COPD or acute lung injury in mice [33,34]. Furthermore, administration of exogenous aspirin-triggered resolvin D1 (AT-RvD1) during the acute phase of infection significantly reduced pneumococcal lung load and pneumonia severity in mice coinfected with *S. pneumoniae* and IAV by limiting excess neutrophil and monocyte recruitment [30]. Therefore, it is reasonable to speculate that AT-RvD1 might become a novel putative therapeutic option for reducing bacterial burden during pneumonia [30], but this warrants rigorous investigation.

*S. pneumoniae* is also one of the most frequent causes of bacterial meningitis, associated with a high mortality rate in addition to brain damage leading to neurological sequelae in up to 50% of human survivors [35]. Astrocytes and microglia, both FPR-expressing cells, play an important role in pathogen recognition and the inflammatory response [36]. Experiments conducted in mFpr1- or mFpr2-deficient mice showed that these receptors were crucial during bacterial meningitis. Specifically, compared with wild-type animals, loss of mFpr1 or mFpr2 resulted in increased mortality of pneumococcal meningitis-infected mFpr knockout mice; increased *S. pneumoniae* bacterial burden in the cerebellum, blood, and spleen; and increased neutrophil and microglia infiltration of the central nervous system (Figure 2A) [9]. In vitro, bacterial supernatants of *S. pneumoniae* and *Neisseria meningitidis* induced FPR2-dependent expression and release of the antimicrobial peptide CRAMP, in addition to the proinflammatory cytokine IL-1b by rat astrocytes and microglia [10]. Of clinical relevance, the antimicrobial human homolog of CRAMP, LL-37, has been consistently found in the cerebrospinal fluid (CSF) of patients with active bacterial meningitis but not in the CSF of healthy controls [37]. Collectively, these findings indicate that FPR deficiency leads to an increased inflammatory response and an unfavorable outcome in response to bacterial meningitis. Thus, presumably, FPRs may be able to play both anti-inflammatory and probactericidal roles in the central nervous system’s immune response against pathogens, but the actual mechanisms underlying these pathways remain to be investigated.

## Polymicrobial Sepsis and Other Infections

Sepsis is a systemic infection caused by microbial invasion of otherwise sterile parts of the body [38]. Substantial impairment of the innate immune system during early sepsis may result in dysregulation of the inflammatory response. Patients with sepsis often die from multiple organ failure, which is caused by excessive lymphocyte apoptosis and dysregulation of pro- and anti-inflammatory cytokine levels [39]. Therefore, effective prevention of mortality from severe sepsis or septic shock requires drugs that enhance the bactericidal activity of phagocytes, inhibit the production of proinflammatory mediators, and prolong adaptive immune responses. Administration of the synthetic FPR agonist WKYMVm during sepsis can effectively reduce lethality in wild-type mice by enhancing bactericidal activity, downregulating proinflammatory cytokines, and inhibiting apoptosis of splenocytes [39]. Moreover, endogenous mFpr2 ligands, such as RvD1 and $LXA_4$, can increase mouse survival during cecal ligation and puncture-induced sepsis by reducing bacterial loads and preventing excessive activation of the inflammatory response (Figure 2A) [40,41]. Nevertheless, the use of these anti-inflammatory and proresolution agents to dampen the inflammatory response during early sepsis could lead to more severe infections, as they have also been reported to decrease cell migration and worsen infection [42]. Therefore, the timing of the production of these endogenous ligands has an impact on sepsis outcomes. By contrast, loss of mFpr2/3 during polymicrobial murine sepsis leads to exacerbation of disease severity, higher levels of proinflammatory cytokines, and reduced monocyte recruitment leading to inadequate bacterial removal compared with wildtype animals (Figure 2A) [43]. In addition, the phagocytic functions of neutrophils have been shown to be impaired in septic mFpr2/3$^{-/-}$ mice compared with wild-type mice, and the absence of mFpr2/3 has been associated with significant injury to distant organs, with a particular effect on the heart and kidneys [43].

*S. aureus* is a major cause of life-threatening infections. *S. aureus* releases both short formylated peptides, which are liberated by all types of bacteria, and phenol-soluble modulins, which are chemotactic peptide ligands for FPR2 and, with much lower efficacy, FPR1, respectively [5]. In vivo, the early local infiltration of leukocytes into *S. aureus* peritonitis infections of mice depends on mFpr2, as knockout of this receptor has resulted in decreased migration relative to wild type, demonstrating a crucial role for this receptor during *S. aureus* infection in mice [18]. Moreover, neutrophil recruitment and IL-1β production during *S. aureus* cutaneous infection of

mice is mFpr1, TLR2, and nucleotide-binding oligomerization domain (NOD) 2 dependent, as determined from experiments using FPR1$^{-/-}$, TLR2$^{-/-}$, and NOD2$^{-/-}$ mice [44]. Overall, IL-1 β seems to be essential for the amplification of the neutrophil response to promote abscess formation and clearance of *S. aureus* skin infection in mice [44].

Another example is *L. monocytogenes*, an opportunistic pathogen that causes severe infections in immunocompromised patients. These infections are typically characterized by several clinical features, including septicemia, brain infection, fetal abortion, and perinatal infection [45]. Of relevance, mFpr1 and mFpr2 play an essential role during superoxide production and early neutrophil recruitment in *Listeria*-infected mouse livers [6]. Specifically, mFpr1-, mFpr2-, or mFrp1/2-deficient mice are more susceptible to *L. monocytogenes* infection than wild-type animals, as evidenced from higher mortality rates in these animals relative to wild-type mice [6,46]. mFpr1$^{-/-}$ mice also harbor higher bacterial loads in the spleen and liver 2 days postinfection compared with wild-type mice. Therefore, the expression of FPRs is critical in the elimination of invading pathogens and ensuing survival of *Listeria*-infected mice [6,46].

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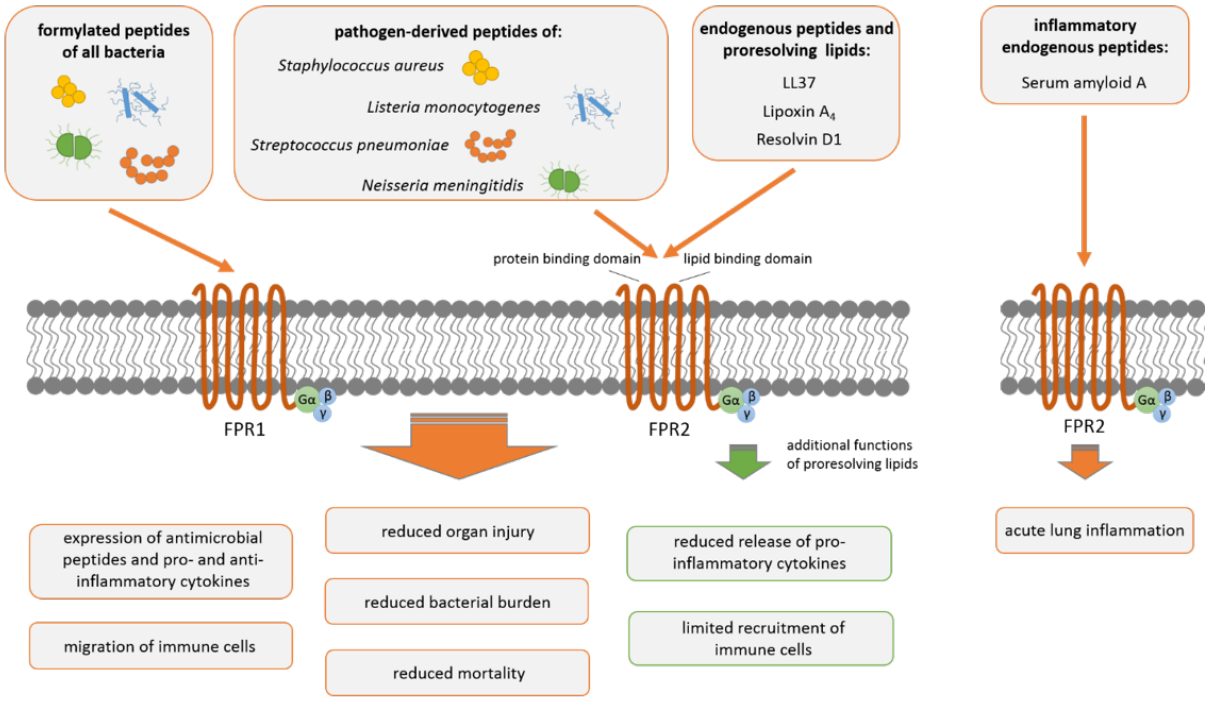

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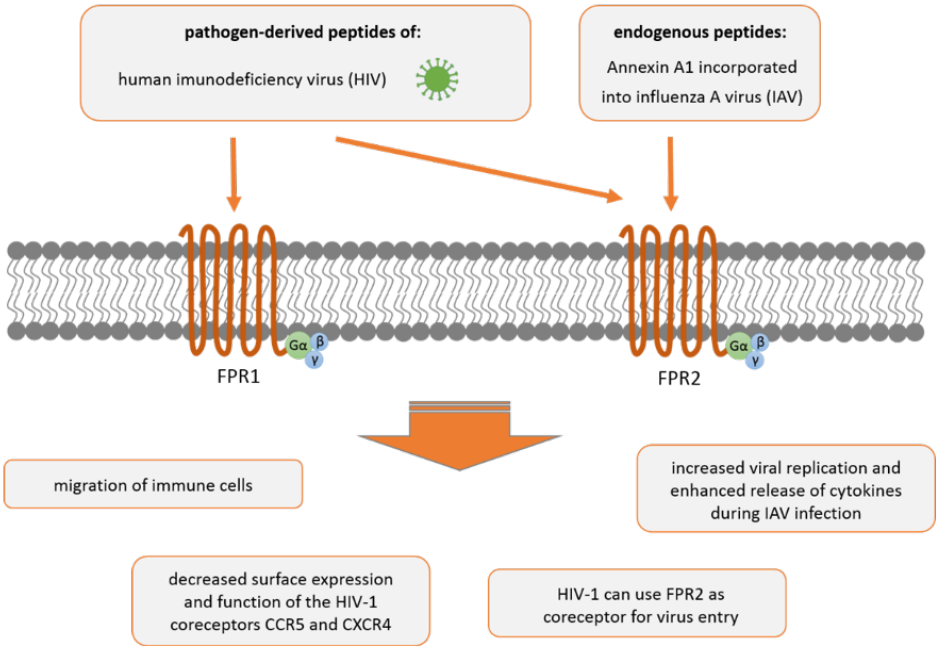


**Figure 2. Formyl-Peptide Receptor (FPR) Activation and Its Consequences in Bacterial and Viral Infection.** (A) Formylated peptides of all bacteria and mitochondria activate FPR1, whereas FPR2 is stimulated by specific endogenous and pathogen-derived ligands [1,2]. Activation of FPRs during infections results in migration of leukocytes to the infection site. Serum amyloid A (SAA) exacerbates infection leading to acute lung inflammation during pneumonia via FPR2 [30–32]. Resolvin D1 (RvD1) and lipoxin $A_4$ ($LXA_4$) can oppose the harmful effect of SAA as they facilitate the resolution of inflammation [33,34]. Binding of these lipids to FPR2 leads, for example, to reduced release of proinflammatory cytokines and limited recruitment of leukocytes to the infection site. (B) Peptides of the envelope proteins of HIV activate FPRs [11–15]. Activation of FPRs during HIV-1 infection leads to decreased surface expression of the HIV-1 coreceptors CCR5 and CXCR4 [53,54]. To circumvent this, HIV-1 can use FPR2 as coreceptor for virus entry [55]. Influenza A virus (IAV) incorporates the endogenous peptide annexin A1 resulting in increased viral replication and release of cytokines during IAV infection [47,49].

## Role of FPRs in Viral Infections

### Influenza Viral Infections

Influenza is one of the most important infectious diseases affecting the respiratory tract and is responsible for seasonal epidemics and sporadic pandemic outbreaks. One study reported that FPR2 might be a new potential target for host-directed influenza A antivirals [47]. IAV is mainly recognized by the PRRs TLR3/7/8, NOD-like

receptor (NLR) NLRP3, and retinoic acid-inducible gene I (RIG-I). Activation of TLR3 and TLR7 by IAV leads to the upregulation of mFpr2 *in vitro* in murine lung epithelial cells and macrophages, and *in vivo* in murine lungs [48]. One study demonstrated **that annexin A1 (ANXA1)**, an anti-inflammatory FPR2 ligand, was increased at the surface of influenza-infected human alveolar cells and was also enriched in **lipid rafts**, the sites of virus budding. ANXA1 was then incorporated into IAV particles during the budding process, thus equipping the virus with an FPR2 ligand on its surface [47]. Furthermore, activation of FPR2 by IAV led to an extracellular signal-regulated kinase (ERK)-dependent increase in viral replication and an enhanced release of cytokines, such as IL-6 and interferon-β (IFN- β), by the infected human alveolar cells *in vitro* and in the bronchoalveolar lavage fluid *in vivo* (Figure 2B) [47]. Accordingly, mice treated with mFpr2 antagonists during IAV infection presented a significantly higher survival rate than untreated mice [47,49]. It was demonstrated that FPR2 antagonists exerted a general antiviral effect during IAV and influenza B virus infections through early inhibition of virus-induced ERK activation [49]. Consistent with this, another group showed that ANXA1-deficient mice were protected from IAV replication and lethal viral infections, as evidenced from decreased weight loss and increased survival [50]. Thus, FPR2 may represent a novel putative target that might be tested in the prevention and clearance of influenza infections. Blocking FPR2 might not only prevent viral replication, but could also temper inflammation [51]. However, it remains to be determined to what extent such findings could be generalizable in humans.

### Human Immunodeficiency Virus Infections

HIV infections constitute a major global health issue, given that it causes acquired immunodeficiency syndrome (AIDS), one of the top ten fatal diseases worldwide, leading to the death of more than one million people each year [52]. The glycoproteins gp120 and gp41 coat the envelope of HIV-1, thus enabling this retrovirus to bind, fuse with the membrane, and infect susceptible host cells. Synthetic peptides derived from gp41 – T20/DP178 and T21/DP107 – and gp120 – V3 and F peptide – have been described as FPR ligands (Table 1) [11–14]. Recently, the first natural proteolytic cleavage product of the HIV-1 envelope protein gp41 – MAT-1 – was reported to induce migration of primary human neutrophils and

monocytes in an FPR2-dependent manner, as pharmacological inhibition of FPR2 abolished migration *in vitro* (Figure 2B) [15]. Moreover, this chemotactic peptide can be cleaved by matriptase, an epithelial serine protease found in the prostate, an important viral reservoir for the dissemination of HIV-1 [15]. However, whether MAT-1 plays a role *in vivo* remains to be determined.

*In vitro* activation of FPR1 or FPR2 by non-HIV-1-derived ligands has been reported to decrease the surface expression and function of the HIV-1 coreceptors CCR5 and CXCR4 on human primary monocytes and human osteosarcoma cells by heterologous desensitization (Figure 2B) [53,54]. Therefore, FPR ligands might inhibit HIV-1 fusion and infection of host cells [53,54]. However, other studies have indicated that FPRs can also enhance HIV-1 entry into target cells [55]. Thus, the overall effect of FPR activation during HIV-1 infection remains elusive and warrants robust investigation to fully clarify the role of FPRs during viral infection *in vivo*.

## Role of FPRs during Sterile Inflammation

FPRs seem to be critical for normal healing of a sterile wound, since they can mediate the first wave of neutrophil infiltration [56]. Specifically, during **sterile inflammation**, neutrophil recruitment is mediated by the release of damage-associated molecular patterns (DAMPs) following the rupture of the plasma membrane [57,58]. Neutrophils migrate out of the circulatory system toward the site of injury, initiating the inflammatory response by releasing a number of proinflammatory mediators, thus amplifying the immune response [58]. Mitochondrial peptides can induce inflammatory processes via FPR1- and FPR2-mediated activation of neutrophils (Figure 3A) [56]. Specifically, in wild-type mice, neutrophils rapidly infiltrate the dermis of a sterile skin wound prior to the initiation of chemokine production by the injured tissue. By contrast, rapid neutrophil infiltration is markedly reduced and wound closure is delayed in mice globally deficient in both mFprs [56].

From another angle, a detrimental effect of FPR-mediated inflammation can be observed in proliferative diabetic retinopathy (PDR), a major complication of diabetes mellitus. Using human pars plana vitrectomy-derived PDR vitreous fluid samples in murine Matrigel plug and chick embryo chorioallantoic membrane assays, FPR1-induced inflammation and neovessel formation have been shown to trigger PDR, which can eventually lead to blindness [59]. Furthermore, traumatic injury can induce

SIRS or a 'cytokine storm' [20]. SIRS is characterized by the release of proinflammatory cytokines into the circulation, leading to marked immune cell activation. Formylated DAMPs released from necrotic cells due to tissue damage can initiate the SIRS response [20]. During SIRS, neutrophils are recruited to distal organs where they can contribute to the development of multiple organ dysfunction, circulatory collapse, and potentially death [60]. In general, FPR1 activation is deemed as a key event in mitochondrial DAMP-induced neutrophil activation [61]. Moreover, formylated mitochondrial peptides can induce **sepsis-like syndrome** and cardiovascular collapse (Figure 3A) in rats undergoing hemorrhagic shock, with increased plasma concentrations of mitochondrial formylated proteins associated with lung damage, relative to rats treated with the FPR2 inhibitor $WRW_4$ (Figure 3A) [20]. Similar results have been observed in acute respiratory distress syndrome (ARDS): mitochondrial-derived formylated peptides (fMITs) were elevated in both bronchoalveolar lavage fluid and serum samples from patients with ARDS [62]. In summary, these data suggest that formylated peptides may potentially link mechanisms of sterile inflammation in trauma, SIRS, and cardiovascular collapse. However, despite unwanted collateral damage induced by overwhelming neutrophil recruitment, the infiltration of neutrophils to a sterile site of injury may also play a significant role in prompting the resolution of inflammation and wound closure [56]. Therefore, well-defined FPR activation and signaling are necessary to ensure an optimal immune response.

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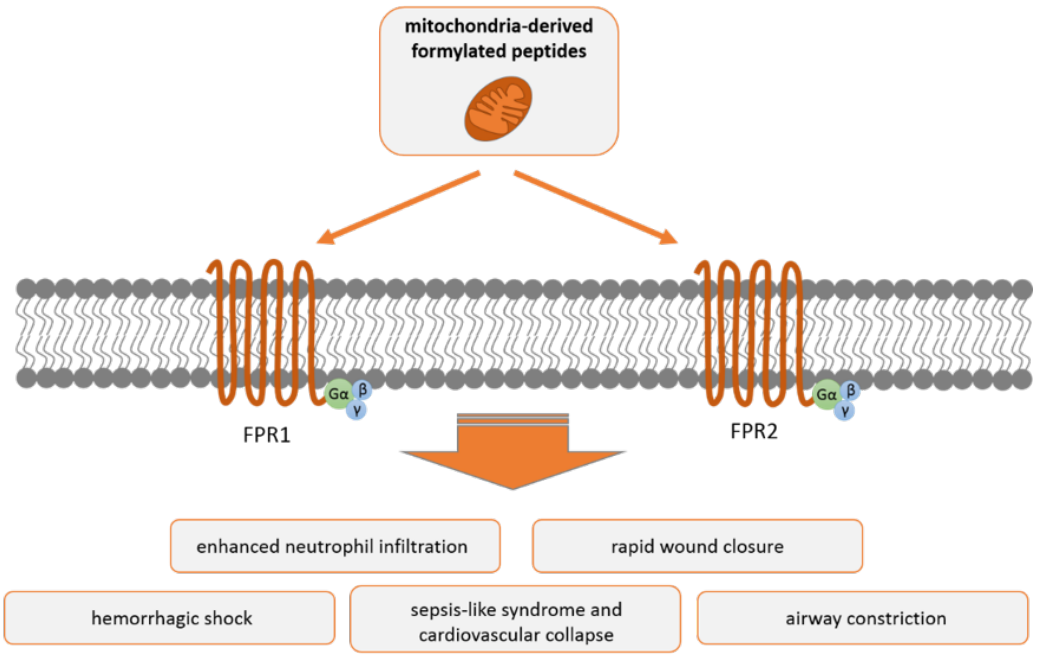

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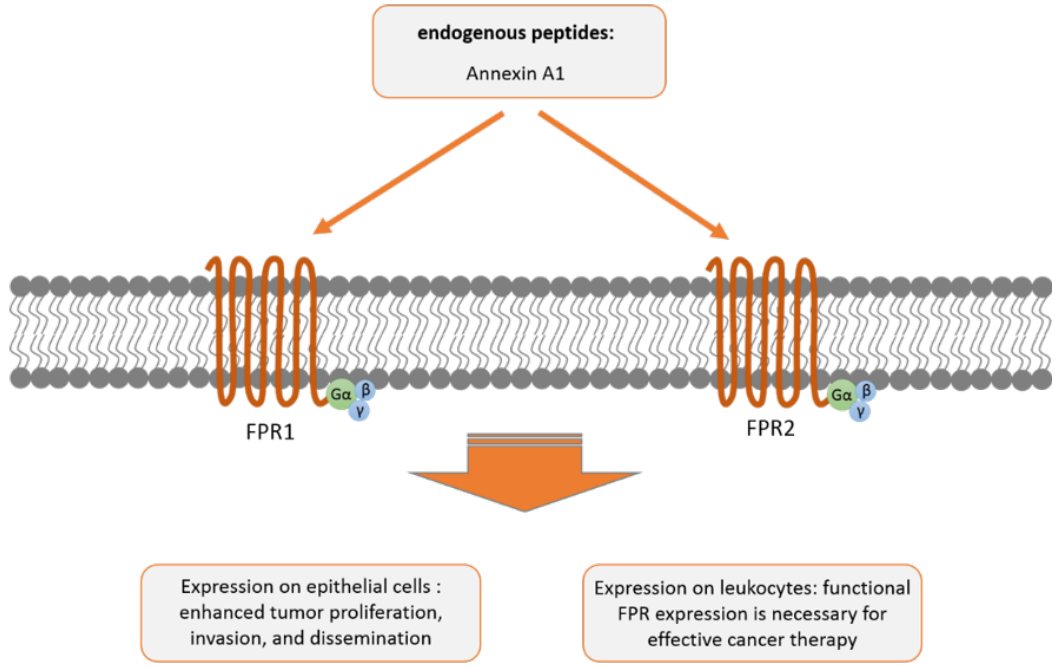


**Figure 3. Formyl-Peptide Receptor (FPR) Activation and Its Consequences in Inflammation and Cancer.** (A) Damage-associated molecular patterns (DAMPs) are released during sterile inflammation. This leads to rapid neutrophil infiltration and wound closure. Excess release of DAMPs by necrotic cells might result in hemorrhagic shock and sepsis-like syndrome [57,58]. (B) During cancer, FPRs play ambivalent roles depending on the cell type expressing these receptors. Expression on epithelial cells leads to enhanced tumor proliferation, invasion, and dissemination after FPR activation by annexin A1 [68]. On leukocytes, functional FPR expression appears to be necessary to achieve effective therapy for certain cancers [26].

## Role of FPRs in Cancer

### Tumor-Promoting Role of FPRs

FPRs have also been implicated in various cancers (Figure 3B). Tumors have learned to exploit FPRs to gain a growth advantage. Indeed, FPR1 has been shown to be selectively expressed by highly malignant human glioblastoma cells and to promote disease progression [25]. Specifically, ANXA1 has been found to be present in xenografts formed by human glioblastoma cells in nude mice; and in addition, mFpr1/ANXA1 double knockdown in nude mice resulted in diminished tumor growth compared with single ANXA1 knockdown [63]. Furthermore, *in vitro* experiments using the human glioblastoma cell line U-87 have shown that activation of the

endogenous ligand ANXA1 released by necrotic tumor cells can lead to FPR1-mediated glioblastoma cell chemotaxis, invasion, as well as production of proangiogenic factors vascular endothelial growth factor and IL-8 [64].

Moreover, FPR1 has been reported to be highly expressed in human astrocytoma cells and can be activated by human mitochondria-derived agonists in U87 cells, while being inhibited by the FPR antagonist CHIPS [65] or, in glioblastoma, by pertussis toxin [22]. High FPR1 expression in neuroblastoma has been documented and significantly associated with worsened survival outcomes in humans [66]. By contrast, knockdown of FPR1 in neuroblastoma cells has been found to delay xenograft tumor development in nude mice [66].

Furthermore, high FPR1 expression has been significantly associated with Stage IV disease, submucosal invasion, serosal invasion, and clinical outcome in gastric cancer [23]. Similarly, FPR1 has been reported to be highly expressed in progressive primary human colorectal cancer, and associated with poor patient prognosis, when compared with distant normal tissues and adjacent non-tumor tissues [24]. Both FPR1 and FPR2 have been associated with tumor progression: in 60 human epithelial ovarian cancer tissue samples, FPR2 expression was correlated with poor prognosis and with promoting invasion and metastasis [67]. An important aspect in FPR-dependent tumor progression represents the release of endogenous FPR ligands, for example, in context with ANXA1 release. The consequence of ANXA1 release is often increased tumor cell proliferation. Hypoxia can regulate ANXA1 expression to support prostate cancer cell invasion and aggressiveness as shown in three different human prostate cancer cell lines via *in vitro* loss- and gain-of-function experiments performed using the RNA interfering siANXA1 and an ANXA1 overexpressing plasmid [68]. Moreover, ANXA1 is frequently overexpressed in human pancreatic carcinoma tissue [69]. However, in addition to ANXA1, the FPR2 ligand LL-37 can increase tumor proliferation: LL-37 is highly expressed in human breast, lung, and ovarian epithelial tumors and experiments *in vitro* have shown that administration of recombinant LL-37 could increase the proliferation, migration, and invasion of malignant ovarian cancer cell line SK-OV-3 through Matrigel-coated inserts relative to pertussis toxin-treated cells [70].

Thus, it seems that high FPR expression in tumor epithelial cells can promote tumor proliferation, invasion, and dissemination. However, the role of FPR expression in

leukocytes may be an important consideration when selecting a treatment strategy for breast cancer patients, as it will be important to assess the functionality of FPRs in these patients when compared to patients with single-nucleotide polymorphisms in FPR1, since adjuvant chemotherapy probably only works with functional FPR1. Evidently, this warrants further investigation.

**Tumor-Suppressive Role of FPRs**

Although there are many examples in which FPR expression correlates with a poor prognosis, this is only one side of the coin. FPR expression can also prevent tumor growth. In six different human primary lung carcinoma samples, independent of tumor classification, all types of FPRs were expressed at lower concentrations in lung cancer tissue compared with surrounding peritumoral tissues [71]. Furthermore, mFpr2, expressed by mouse colonic epithelial cells, has been reported to protect against inflammation-associated tumorigenesis [72]. Colonic epithelial cells in FPR2-deficient mice have been shown to display defects in commensal bacterium dependent homeostasis, since the absence of mFpr2 led to shortened colonic crypts, reduced acute inflammatory responses to dextran sulfate sodium challenge, delayed mucosal restoration after injury, and increased azoxymethane-induced tumorigenesis [72].

However, these are only two examples suggesting a tumor-suppressive role of FPRs, while there are many examples in which FPR expression correlates with tumor growth. It seems that functional FPR expression is necessary for effective cancer therapy. For instance, chemotherapy-induced antitumor immunity has been found to require FPR1 expression in human breast cancer patients and loss-of-function mutations in FPR1 have correlated with poor prognosis and impaired immunosurveillance (Box 3) [27]. Moreover, the FPR1 single-nucleotide polymorphism rs867228 (1037A>C) leads to an amino acid substitution (G346A) in the intracellular C terminus of the protein, which has been shown to be required for signaling [26]. Breast cancer patients bearing this FPR1 loss-of-function allele in either homozygosity or heterozygosity have exhibited reduced overall survival following adjuvant anthracycline-based chemotherapy compared to breast cancer patients with wild-type FPR1 [26]. Similarly, colorectal cancer patients homozygous for this allele have also exhibited decreased overall survival following oxaliplatin

based chemotherapy relative to patients with heterozygous or wild-type FPR1 [27]. In addition, unlike the control cell line CNE2-pLKO.1, ANXA1 knockdown can enhance radioresistance of human nasopharyngeal carcinoma tumor cell line CNE2-sh annexin A1 (knockdown of ANXA1) by reducing intracellular reactive oxygen species concentrations [73].

Nevertheless, it is not clear yet if these effects are primarily mediated via FPRs, although it is reasonable to speculate that they might. Taken together, as various types of cancer can behave in a contradictory manner, it is important to analyze the cancer and organ type, as well as the FPR expression carefully. Subsequently, it may be feasible to assess to what extent FPRs might be exploited as candidate targets for potential cancer treatments.

## Concluding Remarks

Activation of FPRs by formylated bacterial peptides or by host-derived mitochondrial peptides is required for local control of infection and wound healing. Nevertheless, various detrimental effects can result from the release of formylated peptides into the bloodstream, either through conditions such as sepsis or through severe trauma. Thus, inhibitory strategies to prevent FPR activation might be considered as a novel candidate approach for treating sepsis or perhaps SIRS, although evidently, extensive work is necessary to determine this. From another angle, targeting FPRs to treat certain cancer types seems to be more complex, since at least two aspects must be considered. Specifically, since the activation of human leukocytes may require FPR1 expression, functional FPR1 seems to be necessary in humans to achieve effective chemotherapy or radiotherapy [27]. However, increased FPR expression on tumor cells can also enhance proliferation and metastasis, and the mechanisms for these processes remain elusive [24,67] (see Outstanding Questions). To assess potential therapeutic strategies, it is important to understand in which context the activation or inhibition of FPRs is advantageous or detrimental to ameliorating disease processes. Furthermore, as FPRs are expressed on diverse cell types, it will be necessary to elucidate and fully dissect the consequences of FPR activation in different cell types and for different types of diseases including as discussed, infections, cancers, and inflammatory conditions such as SIRS or ARDS [e.g., epithelial cells vs. leukocytes (see Outstanding Questions)].

However, the investigation of such molecules is often difficult, since mouse and human Fprs/FPRs vary significantly in terms of ligand recognition and their activation by specific ligands (Box 4). An important limitation to acknowledge is the extent to which many of the findings described in this review can be generalized to human populations. Indeed, only very few FPR-modulatory peptides have recently progressed into clinical trials. For instance, two Phase I clinical studies showed that the FPR2 agonist ACT-389949 was safe, but was not a viable drug due to rapid receptor desensitization (ClinicalTrials.gov NCT02099071 and NCT02099201) [74]. Moreover, the proteolytic instability of natural peptide ligands has also provided challenges in the development of FPR ligands. Thus, the design of synthetic peptide mimetics that are partially composed of unnatural amino acid-like residues [75] might be considered as a strategy aiming to enhance proteolytic stability and increase bioavailability of these agents in preclinical experiments (see Outstanding Questions). Although FPRs represent long known ancient receptors, not much is known about the consequences of receptor activation and induction of receptor expression, especially in epithelial and endothelial cells. In the light of treatment failures in inflammatory and infectious diseases as well as in cancer, a big challenge in the future will be to increase our understanding of FPR mechanisms of action in the context of these diseases.

## Outstanding Questions

The human immune system can use the same FPRs for the recognition of tissue damage and bacterial infection. How can we tease apart their different mechanisms of action in different contexts?

How can the activation of FPR2 on the one hand promote inflammation and on the other hand lead to resolution of inflammation?

What are the main differences in FPR activation between leukocytes and epithelial cells? What roles do FPRs play in the gut versus skin epithelial cells?

Since FPRs are expressed on macrophages and dendritic cells, what impact do FPRs have on adaptive immunity?

Are FPRs able to discriminate between endogenous and microbe-derived ligands?

How are FPRs able to distinguish between commensal and pathogenic bacteria?

Although it has been shown that various bacteria (e.g., enterococci) other than staphylococci release FPR2 ligands, very little is known about these ligands. What can we learn about these different ligands?

The activation of FPR1 can lead on the one hand to increased tumor proliferation and on the other hand to increased responsiveness to antitumor treatments. In one organ, FPR activation can lead to increased tumor growth but to decreased tumor growth in another. What mechanisms are responsible for these differences? Which molecules are responsible for the increased expression of FPRs in tumor cells? In general, which pathways are responsible for the (up) regulation of FPR expression in tumor cells?

Since specificity of FPRs for different ligands varies not only between receptors but also between species, it remains unclear if animal models adequately represent appropriate models for analyzing specific FPR ligands. Can we envisage better modeling strategies?

Can FPRs be effectively used as putative therapeutic targets to modulate certain inflammatory responses? If so, how? Can we increase the specificity, bioavailability, and stability of FPR ligands or inhibitors?

For which inflammatory diseases can FPR inhibitors or activators be exploited to influence positive disease outcomes?

## Box 1. Formyl-Peptide Receptor 3

Human FPR3 is expressed in monocytes, myeloid and plasmacytoid dendritic cells, some tissue-specific macrophage subpopulations (particularly in the lung, skin, and colon), and eosinophils, but not in human neutrophils [76]. So far, nothing is known about this receptor in context with diseases. Formyl-peptide receptor 3 in mice (mFpr3, also known as Fpr-rs1) is expressed in subsets of sensory neurons as a pathogen sensor [77]. mFpr3 expression in mouse neutrophils is upregulated after stimulation with the bacterial endotoxin lipopolysaccharide. However, the genomes of at least 19 described mouse strains encode a nonfunctional mFpr3 variant. Therefore, it seems that mFpr3 may be dispensable, at least in mice [78]. F2L and humanin [79] are endogenous peptide agonists for FPR3 [80], which is an acetylated 21-amino acid peptide derived from the N terminus of the intracellular heme-binding

protein 1 [81]. N-terminally formylated signal peptide fragments can also activate human and mouse FPR1 and FPR2 at low nanomolar concentrations [82]. In addition, mFpr3 and human FPR3 are also activated by a relatively small subset of signal peptides and require higher concentrations than FPR1 and FPR2 [82]. In contrast to FPR1 and FPR2, resting FPR3 displays a marked level of phosphorylation in stable transfected HEK293T cells, which is only slightly increased upon agonist stimulation [83]. Overall, the biological relevance of FPR3 *in vivo* remains to be determined.

## Box 2. Pathogen-Derived Inhibitors of Formyl-Peptide Receptors

Bacterial pathogens produce potent FPR antagonists. *Bordetella pertussis* produces pertussis toxin, an inhibitor of GPCR-mediated leukocyte chemotaxis [1]. Its A-subunit inactivates the Gαi proteins of FPRs and related receptors by relocating the ADP ribosyl of NAD to Gαi. *Haemophilus influenzae* releases a small, unidentified factor (<15 kDa) that inhibits neutrophil migration toward fMLF [84]. Approximately 60% of clinical *S. aureus* isolates secrete CHIPS. CHIPS inhibits both human FPR1 and the related complement system C5a receptor [85]. CHIPS is by far the most potent antagonist for the FPR1 receptor with a pKd of 7.46 [85]. The *S. aureus* FPR2-inhibitory protein (FLIPr) is 28% identical to CHIPS. The gene is present in approximately 59% of clinical isolates of *S. aureus* and is located together with genes for other virulence factors within a genetic element called ‘immune evasion cluster 2’ [86]. FLIPr-like is produced after *S. aureus* contacts neutrophil granule components and it can inhibit both FPR1 and FPR2 [87]. Cyclosporine A (CsA) is a fungus-derived cyclic undecapeptide with potent immunosuppressive activity [88]. Its analog, cyclosporin H (CsH), lacks immunosuppressive function but can act as an antagonist for the human FPR [89].

## Box 3. Formyl-Peptide Receptor Human Polymorphisms in Human Disease

Single-nucleotide polymorphisms (SNPs) are variations in a single nucleotide at a specific position in the genome. These naturally occur in the human population and are the most frequent type of variation in the human genome. SNPs are often

associated with increased risk of pathological disease development. Currently, more than 200 SNPs leading to amino acid substitutions have been described for FPRs in humans, but only 20 FPR1 SNPs and seven FPR2 SNPs occur in specific amino acid positions with a known function [90].

The following FPR1 mutations have been associated with the development of aggressive periodontitis: N192K in the second extracellular loop [91], F110S [92] and V101L [93] in the third transmembrane domain, and C126W in the second intracellular loop [92]. The N129K mutation disturbs ligand binding and has been implicated in macular degeneration [94] and gastric cancer [95]. The F110S substitution results in decreased receptor expression, disruption of G-protein binding, and chemotaxis failure in response to fMLF [92]. The V101L missense mutation results in increased affinity of FPR1 for both agonists [96] and antagonists [96]. C126W mutant receptors have shown defects in G-protein binding [97]. The L97W mutation, affecting the main ligand-binding site in the first extracellular loop of FPR1, can act in combination with other risk factors to increase the risk of macular degeneration and polypoidal choroidal vasculopathy [94]. The I11T substitution, affecting the N-terminal part of FPR1, has been associated with decreased serum E-selectin levels [98] and an increased risk for high blood pressure with aging [99].

Fewer SNPs are known for FPR2 and, to our knowledge, most of them are not associated with diseases. Nevertheless, an intronic SNP (rs17694990) has been associated with asthma risk [100] and an SNP (rs11666254) located in the promoter of FPR2 correlates with increased sepsis hypersensitivity in patients with major traumatic injuries [101].

## Box 4. The Role of ANXA1 in Cancer

ANXA1 is a 37-kDa calcium- and phospholipid-binding protein expressed in monocytes, macrophages, neutrophils, and epithelial cells. ANXA1 is released from leukocytes at sites of inflammation and is upregulated by anti-inflammatory mediators such as glucocorticoids [102]. Biologically, ANXA1 functions by activating FPRs. One study showed that ANXA1 could activate FPR1 *in vitro* using primary human neutrophils, and induced intracellular calcium release, known to be inhibited by the FPR inhibitor Boc1 [103]. However, ANXA1 and peptides derived from its N-terminal domain can also bind to FPR2 [104]. ANXA1, derived from wound-associated cells,

including leukocytes and epithelial cells, exerts paracrine and autocrine effects on the epithelium, facilitating wound closure and enhancing barrier recovery in human SK-CO15 epithelia cells [102]. Indeed, ANXA1 is externalized by secondary necrotic cells and is cleaved by ADAM10. Supernatants of secondary necrotic cells can lead to chemotaxis of primary human monocytes *in vitro* [105]. Recent research indicates that ANXA1 can function as either a tumor suppressor or a tumor promoter, depending on the particular type of tumor (Figure 3B).

## Glossary

**Acute phase protein serum amyloid A (SAA):** secreted during the acute phase of inflammation predominantly by the liver.

**Annexin A1 (ANXA1):** belongs to the annexin family of $Ca^{2+}$-dependent phospholipid-binding proteins. It suppresses phospholipase A2 and inhibits various leukocyte inflammatory events.

**Heterologous desensitization:** homologous desensitization leaves a cell transiently unresponsive to agents that activate the desensitized receptor, whereas heterologous desensitization leaves a cell transiently unresponsive to agents that activate related receptors.

**Lipid rafts:** subdomains in the plasma membrane that contain high concentrations of cholesterol and glycosphingolipids. They exist as distinct liquid-ordered regions of the membrane that are resistant to extraction with non-ionic detergents.

**Lipoxin $A_4$ ($LXA_4$):** represents a bioactive metabolite of arachidonic acid made by various cell types (e.g., neutrophils). $LXA_4$ is formed during inflammatory responses and contributes to resolving inflammatory responses.

**Matriptase:** enzyme which cleaves various synthetic substrates with Arg or Lys at the P1 position and prefers small side-chain amino acids, such as Ala and Gly, at the P2 position.

**Pathogen-associated molecular patterns:** molecules with conserved patterns derived from pathogens (e.g., lipopolysaccharide) which are recognized by cells of the innate immune system. They are recognized by PRRs in both plants and animals.

**Pattern recognition receptors (PRRs):** germline-encoded host receptors. They are expressed by cells of the innate immune system, such as macrophages and neutrophils, but also by other cell types (e.g., epithelial cells). PRRs identify pathogen-associated molecular patterns, which are associated with microbial pathogens, and damage-associated molecular patterns (DAMPs), which are associated with components of host cells that are released during cell damage.

**Phenol-soluble modulins:** family of protein toxins produced by staphylococci. They are encoded within the core genome and constitute important virulence factors. They activate leukocytes via FPR2.

**Resolvin D1 (RvD1):** endogenous chemical mediator that exerts potent anti-inflammatory and proresolving activities by acting on GPCRs.

**Sepsis-like syndrome:** defined as systemic SIRS in response to an infectious process, whereas sepsis-like syndrome describes SIRS without infection.

**Sterile inflammation:** form of inflammation (in the absence of infection) caused by mechanical trauma, ischemia, stress, or environmental conditions. These conditions induce the secretion of molecular agents collectively termed DAMPs.

**Systemic inflammatory response syndrome (SIRS):** describes the presence of at least two of the following symptoms: abnormal body temperature, heart rate, respiratory rate, and increased white blood cell count.

**Toll-like receptors (TLRs):** play a key role in the innate immune system. These receptors are usually expressed in macrophages as well as dendritic cells and recognize structurally conserved molecules derived from microbes (e.g., lipopolysaccharides or lipopeptides).

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## References


1. Ye, R.D. et al. (2009) International Union of Basic and Clinical Pharmacology. LXXIII. Nomenclature for the formyl peptide receptor (FPR) family. Pharmacol. Rev. 61, 119–161
2. He, H.Q. and Ye, R.D. (2017) The formyl peptide receptors: diversity of ligands and mechanism for recognition. Molecules 22, E455
3. Forsman, H. et al. (2015) Structural changes of the ligand and of the receptor alters the receptor preference for neutrophil activating peptides starting with a formylmethionyl group. Biochim. Biophys. Acta 1853, 192–200
4. Kretschmer, D. et al. (2015) Peptide length and folding state govern the capacity of staphylococcal beta-type phenol-soluble modulins to activate human formyl-peptide receptors 1 or 2. J. Leukoc. Biol. 97, 689–697
5. Kretschmer, D. et al. (2010) Human formyl peptide receptor 2 senses highly pathogenic *Staphylococcus aureus*. Cell Host Microbe 7, 463–473
6. Liu, M. et al. (2012) Formylpeptide receptors are critical for rapid neutrophil mobilization in host defense against *Listeria monocytogenes*. Sci. Rep. 2, 786
7. Bloes, D.A. et al. (2012) *Enterococcus faecium* stimulates human neutrophils via the formyl-peptide receptor 2. PLoS One 7, e39910
8. Betten, A. et al. (2001) A proinflammatory peptide from *Helicobacter pylori* activates monocytes to induce lymphocyte dysfunction and apoptosis. J. Clin. Invest. 108, 1221–1228
9. Oldekamp, S. et al. (2014) Lack of formyl peptide receptor 1 and 2 leads to more severe inflammation and higher mortality in mice with of pneumococcal meningitis. Immunology 143, 447–461
10. Braun, B.J. et al. (2011) The formyl peptide receptor like-1 and scavenger receptor MARCO are involved in glial cell activation in bacterial meningitis. J. Neuroinflammation 8, 11
11. Deng, X. et al. (1999) A synthetic peptide derived from human immunodeficiency virus type 1 gp120 downregulates the expression and function of chemokine receptors CCR5 and CXCR4 in monocytes by activating the 7-transmembrane Gprotein-coupled receptor FPRL1/LXA4R. Blood 94, 1165–1173
12. Shen, W. et al. (2000) Activation of the chemotactic peptide receptor FPRL1 in monocytes phosphorylates the chemokine receptor CCR5 and attenuates cell responses to selected chemokines. Biochem. Biophys. Res. Commun. 272, 276–283
13. Su, S.B. et al. (1999) T21/DP107, a synthetic leucine zipper-like domain of the HIV-1 envelope gp41, attracts and activates human phagocytes by using G-protein-coupled formyl peptide receptors. J. Immunol. 162, 5924–5930
14. Su, S.B. et al. (1999) T20/DP178, an ectodomain peptide of human immunodeficiency virus type 1 gp41, is an activator of human phagocyte N-formyl peptide receptor. Blood 93, 3885–3892
15. Wood, M.P. et al. (2014) The HIV-1 gp41 ectodomain is cleaved by matriptase to produce a chemotactic peptide that acts through FPR2. Immunology 142, 474–483
16. Kantarci, A. et al. (2018) Combined administration of resolvin E1 and lipoxin A4 resolves inflammation in a murine model of Alzheimer's disease. Exp. Neurol. 300, 111–120
17. Dorward, D.A. et al. (2015) The role of formylated peptides and formyl peptide receptor 1 in governing neutrophil function during acute inflammation. Am. J. Pathol. 185, 1172–1184
18. Weiss, E. et al. (2017) Formyl-peptide receptor 2 governs leukocyte influx in local *Staphylococcus aureus* infections. FASEB J. 32, 26–36
19. Wan, M. et al. (2014) Antimicrobial peptide LL-37 promotes bacterial phagocytosis by human macrophages. J. Leukoc. Biol. 95, 971–981
20. Wenceslau, C.F. et al. (2015) Mitochondrial N-formyl peptides induce cardiovascular collapse and sepsis-like syndrome. Am. J. Physiol. Heart Circ. Physiol. 308, H768–H777

21. Wenceslau, C.F. et al. (2016) Mitochondrial N-formyl peptides cause airway contraction and lung neutrophil infiltration via formyl peptide receptor activation. Pulm. Pharmacol. Ther. 37, 49–56
22. Gilder, A.S. et al. (2016) Pertussis toxin is a robust and selective inhibitor of high grade glioma cell migration and invasion. PLoS One 11, e0168418
23. Cheng, T.Y. et al. (2014) Formyl peptide receptor 1 expression is associated with tumor progression and survival in gastric cancer. Anticancer Res. 34, 2223–2229
24. Li, S.Q. et al. (2017) The expression of formyl peptide receptor 1 is correlated with tumor invasion of human colorectal cancer. Sci. Rep. 7, 5918
25. Huang, J. et al. (2010) The G-protein-coupled formylpeptide receptor FPR confers a more invasive phenotype on human glioblastoma cells. Br. J. Cancer 102, 1052–1060
26. Vacchelli, E. et al. (2016) Impact of pattern recognition receptors on the prognosis of breast cancer patients undergoing adjuvant chemotherapy. Cancer Res. 76, 3122–3126
27. Vacchelli, E. et al. (2015) Chemotherapy-induced antitumor immunity requires formyl peptide receptor 1. Science 350, 972–978
28. GBD Diarrhoeal Diseases Collaborators (2017) Estimates of global, regional, and national morbidity, mortality, and aetiologies of diarrhoeal diseases: a systematic analysis for the Global Burden of Disease Study 2015. Lancet Infect Dis. 17, 909–948
29. Gauthier, J.F. et al. (2007) Differential contribution of bacterial N-formyl-methionyl-leucyl-phenylalanine and host-derived CXC chemokines to neutrophil infiltration into pulmonary alveoli during murine pneumococcal pneumonia. Infect. Immun. 75, 5361–5367
30. Wang, H. et al. (2017) Aspirin-triggered resolvin D1 reduces pneumococcal lung infection and inflammation in a viral and bacterial coinfection pneumonia model. Clin. Sci. (Lond.) 131, 2347–2362
31. Anthony, D. et al. (2014) SAA drives proinflammatory heterotypic macrophage differentiation in the lung via CSF-1R-dependent signaling. FASEB J. 28, 3867–3877
32. Anthony, D. et al. (2013) Serum amyloid A promotes lung neutrophilia by increasing IL-17A levels in the mucosa and gammadelta T cells. Am. J. Respir. Crit. Care Med. 188, 179–186
33. Bozinovski, S. et al. (2012) Serum amyloid A opposes lipoxin A4 to mediate glucocorticoid refractory lung inflammation in chronicobstructive pulmonary disease. Proc. Natl. Acad. Sci. U. S. A. 109, 935–940
34. Eickmeier, O. et al. (2013) Aspirin-triggered resolvin D1 reduces mucosal inflammation and promotes resolution in a murine model of acute lung injury. Mucosal Immunol. 6, 256–266
35. Grimwood, K. et al. (2000) Twelve year outcomes following bacterial meningitis: further evidence for persisting effects. Arch. Dis. Child. 83, 111–116
36. Shastri, A. et al. (2013) Innate immunity and neuroinflammation. Mediators Inflamm. 2013, 342931
37. Brandenburg, L.O. et al. (2008) Role of glial cells in the functional expression of LL-37/rat cathelin-related antimicrobial peptide in meningitis. J. Neuropathol. Exp. Neurol. 67, 1041–1054
38. Lever, A. and Mackenzie, I. (2007) Sepsis: definition, epidemiology, and diagnosis. BMJ 335, 879–883
39. Kim, S.D. et al. (2010) The agonists of formyl peptide receptors prevent development of severe sepsis after microbial infection. J. Immunol. 185, 4302–4310
40. Chen, F. et al. (2014) Resolvin D1 improves survival in experimental sepsis through reducing bacterial load and preventing excessive activation of inflammatory response. Eur. J. Clin. Microbiol. Infect. Dis. 33, 457–464
41. Walker, J. et al. (2011) Lipoxin A4 increases survival by decreasing systemic inflammation and bacterial load in sepsis. Shock 36, 410–416
42. Sordi, R. et al. (2013) Dual role of lipoxin A4 in pneumosepsis pathogenesis. Int. Immunopharmacol. 17, 283–292

43. Gobbetti, T. et al. (2014) Nonredundant protective properties of FPR2/ALX in polymicrobial murine sepsis. Proc. Natl. Acad. Sci. U. S. A. 111, 18685–18690
44. Cho, J.S. et al. (2012) Neutrophil-derived IL-1beta is sufficient for abscess formation in immunity against *Staphylococcus aureus* in mice. PLoS Pathog. 8, e1003047
45. Stavru, F. et al. (2011) Cell biology and immunology of *Listeria monocytogenes* infections: novel insights. Immunol. Rev. 240, 160–184
46. Gao, J.L. et al. (1999) Impaired antibacterial host defense in mice lacking the N-formylpeptide receptor. J. Exp. Med. 189, 657–662
47. Tcherniuk, S. et al. (2016) Formyl peptide receptor 2 plays a deleterious role during influenza A virus infections. J. Infect. Dis. 214, 237–247
48. Ampomah, P.B. et al. (2017) Formyl peptide receptor 2 is regulated by RNA mimics and viruses through an IFN-beta-STAT3-dependent pathway. FASEB J. 32, 1468–1478
49. Courtin, N. et al. (2017) Antiviral activity of formyl peptide receptor 2 antagonists against influenza viruses. Antiviral Res. 143, 252–261
50. Arora, S. et al. (2016) Influenza A virus enhances its propagation through the modulation of annexin-A1 dependent endosomal trafficking and apoptosis. Cell Death Differ. 23, 1243–1256
51. Alessi, M.C. et al. (2017) FPR2: a novel promising target for the treatment of influenza. Front. Microbiol. 8, 1719
52. Joint United Nations Programme on HIV/AIDS (2013) Global Report: UNAIDS Report on the Global AIDS Epidemic, Joint United Nations Programme on HIV/AIDS (UNAIDS). http://files.unaids.org/en/media/unaids/contentassets/documents/epidemiology/2013/gr2013/UNAIDS_Global_Report_2013_en.pdf
53. Li, B.Q. et al. (2001) The synthetic peptide WKYMVm attenuates the function of the chemokine receptors CCR5 and CXCR4 through activation of formyl peptide receptor-like 1. Blood 97, 2941–2947
54. Shen, W. et al. (2000) Down-regulation of the chemokine receptor CCR5 by activation of chemotactic formyl peptide receptor in human monocytes. Blood 96, 2887–2894
55. Shimizu, N. et al. (2008) A formylpeptide receptor, FPRL1, acts as an efficient coreceptor for primary isolates of human immunodeficiency virus. Retrovirology 5, 52
56. Liu, M. et al. (2014) Formylpeptide receptors mediate rapid neutrophil mobilization to accelerate wound healing. PLoS One 9, e90613
57. Raoof, M. et al. (2010) Mitochondrial peptides are potent immune activators that activate human neutrophils via FPR-1. J. Trauma 68, 1328–1332; discussion 1332–1334
58. Hauser, C.J. et al. (2010) Mitochondrial damage associated molecular patterns from femoral reamings activate neutrophils through formyl peptide receptors and P44/42 MAP kinase. J. Orthop. Trauma 24, 534–558
59. Rezzola, S. et al. (2017) Inflammation and N-formyl peptide receptors mediate the angiogenic activity of human vitreous humour in proliferative diabetic retinopathy. Diabetologia 60, 719–728
60. Pittman, K. and Kubes, P. (2013) Damage-associated molecular patterns control neutrophil recruitment. J. Innate Immun. 5, 315–323
61. Hazeldine, J. et al. (2015) N-formyl peptides drive mitochondrial damage associated molecular pattern induced neutrophil activation through ERK1/2 and P38 MAP kinase signalling pathways. Injury 46, 975–984
62. Dorward, D.A. et al. (2017) Novel role for endogenous mitochondrial formylated peptide-driven formyl peptide receptor 1 signalling in acute respiratory distress syndrome. Thorax 72, 928–936
63. Yang, Y. et al. (2011) Annexin 1 released by necrotic human glioblastoma cells stimulates tumor cell growth through the formyl peptide receptor 1. Am. J. Pathol. 179, 1504–1512
64. Zhou, Y. et al. (2005) Formylpeptide receptor FPR and the rapid growth of malignant human gliomas. J. Natl. Cancer Inst. 97, 823–835

65. Boer, J.C. et al. (2013) Inhibition of formyl peptide receptor in high-grade astrocytoma by chemotaxis inhibitory protein of *S. aureus*. Br. J. Cancer 108, 587–596
66. Snapkov, I. et al. (2016) The role of formyl peptide receptor 1 (FPR1) in neuroblastoma tumorigenesis. BMC Cancer 16, 490
67. Xie, X. et al. (2017) Formyl peptide receptor 2 expression predicts poor prognosis and promotes invasion and metastasis in epithelial ovarian cancer. Oncol. Rep. 38, 3297–3308
68. Bizzarro, V. et al. (2017) Hypoxia regulates ANXA1 expression to support prostate cancer cell invasion and aggressiveness. Cell Adh. Migr. 11, 247–260
69. Belvedere, R. et al. (2014) Role of intracellular and extracellular annexin A1 in migration and invasion of human pancreatic carcinoma cells. BMC Cancer 14, 961
70. Coffelt, S.B. et al. (2009) Leucine leucine-37 uses formyl peptide receptor-like 1 to activate signal transduction pathways, stimulate oncogenic gene expression, and enhance the invasiveness of ovarian cancer cells. Mol. Cancer Res. 7, 907–915
71. Cattaneo, F. et al. (2015) Expression of formyl-peptide receptors in human lung carcinoma. Anticancer Res. 35, 2769–2774
72. Chen, K. et al. (2013) Formylpeptide receptor-2 contributes to colonic epithelial homeostasis, inflammation, and tumorigenesis. J. Clin. Invest. 123, 1694–1704
73. Liao, L. et al. (2018) Knockdown of annexin A1 enhances radioresistance and inhibits apoptosis in nasopharyngeal carcinoma. Technol. Cancer Res. Treat. 17, 1533034617750309
74. Stalder, A.K. et al. (2017) Biomarker-guided clinical development of the first-in-class anti-inflammatory FPR2/ALX agonist ACT-389949. Br. J. Clin. Pharmacol. 83, 476–486
75. Skovbakke, S.L. et al. (2018) The role of formyl peptide receptors for immunomodulatory activities of antimicrobial peptides and peptidomimetics. Curr. Pharm. Des. 24, 1100–1120
76. Devosse, T. et al. (2009) Formyl peptide receptor-like 2 is expressed and functional in plasmacytoid dendritic cells, tissue-specific macrophage subpopulations, and eosinophils. J. Immunol. 182, 4974–4984
77. Bufe, B. et al. (2012) Formyl peptide receptors from immune and vomeronasal system exhibit distinct agonist properties. J. Biol. Chem. 287, 33644–33655
78. Stempel, H. et al. (2016) Strain-specific loss of formyl peptide receptor 3 in the murine vomeronasal and immune systems. J. Biol. Chem. 291, 9762–9775
79. Harada, M. et al. (2004) N-Formylated humanin activates both formyl peptide receptor-like 1 and 2. Biochem. Biophys. Res. Commun. 324, 255–261
80. Migeotte, I. et al. (2005) Identification and characterization of an endogenous chemotactic ligand specific for FPRL2. J. Exp. Med. 201, 83–93
81. Devosse, T. et al. (2011) Processing of HEBP1 by cathepsin D gives rise to F2L, the agonist of formyl peptide receptor 3. J. Immunol. 187, 1475–1485
82. Bufe, B. et al. (2015) Recognition of bacterial signal peptides by mammalian formyl peptide receptors: a new mechanism for sensing pathogens. J. Biol. Chem. 290, 7369–7387
83. Rabiet, M.J. et al. (2011) N-formyl peptide receptor 3 (FPR3) departs from the homologous FPR2/ALX receptor with regard to the major processes governing chemoattractant receptor regulation, expression at the cell surface, and phosphorylation. J. Biol. Chem. 286, 26718–26731
84. Cundell, D.R. et al. (1993) Inhibition of human neutrophil migration in vitro by low-molecular-mass products of nontypeable *Haemophilus influenzae*. Infect. Immun. 61, 2419–2424
85. Postma, B. et al. (2004) Chemotaxis inhibitory protein of *Staphylococcus aureus* binds specifically to the C5a and formylated peptide receptor. J. Immunol. 172, 6994–7001
86. Prat, C. et al. (2006) A new staphylococcal anti-inflammatory protein that antagonizes the formyl peptide receptor-like 1. J. Immunol. 177, 8017–8026

87. Prat, C. et al. (2009) A homolog of formyl peptide receptor-like 1 (FPRL1) inhibitor from *Staphylococcus aureus* (FPRL1 inhibitory protein) that inhibits FPRL1 and FPR. J. Immunol. 183, 6569–6578
88. Yan, P. et al. (2006) The immunosuppressant cyclosporin A antagonizes human formyl peptide receptor through inhibition of cognate ligand binding. J. Immunol. 177, 7050–7058
89. Stenfeldt, A.L. et al. (2007) Cyclosporin H, Boc-MLF and Boc-FLFLF are antagonists that preferentially inhibit activity triggered through the formyl peptide receptor. Inflammation 30, 224–229
90. Skvortsov, S.S. and Gabdoulkhakova, A.G. (2017) Formyl peptide receptor polymorphisms: 27 most possible ways for phagocyte dysfunction. Biochemistry (Mosc.) 82, 426–437
91. Zhang, Y. et al. (2003) Evaluation of human leukocyte N-formylpeptide receptor (FPR1) SNPs in aggressive periodontitis patients. Genes Immun. 4, 22–29
92. Jones, B.E. et al. (2003) Mutations of F110 and C126 of the formyl peptide receptor interfere with G-protein coupling and chemotaxis. J. Periodontol. 74, 475–484
93. Gunji, T. et al. (2007) Functional polymorphisms of the FPR1 gene and aggressive periodontitis in Japanese. Biochem. Biophys. Res. Commun. 364, 7–13
94. Liang, X.Y. et al. (2014) FPR1 interacts with CFH, HTRA1 and smoking in exudative age-related macular degeneration and polypoidal choroidal vasculopathy. Eye (Lond) 28, 1502–1510
95. Otani, T. et al. (2011) Polymorphisms of the formylpeptide receptor gene (FPR1) and susceptibility to stomach cancer in 1531 consecutive autopsy cases. Biochem. Biophys. Res. Commun. 405, 356–361
96. Wenzel-Seifert, K. and Seifert, R. (2003) Functional differences between human formyl peptide receptor isoforms 26, 98, and G6. Naunyn Schmiedebergs Arch. Pharmacol. 367, 509–515
97. Seifert, R. and Wenzel-Seifert, K. (2001) Defective Gi protein coupling in two formyl peptide receptor mutants associated with localized juvenile periodontitis. J. Biol. Chem. 276, 42043–42049
98. Benachour, H. et al. (2009) Human formyl peptide receptor 1 (FPR1) c.32C>T SNP is associated with decreased soluble Eselectin levels. Pharmacogenomics 10, 951–959
99. El Shamieh, S. et al. (2012) Human formyl peptide receptor 1 C32T SNP interacts with age and is associated with blood pressure levels. Clin. Chim. Acta 413, 34–38
100. Kim, H.J. et al. (2012) Association analysis of formyl peptide receptor 2 (FPR2) polymorphisms and aspirin exacerbated respiratory diseases. J. Hum. Genet. 57, 247–253
101. Zhang, H. et al. (2017) The common promoter polymorphism rs11666254 downregulates FPR2/ALX expression and increases risk of sepsis in patients with severe trauma. Crit. Care 21, 171
102. Leoni, G. and Nusrat, A. (2016) Annexin A1: shifting the balance towards resolution and repair. Biol. Chem. 397, 971–979
103. Walther, A. et al. (2000) A novel ligand of the formyl peptide receptor: annexin I regulates neutrophil extravasation by interacting with the FPR. Mol. Cell 5, 831–840
104. Perretti, M. et al. (2002) Endogenous lipid- and peptide-derived anti-inflammatory pathways generated with glucocorticoid and aspirin treatment activate the lipoxin A4 receptor. Nat. Med. 8, 1296–1302
105. Blume, K.E. et al. (2012) Cleavage of annexin A1 by ADAM10 during secondary necrosis generates a monocytic “find-me” signal. J. Immunol. 188, 135–145
106. Southgate, E.L. et al. (2008) Identification of formyl peptides from *Listeria monocytogenes* and Staphylococcus aureus as potent chemoattractants for mouse neutrophils. J. Immunol. 181, 1429–1437
107. Norling, L.V. et al. (2012) Resolvin D1 limits polymorphonuclear leukocyte recruitment to inflammatory loci: receptor-dependent actions. Arterioscler. Thromb. Vasc. Biol. 32, 1970–1988

108. Yang, D. et al. (2009) Alarmins link neutrophils and dendritic cells. Trends Immunol. 30, 531–537
109. Lacy, P. (2006) Mechanisms of degranulation in neutrophils. Allergy Asthma Clin. Immunol. 2, 98–108
110. Zhao, H.F. et al. (2015) The phosphatidylinositol 3-kinase/Akt and c-Jun N-terminal kinase signaling in cancer: alliance or contradiction? (Review). Int. J. Oncol. 47, 429–436
111. Mocsai, A. et al. (2015) Intracellular signalling during neutrophil recruitment. Cardiovasc. Res. 107, 373–385

# CHAPTER 3

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## Formyl-peptide receptor 2 governs leukocyte influx in local *Staphylococcus aureus* infections

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## Abstract

Leukocytes express formyl-peptide receptors (FPRs), which sense microbe-associated molecular pattern (MAMP) molecules, leading to leukocyte chemotaxis and activation. We recently demonstrated that phenol-soluble modulin (PSM) peptides from highly pathogenic *Staphylococcus aureus* are efficient ligands for the human FPR2. How PSM detection by FPR2 impacts on the course of *S. aureus* infections has remained unknown. We characterized the specificity of mouse FPR2 (mFpr2) using a receptor-transfected cell line, homeobox b8 (Hoxb8), and primary neutrophils isolated from wild-type (WT) or mFpr2$^{-/-}$ mice. The influx of leukocytes into the peritoneum of WT and mFpr2$^{-/-}$ mice was analyzed. We demonstrate that mFpr2 is specifically activated by PSMs in mice, and they represent the first secreted pathogen-derived ligands for the mFpr2. Intraperitoneal infection with *S. aureus* led to lower numbers of immigrated leukocytes in mFpr2$^{-/-}$ compared with WT mice at 3 h after infection, and this difference was not observed when mice were infected with an *S. aureus* PSM mutant. Our data support the hypothesis that the mFpr2 is the functional homolog of the human FPR2 and that a mouse infection model represents a suitable model for analyzing the role of PSMs during infection. PSM recognition by mFpr2 shapes leukocyte influx in local infections, the typical infections caused by *S. aureus*

KEY WORDS: mFpr2 • PSMs • innate immunity • neutrophil • Hoxb8

## Introduction

*Staphylococcus aureus*, particularly community-associated methicillin-resistant *S. aureus*, is an important human pathogen and responsible for local infections, such as superficial skin and wound infections. In addition, *S. aureus* often causes severe systemic infections afflicting particularly immunocompromised and elderly patients (1, 2). Polymorphonuclear neutrophils (PMNs) are of particular importance for the effective clearance of *S. aureus* infections, and they contribute to the massive inflammation typically accompanying *S. aureus* infections (3). How neutrophils and other leukocytes are recruited to local infection sites, in particular, in early phases, has remained incompletely understood. Neutrophils migrate to the focus of infection in response to bacterial molecules, such as formylated peptides. The ability of

neutrophils to detect and respond to such chemotactic MAMP molecules is crucial for host defense against invading bacteria (4). All bacteria release formylated peptides, for example, the N-terminal signal peptides released from secreted proteins (5) as a consequence of the use of formyl-methionine as the first amino acid in bacterial protein biosynthesis. In addition, PSMs, which represent the most abundant peptides secreted by *S. aureus*, are potent neutrophil chemoattractants (6). *S. aureus* produces several PSMs, including the short α-type PSMs (PSMα1–4 and δ-toxin) and the twice-as-long β-type PSMs (PSMβ1, PSMβ2) (7). At low concentrations, PSMs induce recruitment of human neutrophils (6). Furthermore, PSMαs induce the release of TLR2-activating lipoproteins from bacterial cells and at high concentrations, lysis of human cells (8, 9). It has been shown that formyl-peptide- or PSM-induced chemotaxis depends on activation of human FPR1 or -2. The two receptors appear to discriminate between ligands based on peptide length and folding state (10). Short, formylated peptides usually have no defined structure, whereas the longer PSMs adopt α-helical, amphipathic structures, which favor detection by FPR1 or FPR2, respectively (10). Mouse neutrophils also respond to formylated peptides and PSMs, but their repertoire of FPR-related receptors differs from that of human neutrophils. They include eight orthologs (in contrast to three in humans), three of which are expressed on mouse neutrophils (11). mFpr1 and mFpr2 (previously named mFpr-rs2) are expressed on naïve mouse neutrophils. In contrast, mFpr3 (previously named mFpr-rs1) is expressed only in some mouse lineages and only upon stimulation (12, 13). The murine mFpr1 has been shown to respond with high efficiency to short formylated peptides but to prefer the sequence fMIFL over fMLF, the most potent formylated peptide for the human FPR1 (14). mFpr1 is 77% identical to the human FPR1 amino acid sequence, and studies with knockout mice have shown that it represents the functional counterpart to human FPR1 (15). The mouse mFpr2 is structurally most similar to human FPR2; it possesses 76% amino acid sequence identity (11, 16). In spite of the high similarity between these two receptors, mFpr2 responds only very weakly to several peptide agonists that strongly activate human FPR2 (17, 18). However, it has been shown that the FPR2-specific antagonist $WRW_4$ inhibits the activation of the mFpr2 (19). This result suggests that mFpr2 is the functional counterpart of the human FPR2. Despite the sequence similarity between mouse and human FPRs, there are crucial species-specific differences. Inhibitory proteins for human FPR1 (chemotaxis inhibitory protein of *S.*

*aureus*) or FPR2 are secreted by many *S. aureus* strains and have been shown to be specific for human FPRs but to be inactive against mFprs (20–22). The unclear relation between human FPR and mFPR homologs has impeded the analysis of the role of the PSM-sensing receptor in infection in mouse models (11). To clarify the role of FPR2 in *S. aureus* infections, we compared the ligand specificities of human FPRs and mFprs and found mFpr2 to respond strongly and specifically to all *S. aureus* PSMs but not to short formylated peptides. In contrast, mFpr1 was not activated by PSMs but by formyl-Met-Ile-Phe-Leu (fMIFL) (14). When mice were infected intraperitoneally with *S. aureus*, lack of mFpr2 led to strongly reduced numbers of immigrated leukocytes at early but not in later stages of infection, indicating that this receptor may play a crucial role in the clearance of superficial, early infections.

## Materials and Methods

**Cells and bacteria:** The plasmid pQCXIN-EF1a was used for transfection of rat basophilic leukemia (RBL; RBL-2H3, CRL 1593; American Type Culture Collection, Manassas, VA, USA; and subclone; gift from Peter Monk, The University of Sheffield, Sheffield, United Kingdom) cells. mFpr cDNAs (16) were cloned in plasmid pcDND3.1 (Thermo Fisher Scientific,Waltham,MA, USA). The cDNAs of mFpr1 and mFpr2 were amplified by PCR with primers containing restriction sites for NotI, BamHI, and PacI (Supplemental Table 1). cDNAs of Fprs were cloned into pQCXIN-EF1a via compatible restriction sites to yield translational fusions with an N-terminal FLAG-tag and maintained in *Escherichia coli* under ampicillin selection. pQCXINEF1a-mFpr1 and pQCXIN-EF1a-mFpr2 were isolated using an endotoxin-free isolation kit from Qiagen (Germantown, MD, USA) and further used for transfection of RBL-2H3 cells using nucleofector technology. Transfection efficiency was analyzed via an antibody against the FLAG-tag (mouse anti-FLAG, isotype control mouse IgG1, secondary antibody anti-mouse IgG-phycoerythrin; MilliporeSigma, Billerica, MA, USA), expressed as part of the mFpr receptors on the surface of the receptor-transfected RBL cells.

Strain USA300 (23) and the isogenic PSM deletion mutant (USA300Δαβδ) used for mouse stimulation and infection experiments have recently been described in detail (24). Bacterial culture supernatants were obtained by centrifugation of overnight

cultures grown in tryptic soy broth and filtered through 0.2-µm pore filters (MilliporeSigma).

**Synthetic peptides:** The PSM peptides were kindly provided by Stefan Stevanovic (University of Tübingen). fMIFL was synthesized by EMC Microcollections (Tübingen, Germany).

**Isolation of primary neutrophils**: Mouse leukocytes were obtained from the peritoneum of 12-wk-old female C57BL/6 mice (Envigo, Somerset, NJ, USA) or from mFpr2$^{-/-}$ mice with a genetic C57BL/6 background (bred in the animal facilities of the University Hospital Tübingen) (25) upon inducing an intraperitoneal inflammatory reaction by repeated casein injection, as previously described (26). All mice were held under specific pathogen-free conditions and provided food and water ad libitum. Animal experiments were performed in strict accordance with the European Health Law of the Federation of Laboratory Animal Science Associations. The protocol was approved by the Regierungspräsidium Tübingen (Anzeige 17.12.14)

**Hoxb8 cell lines:** Progenitors were derived from the bone marrow of the mouse strains mentioned above. The progenitors were retrovirally transduced with estrogen-regulated Hoxb8 and selected for 4 wk in the presence of stem cell factor (SCF) to generate neutrophil progenitor cell lines (27). Polyclonal progenitor cell lines were cultured in Opti-MEM + GlutaMax medium (Thermo Fisher Scientific), supplemented with 10% fetal calf serum, 30 µM 2-ME, 1 µM β-estradiol (MilliporeSigma), and 1% supernatant from SCF-producing Chinese hamster ovary cells. The SCF-producing cell line was kindly provided by Hans Häcker (St. Jude Children's Research Hospital, Memphis, TN, USA). Differentiation was induced by β-estradiol removal.

**Calcium flux assay:** Calcium ion fluxes in primary mouse neutrophils, Hoxb8, and RBL cells were analyzed by stimulating cells loaded with Fluo-3-acetoxymethyl (Thermo Fisher Scientific) and monitoring fluorescence with a FACSCalibur flowcytometer or LSRFortessa (BD Biosciences, San Jose, CA, USA), as described recently (6). Synthetic chemoattractants were used at concentrations in the linear range of the dose-response curves. Measurements of 2000 events were performed, and calcium flux was expressed as relative fluorescence.

**MIP2 production and chemotaxis:** Macrophage inflammatory protein 2 (MIP2) was measured using an ELISA kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. Hoxb8 neutrophils were primed with 100 ng/ml of the

synthetic diacylated lipopeptide S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-(R)-cysteine cysteineserine-lysine 4 (Thermo Fisher Scientific) for 24 h. Hoxb8 neutrophils were incubated with indicated concentrations of the different peptides for 5 h at 37°C, 5% $CO_2$. The cells were centrifuged for 10 min at 250 g and 4°C, and cell supernatants were stored at -20°C until use.

Chemotaxis of primary or Hoxb8 neutrophils toward staphylococcal supernatants or synthetic peptides was determined by using fluorescence-labeled neutrophils that migrated through a 3-µm-pore polycarbonate Transwell filter, as recently described (26). Synthetic chemoattractants were used at concentrations in the linear range of the dose-response curves. Neutrophil migration from the upper to the lower Transwell chamber was only observed when the peptides were added to the lower chamber. When an even PSM concentration was present in the two chambers, no such migration was observed, thereby confirming that PSMs induce chemotactic migration in neutrophils. The relative fluorescence measured was corrected for the buffer control.

**Degranulation assay:** RBL cells were preincubated with 10 µM cytochalasin B in HBSS + 0.1% bovine serum albumin + 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid for 15 min on ice, followed by 15 min at 37°C. Subsequently, the cells were stimulated for 10 min with the indicated amounts of agonists at 37°C. Triton X-100 (0.1%) was used as a positive control. The reaction was terminated by addition of ice-cold PBS, and the supernatants were collected by centrifugation. The amount of released β-glucuronidase was quantified by incubating 20 µl supernatant with 20 µl 10 mM 4-methylumbelliferyl β-D-glucuronide hydrate in 0.1 M sodium acetate (pH 4.0) and 0.1% Triton X-100 for 15 min at 37°C. The reaction was terminated by addition of 300 µl, 50 mM glycine and 5 mM EDTA in $H_2O$ (pH 10.4). Fluorescence was measured at 365 nm excitation and 460 nm emission wavelengths.

**Cytotoxicity assay:** RBL cells ($10^5$/ml) or Hoxb8 cells ($10^6$/ml) were incubated for 1 h in a humidified 37°C incubator with PSMα2, PSMα3, or δ-toxin at final concentrations of 0.5, 2, or 4 µM, respectively, in Roswell Park Memorial Institute (RPMI; 1640; Merck; Darmstadt, Germany) medium without phenol red, and release of cytoplasmic lactate dehydrogenase was determined in triplicate (Roche Applied Science, Mannheim, Germany). The percentage lactate dehydrogenase release was

calculated relative to that observed in detergent-lysed cells (maximal lysis) as a measure for cell lysis as described in Kretschmer et al. (6).

**Peptide-binding assay:** Hoxb8 cells ($10^5$) were incubated with increasing concentrations of 5-carboxytetramethylrhodamine-labeled PSMα3 for 2 h at 37°C under agitation. Cells were washed with ice-cold PBS containing 0.1% human serum albumin, and cell-associated fluorescence was measured using a FACSCalibur flow cytometer, as described in Kretschmer et al. (6).

**Mouse infection assay:** In the mouse peritonitis model, $4x10^8$ (for 3 h) or $5x10^7$ (for 24 h) colony-forming units of live *S. aureus* USA300 wild type (WT) or USA300ΔαβδΔ were injected into the peritoneum of 6- to 8-wk-old female C57BL/6 WT or mFpr2$^{-/-}$ mice. Three or 24 h after infection, the mice were euthanized with $CO_2$. Subsequently, peritoneal exudates were collected, and leukocytes were stained and counted as previously described (9). FITC conjugated α-mouse Ly6G antibody, a marker for neutrophils, and a corresponding isotype control were purchased from BD Biosciences. A phycoerythrin-labeled α-mouse F4/80 antibody, a marker for macrophages, and the corresponding isotype control were purchased from Bio-Rad Laboratories (Hercules, CA, USA) and Thermo Fisher Scientific, respectively. Samples were analyzed on a FACSCalibur flow cytometer (Supplemental Fig. 4). All mice were held under specific pathogen-free conditions and provided food and water ad libitum. Animal experiments were performed in strict accordance with the European Health Law of the Federation of Laboratory Animal Science Associations. The protocol was approved by the Regierungspräsidium Tübingen (H4/09)

**Statistics:** Statistical analysis was performed using Graph Pad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). Unpaired 2-tailed Student's t test was used to compare 2 data groups unless otherwise noted.

## Results

### PSMs induce calcium release and degranulation in mFpr2-expressing RBL cells

mFpr1 and mFpr2 are regarded as the murine orthologs of human FPR1 and FPR2, respectively, but a number of studies have suggested that mFprs exhibit less-defined ligand-binding properties than human FPRs (28). To analyze if mFprs differ in their

capacities to discriminate between short formylated peptides and PSMs from human FPRs, RBL cells were stably transfected with mFpr1 or mFpr2 and found to express the receptors (Supplemental Fig. 1). mFpr1- and mFpr2-transfected but not untransfected RBL cells responded to fMIFL and PSMs, respectively, by mobilization of calcium from intracellular stores and by release of granule components (Fig. 1), thereby confirming that the two receptors were functionally expressed. When these cells were stimulated with PSMα2 or -α3 or δ-toxin, we observed degranulation (Fig. 1A, B) and strong calcium influx (Fig. 1C, D and Supplemental Fig. 2) in mFpr2-transfected RBL cells but hardly any response in mFpr1-transfected or in control cells. mFpr2-transfected cells did not respond to fMIFL, which confirms previous studies (14, 29) and indicates that mFpr1 and mFpr2 have similar capacities to discriminate between short formylated peptides and PSMs as the human homologs. At very high PSM concentrations (above 1 µM), some unspecific activation was observed (data not shown), which was most probably a result of the toxicity of the peptides (Supplemental Fig. 3).

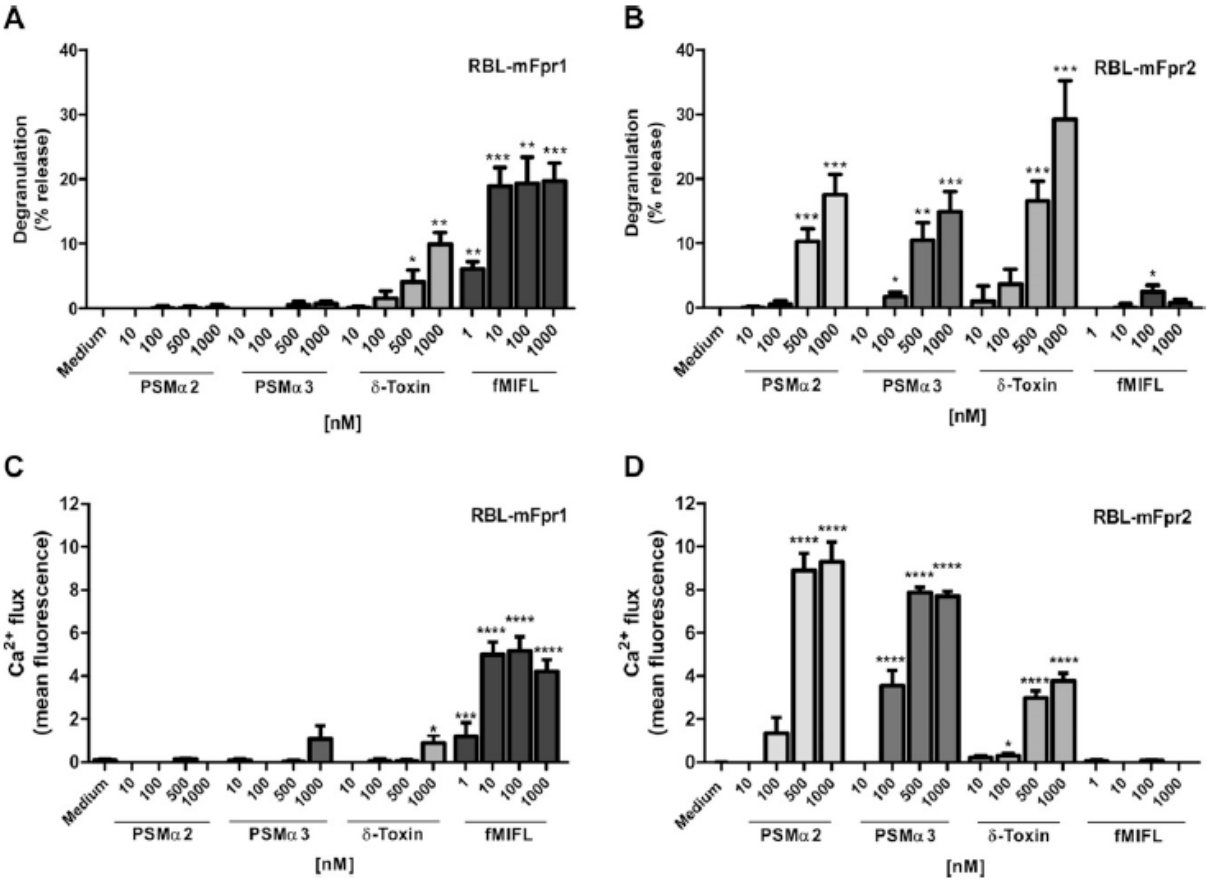

**Figure 1: PSMs induce specific activation and degranulation of mFpr2-expressing RBL cells.** PSMs specifically do not activate mFpr1–transfected RBL cells (A, C), but activate mFpr2 (B, D) at indicated concentrations. Data represent means ± SEM of at least 3 independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001 vs. corresponding medium control, as calculated by Student's t test.

Human FPR2 has been reported to respond only to culture filtrates of PSM-secreting (WT) *S. aureus* but not of PSM-deficient mutants (6, 30). mFpr2 was found to respond in a similar way, because mFpr2-transfected RBL cells were only activated by culture filtrates of the *S. aureus* USA300 WT but not of the isogenic mutant lacking the PSMα and -β and δ-toxin genes (Δαβδ; Fig. 2). mFpr1-transfected RBL cells hardly responded to culture filtrates of *S. aureus*, which are known to contain formylated peptides at concentrations that did not cause toxicity (below 0.375%). Similar observations were previously made with human FPR1, which demonstrated that short formylated peptides are, by far, less concentrated in *S. aureus* culture filtrates than PSMs. Thus, PSMs have similar agonist properties for mouse and human FPRs.

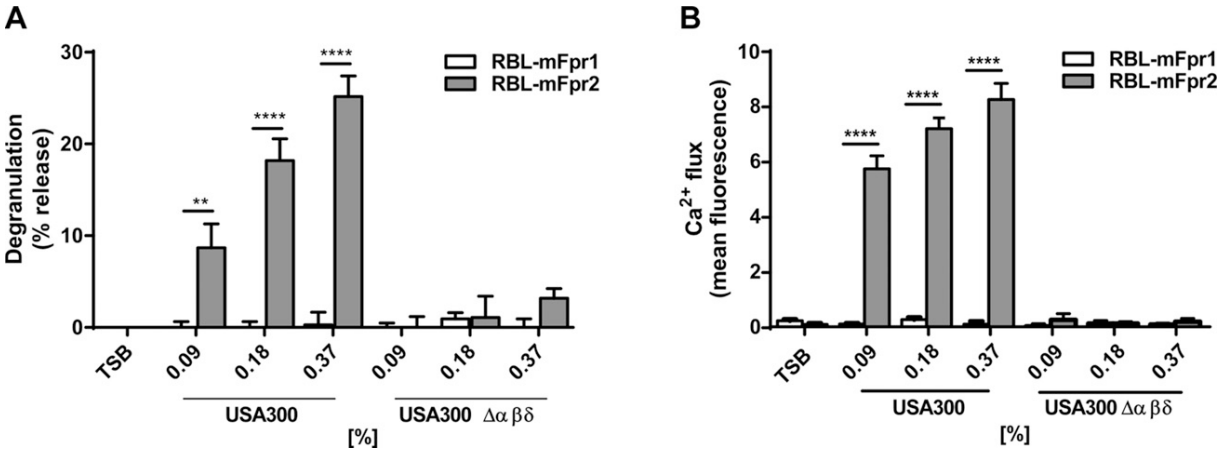


**Figure 2: Culture filtrates from PSM-expressing *S. aureus* induce specific activation and degranulation of mFpr2-expressing RBL cells.** Culture filtrates of PSM-expressing *S. aureus* USA300 WT, but not from an isogenic PSM deletion mutant, induce degranulation (A) and calcium influx (B) in mFpr2, but not mFpr1, transfected RBL cells at indicated concentrations. Data represent means ± SEM of at least 3 independent experiments. TSB, tryptic soy broth. **P < 0.01; ****P < 0.0001 vs. corresponding medium control, as calculated by Student's t test.

### Mouse neutrophils are activated by PSMs via mFpr2

To confirm these findings with primary cells, neutrophils were isolated from WT and mFpr2$^{-/-}$ mice, and intracellular calcium mobilization was monitored after stimulation with mFpr1 and mFpr2 ligands. Indeed, WT neutrophils were activated by fMIFL, PSM$\alpha$2, and PSM$\alpha$3. In contrast, neutrophils from mFpr2$^{-/-}$ mice only responded to fMIFL but not to PSMs (Fig. 3A). Likewise, PSM$\alpha$3 and fMIFL were chemoattractive for neutrophils of WT mice, whereas only fMIFL attracted neutrophils of mFpr2$^{-/-}$ mice (Fig. 3C). Thus, primary mouse neutrophils respond to Fpr agonists in a similar way as Fpr-transfected RBL cells. When neutrophils from WT mice were stimulated with *S. aureus* culture filtrates, only samples of the *S. aureus* parental strain but not the isogenic PSM mutant elicited calcium ion influx at concentrations below toxicity (below 0.375%). None of the *S. aureus* culture filtrates activated mFpr2$^{-/-}$ neutrophils (Fig. 3B). These findings confirm that only the mFpr2 detects PSM peptides and that insufficient amounts of mFpr1 ligands are present in *S. aureus* culture filtrates.

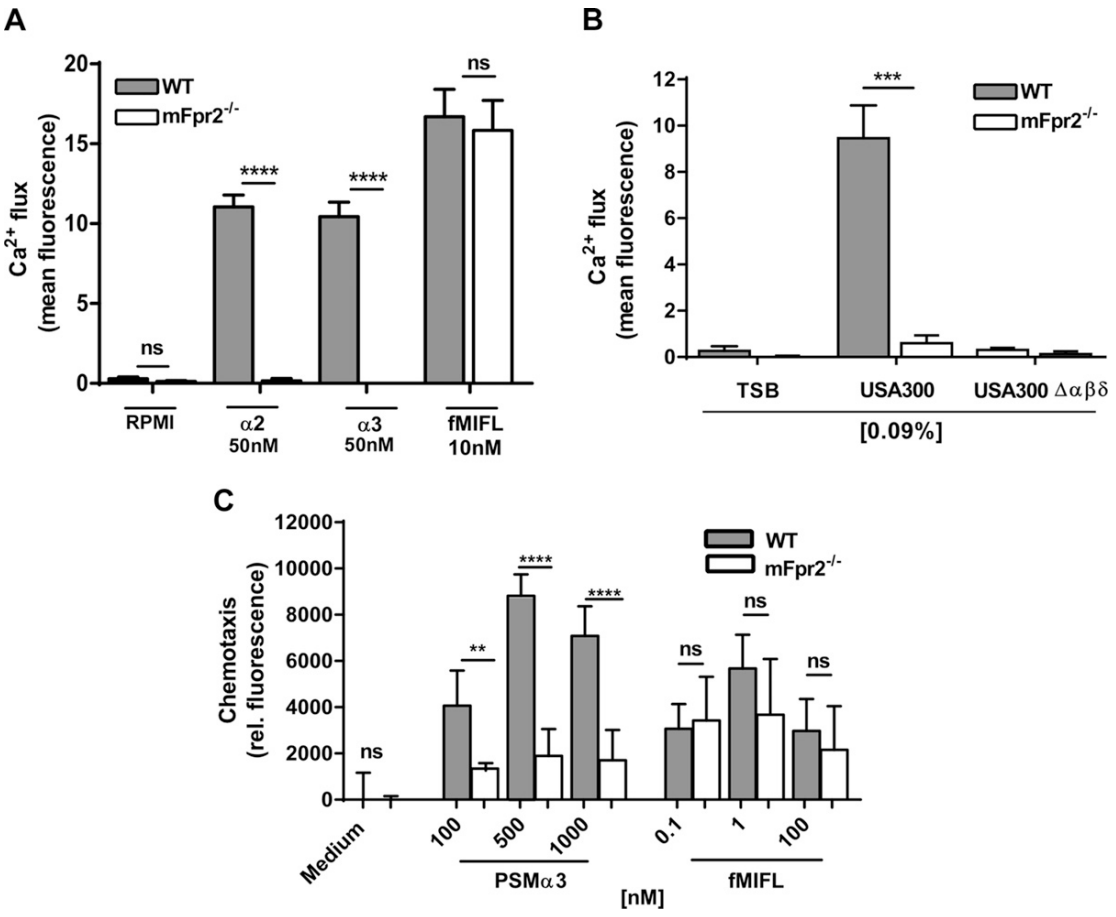

**Figure 3: Primary neutrophils of mice are specifically activated by PSMs via mFpr2.** PSMs induce calcium influx in primary neutrophils of mice. Calcium influx is induced by PSMα2, PSMα3, and fMIFL (A) or culture filtrates of USA300 WT or its isogenic PSM mutant (0.09%; culture filtrates diluted in RPMI 1640) in neutrophils isolated from WT or mFpr2$^{-/-}$ mice (B). Migration is induced by PSMα3 or fMIFL (C) in neutrophils of WT or mFpr2$^{-/-}$ mice. Data represent means ± SEM of at least 3 independent experiments. **P < 0.01; ***P < 0.001; ****P < 0.0001; ns, not significantly different vs. mFpr2$^{-/-}$ neutrophils, as calculated by Student's t test.

## Hoxb8 neutrophils expressing mFpr2 behave like primary neutrophils

In contrast to human neutrophils, mouse neutrophils cannot be isolated in sufficient numbers from blood (31) but need to be collected from the mouse peritoneum upon intraperitoneal injection of casein, which leads to priming of the cells and may alter their responses to inflammatory stimuli (32). To overcome these problems, neutrophil precursor cells from the bone marrow of WT or mFpr2$^{-/-}$ mice were immortalized by stable transfection with estrogen-regulated transcription factor Hoxb8, according to an established protocol (27). The resulting Hoxb8 cells remain immortal as long as estrogen is added to growth medium, but they differentiate into mature neutrophils within 4 d in the absence of estrogen. Hoxb8 neutrophils have previously been shown to behave like primary neutrophils in terms of activation, chemotaxis, opsonophagocytosis, and killing (33).

Stimulation of Hoxb8 neutrophils with various concentrations of fMIFL, PSMα2, PSMα3, or δ-toxin induced calcium influx in WT Hoxb8 cells, whereas only fMIFL was able to stimulate calcium influx in mFpr2$^{-/-}$ Hoxb8 neutrophils (Fig. 4A, B). Furthermore, fluorescent-labeled PSMα3 showed significant, stronger binding to WT Hoxb8 neutrophils than to mFpr2$^{-/-}$ Hoxb8 cells (Fig. 4C). Moreover, culture filtrates from the *S. aureus* parental strain activated only WT Hoxb8, whereas those from the PSM mutant activated neither WT nor mFpr2$^{-/-}$ Hoxb8 (Fig. 6A) at concentrations below the toxicity threshold. These findings support the results with primary neutrophils isolated from the peritoneum of mice and demonstrate that the Hoxb8 cells behave like primary mouse neutrophils.

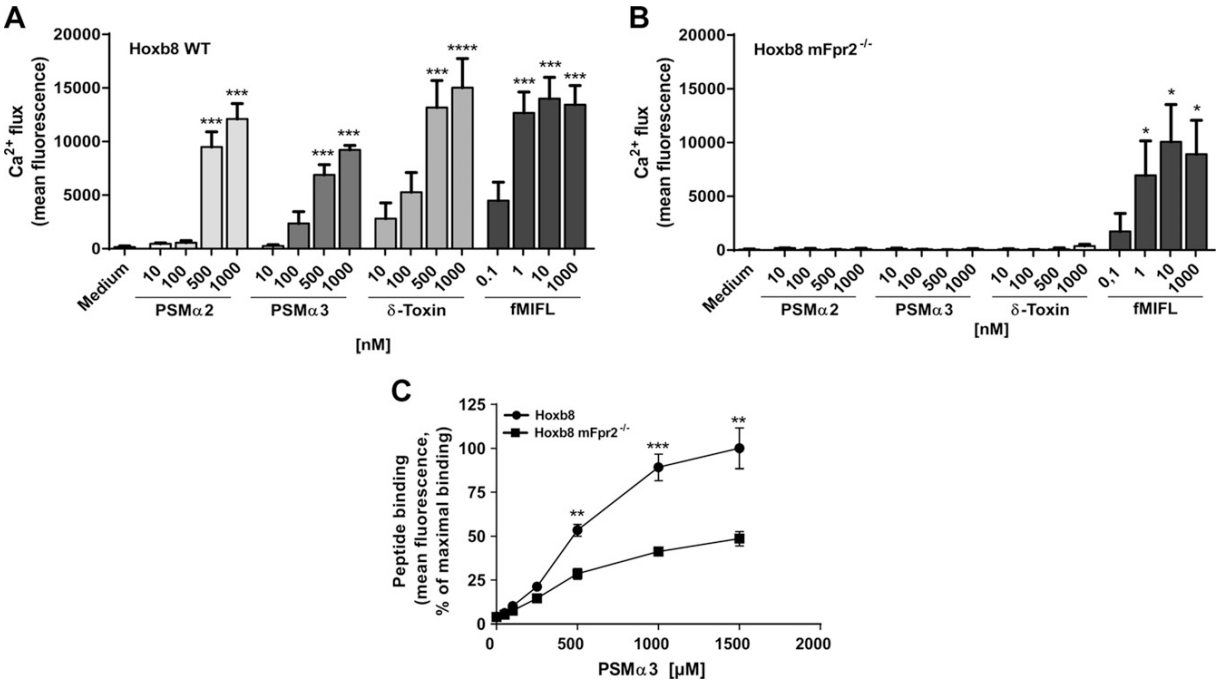


**Figure 4: Hoxb8 neutrophils expressing mFpr2 are activated by PSMs.** Calcium influx is induced by PSMα2, PSMα3, δ-toxin, or fMIFL in WT Hoxb8 (A) or mFpr2$^{-/-}$ Hoxb8 (B). 5-Carboxytetramethylrhodamine-labeled PSMα3 binds specifically to mFpr2-expressing Hoxb8 cells (C). Data represent means ± SEM of at least 3 independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001 vs. corresponding medium control (A, B) or vs. mFpr2$^{-/-}$ Hoxb8 cells (C), as calculated by Student's t test.

CD11b is important for effective phagocytosis of pathogens, and its expression can be used as a marker for neutrophil activation. Hoxb8 neutrophils were then used to study up-regulation of CD11b expression, chemotaxis, and chemokine release. For chemotaxis, we observed the typical bell-shaped curves for all tested peptides (data not shown) and show the optimal concentrations in Fig. 5. Stimulation of WT and mFpr2$^{-/-}$ Hoxb8 with fMIFL induced CD11b up-regulation, chemotaxis, and cytokine release in both cell types (Fig. 5). In contrast, PSMs induced robust activation of WT but no or only very weak activation of mFpr2$^{-/-}$ Hoxb8 in all three assays (Fig. 5). Furthermore, only WT Hoxb8 but not mFpr2$^{-/-}$ Hoxb8 responded to *S. aureus* WT culture filtrates. Supernatants from the *S. aureus* PSM mutant had no activity for any of the Hoxb8 neutrophils (Fig. 6B).

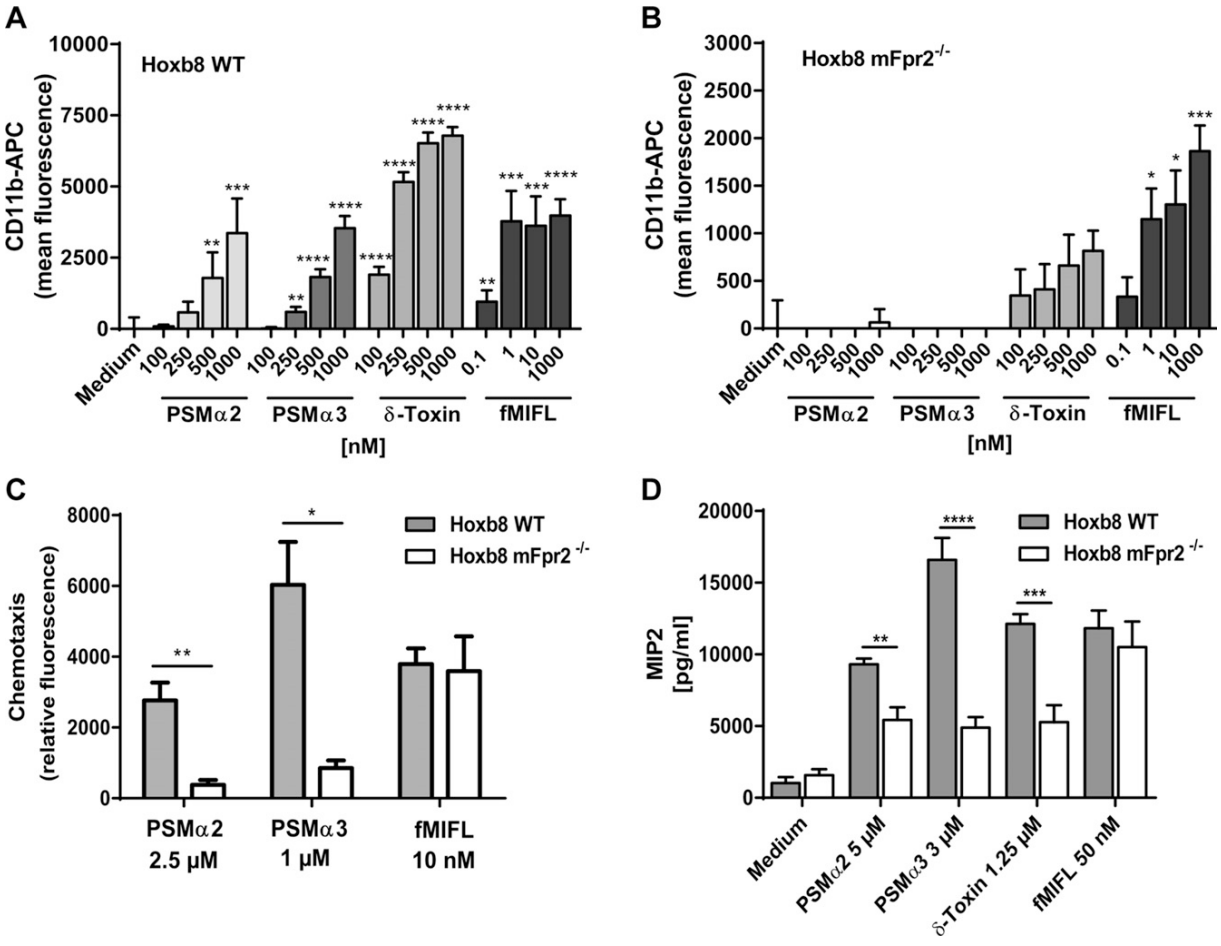


**Figure 5: Hoxb8 neutrophils expressing mFpr2 up-regulate CD11b and migrate toward and release MIP2 upon PSM stimulation.** PSMs (α2, α3, δ-toxin) and fMIFL induce CD11b up-regulation in WT Hoxb8 cells (A) or mFpr2$^{-/-}$ Hoxb8 cells (B). Migration is induced by 2.5 µM PSMα2, 1 µM PSMα3, or 10 nM fMIFL in WT Hoxb8 or mFpr2$^{-/-}$ Hoxb8 cells (C). MIP2 secretion is induced by PSMs and fMIFL in WT and mFpr2$^{-/-}$ Hoxb8 cells (D). APC, allophycocyanin. Data represent means ± SEM of at least 3 independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001 vs. corresponding medium control (A, B) or vs. mFpr2$^{-/-}$ neutrophils (C, D), as calculated by Student's t test.

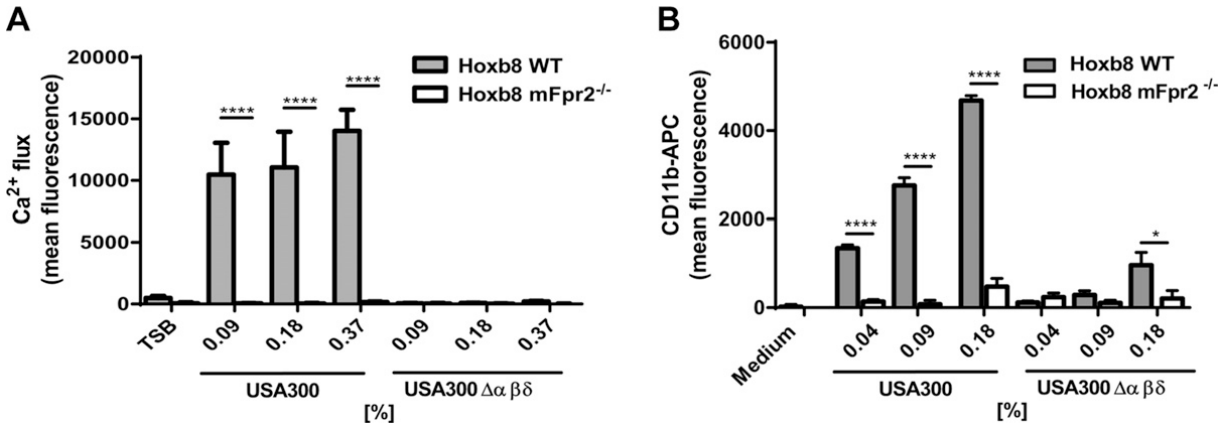


**Figure 6: Hoxb8 neutrophils expressing mFpr2 are activated through culture filtrates of PSM-expressing USA300.** Culture filtrates of PSM-expressing *S. aureus* USA300, but not from an isogenic PSM deletion mutant, induce calcium influx (A) and CD11b up-regulation (B) in WT but not in Hoxb8 mFpr2$^{-/-}$ cells at indicated concentrations. Data represent means ± SEM of at least 3 independent experiments. *P < 0.05; ****P < 0.0001 vs. mFpr2$^{-/-}$ neutrophils, as calculated by Student's t test.

## Early leukocyte recruitment in peritoneal *S. aureus* infection is mediated via mFpr2

The mFpr2 receptor behaved like the human FPR2 in all tested parameters so far in response to *S. aureus* PSMs, indicating that mFpr2$^{-/-}$ mice could be useful for studying the role of mFpr2 in *S. aureus* infection. To analyze if local leukocyte recruitment after *S. aureus* infection is influenced by mFpr2, WT and mFpr2$^{-/-}$ mice were infected intraperitoneally for 3 h with USA300 or the isogenic PSM mutant. Subsequently, the numbers of infiltrated granulocytes and macrophages were determined. Notably, >2-fold lower numbers of granulocytes and macrophages were detected in the peritoneum of mFpr2$^{-/-}$ mice compared with WT mice after infection with USA300. In contrast, the isogenic PSM mutant caused lower numbers of macrophages compared with infection with the parental strain, and there were no differences in leukocyte counts between WT and mFpr2$^{-/-}$ mice (Fig. 7). Twenty-four hours after infection, no difference between WT and mFpr2$^{-/-}$ mice was observed in the recruitment of neutrophils and macrophages, neither with the PSM producing USA300 nor with the isogenic PSM mutant (Fig. 7). Thus, mFpr2 plays an important role in recruiting leukocytes in early phases of local *S. aureus* infections.

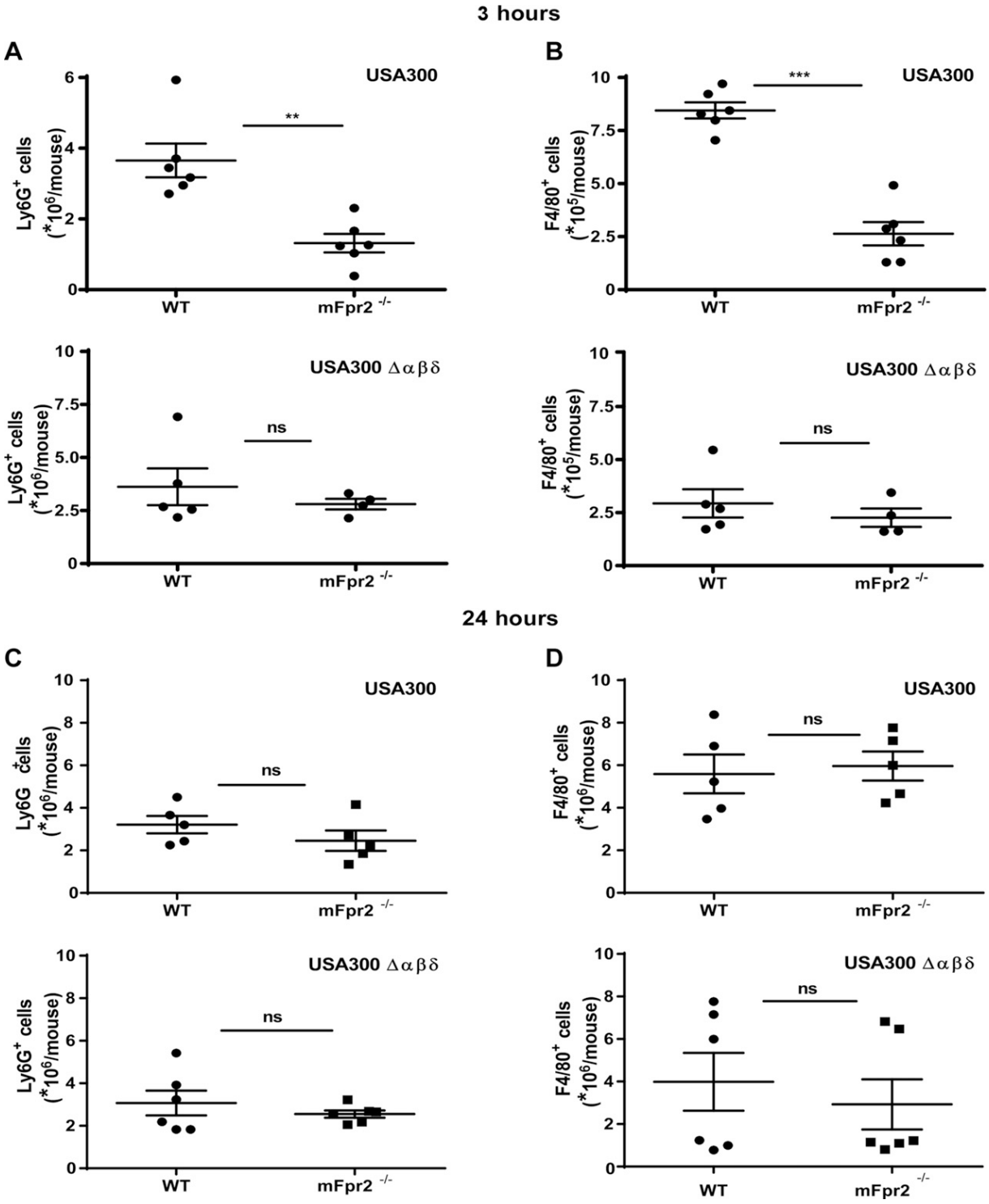


**Figure 7: Leukocyte infiltration into the peritoneum of WT or mFpr2$^{-/-}$ mice, induced by *S. aureus* USA300.** Infiltration of granulocytes (A) or macrophages (B) into the peritoneum of WT or mFpr2$^{-/-}$ mice, 3 h after intraperitoneal infection with USA300 WT or USA300Δαβδ. Infiltration of granulocytes (C) or macrophages (D) into the peritoneum of WT or mFpr2$^{-/-}$ mice, 24 h after intraperitoneal infection with USA300 WT or USA300Δαβδ. Data represent mean values of 4–6 mice. **P < 0.01; ***P < 0.001; ns, not significantly different (WT vs. mFpr2$^{-/-}$ mice), as calculated by Student's t test.

## Discussion

*S. aureus* infections belong to the most frequent infectious diseases (2). Most of them remain superficial, for example, as minor skin or wound infections, and are often hardly recognized, because they are quickly and efficiently cleared by the innate immune system (34). Which mechanisms contribute to the detection and eradication of such infections has remained insufficiently explored. Innate immunity also contributes to severe infections, such as septicemia and pneumonia, which are always associated with massive inflammation, and it instructs the adaptive immune system if infections cannot easily be cured (34). Staphylococcal lipoproteins recognized by the TLR2 receptor are regarded as major MAMPs in *S. aureus* infections (35). In addition, other bacterial molecules, including peptidoglycan (36), DNA, RNA (37, 38), formylated peptides (26), and PSMs (6, 9), are thought to shape the course of *S. aureus* infections, but in which instances and to which extent they may instruct innate immunity remain elusive. Our study demonstrates that FPR2 and its staphylococcal ligands are crucial players in early *S. aureus* infections.

In contrast to other pattern recognition receptors, FPRs differ largely between mammalian species with different numbers of orthologs and specificities (12, 39, 40), probably because FPR peptide ligands can more easily be altered by pathogens than nonprotein MAMPs, which may provoke fast receptor coevolution. The FPR gene cluster has probably expanded and diversified to optimize the recognition of a wide range of peptides. A recent study supported this hypothesis and suggested that FPR genes have been subject to positive selection in the ongoing contact with pathogens (41). The mouse mFpr1 receptor has been clearly identified as the homolog of human FPR1, and mFpr1 knockout mice have been used to demonstrate that it contributes to the course of systemic *Listeria monocytogenes* infections (42, 43). Whether FPR1 also contributes to clearance of superficial infections remains to be analyzed. The association of FPR1 polymorphisms with more severe cases of periodontitis suggests that it contributes to the clearance of local infections (44).

The identity of the functional mFpr2 homolog has been more difficult to elucidate, as mice have several close FPR2 homologs, as outlined before (16). Nevertheless, recent studies supported the notion that mFpr2 has similar ligand specificities as human FPR2 (29). Interestingly, mFpr3, which has been proposed as a third FPR on the surface of mouse neutrophils, is functionally expressed only in some mouse

strains (13). Our used mouse strain belongs to the mice, which express functional mFpr3, but our data demonstrate that only mFpr2 senses *S. aureus* PSMs. Moreover, mFpr2 responds to *S. aureus* culture filtrates much stronger than mFpr1, which reflects similar findings with human FPR1 and FPR2 (45). mFpr2 has been found to contribute to the severity of systemic *L. monocytogenes* infections, although ligands from Listeria that activate FPR2 or mFpr2 have not been reported yet (42). Additionally, it has been shown that lipoxin $A_4$, an endogenous anti-inflammatory ligand of mFpr2, inhibits leukocyte recruitment in a pneumosepsis model in early sepsis, and the blocking of mFpr2 has exhibited beneficial effects to the host, increasing leukocyte migration, reducing bacterial load, and culminating with a survival increase (46). In contrast, we recently found that mFpr2 does not contribute to systemic *S. aureus* infection, probably as neutrophil chemotaxis is more relevant in local than in systemic infection (8). Our current study demonstrates that mFpr2 has a profound impact on early stages of local *S. aureus* infections governing the numbers of immigrated neutrophils and monocytes. mFpr2 did not seem to play a major role at later stages of infection, suggesting that other pattern recognition receptors may have more pronounced roles if a local *S. aureus* infection cannot be cleared rapidly. Interestingly, it has been shown that polymicrobial sepsis of Fpr2/3$^{-/-}$ mice that lack these receptors leads to an exacerbation of sepsis (47). It has been observed that the loss of Fpr2/3 correlates with increased plasma cytokine and chemokine levels, increased PMN amount, and reduced killing of *E. coli* by PMNs. It could be speculated that reduced activity of PMNs in these knockout mice is compensated by recruitment of more PMNs. As the authors used only double-knockout mice, the observed effects could, at least partially, be mediated by mFpr3 (47). Human FPR2 polymorphisms have been reported, but it is not clear yet if these may be associated with increased frequency or severity of superficial *S. aureus* infections. Nevertheless, such polymorphisms have been found to affect allergic diseases, such as chronic urticaria (48) and asthma (49), which are also associated with increased rates of *S. aureus* colonization (50). Thus, FPR2 function may affect *S. aureus* skin and airway colonization and infection.

It remains unclear which role FPR2 may play in infections caused by other pathogens. Culture filtrates of *Enterococcus faecalis*, *Enterococcus faecium* (45), and *L. monocytogenes* (51) contain FPR2 agonists whose identity has not been revealed yet, suggesting that FPR2 may be important for a wider range of bacterial pathogens

and providing a potential explanation for the presence of an FPR2 homolog in mice, which seem to be less frequently infected by *S. aureus* than humans. Superficial, difficult-to-treat skin infections caused by community-associated methicillin-resistant *S. aureus* strains, such as USA300, have strongly increased in recent years (52). The targeting of FPR2 by immunomodulatory drugs may become a new strategy for treating such infections.

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## Author contributions

E. Weiss, A. Peschel, and D. Kretschmer designed research and wrote the paper; F. D. von Loewenich and J. Liese prepared Hoxb8 cells; D. Hanzelmann, B. Fehlhaber, and A. Klos prepared transfected RBL cells; and E. Weiss and D. Kretschmer conducted experiments.

## References

1. van Belkum, A., Melles, D. C., Nouwen, J., van Leeuwen, W. B., van Wamel, W., Vos, M. C., Wertheim, H. F., and Verbrugh, H. A. (2009) Co-evolutionary aspects of human colonisation and infection by Staphylococcus aureus. *Infect Genet Evol* **9**, 32-47
2. Thammavongsa, V., Kim, H. K., Missiakas, D., and Schneewind, O. (2015) Staphylococcal manipulation of host immune responses. *Nat Rev Microbiol* **13**, 529-543
3. Foster, T. J. (2004) The Staphylococcus aureus "superbug". *J Clin Invest* **114**, 1693-1696
4. Bloes, D. A., Kretschmer, D., and Peschel, A. (2015) Enemy attraction: bacterial agonists for leukocyte chemotaxis receptors. *Nat Rev Microbiol* **13**, 95-104
5. Bufe, B., Schumann, T., Kappl, R., Bogeski, I., Kummerow, C., Podgorska, M., Smola, S., Hoth, M., and Zufall, F. (2015) Recognition of bacterial signal peptides by mammalian formyl peptide receptors: a new mechanism for sensing pathogens. *J Biol Chem* **290**, 7369-7387

6. Kretschmer, D., Gleske, A. K., Rautenberg, M., Wang, R., Koberle, M., Bohn, E., Schoneberg, T., Rabiet, M. J., Boulay, F., Klebanoff, S. J., van Kessel, K. A., van Strijp, J. A., Otto, M., and Peschel, A. (2010) Human formyl peptide receptor 2 senses highly pathogenic Staphylococcus aureus. *Cell Host Microbe* **7**, 463-473
7. Rautenberg, M., Joo, H. S., Otto, M., and Peschel, A. (2011) Neutrophil responses to staphylococcal pathogens and commensals via the formyl peptide receptor 2 relates to phenol-soluble modulin release and virulence. *FASEB J* **25**, 1254-1263
8. Hanzelmann, D., Joo, H. S., Franz-Wachtel, M., Hertlein, T., Stevanovic, S., Macek, B., Wolz, C., Gotz, F., Otto, M., Kretschmer, D., and Peschel, A. (2016) Toll-like receptor 2 activation depends on lipopeptide shedding by bacterial surfactants. *Nat Commun* **7**, 12304
9. Wang, R., Braughton, K. R., Kretschmer, D., Bach, T. H., Queck, S. Y., Li, M., Kennedy, A. D., Dorward, D. W., Klebanoff, S. J., Peschel, A., DeLeo, F. R., and Otto, M. (2007) Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. *Nat Med* **13**, 1510-1514
10. Kretschmer, D., Rautenberg, M., Linke, D., and Peschel, A. (2015) Peptide length and folding state govern the capacity of staphylococcal beta-type phenol-soluble modulins to activate human formyl-peptide receptors 1 or 2. *J Leukoc Biol* **97**, 689-697
11. Ye, R. D., Boulay, F., Wang, J. M., Dahlgren, C., Gerard, C., Parmentier, M., Serhan, C. N., and Murphy, P. M. (2009) International Union of Basic and Clinical Pharmacology. LXXIII. Nomenclature for the formyl peptide receptor (FPR) family. *Pharmacol Rev* **61**, 119-161
12. Gao, J. L., Chen, H., Filie, J. D., Kozak, C. A., and Murphy, P. M. (1998) Differential expansion of the N-formylpeptide receptor gene cluster in human and mouse. *Genomics* **51**, 270-276
13. Stempel, H., Jung, M., Perez-Gomez, A., Leinders-Zufall, T., Zufall, F., and Bufe, B. (2016) Strain-specific Loss of Formyl Peptide Receptor 3 in the Murine Vomeronasal and Immune Systems. *J Biol Chem* **291**, 9762-9775
14. Southgate, E. L., He, R. L., Gao, J. L., Murphy, P. M., Nanamori, M., and Ye, R. D. (2008) Identification of formyl peptides from Listeria monocytogenes and Staphylococcus aureus as potent chemoattractants for mouse neutrophils. *J Immunol* **181**, 1429-1437
15. Gao, J. L., and Murphy, P. M. (1993) Species and subtype variants of the N-formyl peptide chemotactic receptor reveal multiple important functional domains. *J Biol Chem* **268**, 25395-25401
16. Hartt, J. K., Barish, G., Murphy, P. M., and Gao, J. L. (1999) N-formylpeptides induce two distinct concentration optima for mouse neutrophil chemotaxis by differential interaction with two N-formylpeptide receptor (FPR) subtypes. Molecular characterization of FPR2, a second mouse neutrophil FPR. *J Exp Med* **190**, 741-747
17. Liang, T. S., Wang, J. M., Murphy, P. M., and Gao, J. L. (2000) Serum amyloid A is a chemotactic agonist at FPR2, a low-affinity N-formylpeptide receptor on mouse neutrophils. *Biochem Biophys Res Commun* **270**, 331-335
18. Tiffany, H. L., Lavigne, M. C., Cui, Y. H., Wang, J. M., Leto, T. L., Gao, J. L., and Murphy, P. M. (2001) Amyloid-beta induces chemotaxis and oxidant stress by acting at formylpeptide receptor 2, a G protein-coupled receptor expressed in phagocytes and brain. *J Biol Chem* **276**, 23645-23652
19. Onnheim, K., Bylund, J., Boulay, F., Dahlgren, C., and Forsman, H. (2008) Tumour necrosis factor (TNF)-alpha primes murine neutrophils when triggered via formyl peptide receptor-related sequence 2, the murine orthologue of human formyl peptide receptor-like 1, through a process involving the type I TNF receptor and subcellular granule mobilization. *Immunology* **125**, 591-600
20. de Haas, C. J., Veldkamp, K. E., Peschel, A., Weerkamp, F., Van Wamel, W. J., Heezius, E. C., Poppelier, M. J., Van Kessel, K. P., and van Strijp, J. A. (2004) Chemotaxis inhibitory protein of Staphylococcus aureus, a bacterial antiinflammatory agent. *J Exp Med* **199**, 687-695
21. Prat, C., Bestebroer, J., de Haas, C. J., van Strijp, J. A., and van Kessel, K. P. (2006) A new staphylococcal anti-inflammatory protein that antagonizes the formyl peptide receptor-like 1. *J Immunol* **177**, 8017-8026

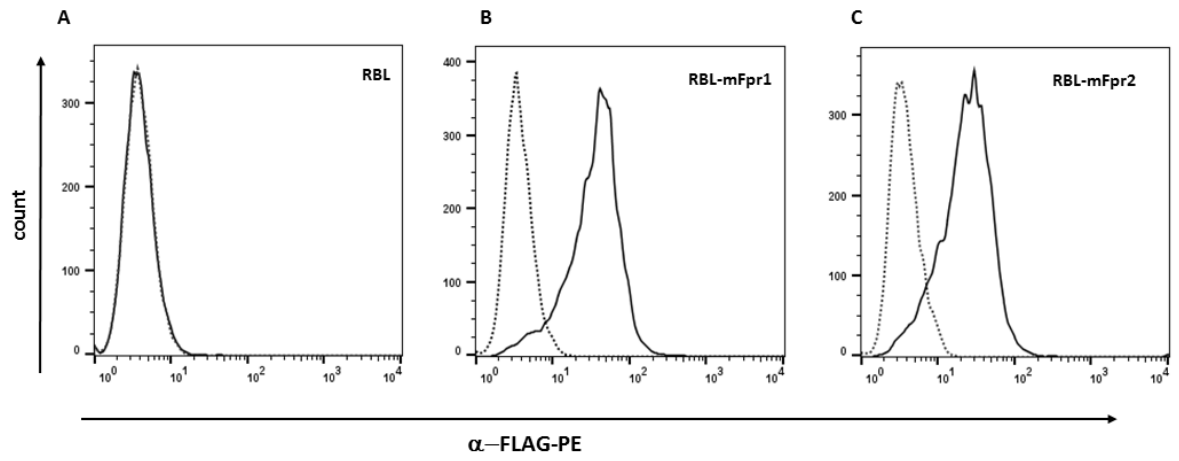
22. Prat, C., Haas, P. J., Bestebroer, J., de Haas, C. J., van Strijp, J. A., and van Kessel, K. P. (2009) A homolog of formyl peptide receptor-like 1 (FPRL1) inhibitor from Staphylococcus aureus (FPRL1 inhibitory protein) that inhibits FPRL1 and FPR. *J Immunol* **183**, 6569-6578
23. Diep, B. A., and Otto, M. (2008) The role of virulence determinants in community-associated MRSA pathogenesis. *Trends Microbiol* **16**, 361-369
24. Joo, H. S., Cheung, G. Y., and Otto, M. (2011) Antimicrobial activity of community-associated methicillin-resistant Staphylococcus aureus is caused by phenol-soluble modulin derivatives. *J Biol Chem* **286**, 8933-8940
25. Chen, K., Le, Y., Liu, Y., Gong, W., Ying, G., Huang, J., Yoshimura, T., Tessarollo, L., and Wang, J. M. (2010) A critical role for the g protein-coupled receptor mFPR2 in airway inflammation and immune responses. *J Immunol* **184**, 3331-3335
26. Durr, M. C., Kristian, S. A., Otto, M., Matteoli, G., Margolis, P. S., Trias, J., van Kessel, K. P., van Strijp, J. A., Bohn, E., Landmann, R., and Peschel, A. (2006) Neutrophil chemotaxis by pathogen-associated molecular patterns--formylated peptides are crucial but not the sole neutrophil attractants produced by Staphylococcus aureus. *Cell Microbiol* **8**, 207-217
27. Wang, G. G., Calvo, K. R., Pasillas, M. P., Sykes, D. B., Hacker, H., and Kamps, M. P. (2006) Quantitative production of macrophages or neutrophils ex vivo using conditional Hoxb8. *Nat Methods* **3**, 287-293
28. Dahlgren, C., Gabl, M., Holdfeldt, A., Winther, M., and Forsman, H. (2016) Basic characteristics of the neutrophil receptors that recognize formylated peptides, a danger-associated molecular pattern generated by bacteria and mitochondria. *Biochem Pharmacol* **114**, 22-39
29. Skovbakke, S. L., Winther, M., Gabl, M., Holdfeldt, A., Linden, S., Wang, J. M., Dahlgren, C., Franzyk, H., and Forsman, H. (2016) The peptidomimetic Lau-(Lys-betaNSpe)6-NH2 antagonizes formyl peptide receptor 2 expressed in mouse neutrophils. *Biochem Pharmacol* **119**, 56-65
30. Kretschmer, D., Nikola, N., Durr, M., Otto, M., and Peschel, A. (2012) The virulence regulator Agr controls the staphylococcal capacity to activate human neutrophils via the formyl peptide receptor 2. *J Innate Immun* **4**, 201-212
31. Swamydas, M., Luo, Y., Dorf, M. E., and Lionakis, M. S. (2015) Isolation of Mouse Neutrophils. *Curr Protoc Immunol* **110**, 3 20 21-23 20 15
32. Rzodkiewicz, P., Wojtecka-Lukasik, E., Szukiewicz, D., Schunack, W., and Maslinski, S. (2010) Antihistaminic drugs modify casein-induced inflammation in the rat. *Inflamm Res* **59 Suppl 2**, S187-188
33. McDonald, J. U., Cortini, A., Rosas, M., Fossati-Jimack, L., Ling, G. S., Lewis, K. J., Dewitt, S., Liddiard, K., Brown, G. D., Jones, S. A., Hallett, M. B., Botto, M., and Taylor, P. R. (2011) In vivo functional analysis and genetic modification of in vitro-derived mouse neutrophils. *FASEB J* **25**, 1972-1982
34. Belkaid, Y., and Tamoutounour, S. (2016) The influence of skin microorganisms on cutaneous immunity. *Nat Rev Immunol* **16**, 353-366
35. Kawai, T., and Akira, S. (2010) The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol* **11**, 373-384
36. Xie, X., Wang, L., Gong, F., Xia, C., Chen, J., Song, Y., Shen, A., and Song, J. (2012) Intracellular Staphylococcus aureus-induced NF-kappaB activation and proinflammatory responses of P815 cells are mediated by NOD2. *J Huazhong Univ Sci Technolog Med Sci* **32**, 317-323
37. Bergstrom, B., Aune, M. H., Awuh, J. A., Kojen, J. F., Blix, K. J., Ryan, L., Flo, T. H., Mollnes, T. E., Espevik, T., and Stenvik, J. (2015) TLR8 Senses Staphylococcus aureus RNA in Human Primary Monocytes and Macrophages and Induces IFN-beta Production via a TAK1-IKKbeta-IRF5 Signaling Pathway. *J Immunol* **195**, 1100-1111
38. Kruger, A., Oldenburg, M., Chebrolu, C., Beisser, D., Kolter, J., Sigmund, A. M., Steinmann, J., Schafer, S., Hochrein, H., Rahmann, S., Wagner, H., Henneke, P., Hornung, V., Buer, J., and Kirschning, C. J. (2015) Human TLR8 senses UR/URR motifs in bacterial and mitochondrial RNA. *EMBO Rep* **16**, 1656-1663

39. Snyderman, R., and Pike, M. C. (1980) N-Formylmethionyl peptide receptors on equine leukocytes initiate secretion but not chemotaxis. *Science* **209**, 493-495
40. Oseas, R. S., Boxer, L. A., Butterick, C., and Baehner, R. L. (1980) Differences in polymorphonuclear leukocyte aggregating responses among several species in response to chemotactic stimulation. *J Lab Clin Med* **96**, 213-221
41. Muto, Y., Guindon, S., Umemura, T., Kohidai, L., and Ueda, H. (2015) Adaptive evolution of formyl peptide receptors in mammals. *J Mol Evol* **80**, 130-141
42. Liu, M., Chen, K., Yoshimura, T., Liu, Y., Gong, W., Wang, A., Gao, J. L., Murphy, P. M., and Wang, J. M. (2012) Formylpeptide receptors are critical for rapid neutrophil mobilization in host defense against Listeria monocytogenes. *Sci Rep* **2**, 786
43. Gao, J. L., Lee, E. J., and Murphy, P. M. (1999) Impaired antibacterial host defense in mice lacking the N-formylpeptide receptor. *J Exp Med* **189**, 657-662
44. Zhang, Y., Syed, R., Uygar, C., Pallos, D., Gorry, M. C., Firatli, E., Cortelli, J. R., VanDyke, T. E., Hart, P. S., Feingold, E., and Hart, T. C. (2003) Evaluation of human leukocyte N-formylpeptide receptor (FPR1) SNPs in aggressive periodontitis patients. *Genes Immun* **4**, 22-29
45. Bloes, D. A., Otto, M., Peschel, A., and Kretschmer, D. (2012) Enterococcus faecium stimulates human neutrophils via the formyl-peptide receptor 2. *PLoS One* **7**, e39910
46. Sordi, R., Menezes-de-Lima, O., Jr., Horewicz, V., Scheschowitsch, K., Santos, L. F., and Assreuy, J. (2013) Dual role of lipoxin A4 in pneumosepsis pathogenesis. *Int Immunopharmacol* **17**, 283-292
47. Gobbetti, T., Coldewey, S. M., Chen, J., McArthur, S., le Faouder, P., Cenac, N., Flower, R. J., Thiemermann, C., and Perretti, M. (2014) Nonredundant protective properties of FPR2/ALX in polymicrobial murine sepsis. *Proc Natl Acad Sci U S A* **111**, 18685-18690
48. Yang, E. M., Kim, S. H., Kim, N. H., and Park, H. S. (2010) The genetic association of the FPRL1 promoter polymorphism with chronic urticaria in a Korean population. *Ann Allergy Asthma Immunol* **105**, 96-97
49. Kim, H. J., Cho, S. H., Park, J. S., Lee, T. H., Lee, E. J., Kim, Y. H., Uh, S. T., Chung, I. Y., Kim, M. K., Choi, I. S., Park, B. L., Shin, H. D., and Park, C. S. (2012) Association analysis of formyl peptide receptor 2 (FPR2) polymorphisms and aspirin exacerbated respiratory diseases. *J Hum Genet* **57**, 247-253
50. Sharma, A. D. (2012) Role of Nasal Carriage of Staphylococcus aureus in Chronic Urticaria. *Indian J Dermatol* **57**, 233-236
51. Rabiet, M. J., Huet, E., and Boulay, F. (2005) Human mitochondria-derived N-formylated peptides are novel agonists equally active on FPR and FPRL1, while Listeria monocytogenes-derived peptides preferentially activate FPR. *Eur J Immunol* **35**, 2486-2495
52. Otto, M. (2013) Community-associated MRSA: what makes them special? *Int J Med Microbiol* **303**, 324-330

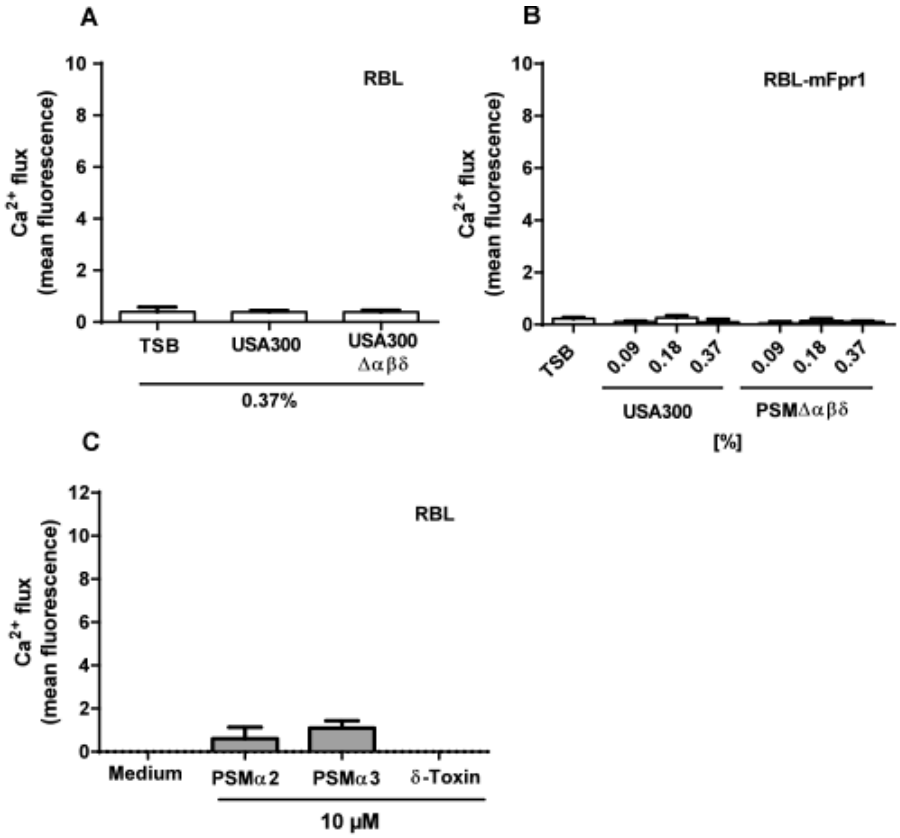
## Supplement

**Supplemental Table 1: Primer used in this study.**

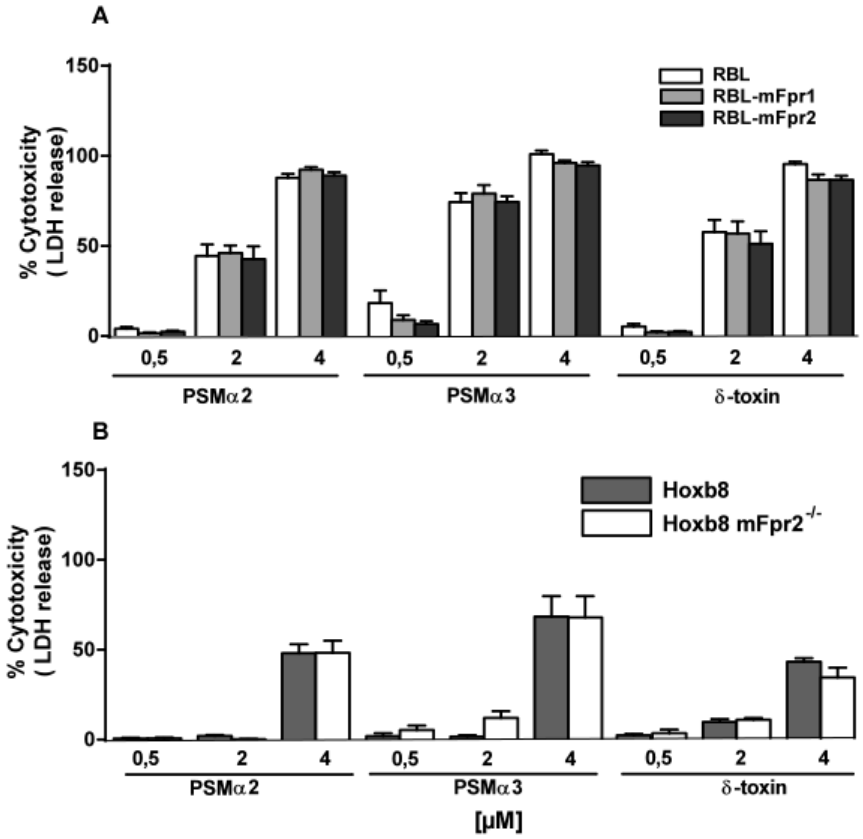
| Primer Description | Primer Sequence |
|---|---|
| mFPR P1-NotI | aaaaaaaaaagcggccgccgccaccatggattataaagatgatgatgataaa |
| mFPR P2-BamH1 | aaaaaaaaaaggatccttacattgcatttaaagtgtttt |
| mFpr-rs2 / mFPR2 P1-NotI | aaaaaaaaaagcggccgccgccaccatggattataaagatgatgatgataaa |
| mFpr-rs2 / mFPR2 P2-PacI | aaaaaaaaaattaattaattatggggcctttaactcaatgt |
| Sequencing P1 pQCXIN-EF1α | aagttaggccagcttggcact |
| Sequencing P2 pQCXIN-EF1α | aagcggcttcggccagtaacgtta |



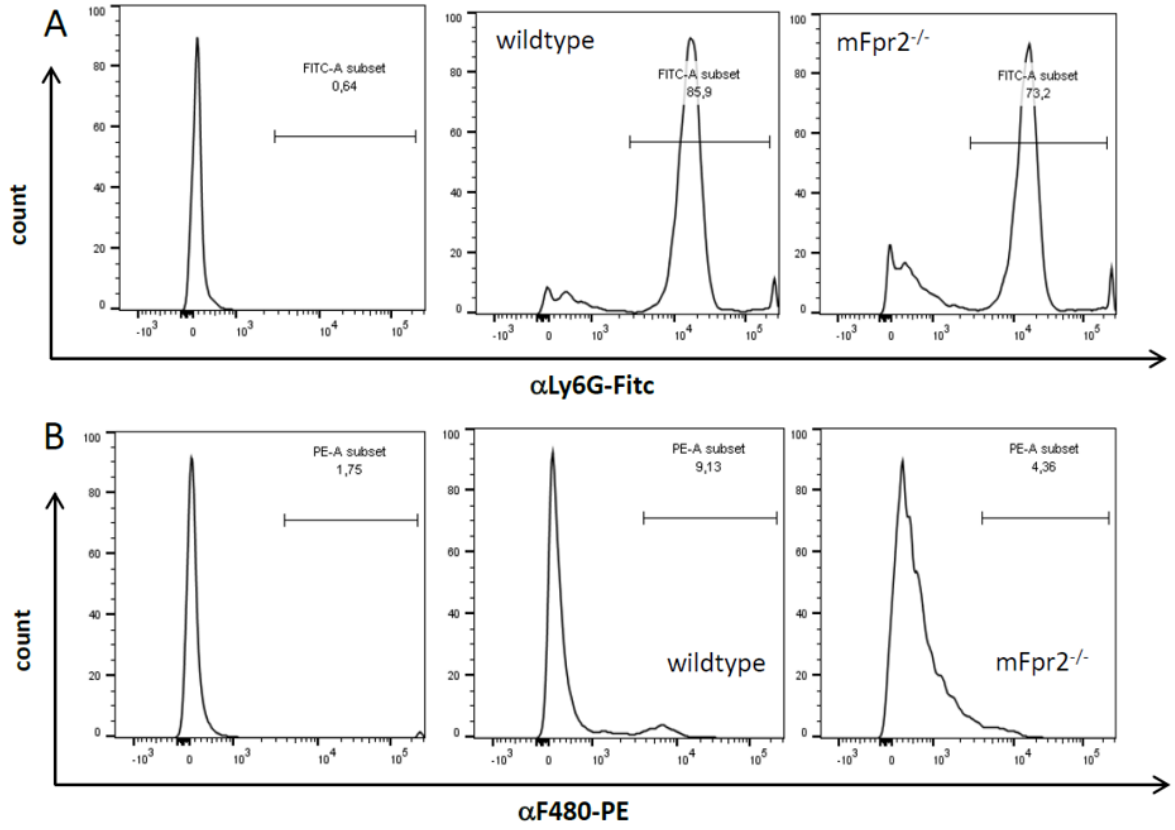

**Supplemental Figure 1: Expression of mFpr1 or mFpr2 in RBL cells.** Expression of a FLAG-tag in RBL cells transfected either with the empty vector (A), mFpr1 (B) or mFpr2 (C). Isotype control antibodies are shown as dotted lines; specific antibodies as solid lines. One experiment representative for three independent experiments is given.




**Supplemental Figure 2: PSMs induce no activation of mFpr1 transfected RBL cells or RBL control cells with empty vector.** Calcium influx induced by culture filtrates of USA300 wild type or USA300Δαβδ in RBL cells transfected with an empty vector (A), mFpr1 transfected RBL cells (B), or calcium flux induced by PSMs in RBL cells transfected with an empty vector (C); Data represent means ± SEM of at least three independent experiments.




**Supplemental Figure 3: PSMs induce stronger lysis of RBL cells than of Hoxb8 neutrophils**. LDH release is induced by 4 µM PSMα2, α3, or δ-toxin of RBL cells, stably transfected either with empty vector, with mFpr1 or mFpr2 (A) LDH release induced by 4 µM PSMα2, PSMα3 or δ-toxin in wild-type or mFpr2$^{-/-}$ Hoxb8 cell**s.** Data represent means ± SEM of at least three independent experiments.




**Supplemental Figure 4: Gating strategies for peritoneal neutrophils and macrophages.** The overall number of cells in the peritoneal cavity was determined microscopically (Zeiss, Jena) via a Neubauer hemocytometer. The number of neutrophils (A) or macrophages (B) was calculated with the following mathematical formula: cell number*leucocytes (%)*Ly6G$^{+}$cells (%) or cell number*leucocytes (%)*F4/80$^{+}$ cells (%), respectively.

# CHAPTER 4

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## Costimulation of FPR1 and FPR2 leads to a synergistic release of chemokines in neutrophils

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## Abstract

Formyl-peptide receptors (FPRs) are crucial pattern recognition receptors (PRRs) governing leukocyte chemotaxis and release of reactive oxygen species (ROS), antimicrobial peptides (AMPs), and cytokines and chemokines in response to microbe-associated molecular patterns (MAMPs). FPR1 senses short formylated peptides produced by all kinds of bacteria, while FPR2 is activated by phenol-soluble modulins (PSM) from highly pathogenic staphylococci. In this study we analyzed the impact of co-activation of both FPRs on different cellular functions of human neutrophils and monocytes. Whereas cellular functions of neutrophils like chemotaxis, calcium influx, and degranulation were not be affected, we could show that co-stimulation of FPR1 and FPR2 leads to a synergistic release of the chemokine IL-8 by human neutrophils. Interestingly, this effect could be observed only in neutrophils but not in monocytes. Our data support an important role for neutrophils and the FPRs during the onset of inflammation. Their analysis could be helpful for the understanding of the regulation of inflammation and may enable novel therapeutic strategies to minimize host injury following bacterial infection.

## Introduction

Formyl-peptide receptors (FPRs) are pattern recognition receptors (PRRs) highly expressed by immune cells, such as neutrophils and monocytes. They belong to the family of seven-transmembrane G protein-coupled receptors (GPCRs), which are involved in host defense against bacterial infections and in the clearance of damaged cells [1]. FPRs sense microbe-associated molecular patterns (MAMPs) and thereby discriminate between self and foreign. Human neutrophils express FPR1 and FPR2, which are activated by short formylated peptides or amphipathic, α-helical peptides, respectively [2]. FPR1 generally recognizes short peptides initiated with N-formyl-methionine, which are cleavage products of bacterial and mitochondrial proteins. One of the most potent FPR1 ligands represents the peptide N-formyl-methionyl-leucyl-phenylalanine (fMLF) [3]. In contrast, FPR2 is a low-affinity receptor for fMLF, yet many endogenous ligands have been described for FPR2. In addition, we have previously shown that phenol-soluble modulins (PSMs) of highly pathogenic *Staphylococcus aureus* are also ligands for FPR2 [4]. We show here that activation of

FPRs results in a strong calcium influx into the cytosol, migration of immune cells to the infection site, and release of reactive oxygen species (ROS), antimicrobial peptides (AMPs), and chemokines.

The main chemokine released by human neutrophils represents interleukin (IL)-8. It can bind to the two GPCRs CXCR1 and CXCR2, but it has been found that chemotactic signaling occurs primarily via CXCR1 [5]. In contrast to FPR ligands, which belong to the end-target chemoattractants, IL-8 represents an intermediate chemoattractant. The classification of chemoattractants is based on their intracellular signaling hierarchy. Neutrophils prefer end-target chemoattractants over intermediate chemoattractants, which ensures guided migration to the infection site [6]. Although both groups of chemoattractants stimulate neutrophil chemotaxis, the end-target chemoattractants, such as fMLF and C5a, have the additional capability of eliciting cytotoxic responses including release of AMPs and ROS [7]. During infection chemokines play an essential role for the recruitment of neutrophils and subsequent killing of pathogens. However, failure to remove invading pathogens can lead to systemic infection and hyperactive inflammatory responses termed systemic inflammatory response syndrome (SIRS), which is mediated by cytokines and chemokines [8, 9]. Blocking of CXCR1/2 reversed several criteria of established SIRS in septic mice and functional loss of CXCR1/2 protected mice from mortality due to SIRS or sepsis [10, 11]. As blocking of CXCR1/2 does not influence other chemokine receptors, the effects may be considered immunomodulatory rather than immunosuppressive. During the lethal outcome of severe sepsis neutrophils show impaired recruitment because of prolonged exposure to high amounts of chemokines leading to receptor desensitization, especially of CXCR2 [12, 13]. In this case loss of CXCR2 correlates with bad prognosis [14].

We investigated here, which role individual or simultaneous stimulation of FPR1 and FPR2 has regarding calcium influx, migration, degranulation, ROS release, and release of chemokines. We could show that activation of both FPRs results in a strong, non-synergistic intracellular calcium influx, migration of neutrophils, degranulation, and release of ROS. Although culture filtrates of *S. aureus* USA300 induce a strong release of the chemokine IL-8 even at very high dilutions, high amounts of synthetic PSMs are needed to induce IL-8 release by human neutrophils. Furthermore, at the same concentrations culture filtrates of an isogenic PSM mutant do not induce chemokine release by neutrophils. Thus, we supposed that besides

PSMs additional ligands may contribute to the release of IL-8 induced by culture filtrates of USA300. Therefore, we analyzed if the simultaneous activation of FPR1 with FPR2 results in a higher chemokine release compared to PSMs alone. Indeed, costimulation of FPR1 and FPR2 results in synergistic IL-8 release by neutrophils.

## Materials & Methods

**Synthetic peptides:** PSMα3 was kindly provided by Stefan Stevanovic (Immunology Department, University of Tübingen), fMFL was purchased from Sigma, recombinant human C5a was from R&D.

**Isolation of human neutrophils:** Human neutrophils were isolated from healthy blood donors by density gradient centrifugation as described recently [4] and suspended in RPMI + 2 % HSA + 2 mM Sodiumpyruvat + 10 mM HEPES.

**Isolation of human monocytes:** Peripheral blood mononuclear cells (PBMCs) were isolated from healthy blood donors by density gradient centrifugation. Human monocytes were isolated with CD14 MicroBeads by using the MACS® Technology of Miltenyi Biotech according to the manufacturer's instructions. Cells were suspended in RPMI.

**Calcium flux assay:** Human neutrophils were analyzed by stimulating cells loaded with Fluo-3-AM (Molecular Probes) and monitoring fluorescence with a FACSCalibur flow cytometer (Becton Dickinson) as described recently [4]. Measurements of 2,000 events were performed and calcium flux was expressed as mean fluorescence intensity.

**Chemotaxis assay:** Neutrophils were labeled with BCECF, AM (Life Technologies) and seeded in 3 µm pore size ThinCerts (greiner bio-one). The compartments below the cell culture inserts contained stated peptides. After 80 minutes the cell culture inserts were removed and the fluorescence intensity of the migrated neutrophils in the lower compartments was measured with a BMG Labtech CLARIOstar plate reader. The percentages of migrated neutrophils were calculated towards a positive control (neutrophils were just seeded in the lower compartment).

**Oxidative burst:** Neutrophils were incubated with the specified ligands and 100 µM luminol in HBSS + 0.05 % HSA. Luminescence was measured every three minutes

with a BMG Labtech CLARIOstar plate reader for a total time of one hour. Oxidative burst was expressed as chemoluminescence avtivity. Medium control was zero.

**Degranulation assay:** Neutrophils were stimulated with the indicated peptides and the release of β-glucuronidase was measured as described recently [15]. Degranulation was calculated as percentage of positive control (TritonX-100).

**IL-8 release:** Neutrophils were incubated with the indicated agonists for 5 hours at 37°C, monocytes were incubated for 24 hours. For the IL-8 kinetic neutrophils were incubated for the indicated time points with fMLF and PSMα3. The supernatants were collected and released IL-8 was measured using an ELISA kit (R&D Systems) according to the manufacturer's instructions.

**Statistics:** Statistical analysis were performed using Graph Pad Prism 6.01. Two-tailed t-test was used to compare two data groups. To compare multiple groups two-way ANOVA was used.

## Results

### Costimulation of FPR1 and FPR2 of human neutrophils did not enhance several cellular functions

It has been shown that short formylated peptides and PSMs are ligands for the FPR1 and the FPR2, respectively [4, 16]. Neutrophils respond to these ligands with a strong calcium influx, chemotaxis, and release of reactive oxygen species (ROS), antimicrobial peptides (AMPs) by degranulation, and chemokines. As just very few is known about the downstream signaling of the FPRs we analyzed the response of human neutrophils costimulated with FPR1 and FPR2 ligands. Our data demonstrate that neutrophils stimulated with fMLF as FPR1 ligand or PSMα3 as FPR2 ligand did not respond with significant higher migration or degranulation when they were stimulated with both ligands compared to activation of just FPR1 or FPR2 (figure 1B, D). Costimulated neutrophils responded with a higher calcium influx and enhanced release of ROS compared to cells stimulated with fMLF or PSMα3 alone (figure 1A, C), but the effect was not even additive. Thus, coactivation of FPR1 and FPR2 did not result in higher migration of neutrophils and degranulation compared to

stimulation of FPR1 or FPR2 alone and just in a very slight enhancement of ROS production as well as of calcium influx into the cytosol

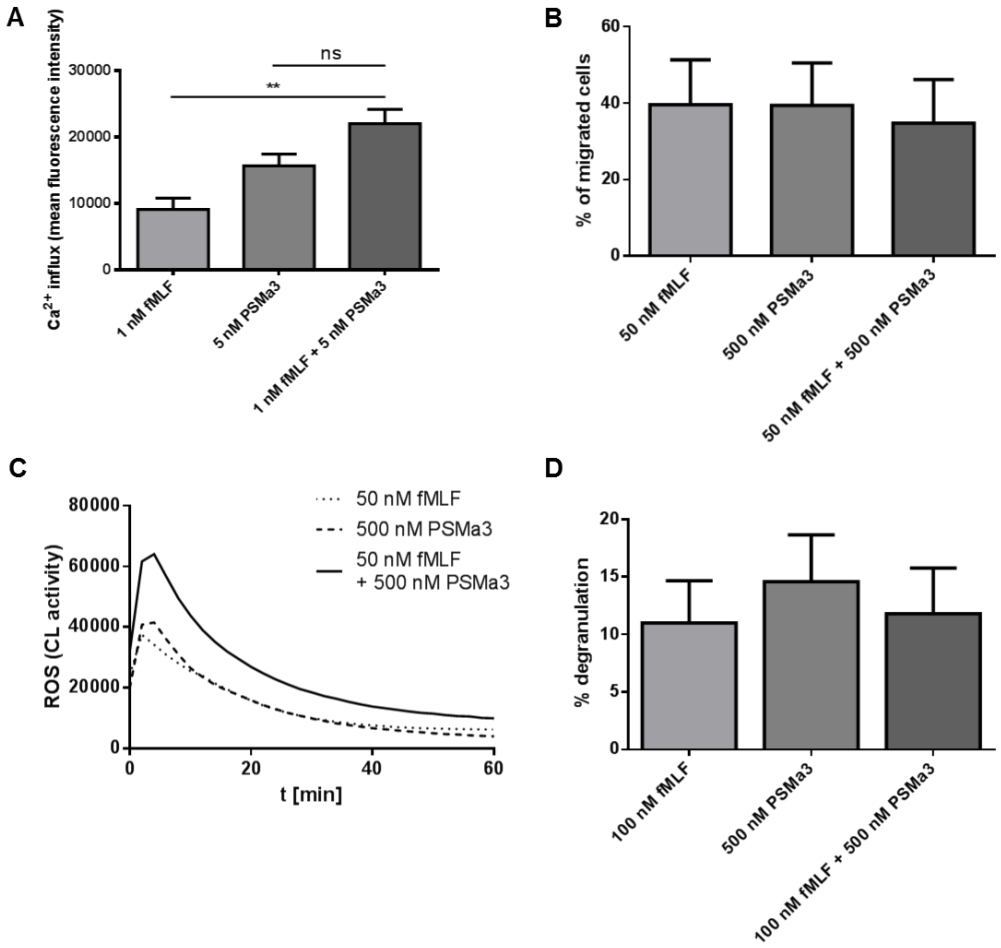
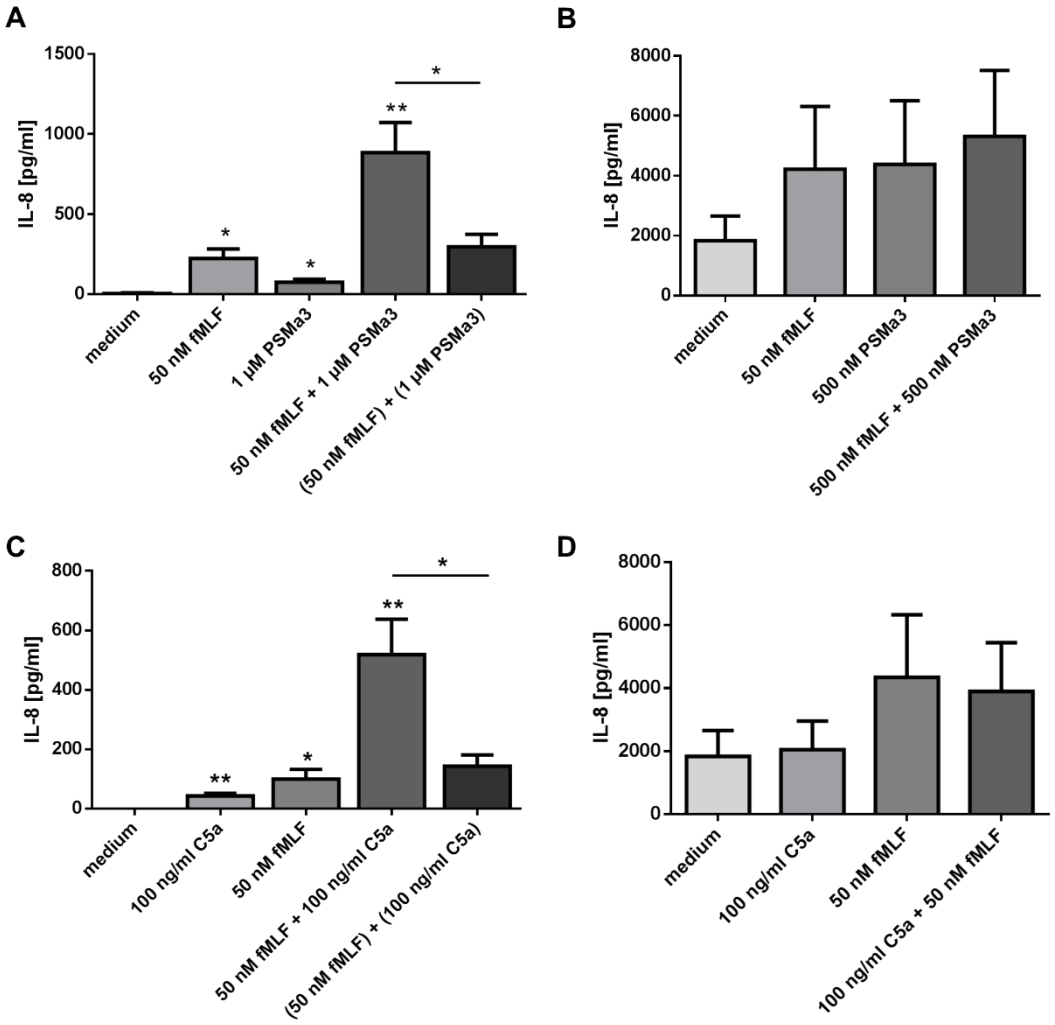



**Figure 1:** Costimulation of neutrophils with fMLF and PSMα3 did not result in a synergistic response during A) calcium flux or C) oxidative burst or even in an enhanced response for B) chemotaxis or D) degranulation of neutrophils. Data represent means ± SEM of at least three independent experiments.

## The release of IL-8 is synergistically enhanced in neutrophils, but not monocytes costimulated with FPR1 and FPR2 ligands

Neutrophils are very important producers of the chemokine IL-8. They are the first immune cells at the infection site and by releasing high amounts of this chemokine they amplify their own recruitment and induce migration of monocytes to the infection site. Costimulation of neutrophils with fMLF and PSMα3 resulted in a synergistic release of IL-8 compared to neutrophils stimulated with just the FPR1 or FPR2 ligand (figure 2A). This effect seems not to be limited to these two receptors as

costimulation with C5a, ligand of a related GPCR, resulted also in a synergistic release of IL-8 (figure 2C, E). Monocytes costimulated with FPR1 and FPR2 ligands (figure 2B) or C5a (figure 2D, F) did not respond with synergistic release of IL-8. By analyzing the synergistic release of IL-8 by human neutrophils over time we could show a strong increase of IL-8 between one and five hours when the cells were stimulated with fMLF and PSMα3 simultaneously (figure 3). The amount of IL-8 released by neutrophils, which were stimulated with fMLF or PSMα3 alone increased just very weakly. In summary, we could show that FPR1 and FPR2 of neutrophils act synergistically during the release of the chemokine IL-8 and this effect increases over time.



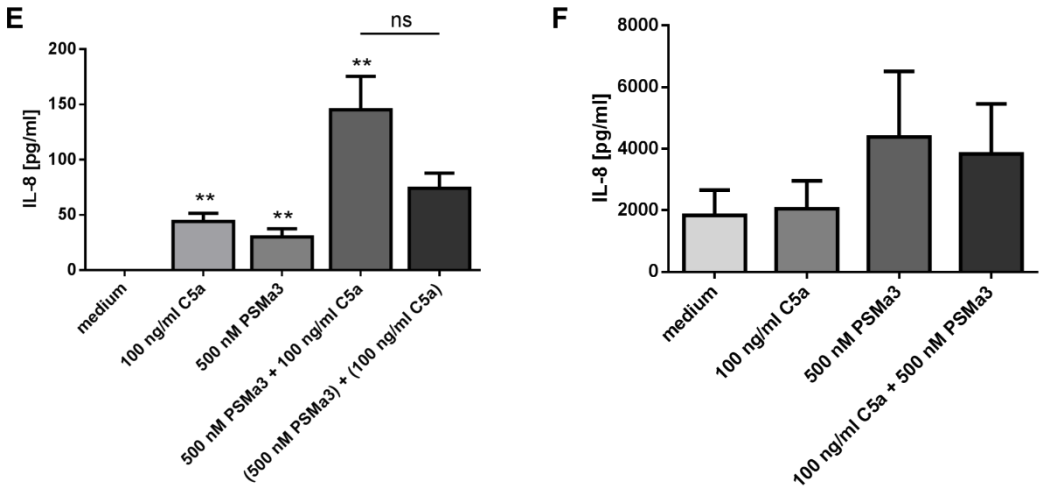



**Figure 2:** IL-8 released by A, C, E) neutrophils or B, D, F) monocytes stimulated with the depicted concentrations of A, B) fMLF and PSMα3, C, D) C5a and fMLF or E, F) C5a and PSMα3. Data represent means ± SEM of at least three independent experiments. *P < 0.05; **P < 0.01 vs. corresponding medium control or as indicated calculated by Student's t test.

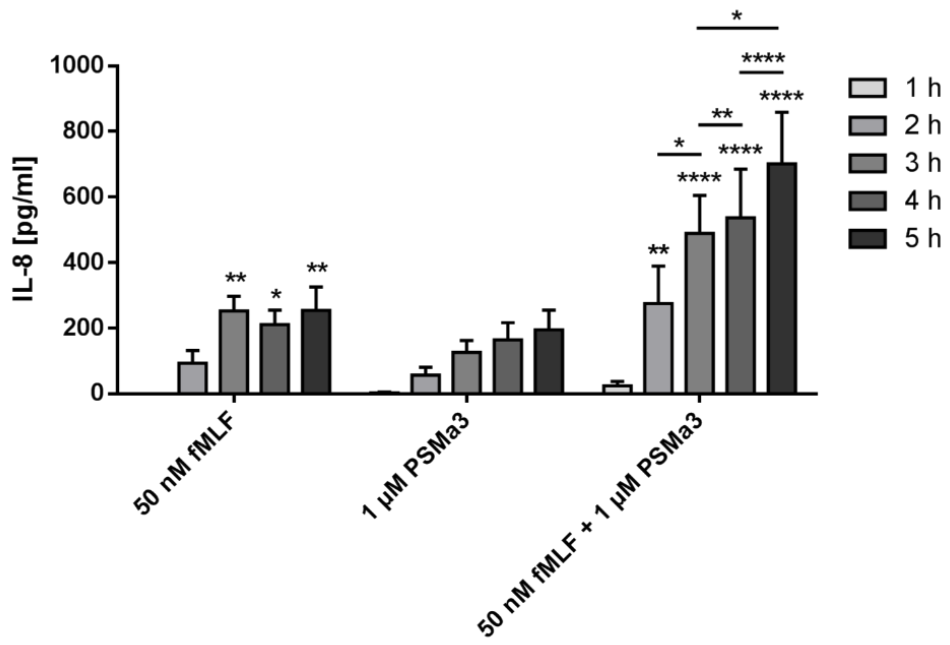



**Figure 3:** Kinetic of released IL-8 by neutrophils stimulated with fMLF and PSMα3 up to five hours. Data represent means ± SEM of at least three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 vs. 1 hour time point or as indicated calculated by two-way ANOVA.

## Discussion

FPRs play an important role for the recognition and eradication of invading pathogens. Besides the capacity of FPR1 to detect formylated peptides of bacteria in general, phagocytes are additionally equipped with FPR2 which gets activated by specific bacterial ligands like the staphylococcal PSMs. Here we could show that costimulation of FPR1 and FPR2 by the short formylated peptide fMLF and PSMα3, respectively, results in a synergistic release of the chemokine IL-8 by human neutrophils. This effect was not observable with human monocytes. Additionally, further cellular responses of neutrophils like intracellular calcium influx, chemotaxis, and the release of ROS, and degranulation are not affected. As the synergistic release of IL-8 increases dramatically over time we hypothesize that this effect might be transcriptionally regulated. In contrast, the cellular responses, which could not be enhanced by costimulation of FPR1 and FPR2 are thought to be not transcriptionally regulated, which further supports our hypothesis.

For the simultaneous activation of TLR4 by LPS and FPR1 by fMLF it was already shown that it results in a synergistic release of chemokines and cytokines by human monocytes compared to a single stimulation of just one of these receptors. The authors could show that this effect is induced by phosphorylation of different serine residues of the NFκB subunit p65. This finally results in synergistic activation of NFκB leading to a boosted inflammatory response [17]. NFκB is a dimeric transcription factor which is essential for IL-8 gene expression in all studied cell types [18]. Enhanced transcription might be induced by additional phosphorylation of the NFκB subunits as well as binding of coactivators [19]. Unlike NFκB, which is essential for transcriptional activation, the activating protein (AP)-1 and CAAT/enhancer-binding protein (C/EBP) are just required for maximal gene expression [20]. Downstream of FPRs three mitogen-activated protein kinase (MAPK) pathways contribute to IL-8 gene expression, the extracellular-regulated protein kinase (ERK), JUN-N-terminal protein kinase (JNK), and p38 MAPK cascades [18]. Further research will focus on the elucidation of the signal pathway behind the synergistic IL-8 release after simultaneous FPR1 and FPR2 activation in case of neutrophils.

For migration of neutrophils it was already shown that predominantly CXC chemokines, which bind to chemokine receptors other than those for IL-8, can synergize with IL-8 in chemotaxis assays. Additionally, also CC chemokines can

synergize with IL-8 [21]. This observation was absent for intracellular calcium concentrations, which could not be synergistically enhanced by costimulation compared to stimulation with IL-8 alone. The postulated mechanism behind is an enhanced protein kinase activity and/or enhanced production of second messengers [21]. As we could not observe a synergistic effect for FPR1 and FPR2 during chemotaxis this process might be limited to other chemokine receptors or the two stimulated receptors need to share less amino acid sequence homology.

Our data demonstrate that *S. aureus* induces a strong release of chemokines by human neutrophils by activating FPR1 and FPR2 synergistically. Further research will focus on the mechanism which could lead to a better understanding of the regulation of inflammation. This may suggest novel therapeutic strategies to minimize host injury following bacterial infection.

## References


1. Ye, R.D., et al., *International Union of Basic and Clinical Pharmacology. LXXIII. Nomenclature for the formyl peptide receptor (FPR) family.* Pharmacol Rev, 2009. **61**(2): p. 119-61.
2. Kretschmer, D., et al., *Peptide length and folding state govern the capacity of staphylococcal beta-type phenol-soluble modulins to activate human formyl-peptide receptors 1 or 2.* J Leukoc Biol, 2015. **97**(4): p. 689-97.
3. Marasco, W.A., 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): p. 5430-9.
4. Kretschmer, D., et al., *Human formyl peptide receptor 2 senses highly pathogenic Staphylococcus aureus.* Cell Host Microbe, 2010. **7**(6): p. 463-73.
5. Hammond, M.E., et al., *IL-8 induces neutrophil chemotaxis predominantly via type I IL-8 receptors.* J Immunol, 1995. **155**(3): p. 1428-33.
6. Kolaczkowska, E. and P. Kubes, *Neutrophil recruitment and function in health and inflammation.* Nat Rev Immunol, 2013. **13**(3): p. 159-75.
7. Ye, R.D., *Editorial: Biased agonism in chemoattractant receptor signaling.* J Leukoc Biol, 2010. **87**(6): p. 959-61.
8. Bone, R.C., *Toward a theory regarding the pathogenesis of the systemic inflammatory response syndrome: what we do and do not know about cytokine regulation.* Crit Care Med, 1996. **24**(1): p. 163-72.
9. Dinarello, C.A., J.A. Gelfand, and S.M. Wolff, *Anticytokine strategies in the treatment of the systemic inflammatory response syndrome.* JAMA, 1993. **269**(14): p. 1829-35.
10. Kaneider, N.C., et al., *Reversing systemic inflammatory response syndrome with chemokine receptor pepducins.* Nat Med, 2005. **11**(6): p. 661-5.
11. Ness, T.L., et al., *Immunomodulatory role of CXCR2 during experimental septic peritonitis.* J Immunol, 2003. **171**(7): p. 3775-84.
12. Rios-Santos, F., et al., *Down-regulation of CXCR2 on neutrophils in severe sepsis is mediated by inducible nitric oxide synthase-derived nitric oxide.* Am J Respir Crit Care Med, 2007. **175**(5): p. 490-7.

13. Arraes, S.M., et al., *Impaired neutrophil chemotaxis in sepsis associates with GRK expression and inhibition of actin assembly and tyrosine phosphorylation.* Blood, 2006. **108**(9): p. 2906-13.
14. Tavares-Murta, B.M., et al., *Failure of neutrophil chemotactic function in septic patients.* Crit Care Med, 2002. **30**(5): p. 1056-61.
15. Weiss, E., et al., *Formyl-peptide receptor 2 governs leukocyte influx in local Staphylococcus aureus infections.* FASEB J, 2018. **32**(1): p. 26-36.
16. Boulay, F., et al., *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): p. 11123-33.
17. Chen, L.Y., et al., *Synergistic induction of inflammation by bacterial products lipopolysaccharide and fMLP: an important microbial pathogenic mechanism.* J Immunol, 2009. **182**(4): p. 2518-24.
18. Hoffmann, E., et al., *Multiple control of interleukin-8 gene expression.* J Leukoc Biol, 2002. **72**(5): p. 847-55.
19. Schmitz, M.L., S. Bacher, and M. Kracht, *I kappa B-independent control of NF-kappa B activity by modulatory phosphorylations.* Trends Biochem Sci, 2001. **26**(3): p. 186-90.
20. Persson, E., et al., *Increased expression of interleukin-6 by vasoactive intestinal peptide is associated with regulation of CREB, AP-1 and C/EBP, but not NF-kappaB, in mouse calvarial osteoblasts.* Bone, 2005. **37**(4): p. 513-29.
21. Gouwy, M., et al., *Synergy between proinflammatory ligands of G protein-coupled receptors in neutrophil activation and migration.* J Leukoc Biol, 2004. **76**(1): p. 185-94.

# CHAPTER 5

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## Always hungry: Formyl-peptide receptor activation augments phagocytosis of *Staphylococcus aureus* by human neutrophils

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**Manuscript in preparation**

## Abstract

Formyl-peptide receptors (FPRs) are important pattern recognition receptors regulating the inflammatory immune response. They are strongly expressed on phagocytes and their activation leads primarily to migration of phagocytes to the infection site. Since it has been shown that the antimicrobial cathelicidin LL-37 enhances phagocytosis in a FPR2-dependent manner, we analyzed if FPR activation by bacterial ligands amplifies neutrophil phagocytosis.

Incubation of neutrophils with FPR ligands during phagocytosis led to an increase in engulfed *S. aureus* and synergistic release of IL-8 compared to phagocytosis without FPR ligands. The increased phagocytosis is mediated via upregulation of complement receptor 1 (CD35) and 3 (CD11b) as well as FCγ receptor I (CD64). Enhanced IL-8 release led to stronger chemotaxis of neutrophils, thereby changing the ratio of neutrophils versus pathogens. Interestingly, we observed that the enhanced phagocytosis could be of favor either for killing or survival of *S. aureus* depending on the amount of bacteria to neutrophils and the time period of killing. Furthermore, although lack of FPR2 resulted in less phagocytosis of *S. aureus* in a mouse infection model, better killing of PSM releasing *S. aureus* could be observed.

We could demonstrate that activation of FPRs play an important role during phagocytosis of *S. aureus*. Elucidation of the *in vivo* relevance could help to find novel therapeutic targets for the clearance of infections.

**Keywords:** *Staphylococcus aureus*, innate immunity, neutrophils, phagocytosis, bacterial infection, complement receptor, FCγ receptor, killing, FPR receptors, phenol soluble modulins

## Introduction

*Staphylococcus aureus* is a major human pathogen causing a variety of diseases such as local skin infections, sepsis, endocarditis, pneumonia, and toxic shock syndrome (Lowy, 1998). Neutrophils are the first line of defense against invading microorganisms as they represent about 50% to 70% of circulating human leukocytes. In response to chemotactic signals, they rapidly migrate from the blood stream into the tissues (Kolaczkowska and Kubes, 2013). Such chemotactic microbe associated molecular pattern (MAMP) molecules released by *S. aureus* include formylated peptides (Bufe et al., 2015) and phenol-soluble modulins (PSMs) (Kretschmer et al., 2010). They activate formyl-peptide receptor (FPR) 1 and 2, respectively (Kretschmer et al., 2015). Besides their ability to recruit human neutrophils, FPR ligands of *S. aureus* also induce the release of reactive oxygen species (ROS) (Liles et al., 2001), antimicrobial peptides (AMPs), and chemokines (Weiss et al., 2017), and, receptor-independently, the formation of NETs (Bjornsdottir et al., 2017) by neutrophils. If the activation of FPRs on the surface of human neutrophils also regulates phagocytosis of *S. aureus* by these professional phagocytes is still unknown.

It has already been shown that stimulation of human neutrophils with the FPR1 ligand fMLF enhances the phagocytosis of microspheres (Ogle et al., 1990) and *Aspergillus fumigatus* (Richardson and Patel, 1995). Ogle et al. suggested that a higher expression of complement receptor (CR) 1 by fMLF is required but not sufficient for the enhanced phagocytosis. Human neutrophils express on their surface two complement receptors – CR1 and CR3 – which recognize pathogens opsonized by complement factor C3b (van Kessel et al., 2014). Opsonization of pathogens with IgG antibodies is recognized by FCγ receptors on the surface of neutrophils. Normal peripheral blood neutrophils express FCγRII and FCγRIIIB and, during systemic infections and sepsis, also FCγRI (Hoffmann, 2009, Wang et al., 2015, van Kessel et al., 2014). Recognition of opsonized pathogens by the complement receptors and the FCγ receptors is mandatory for phagocytosis. *S. aureus* evolved many strategies to avoid phagocytosis, for example staphylococcal protein A (SpA), which binds the Fc part of IgG antibodies and thereby prevents the recognition of bacteria by FCγ receptors (Spaan et al., 2013).

Phagocytosis is not only an important mechanism initiating intracellular killing of pathogens but also triggering the release of chemokines by neutrophils (Arnold and Konig, 1998, Bazzoni et al., 1991) and monocytes (Friedland et al., 2001, Kang et al., 2011). Kang et al. showed that the amount of phagocytosed *S. aureus* by human monocytes correlates with the concentration of IL-8 released by these cells. IL-8 is a host-derived, intermediate chemokine, which recruits neutrophils from the blood stream into the tissues, whereas end-target chemoattractants like fMLF guide neutrophils within the tissues to the infection site (Heit et al., 2002). Thus, phagocytosis seem to be an important enhancer of the recruitment of neutrophils to the infection site.

We analyzed in this work the role of FPR1 and FPR2 activation during phagocytosis and killing of *S. aureus* by human neutrophils. We could show that stimulation of these two receptors significantly enhances phagocytosis. Our results demonstrate that FPR activation leads to higher expression of CR1 and CR3 as well as FCyRI. Interestingly, enhanced phagocytosis leads depending on the amount of bacteria to neutrophils and the duration of bacterial killing either to enhanced killing or survival of bacteria. Furthermore, we could show that enhanced phagocytosis induced by the FPR ligands leads to a synergistic release of IL-8 by human neutrophils followed by higher neutrophil recruitment compared to phagocytosis without FPR stimulation. This would lead to a ratio shift between neutrophils and bacteria at the infection site and thereby to higher killing of the pathogen.

## Materials and Methods

**Isolation of human neutrophils:** Human neutrophils were isolated from healthy blood donors by density gradient centrifugation as described recently (Kretschmer et al., 2010).

**Phagocytosis assay:** *S. aureus* USA300 (Diep and Otto, 2008), *S. epidermidis* 1457 (Mack et al., 1992), *S. lugdunensis* IVK28 (Zipperer et al., 2016) and a clinical isolate of *L. monocytogenes* (from the strain collection of the diagnostics unit of the Medical Microbiology and Hygiene department, University of Tübingen) were grown over night in tryptic soy broth (TSB) medium, *E. coli* BK2324 (Bloes et al., 2012) was grown in Lennox broth (LB) medium. Bacteria were labeled with CFSE (Sigma), heat-

inactivated for 20 min at 70°C and opsonized with 10 % human pooled serum (Hospital Tübingen) in RPMI for one hour at 37°C. Opsonized bacteria and human neutrophils were seeded in a 96-well-plate at a ratio of 5 bacteria per neutrophil (multiplicity of infection (MOI) 5). FPR-ligands (fMLF from Sigma; PSM peptides were kindly provided by Stefan Stevanovic, Immunology Department, University of Tübingen) were added at indicated concentrations. After one hour incubation at 37°C the neutrophils were fixed with 3.7 % formaldehyde. For the inhibition of the FPRs the neutrophils were incubated with 1.25 µg/ml CHIPS (kindly provided by Kok van Kessel, Bacterial Infections and Immunity Department, University of Utrecht) or 22.5 µM $WRW_4$ (synthesized by EMC microcollections) for 20 minutes at room temperature prior the phagocytosis. The fluorescence intensity of the neutrophils was determined with a BD FACSCalibur and the phagocytic index (= % CFSE positive neutrophils x CFSE fluorescent mean) was calculated.

**Expression of complement- and FCγ-receptors:** Neutrophils were seeded in a 96-well-plate and stimulated with fMLF or PSM peptides at the indicated concentrations for one hour. The supernatant was discarded and the neutrophils were incubated with PE-labeled antibodies against CD11b (BD Pharmingen), CD35 (Miltenyi Biotech), CD64 (Miltenyi Biotech) or an IgG isotyp control (Miltenyi Biotech) for 30 minutes on ice. The neutrophils were fixed with 3.7 % formaldehyde and the fluorescence intensity of the neutrophils was determined with a BD FACSCalibur. Mean fluorescence intensity (= % PE positive neutrophils x PE fluorescent mean) was calculated.

**Phagocytosis assay with blocking antibodies:** Neutrophils were seeded in a 96-well-plate and stimulated with 500 nM fMLF or PSMα3 for 30 minutes. LEAF™ purified anti-human antibodies from BioLegend (CD11b clone ICRF44 (10 µg/ml); CD35 clone E11 (20 µg/ml); CD64 clone 10.1 (2.5 µg/ml)) were used to functionally block the complement- and FCγ-receptors for 15 minutes. Afterwards CFSE-labeled bacteria were added similar to the phagocytosis assay. After one hour incubation at 37°C the neutrophils were fixed with 3.7 % formaldehyde, the fluorescence intensity of the neutrophils was determined with a BD FACSCalibur and the phagocytic index was calculated.

**IL-8 release:** Neutrophils were incubated with opsonized or non-opsonized heat-inactivated USA300 (MOI 5) and 50 nM fMLF or 1 µM PSMα3 for 5 hours at 37°C.

For the inhibition of the FPRs neutrophils were previously incubated with 1.25 µg/ml CHIPS or 22.5 µM $WRW_4$ for 20 minutes at room temperature. The supernatants were collected and released IL-8 was measured using an ELISA kit (R&D Systems) according to the manufacturer's instructions.

**Chemotaxis assay:** Neutrophils were labeled with BCECF, AM (Life Technologies) and seeded in 3 µm pore size ThinCerts (greiner bio-one). The compartments below the cell culture inserts contained supernatants of neutrophils stimulated for IL-8 release. After two hours the cell culture inserts were removed and the fluorescence intensity of the migrated neutrophils in the lower compartments was measured with a BMG Labtech CLARIOstar plate reader. The percentages of migrated neutrophils were calculated compared to a positive control (neutrophils were just seeded in the lower compartment).

**Bacterial killing assay:** *S. aureus* USA300 was grown over night in TSB medium. Bacteria were washed three times with PBS and opsonized with 10 % human pooled serum (Hospital Tübingen) in RPMI for one hour at 37°C.

For the bacterial killing assay with FPR-ligands neutrophils and bacteria were seeded in a 24-well-plate at MOI 0.1 or MOI 5 and incubated for 60 min together either with 500 nM fMLF or 1 µM PSMα3. For the bacterial killing assay with FPR-inhibitors neutrophils were incubated with 1.25 µg/ml CHIPS or 22.5 µM $WRW_4$ for 20 minutes at room temperature seeded with bacteria in a 24-well-plate at MOI 0.1 or MOI 5 and incubated for 60 min. For the kinetic neutrophils and bacteria were seeded in a 24-well-plate at the indicated MOIs.

On the hour 100 µl of each sample were collected and the neutrophils were lysed with $ddH_2O$ for 15 min at 4°C, 1000 rpm. Serial dilutions of the samples were plated on TSA plates with an EDDY Jet. On the next day CFUs were counted with Flash & Go.

**Mouse infection assay:** In the mouse peritonitis model $5x10^8$ CFUs of live *S. aureus* USA300 wild type or USA300 ΔαβδΔαβδ were injected into the peritoneum of 6-8 weeks-old female C57/BL6 wild type or mFpr2$^{-/-}$ mice. Six hours after infection the mice were euthanized with $CO_2$. Subsequently, peritoneal exudates were collected and leukocytes were stained and counted as described previously (Wang et al., 2007). APC-conjugated anti-mouse Ly6G antibody, PE-Vio770-conjugated anti-mouse F4/80 antibody, and the corresponding isotype controls were purchased from Miltenyi

Biotech. Samples were analyzed with a FACSFortessa flow cytometer. Furthermore, spleen, kidney, and liver of the animals were collected, homogenized, and plated on TSA plates for CFU determination. All mice were held under specific pathogen-free conditions, provided food and water *ad libitum*. Animal experiments were performed in strict accordance with the European Health Law of the Federation of Laboratory Animal Science Associations. The protocol was approved by the Regierungspräsidium Tübingen (IMIT 1/18).

**Statistics:** Statistical analysis were performed using Graph Pad Prism 6.01. Two-tailed t-test was used to compare two data groups. To compare multiple groups two-way ANOVA was used.

## Results

### FPR ligands enhance the phagocytosis of *S. aureus* by human neutrophils

It has already been shown that short formylated peptides and PSMs are ligands for FPR1 and the FPR2, respectively (Kretschmer et al., 2010, Boulay et al., 1990). Neutrophils respond to these ligands mainly by migration to the infection site. As neutrophils are professional phagocytes we analyzed if the activation of FPRs is also somehow involved in the uptake of *S. aureus* USA300. To study this we analyzed the phagocytosis of serum-opsonized USA300 by human neutrophils, which were simultaneously stimulated with different FPR ligands. We could show that fMLF, the most potent ligand of the human FPR1, induced a dose -dependent increase of phagocytosed USA300 (figure 1A). Also different α-type PSMs (PSMα2, PSMα3, δ-toxin) enhanced phagocytosis (figure 1 B-D). Inhibition of FPR1 with CHIPS or FPR2 with $WRW_4$ showed that the increased phagocytosis through fMLF and the α-type PSMs is FPR1- and FPR2-dependent, respectively (figure 1E, F).

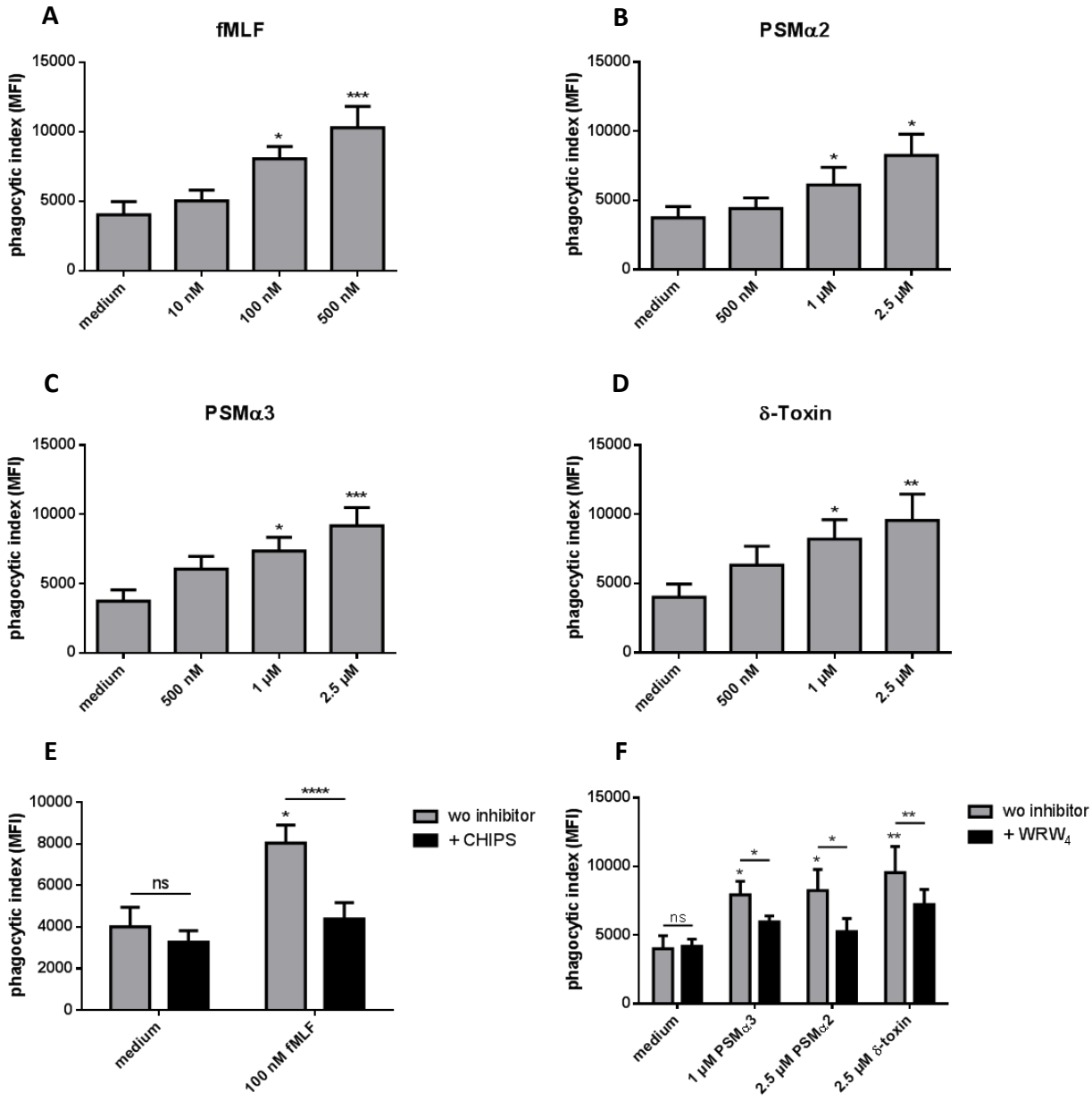
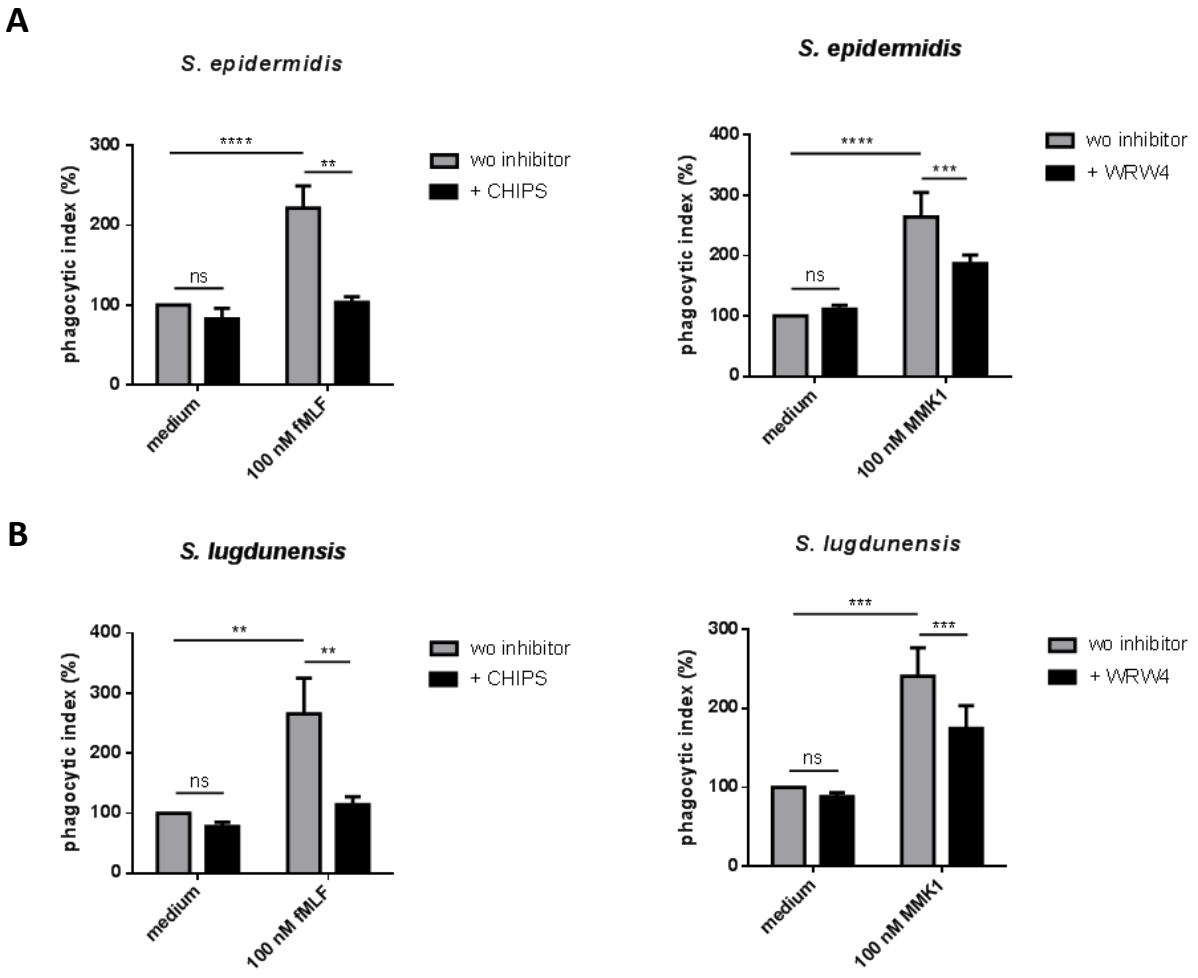



**Figure 1: FPR ligands enhance the phagocytosis of USA300 by human neutrophils receptor-dependent.** A) fMLF, B) PSMa2, C) PSMa3 and D) d-toxin induced concentration-dependently an increase of the phagocytosis of *S. aureus* USA300 by human neutrophils. The enhanced phagocytosis by E) fMLF could be blocked by the FPR1 inhibitor CHIPS and the higher phagocytosis by F) α-type PSMs could be blocked by the FPR2 inhibitor $WRW_4$. Data represent means ± SEM of at least three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns not significantly different vs. corresponding medium control calculated by Student´s t test (A-D) or vs. inhibitor treated cells calculated by two-way ANOVA (E, F).

## FPR activation enhances phagocytosis of Gram-positive and Gram-negative bacteria

To analyze if the upregulated phagocytosis of *S. aureus* by human neutrophils stimulated with FPR ligands is specific for *S. aureus* or a common mechanism for all bacteria, we repeated the phagocytosis assay with the following Gram-positive and Gram-negative bacteria. We used the Gram-positive *Staphylococcus epidermidis*, *Staphylococcus lugdunensis*, *Listeria monocytogenes*, and the Gram-negative *Escherichia coli*. For all tested bacteria we observed higher phagocytosis when neutrophils were costimulated with FPR-ligands compared to non-stimulated neutrophils (figure 2). Thus, the augmented phagocytosis induced by FPR-ligands seems to be a common mechanism for different kinds of bacteria.



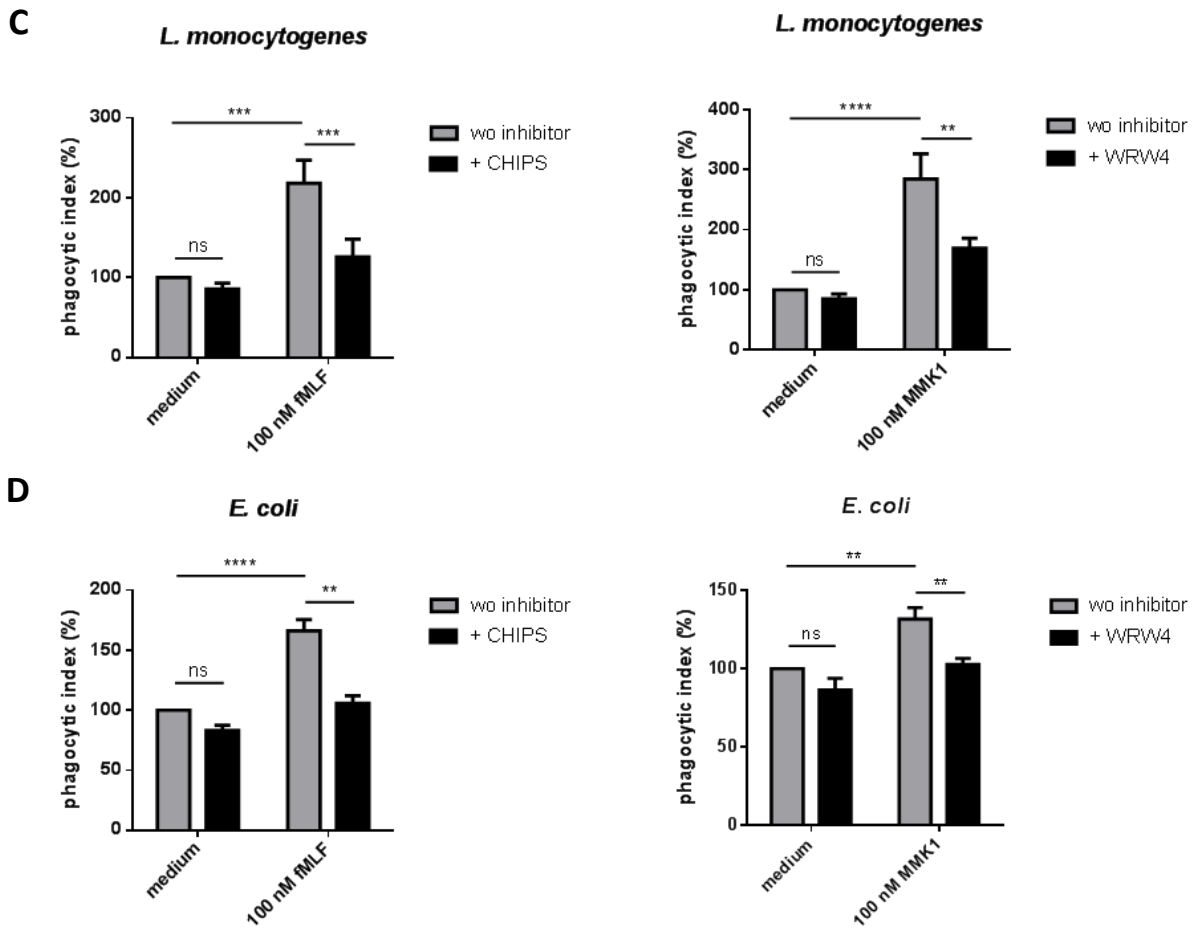



**Figure 2: The phagocytosis of gram-positive and gram-negative bacteria by human neutrophils is enhanced by FPR-ligands.** Phagocytosis of A*) S. epidermidis*, B) *S. lugdunensis*, C) *L. monocytogenes* and D) *E. coli* was enhanced by fMLF or MMK1 and could be inhibited through CHIPS or $WRW_4$, respectively. Data represent means ± SEM of at least three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns not significantly different vs. corresponding medium control or inhibitor-treated cells calculated by two-way ANOVA.

## Complement receptors and FCγRI are responsible for the increased phagocytosis

It has been shown that stimulation of the FPRs with fMLF or α-type PSMs leads to a higher expression of CD11b on the surface of human neutrophils (Durr et al., 2006, Kretschmer et al., 2012). CD11b is part of the complement receptor 3 (CR3), which recognizes C3b-opsonized pathogens. Besides CR3 neutrophils express a second complement receptor, CR1 (CD35), which also recognizes C3b. Pathogens opsonized with IgG antibodies are recognized by FCγ receptors on the surface of neutrophils. Normal peripheral blood neutrophils express FCγRII (CD32) and FCγRIIIB (CD16) and during systemic infections and sepsis, also FCγRI (CD64) (Hoffmann, 2009, Wang et al., 2015).

Our data show that stimulation of neutrophils with the FPR1 ligand fMLF or the FPR2 ligand PSMα3 led to a significantly higher expression of CD11b, CD35, and CD64 (figure 3) but not of the FCγ receptors CD16 or CD32 (supplemental figure 1). This upregulation could completely be inhibited through preincubation with the FPR1 inhibitor CHIPS or the FPR2 inhibitor $WRW_4$, which shows that this process is FPR-dependent (figure 3). To correlate the higher expression of the complement receptors and the FCγRI by the FPR ligands with the enhanced phagocytosis of USA300 under these conditions we repeated the phagocytosis assay with functional blocking antibodies against the involved complement receptors and the FCγRI. These data show that blocking of CD11b together with either CD35 or CD64 reversed almost completely the enhanced phagocytosis by the FPR-ligands (figure 4).

Thus, we could show that FPR ligands of *S. aureus* induce enhanced expression of complement receptors and the FCγRI on the surface of neutrophils, which leads to higher uptake of bacteria by these professional phagocytes.

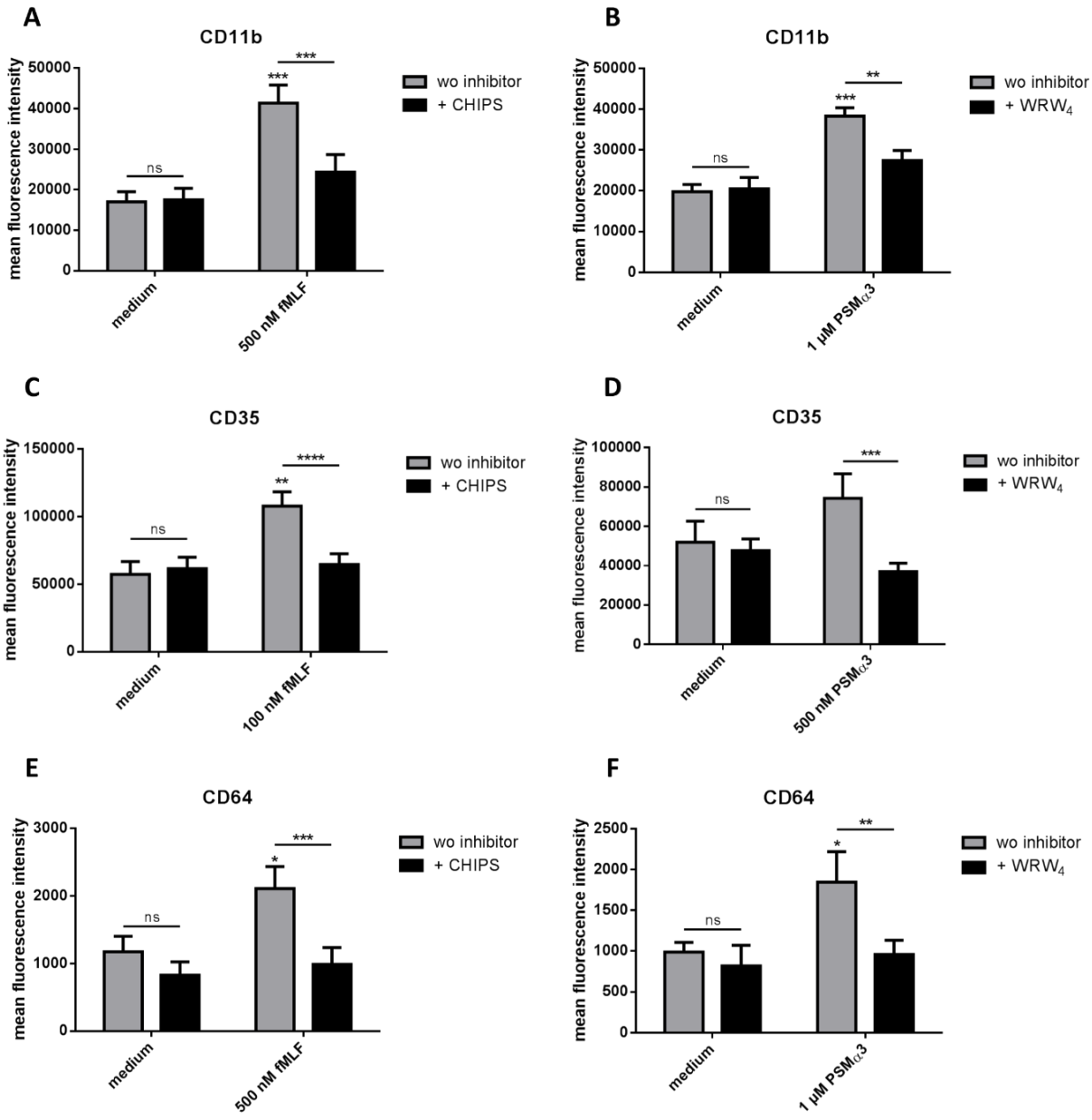



**Figure 3: FPR ligands induce receptor-dependently the upregulation of complement receptors and the FCγRI (CD64).** Enhanced expression of A, B) CD11b, C, D) CD35 and E, F) CD64 by A, C, E) fMLF or B, D, F) PSMα3 were FPR1- or FPR2-dependent, respectively. Data represent means ± SEM of at least three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns not significantly different vs. corresponding medium control or inhibitor-treated cells calculated by two-way ANOVA.

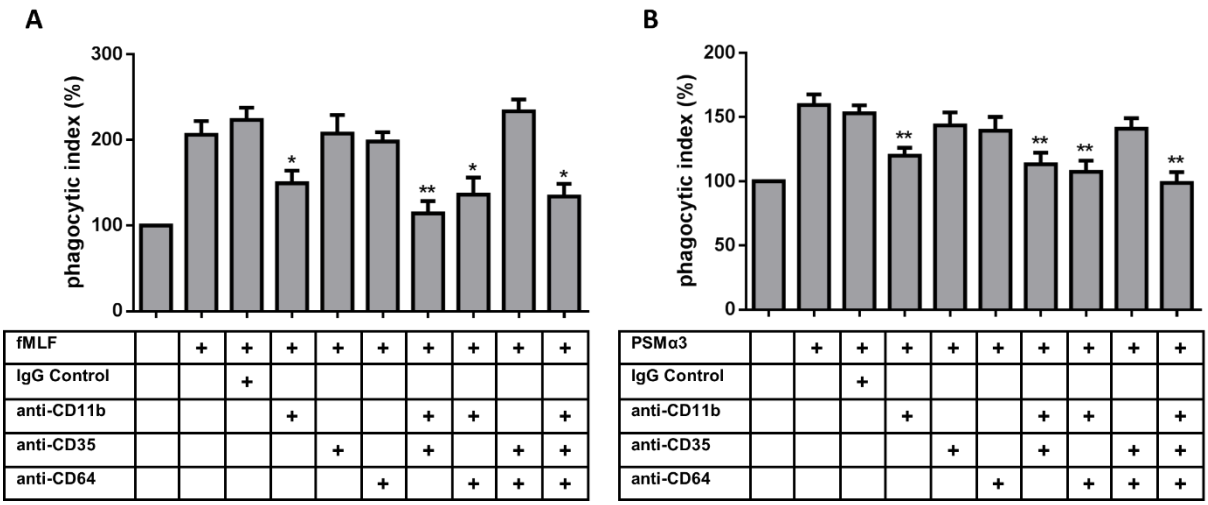



**Figure 4: The enhanced phagocytosis by FPR ligands is complement receptor and FCγRI-dependent.** Incubation of human neutrophils with A) fMLF or B) PSMα3 led to enhanced phagocytosis of USA300, which can be blocked by antibodies against CD11b, CD35, and/or CD64. Data represent means ± SEM of at least three independent experiments. *P < 0.05, **P < 0.01 vs. corresponding medium control calculated by Student´s t test.

## FPR activation and phagocytosis synergistically amplify neutrophil recruitment

We analyzed the biological consequence of the enhanced phagocytosis by FPR ligands. We could show that phagocytosis of opsonized *S. aureus* led to a significant higher amount of IL-8 released by neutrophils compared to non-opsonized bacteria (figure 5 A-D). This supports previously publications showing a correlation between phagocytosis and the amount of released IL-8 by neutrophils and monocytes (Bazzoni et al., 1991, Kang et al., 2011). Moreover, we could show that stimulation of neutrophils with FPR ligands during phagocytosis of *S. aureus* led to a synergistic IL-8 release compared to neutrophils, which were incubated with opsonized bacteria or with FPR ligands alone (figure 5 A, B). This effect is phagocytosis-dependent, as it was not observed with non-opsonized bacteria (figure 5 C, D). Inhibition of FPR1 also abrogate this effect, whereas inhibition of FPR2 has no influence on IL8 release (figure 5 E, F).

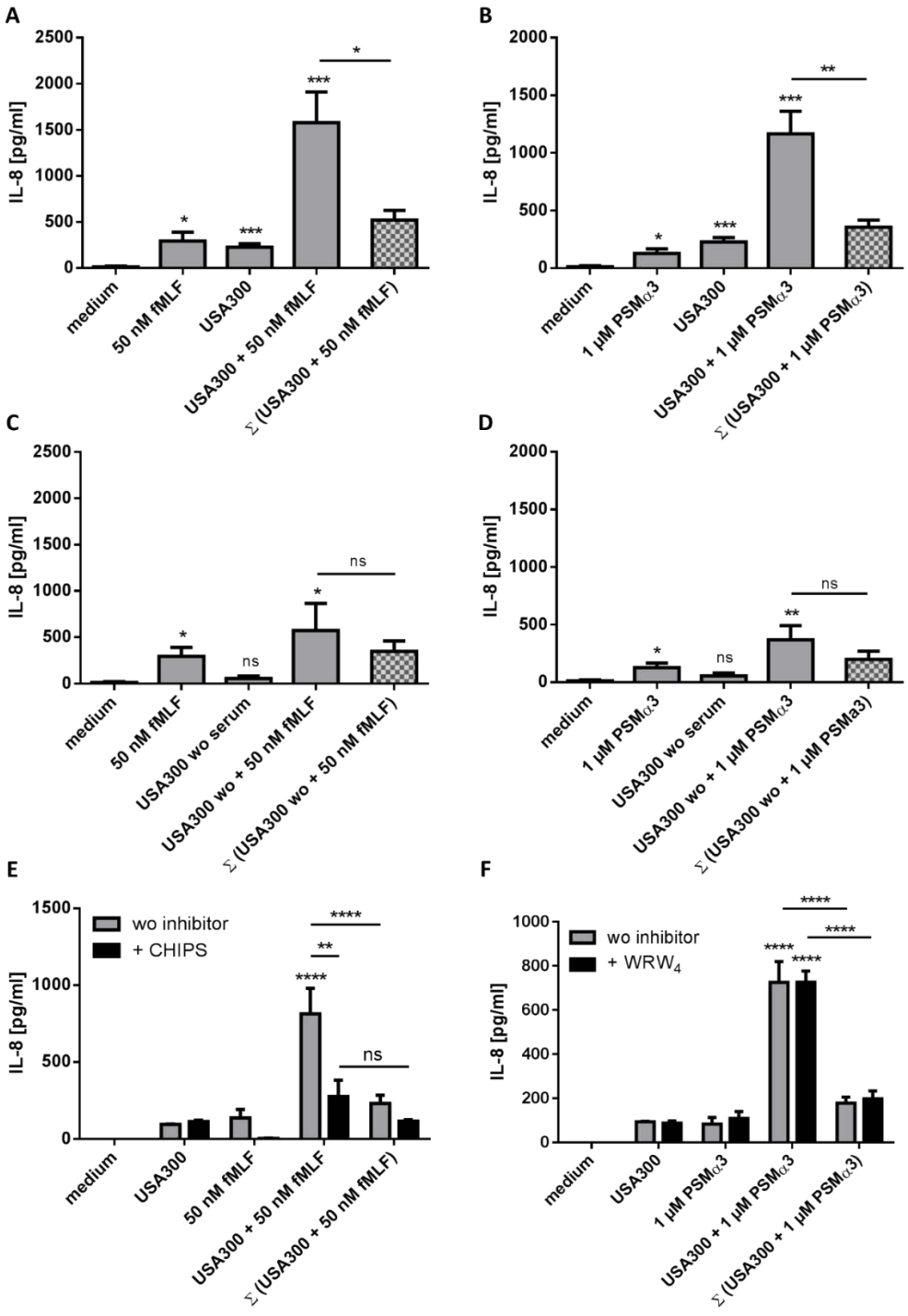



**Figure 5: The enhanced phagocytosis by FPR-ligands leads to synergistic IL-8 release.** Stimulation of human neutrophils with A) fMLF or B) PSMα3 during phagocytosis of USA300 resulted in a synergistic release of IL-8. C, D) The synergistic IL-8 release was abrogated using non-opsonized bacteria. E) Inhibition of the FPR1 by CHIPS blocked the synergistic IL-8 release by fMLF. F) Inhibition of the FPR2 by WRW4 had no effect on the PSMα3-enhanced IL-8 release. Data represent means ± SEM of at least three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns not significantly different vs. corresponding medium control or as indicated calculated by two-way ANOVA.

To check if the synergistically released IL-8 leads to higher recruitment of neutrophils, we used supernatants of neutrophils, which were costimulated with FPR ligands during phagocytosis for a chemotaxis assay. We could show that supernatants of FPR-stimulated and phagocytosing neutrophils induced significantly higher migration of neutrophils compared to supernatants collected from neutrophils, which were stimulated only with FPR ligands or phagocytosed bacteria (figure 6 A, B). The supernatants of neutrophils, which were incubated with non-opsonized bacteria induced almost no migration of neutrophils (figure 6 A, B).

Thus, we could demonstrate that FPR ligands induce a synergistic release of IL-8 during phagocytosis of bacteria and thereby significantly more migration of neutrophils to the infection site.

**Enhanced phagocytosis of *S. aureus* leads indirectly to higher bacterial killing**

Then we analyzed, if the significant higher migration of neutrophils after stimulating phagocytosis of *S. aureus* by different FPR ligands has an effect on bacterial killing by human neutrophils. We challenged USA300 and human neutrophils at different multiplicities of infection (MOIs) for about four hours and determined bacterial and neutrophil survival at different time points. Our data show, that during the first two hours around 50% of bacteria were killed by human neutrophils independently of the used MOIs (figure 6C). However, we observed a clear difference in neutrophil survival. If bacteria outnumbered neutrophils, a rapid destruction of neutrophils occurred (supplemental figure 2). In contrast, when neutrophils outnumbered bacteria neutrophils survived (supplemental figure 2). Destruction of neutrophils led to outgrow of bacteria and increased survival of USA300 after four hours (figure 6C, D). Under the conditions where the neutrophils were still intact after two hours bacteria were almost completely killed after four hours (figure 6C, D). Our data show that the amount of neutrophils to bacteria is highly important for the killing of *S. aureus* and that a fast recruitment of neutrophils to the infection site could avoid progression of infection.

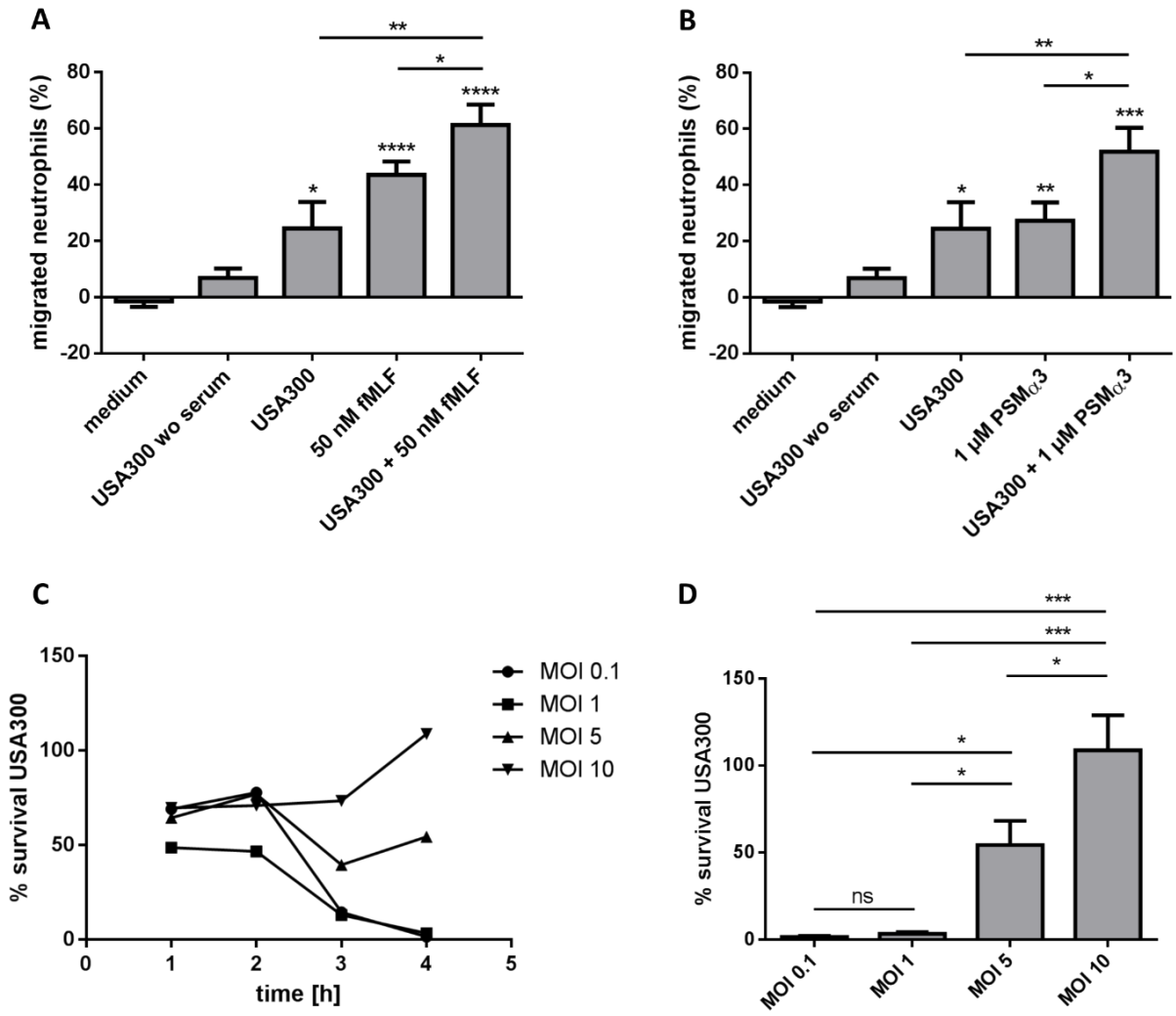
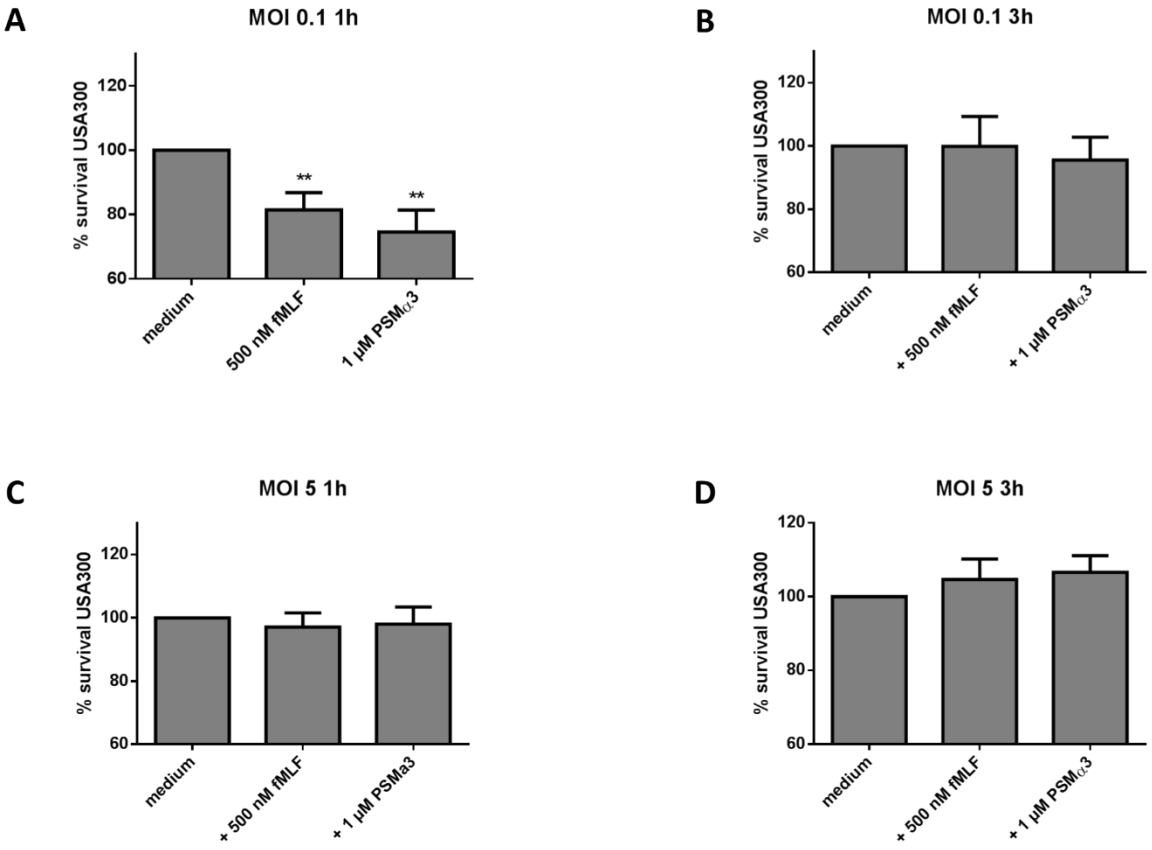



**Figure 6: The phagocytosis-induced synergistic IL-8 release leads to significant higher migration of human neutrophils followed by better killing of USA300.** The synergistic release of IL-8 by A) fMLF or B) PSMα3 resulted in significant higher migration of neutrophils. C) Killing of USA300 over time depended on the ratio between neutrophils and bacteria. D) After four hours all bacteria were killed, if neutrophils outnumbered bacteria. At higher MOIs bacteria survived neutrophil killing. Data represent means ± SEM of at least three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns not significantly different vs. corresponding medium control or as indicated calculated by two-way ANOVA. MOI multiplicity of infection.

**The enhanced phagocytosis could be an advantage or disadvantage for *S. aureus* killing**

Our data indicate that at low MOIs and after short time periods the enhanced phagocytosis induced by FPR ligands led to higher killing of *S. aureus* by human neutrophils (figure 7A). Inhibition of FPRs by CHIPS for FPR1 or WRW4 for FPR2 resulted in higher survival of USA300 (figure 7E). In contrast, at higher MOIs when bacteria outnumbered neutrophils, and at later time points the beneficial effect of the enhanced phagocytosis was reverted. Stimulation of neutrophils during bacterial killing with FPR ligands resulted in slightly better survival of *S. aureus* (figure 7D), whereas inhibition of the FPRs led to increased bacterial killing (figure 7H). These data show that *S. aureus* promotes phagocytosis to survive within neutrophils. Since it has been shown that *S. aureus* is able to escape from the phagosome and consequently from phagocytes by PSM-secretion (Munzenmayer et al., 2016) this novel mechanism could explain, how *S. aureus* uses extracellular PSM secretion and FPR activation for its own purposes.



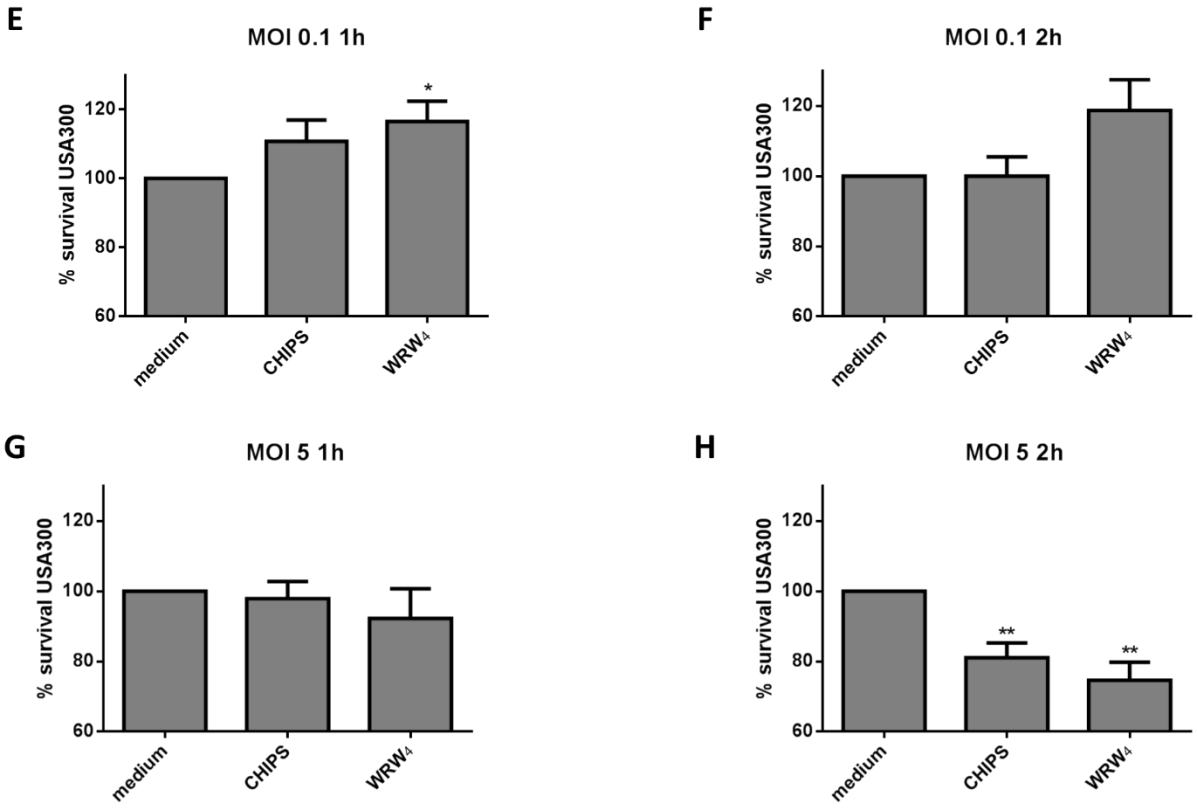



**Figure 7: The enhanced phagocytosis could be beneficial for *S. aureus* killing or survival.** Human neutrophils were incubated with A-D) FPR ligands or E-H) FPR inhibitors during killing of USA300. A) FPR ligands were beneficial for the immune system at low MOIs and early time points, whereas H) FPR inhibitors led to higher bacterial killing at high MOI and later time points. E) FPR inhibitors were beneficial for *S. aureus* at low MOI and early time points, whereas D) FPR ligands led to less bacterial killing at high MOI and late time points. Data represent means ± SEM of at least three independent experiments. *P < 0.05, **P < 0.01 vs. corresponding medium control calculated by Student´s t test.

## mFpr2 is involved in phagocytosis of *S. aureus in vivo*

Before we analyzed the role of mFprs *in vivo* during phagocytosis we analyzed, if complement and FCγ receptors are also upregulated in mouse neutrophils after stimulation with FPR ligands. For human neutrophils we showed increased expression of CD11b, CD35, and CD64 upon stimulation with FPR1 or FPR2 ligands (figure 3). Similar results were obtained with mouse neutrophils. Stimulation of mFpr1 with fMIFL or mFpr2 with PSMα3 resulted in upregulation of CD11b (figure 8A), CD21/35 (figure 8B), CD64 (figure 8C), and for mouse neutrophils also of CD16/32 (figure 8D). Accordingly, mouse neutrophils without mFpr2 did not respond by receptor upregulation anymore after PSMα3 stimulation (figure 8).

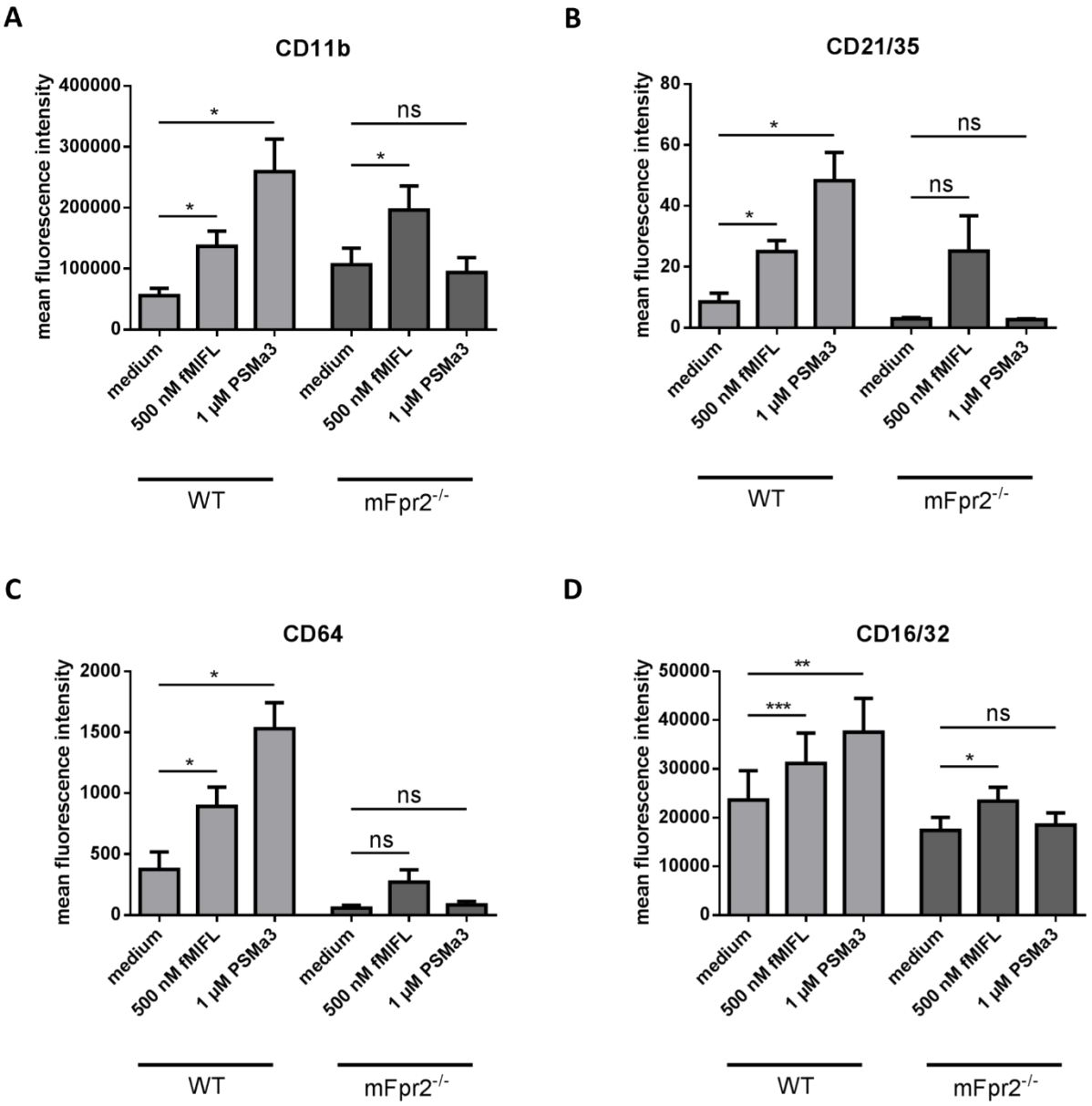



**Figure 8: FPR ligands induce receptor dependent higher expression of complement receptors and FCγ receptors in mouse neutrophils.** Hoxb8 WT mouse neutrophils were incubated with fMIFL or PSMα3, which led to higher expression of A) CD11b, B) CD21/CD35, C) CD64, and D) CD16/32. Hoxb8 mFpr2$^{-/-}$ neutrophils showed similar results stimulated with fMIFL, but did not respond to PSMα3. Data represent means ± SEM of at least three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ns not significantly different vs. corresponding medium control calculated by Student´s t test.

Then we analyzed, which consequences FPR activation may have for phagocytosis during peritoneal infection. Therefore, wild type (WT) and mFpr2$^{-/-}$ mice were infected with USA300 WT or an isogenic PSM-mutant (Δαβδ). Neutrophils were isolated from peritoneum and analyzed for internalized *S. aureus*. Significantly more neutrophils of

WT animals phagocytosed bacteria when mice were infected with USA300 WT compared to neutrophils of animals without mFpr2$^{-/-}$ or when WT mice were infected with an isogenic PSM mutant (figure 9A). The enhanced phagocytosis in WT animals infected with USA300 WT resulted in higher CFUs in the liver compared to animals without mFpr2 or mice infected with USA300 Δαβδ (figure 9B). These findings support the *in vitro* data and show that *S. aureus* uses neutrophils *in vivo* to protect its own killing and for dissemination.

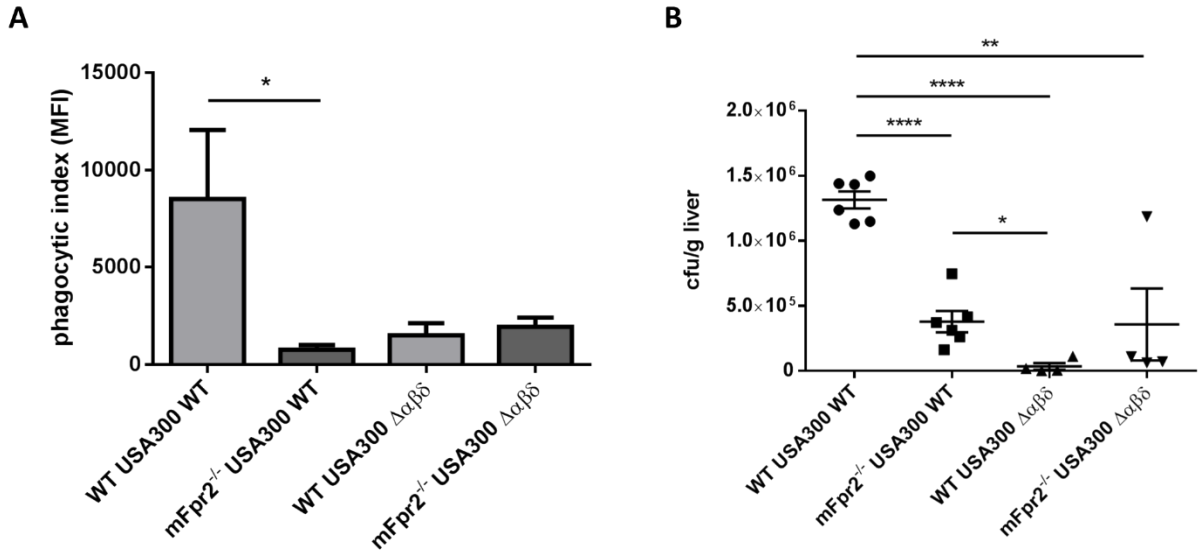



**Figure 9: *In vivo* phagocytosis and bacterial killing is mFpr2 and PSM-dependent.** WT and mFpr2 $^{-/-}$ mice were infected i.p. with USA300 WT or USA300 Δαβδ. A) Neutrophils of WT mice infected with USA300 WT phagocytosed significant more bacteria than neutrophils of mFpr2$^{-/-}$ mice infected with USA300 WT or mice infected with USA300 Δαβδ. B) Higher CFUs were detected in the liver of WT mice infected with USA300 WT compared to mFpr2$^{-/-}$ mice infected with USA300 WT or mice infected with USA300 Δαβδ. Data represent mean values of six mice ± SEM. *P < 0.05, **P < 0.01, ****P < 0.0001 as indicated calculated by Student´s t test.

## Discussion

Neutrophils represent the first line of defense during infections and they are professional phagocytes of the innate immune system. Here we could show that FPR ligands play an important role for the phagocytosis of Gram-positive and Gram-negative bacteria by human neutrophils. Stimulation of FPRs resulted in higher expression of complement and FCγ receptors on the surface of neutrophils resulting in higher phagocytosis of bacteria. Already in the early 90´s it has been shown that stimulation of human neutrophils with fMLF enhances CR1 expression and

phagocytosis. The authors speculated that the higher expression of CR1 is required but not sufficient for the enhanced phagocytosis (Ogle et al., 1990). Here we could demonstrate that not just a higher expression of CR1, but also of CR3 and FCγRI are the reasons for the enhanced phagocytosis, as blocking of these receptors abrogated this effect.

Phagocytosis itself results in the release of the chemokine IL-8 by neutrophils and monocytes (Arnold and Konig, 1998, Bazzoni et al., 1991, Kang et al., 2011). Here we showed that activation of the FPRs during phagocytosis results in a synergistic release of IL-8, which results in significantly stronger recruitment of neutrophils. Phagocytosis and FPR activation both induce activation of the transcription factor NFκB resulting in expression of IL-8 (McDonald and Cassatella, 1997, Chen et al., 2009). It was shown that activation of different receptors, which both activate NFκB could result in synergistic activation of this transcription factor. Phosphorylation of different serine residues of p65 is involved in this process (Chen et al., 2009). Further research will elucidate the mechanism behind the synergistic IL-8 release by FPR activation and phagocytosis and if a similar mechanism is involved.

The enhanced phagocytosis of bacteria upon activation of FPRs might result in higher bacterial killing within neutrophils. Our study shows, that this process is much more sophisticated. We showed *in vitro* that the ratio of bacteria to neutrophils and the duration of bacterial killing have a high impact on the outcome of bacterial elimination. Additionally, our *in vivo* data demonstrate that higher phagocytosis results in a higher amount of bacteria in the liver. Thus, our data indicate that *S. aureus* may use neutrophils as Trojan horse. This bacterium has evolved many strategies to resist AMPs and ROS within the phagosome (Spaan et al., 2013) and to lyse neutrophils from within (DuMont et al., 2013, Munzenmayer et al., 2016, Surewaard et al., 2013). Staphylococcal PSMs play a crucial role for the destruction of the phagosome, whereas the bicomponent pore-forming toxin LukAB destroys the cytoplasmic membrane of host cells (Munzenmayer et al., 2016). Furthermore, phagocytosis of *S. aureus* also results in upregulation of the “don´t eat me signal” CD47 on the surface of neutrophils preventing the uptake of bacteria-containing neutrophils by macrophages (Greenlee-Wacker et al., 2014). The hypothesis that *S. aureus* uses neutrophils to reach distant organs and to induce systemic infections (Gresham et al., 2000, Lehar et al., 2015, Thwaites and Gant, 2011) is further supported by some studies, which show that neutropenic cancer patients, which have

decreased numbers of neutrophils, are rarely affected by *S. aureus* bacteremia compared to Gram-negative bacteremia (Velasco et al., 2006, Venditti et al., 2003). In contrast, neutropenic cancer patients are more often affected by polymicrobial infections (Velasco et al., 2006). Additionally, non-neutropenic cancer patients affected by *S. aureus* bacteremia developed more frequent severe sepsis or septic shock, they developed metastatic infections, and overall mortality was significantly higher compared to neutropenic cancer patients (Venditti et al., 2003).

In conclusion, this study demonstrates that FPRs play an important role for the phagocytosis of bacteria. Depending on the amount of bacteria to neutrophils and the duration of bacterial killing the enhanced phagocytosis induced by FPR ligands has different outcomes. During local infections with low numbers of bacteria, activation of FPRs might promote bacterial killing, whereas during systemic infections or abscesses, with a high bacterial amount, FPR activation might be harmful. It could be speculated that the development of systemic infections depends on FPRs, as inhibition or absence of these receptors during *in vitro* or *in vivo* bacterial killing with a high bacterial amount led to less survival of *S. aureus*. Thus, FPRs could be a central therapeutic target for prevention of sepsis development. Resolution of local infections with low numbers of *S. aureus* could be therapeutically supported by administration of FPR ligands, whereas resolution of systemic infections could be supported by FPR inhibitors. Further research should focus on the role of FPRs during local and systemic infections and their development.

## References

ARNOLD, R. & KONIG, W. 1998. Interleukin-8 release from human neutrophils after phagocytosis of Listeria monocytogenes and Yersinia enterocolitica. *J Med Microbiol,* 47**,** 55-62.

BAZZONI, F., CASSATELLA, M. A., ROSSI, F., CESKA, M., DEWALD, B. & BAGGIOLINI, M. 1991. Phagocytosing neutrophils produce and release high amounts of the neutrophil-activating peptide 1/interleukin 8. *J Exp Med,* 173**,** 771-4.

BJORNSDOTTIR, H., DAHLSTRAND RUDIN, A., KLOSE, F. P., ELMWALL, J., WELIN, A., STYLIANOU, M., CHRISTENSON, K., URBAN, C. F., FORSMAN, H., DAHLGREN, C., KARLSSON, A. & BYLUND, J. 2017. Phenol-Soluble Modulin alpha Peptide Toxins from Aggressive Staphylococcus aureus Induce Rapid Formation of Neutrophil Extracellular Traps through a Reactive Oxygen Species-Independent Pathway. *Front Immunol,* 8**,** 257.

BLOES, D. A., OTTO, M., PESCHEL, A. & KRETSCHMER, D. 2012. Enterococcus faecium stimulates human neutrophils via the formyl-peptide receptor 2. *PLoS One,* 7**,** e39910.

BOULAY, F., TARDIF, M., BROUCHON, L. & VIGNAIS, P. 1990. The human N-formylpeptide receptor. Characterization of two cDNA isolates and evidence for a new subfamily of G-protein-coupled receptors. *Biochemistry,* 29**,** 11123-33.

BUFE, B., SCHUMANN, T., KAPPL, R., BOGESKI, I., KUMMEROW, C., PODGORSKA, M., SMOLA, S., HOTH, M. & ZUFALL, F. 2015. Recognition of bacterial signal peptides by mammalian formyl peptide receptors: a new mechanism for sensing pathogens. *J Biol Chem,* 290**,** 7369-87.

CHEN, L. Y., PAN, W. W., CHEN, M., LI, J. D., LIU, W., CHEN, G., HUANG, S., PAPADIMOS, T. J. & PAN, Z. K. 2009. Synergistic induction of inflammation by bacterial products lipopolysaccharide and fMLP: an important microbial pathogenic mechanism. *J Immunol,* 182**,** 2518-24.

DIEP, B. A. & OTTO, M. 2008. The role of virulence determinants in community-associated MRSA pathogenesis. *Trends Microbiol,* 16**,** 361-9.

DUMONT, A. L., YOONG, P., SUREWAARD, B. G., BENSON, M. A., NIJLAND, R., VAN STRIJP, J. A. & TORRES, V. J. 2013. Staphylococcus aureus elaborates leukocidin AB to mediate escape from within human neutrophils. *Infect Immun,* 81**,** 1830-41.

DURR, M. C., KRISTIAN, S. A., OTTO, M., MATTEOLI, G., MARGOLIS, P. S., TRIAS, J., VAN KESSEL, K. P., VAN STRIJP, J. A., BOHN, E., LANDMANN, R. & PESCHEL, A. 2006. Neutrophil chemotaxis by pathogen-associated molecular patterns--formylated peptides are crucial but not the sole neutrophil attractants produced by Staphylococcus aureus. *Cell Microbiol,* 8**,** 207-17.

FRIEDLAND, J. S., CONSTANTIN, D., SHAW, T. C. & STYLIANOU, E. 2001. Regulation of interleukin-8 gene expression after phagocytosis of zymosan by human monocytic cells. *J Leukoc Biol,* 70**,** 447-54.

GREENLEE-WACKER, M. C., RIGBY, K. M., KOBAYASHI, S. D., PORTER, A. R., DELEO, F. R. & NAUSEEF, W. M. 2014. Phagocytosis of Staphylococcus aureus by human neutrophils prevents macrophage efferocytosis and induces programmed necrosis. *J Immunol,* 192**,** 4709-17.

GRESHAM, H. D., LOWRANCE, J. H., CAVER, T. E., WILSON, B. S., CHEUNG, A. L. & LINDBERG, F. P. 2000. Survival of Staphylococcus aureus inside neutrophils contributes to infection. *J Immunol,* 164**,** 3713-22.

HEIT, B., TAVENER, S., RAHARJO, E. & KUBES, P. 2002. An intracellular signaling hierarchy determines direction of migration in opposing chemotactic gradients. *J Cell Biol,* 159**,** 91-102.

HOFFMANN, J. J. 2009. Neutrophil CD64: a diagnostic marker for infection and sepsis. *Clin Chem Lab Med,* 47**,** 903-16.

KANG, H. J., HA, J. M., KIM, H. S., LEE, H., KUROKAWA, K. & LEE, B. L. 2011. The role of phagocytosis in IL-8 production by human monocytes in response to lipoproteins on Staphylococcus aureus. *Biochem Biophys Res Commun,* 406**,** 449-53.

KOLACZKOWSKA, E. & KUBES, P. 2013. Neutrophil recruitment and function in health and inflammation. *Nat Rev Immunol,* 13**,** 159-75.

KRETSCHMER, D., GLESKE, A. K., RAUTENBERG, M., WANG, R., KOBERLE, M., BOHN, E., SCHONEBERG, T., RABIET, M. J., BOULAY, F., KLEBANOFF, S. J., VAN KESSEL, K. A., VAN STRIJP, J. A., OTTO, M. & PESCHEL, A. 2010. Human formyl peptide receptor 2 senses highly pathogenic Staphylococcus aureus. *Cell Host Microbe,* 7**,** 463-73.

KRETSCHMER, D., NIKOLA, N., DURR, M., OTTO, M. & PESCHEL, A. 2012. The virulence regulator Agr controls the staphylococcal capacity to activate human neutrophils via the formyl peptide receptor 2. *J Innate Immun,* 4**,** 201-12.

KRETSCHMER, D., RAUTENBERG, M., LINKE, D. & PESCHEL, A. 2015. Peptide length and folding state govern the capacity of staphylococcal beta-type phenol-soluble modulins to activate human formyl-peptide receptors 1 or 2. *J Leukoc Biol,* 97**,** 689-97.

LEHAR, S. M., PILLOW, T., XU, M., STABEN, L., KAJIHARA, K. K., VANDLEN, R., DEPALATIS, L., RAAB, H., HAZENBOS, W. L., MORISAKI, J. H., KIM, J., PARK, S., DARWISH, M., LEE, B. C., HERNANDEZ, H., LOYET, K. M., LUPARDUS, P., FONG, R., YAN, D., CHALOUNI, C., LUIS, E., KHALFIN, Y., PLISE, E., CHEONG, J., LYSSIKATOS, J. P., STRANDH, M., KOEFOED, K., ANDERSEN, P. S., FLYGARE, J. A., WAH TAN, M., BROWN, E. J. & MARIATHASAN, S. 2015. Novel antibody-antibiotic conjugate eliminates intracellular S. aureus. *Nature,* 527**,** 323-8.

LILES, W. C., THOMSEN, A. R., O'MAHONY, D. S. & KLEBANOFF, S. J. 2001. Stimulation of human neutrophils and monocytes by staphylococcal phenol-soluble modulin. *J Leukoc Biol,* 70**,** 96-102.

LOWY, F. D. 1998. Staphylococcus aureus infections. *N Engl J Med,* 339**,** 520-32.

MACK, D., SIEMSSEN, N. & LAUFS, R. 1992. Parallel induction by glucose of adherence and a polysaccharide antigen specific for plastic-adherent Staphylococcus epidermidis: evidence for functional relation to intercellular adhesion. *Infect Immun,* 60**,** 2048-57.

MCDONALD, P. P. & CASSATELLA, M. A. 1997. Activation of transcription factor NF-kappa B by phagocytic stimuli in human neutrophils. *FEBS Lett,* 412**,** 583-6.

MUNZENMAYER, L., GEIGER, T., DAIBER, E., SCHULTE, B., AUTENRIETH, S. E., FRAUNHOLZ, M. & WOLZ, C. 2016. Influence of Sae-regulated and Agr-regulated factors on the escape of Staphylococcus aureus from human macrophages. *Cell Microbiol,* 18**,** 1172-83.

OGLE, J. D., NOEL, J. G., SRAMKOSKI, R. M., OGLE, C. K. & ALEXANDER, J. W. 1990. Effects of chemotactic peptide f-Met-Leu-Phe (FMLP) on C3b receptor (CR1) expression and phagocytosis of microspheres by human neutrophils. *Inflammation,* 14**,** 337-53.

RICHARDSON, M. D. & PATEL, M. 1995. Stimulation of neutrophil phagocytosis of Aspergillus fumigatus conidia by interleukin-8 and N-formylmethionyl-leucylphenylalanine. *J Med Vet Mycol,* 33**,** 99-104.

SPAAN, A. N., SUREWAARD, B. G., NIJLAND, R. & VAN STRIJP, J. A. 2013. Neutrophils versus Staphylococcus aureus: a biological tug of war. *Annu Rev Microbiol,* 67**,** 629-50.

SUREWAARD, B. G., DE HAAS, C. J., VERVOORT, F., RIGBY, K. M., DELEO, F. R., OTTO, M., VAN STRIJP, J. A. & NIJLAND, R. 2013. Staphylococcal alpha-phenol soluble modulins contribute to neutrophil lysis after phagocytosis. *Cell Microbiol,* 15**,** 1427-37.

THWAITES, G. E. & GANT, V. 2011. Are bloodstream leukocytes Trojan Horses for the metastasis of Staphylococcus aureus? *Nat Rev Microbiol,* 9**,** 215-22.

VAN KESSEL, K. P., BESTEBROER, J. & VAN STRIJP, J. A. 2014. Neutrophil-Mediated Phagocytosis of Staphylococcus aureus. *Front Immunol,* 5**,** 467.

VELASCO, E., BYINGTON, R., MARTINS, C. A., SCHIRMER, M., DIAS, L. M. & GONCALVES, V. M. 2006. Comparative study of clinical characteristics of neutropenic and non-neutropenic adult cancer patients with bloodstream infections. *Eur J Clin Microbiol Infect Dis,* 25**,** 1-7.

VENDITTI, M., FALCONE, M., MICOZZI, A., CARFAGNA, P., TAGLIETTI, F., SERRA, P. F. & MARTINO, P. 2003. Staphylococcus aureus bacteremia in patients with hematologic malignancies: a retrospective case-control study. *Haematologica,* 88**,** 923-30.

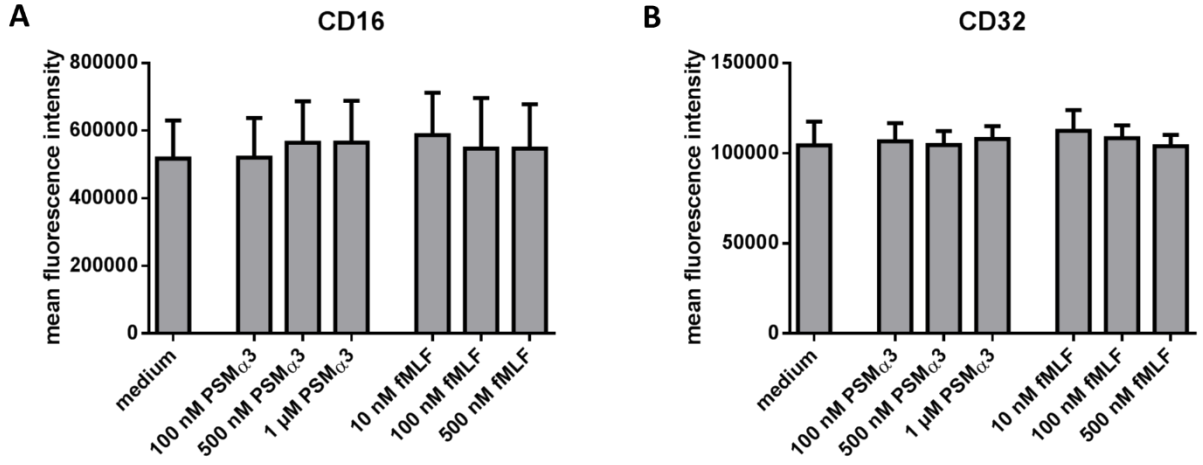
WANG, R., BRAUGHTON, K. R., KRETSCHMER, D., BACH, T. H., QUECK, S. Y., LI, M., KENNEDY, A. D., DORWARD, D. W., KLEBANOFF, S. J., PESCHEL, A., DELEO, F. R. & OTTO, M. 2007. Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. *Nat Med,* 13**,** 1510-4.

WANG, X., LI, Z. Y., ZENG, L., ZHANG, A. Q., PAN, W., GU, W. & JIANG, J. X. 2015. Neutrophil CD64 expression as a diagnostic marker for sepsis in adult patients: a meta-analysis. *Crit Care,* 19**,** 245.

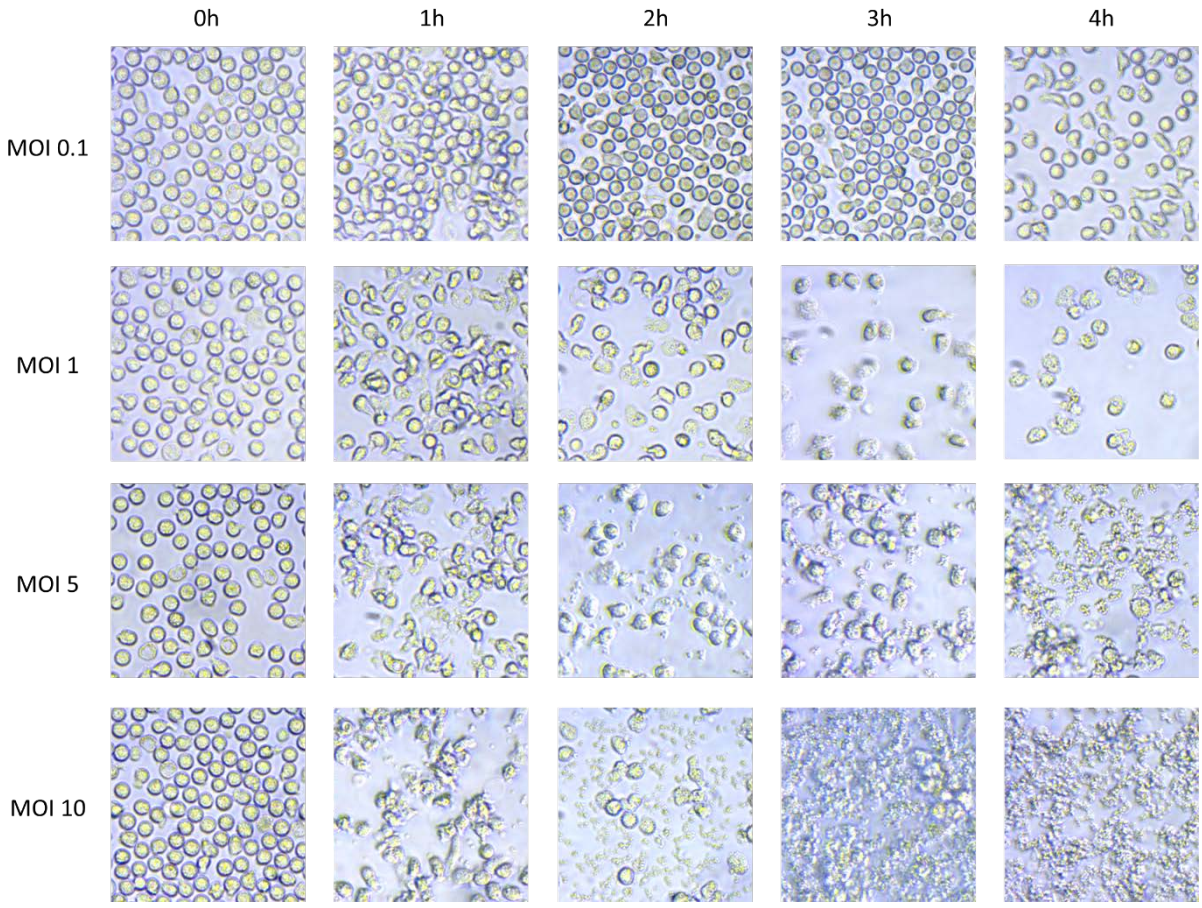
WEISS, E., HANZELMANN, D., FEHLHABER, B., KLOS, A., VON LOEWENICH, F. D., LIESE, J., PESCHEL, A. & KRETSCHMER, D. 2017. Formyl-peptide receptor 2 governs leukocyte influx in local Staphylococcus aureus infections. *FASEB J.*

ZIPPERER, A., KONNERTH, M. C., LAUX, C., BERSCHEID, A., JANEK, D., WEIDENMAIER, C., BURIAN, M., SCHILLING, N. A., SLAVETINSKY, C., MARSCHAL, M., WILLMANN, M., KALBACHER, H., SCHITTEK, B., BROTZ-OESTERHELT, H., GROND, S., PESCHEL, A. & KRISMER, B. 2016. Human commensals producing a novel antibiotic impair pathogen colonization. *Nature,* 535**,** 511-6.

## Supplement




**Supplemental figure 1: FPR-ligands does not induce upregulation of FCγRIIIB (CD16) and FCγRII (CD32).** Indicated concentrations of fMLF and PSMα3 does not induce an altered expression of A) CD16 or B) CD32 on the surface of human neutrophils.




**Supplemental figure 2: Destruction of human neutrophils by USA300.** Human neutrophils and USA300 were incubated at indicated MOIs. At indicated time points neutrophils were visualized with a Carl Zeiss Primovert Microscope and pictures were taken with an Axiocam ERc 5s.

# CHAPTER 6

## General discussion

## FPR2 is crucial for rapid leukocyte recruitment in local *S. aureus* infections

Recently, it was shown that the phenol soluble modulins (PSMs) of *S. aureus* are potent ligands of FPR2. As staphylococcal virulence correlates with PSM levels, FPR2 may equip leukocytes to distinguish between high-pathogenic, low-pathogenic, and commensal staphylococci, thereby enabling the innate immune system to adjust immune responses appropriately. For example, PSMs of highly virulent *S. aureus* USA300 induce much higher activation of FPR2 than PSMs of virulent *S. lugdunensis* or the opportunistic pathogen *S. epidermidis* [1]. *S. aureus* produces two inhibitors of FPR2 – FPR-like 1 inhibitory protein (FLIPr) and FLIPr-like – to evade immune cell responses, showing the importance of FPR2 for the host during *S. aureus* infection [2, 3].

As most of the *in vivo* infection experiments are performed in mice we addressed the question if mice express a homolog receptor to human FPR2 and if this receptor also senses PSMs. The mouse Fpr1 has been identified as the homolog of human FPR1 and its importance was shown for the course of systemic *Listeria monocytogenes* infections [4, 5]. It has been shown that mFpr1 responds with high efficiency to short formylated peptides but to prefer the sequence fMIFL over fMLF, the most potent formylated peptide for the human FPR1 [6]. In contrast, the identity of the functional mouse Fpr2 homolog has been more difficult to elucidate, as mice express several close FPR2 homologs [7, 8]. The mouse Fpr2, previously named mFpr-rs2, shows the highest sequence similarity to the human FPR2 [8], but mFpr2 responds only very weakly to several peptide agonists that strongly activate human FPR2 [9, 10]. However, it has been shown that the FPR2-specific antagonist $WRW_4$ inhibits the activation of the mFpr2 [11]. Despite the sequence similarity between mouse and human FPRs, there are crucial species-specific differences. Many *S. aureus* strains secret inhibitory proteins against human FPR1 (CHIPS) and FPR2 (FLIPr), which are inactive against mouse Fprs [2, 3, 12]. The unclear relation between human and mouse FPR homologs has impeded the analysis of the role of the PSM-sensing receptor in *in vivo* infections.

In this study we show that mouse neutrophils respond to PSMs by migration, release of chemokines, and upregulation of the complement receptor 3 (CD11b), thereby, showing similar functional activation than human neutrophils [13]. Sensing of PSMs

by mouse neutrophils is mediated by the mouse Fpr2 (formerly mFpr-rs2), as mFpr2 knockout neutrophils did not respond to PSMs. Additionally, stable mFpr2-transfected cells respond to PSMs by degranulation, whereas mock-transfected or mFpr1-transfected cells were not activated. Thus, our data support the hypothesis that the mouse Fpr2 is the functional homolog of the human FPR2 and that a mouse infection model may be a suitable model for analyzing the role of PSMs and FPRs during *S. aureus* infection.

Appropriately, mFpr2 has been found to contribute to the severity of sepsis and systemic *L. monocytogenes* infections. Activation of this receptor during sepsis effectively prevents lethality in mice by enhancing bacterial activity, downregulating pro-inflammatory cytokines, and by its anti-apoptotic effect on immune cells [14]. Furthermore, loss of Fpr2/3 during polymicrobial murine sepsis leads to exacerbation of disease severity, higher levels of pro-inflammatory cytokines, and reduced monocyte recruitment leading to inadequate bacterial removal. Additionally, phagocyte functions of neutrophils are impaired in mFpr2/3$^{-/-}$ mice compared to WT mice during sepsis and the absence of mFpr2/3 is associated with major changes in distant organ injury, with a particular effect on heart and kidney [15]. mFpr1 and mFpr2 play also essential roles during early neutrophil recruitment in *Listeria*-infected mouse livers and for the production of superoxide. mFpr-deficient mice are more susceptible to *L. monocytogenes*, as shown by higher mortality of these animals [5]. Thus, expression of FPRs is critical for elimination of invading pathogens and survival of infected mice.

Our study demonstrates a crucial role for mFpr2 during early leukocyte recruitment in local *S. aureus* infections. Intraperitoneal infection of mice with *S. aureus* resulted in less recruitment of leukocytes to the infection site in mFpr2$^{-/-}$ mice compared to wild-type mice 3 h post infection. At later stages of infection mFpr2 does not seem to play a role, suggesting the involvement of other chemokine receptors. The endogenous chemokines CXCL1 and CXCL2 are released in a time frame far behind the first wave of infiltrated leukocytes [5]. The activation of the innate immunity by other bacterial ligands than PSMs may contribute to the release of endogenous chemokines and thereby enabling a proper immune response in absence of mFpr2. Additionally, it was shown that blocking of mFpr2 leads to higher expression of the chemokine receptor CCR4 in macrophages [16]. Thus, also an altered expression profile of chemokine receptors in mFpr2$^{-/-}$ mice could abolish the effect of mFpr2 at

later stages. Nevertheless, for the rapid recruitment of leukocytes mFpr2 seems to be mandatory.

## Chemokine release by neutrophils is synergistically enhanced by costimulation of FPR1 and FPR2

Besides their chemotactic function, the FPRs play also an important role for onset of inflammation and recruitment of further immune cells. Neutrophils release IL-8 upon stimulation of FPRs. Additionally, IL-8 activates its own expression in an autocrine feedback loop [17, 18]. Together, this leads to amplification of neutrophil infiltration to the infection site. IL-8 is one of the major chemokines released by leukocytes and it induces primarily migration of neutrophils. Furthermore, it has an impact on monocyte adhesion to endothelial cells, which is necessary for subsequent transmigration through the endothelial barrier [19].

This study demonstrates that coactivation of the both FPRs – FPR1 and FPR2 – and the C5a receptor (C5aR) results in a synergistic release of IL-8, but has no such effect on other cellular functions of human neutrophils. Interestingly, monocytes did not respond with a synergistic release of IL-8 upon costimulation. Thus, we could speculate about the special role of neutrophils during the onset of inflammation. As neutrophils are the first cells at the infection site, it might be possible that they need more than one activation signal to release a high amount of chemokines. This could prevent hyper-inflammation. In contrast, monocytes are recruited later to the infection site to support resolution of infection. Thus, these cells might not need such a high threshold for production of chemokines as inflammation is already initiated. Especially the role of neutrophils during sepsis would be important to study further. A feature of sepsis is dysregulated production of cytokines and chemokines leading to hyper-inflammation followed by a hypo-inflammatory phase [20, 21].

As we could already show in this study that the expression of mFpr2 is important for rapid leukocyte infiltration in local *S. aureus* infections, the synergistic release of IL-8 might play a role in this scenario. Although, our data showed just a role for mFpr2 in the rapid recruitment of leukocytes and IL-8 is produced at later time points, the activation of both FPRs might be important for adequate production of this chemokine.

The transcriptional regulation of IL-8 expression in neutrophils needs to be analyzed further and compared to the regulation in monocytes. The signaling pathways involved in IL-8 expression include the extracellular-regulated kinase (ERK), JUN-N-terminal protein kinase (JNK), and p38 MAPK cascades [22]. ERK1 and ERK2 regulate differentiation and proliferation in many cell types and inhibitors of ERKs are being explored as anticancer agents. JNK1, 2, and 3 are critical regulators of cytokine transcription and programmed cell death, and JNK inhibitors may be effective in control of rheumatoid arthritis and cancer. The p38 MAPKs are activated by inflammatory cytokines and environmental stresses and they are key regulators of cytokine expression and thereby for the inflammatory response. p38 MAPKs may contribute to diseases like asthma and autoimmunity [23]. How these signaling cascades contribute to the synergistic release of IL-8 by neutrophils needs to be elucidated. Therefore, neutrophils will be challenged with combinations of ligands of different receptors to check which of them are able to induce synergistic IL-8 release. Furthermore, inhibitors against molecules of the signaling pathways involved in IL-8 release will help to clarify the involvement of these pathways. For costimulation of FPR1 and TLR4 it was already shown that it results in a synergistic release of chemokines and cytokines by human monocytes compared to a single stimulation of just one of these receptors. This effect is induced by phosphorylation of different serine residues of the NFκB subunit p65, which finally results in synergistic activation of NFκB leading to a boosted inflammatory response [24]. Coactivation of the FPRs might also lead to enhanced transcription of IL-8 by several phosphorylations of the NFκB subunits. Additionally, binding of NFκB coactivators might also be involved in enhanced transcription.

Further research will focus on the role of the synergistic IL-8 release by human neutrophils and if it is of favor or harmful for the host during local infections and systemic infections such as sepsis.

## *S. aureus* uses neutrophils as Trojan horses by enhancing its own phagocytosis via FPRs

Neutrophils are professional phagocytes of the innate immune system and the first line of defense during infections. Upon binding of opsonized particles to complement

receptors (CR) and FCγ receptors (FCγR) neutrophils engulf these particles [25]. Intracellularly the formed phagosome fuses with different granules, which provide microbicidal peptides and proteolytic enzymes, as well as numerous membrane-bound proteins that contribute to pathogen elimination [26].

Here we could demonstrate that the FPRs play an important role for the phagocytosis of gram-positive and gram-negative bacteria. Activation of one of these receptors results in significantly higher phagocytosis compared to unstimulated neutrophils. The mechanism behind is a higher expression of the complement receptors CR1 and CR3 and the FCγRI upon FPR stimulation. Higher expression of these receptors on the surface of phagocytes might result in more binding of opsonized particles and higher subsequent phagocytosis. A similar mechanism was already shown for macrophages stimulated with contents of neutrophil granules. Heparin-binding protein (HBP) and human neutrophil peptides 1-3 (HNP1-3) enhance the expression of FCγRI and FCγRII leading to higher phagocytosis of opsonized *S. aureus*. In this context HNP1-3 and HBP activate macrophages in a paracrine manner to produce TNFα and IFN-γ, which are activators of macrophages function and responsible for the enhanced phagocytosis by HNP1-3 and HBP [27]. For the endogenous FPR2 ligand LL-37 it was also shown that it enhances the expression of FCγRI and FCγRII in macrophages leading to higher phagocytosis of IgG-opsonized *S. aureus* and *E. coli* [28]. The mechanisms involved in the enhanced phagocytosis in neutrophils induced by FPR activation needs to be studied further. TNFα and IFN-γ might not be involved, as neutrophils release no or just very low amounts of these cytokines. But it might be possible that the activation of the FPRs itself directly leads to a higher expression of complement receptors and FCγRI.

Since the early 90s it is known that phagocytosis of IgG-opsonized yeast [29], *Listeria monocytogenes,* and *Yersinia enterocolitica* [30] by human neutrophils leads to release of IL-8 by these cells. For *S. aureus* it was shown that the activation of intracellular TLR2 by lipopetides is involved in IL-8 release by human monocytes during phagocytosis [31]. This study confirms the phagocytosis-dependent release of IL-8 by human neutrophils. Non-opsonized *S. aureus*, which is not phagocytosed does not induce IL-8 release by neutrophils. Furthermore, activation of FPRs by secreted peptides of *S. aureus* during phagocytosis of bacteria results in a synergistic release of IL-8, which is absent with non-opsonized *S. aureus*. Phagocytosis itself activate transcription factor NFκB, which is necessary for IL-8 expression [32, 33].

We could show in this study a synergistic IL-8 release by coactivation of FPR1 and FPR2, which is probably due to synergistic activation of NFκB. A similar mechanism might be involved in the synergistic IL-8 release during phagocytosis of *S. aureus* and stimulation of one of the FPRs. The high amount of released IL-8 is biological active as it results in significantly higher migration of neutrophils to the infection site. This might have an impact on clearance of infection. We could demonstrate that killing of *S. aureus* by neutrophils is clearly time- and ratio-dependent and the first two hours are highly important for the outcome. Superior numbers of neutrophils promote complete bacterial clearance showing the importance for rapid neutrophil recruitment to the infection site.

The role of the FPRs during bacterial killing by human neutrophils is much more complex. This study shows that *in vitro* the ratio of bacteria to neutrophils and the duration of bacterial killing have a high impact on the outcome. Activation of the FPRs seems to be of favor for the immune system during the rapid clearance of a low amount of bacteria. On the other hand for a high amount of bacteria and at later time points blocking of FPRs benefits bacterial killing by neutrophils. These data are confirmed *in vivo* where mice received a high amount of bacteria for a longer period. Although, absence of mFpr2 resulted in less phagocytosis of *S. aureus* by mouse neutrophils, these animals showed lower bacterial burden than WT animals with functional mFpr2. Our data support the hypothesis that *S. aureus* uses leukocytes as Trojan horses. *S. aureus* has evolved many strategies to resist AMPs and ROS within the phagosome and thereby to survive intracellularly [34]. Furthermore, several studies showed that *S. aureus* is able to escape phagosomal killing by lysing phagocytes from within [35-37]. Staphylococcal PSMs play an important role for destruction of the phagosome, whereas the bicomponent pore-forming toxin LukAB destroys the cytoplasmic membrane of host cells and thereby sets *S. aureus* free [36]. Besides its ability to survive within and destruct phagocytes, *S. aureus* also prevents eradication of bacteria-containing neutrophils by macrophages. Phagocytosis of *S. aureus* by neutrophils results in upregulation of the “don´t eat me signal” CD47 on the surface of neutrophils. Thereby, bacteria containing neutrophils resist uptake by macrophages and persist extracellularly [38]. Deficiency in CD47 results in lower bacterial burden and increased survival of *S. aureus*-infected mice [39]. Several publications already speculated that *S. aureus* uses neutrophils to reach distant organs and to induce systemic infections [39-41]. This is further supported, as

neutropenic patients, which have decreased numbers of neutrophils, are rarely affected by *S. aureus* bacteremia [42, 43]. This study demonstrates that FPRs might play an important role during local infections with high bacterial burden, like abscesses, to promote *S. aureus* survival and dissemination. Thus, FPRs could be a central factor for the development of sepsis and thereby a target for therapy. Further research should focus on the role of FPRs during sepsis development. Although, it was shown that FPRs prevent development of severe sepsis this studies used the cecal ligation and puncture (CLP) sepsis mouse model [14, 15]. This method leads directly to the onset of sepsis without previous local infections. A model, which shows the development of sepsis from an abscess would be much more interesting and relevant in this context. Nevertheless, during local infections with low bacteria load, which could be rapidly cleared, the immune system benefits from the activation of FPRs.

## References


1. Rautenberg, M., et al., *Neutrophil responses to staphylococcal pathogens and commensals via the formyl peptide receptor 2 relates to phenol-soluble modulin release and virulence.* FASEB J, 2011. **25**(4): p. 1254-63.
2. Prat, C., et al., *A new staphylococcal anti-inflammatory protein that antagonizes the formyl peptide receptor-like 1.* J Immunol, 2006. **177**(11): p. 8017-26.
3. Prat, C., et al., *A homolog of formyl peptide receptor-like 1 (FPRL1) inhibitor from Staphylococcus aureus (FPRL1 inhibitory protein) that inhibits FPRL1 and FPR.* J Immunol, 2009. **183**(10): p. 6569-78.
4. Gao, J.L., E.J. Lee, and P.M. Murphy, *Impaired antibacterial host defense in mice lacking the N-formylpeptide receptor.* J Exp Med, 1999. **189**(4): p. 657-62.
5. Liu, M., et al., *Formylpeptide receptors are critical for rapid neutrophil mobilization in host defense against Listeria monocytogenes.* Sci Rep, 2012. **2**: p. 786.
6. Southgate, E.L., et al., *Identification of formyl peptides from Listeria monocytogenes and Staphylococcus aureus as potent chemoattractants for mouse neutrophils.* J Immunol, 2008. **181**(2): p. 1429-37.
7. Gauthier, J.F., et al., *Differential contribution of bacterial N-formyl-methionyl-leucyl-phenylalanine and host-derived CXC chemokines to neutrophil infiltration into pulmonary alveoli during murine pneumococcal pneumonia.* Infect Immun, 2007. **75**(11): p. 5361-7.
8. Ye, R.D., et al., *International Union of Basic and Clinical Pharmacology. LXXIII. Nomenclature for the formyl peptide receptor (FPR) family.* Pharmacol Rev, 2009. **61**(2): p. 119-61.
9. Liang, T.S., et al., *Serum amyloid A is a chemotactic agonist at FPR2, a low-affinity N-formylpeptide receptor on mouse neutrophils.* Biochem Biophys Res Commun, 2000. **270**(2): p. 331-5.
10. Tiffany, H.L., et al., *Amyloid-beta induces chemotaxis and oxidant stress by acting at formylpeptide receptor 2, a G protein-coupled receptor expressed in phagocytes and brain.* J Biol Chem, 2001. **276**(26): p. 23645-52.

11. Onnheim, K., et al., *Tumour necrosis factor (TNF)-alpha primes murine neutrophils when triggered via formyl peptide receptor-related sequence 2, the murine orthologue of human formyl peptide receptor-like 1, through a process involving the type I TNF receptor and subcellular granule mobilization.* Immunology, 2008. **125**(4): p. 591-600.
12. de Haas, C.J., et al., *Chemotaxis inhibitory protein of Staphylococcus aureus, a bacterial antiinflammatory agent.* J Exp Med, 2004. **199**(5): p. 687-95.
13. Kretschmer, D., et al., *Human formyl peptide receptor 2 senses highly pathogenic Staphylococcus aureus.* Cell Host Microbe, 2010. **7**(6): p. 463-73.
14. Kim, S.D., et al., *The agonists of formyl peptide receptors prevent development of severe sepsis after microbial infection.* J Immunol, 2010. **185**(7): p. 4302-10.
15. Gobbetti, T., et al., *Nonredundant protective properties of FPR2/ALX in polymicrobial murine sepsis.* Proc Natl Acad Sci U S A, 2014. **111**(52): p. 18685-90.
16. Liu, Y., et al., *Cell surface receptor FPR2 promotes antitumor host defense by limiting M2 polarization of macrophages.* Cancer Res, 2013. **73**(2): p. 550-60.
17. Browning, D.D., et al., *Autocrine regulation of interleukin-8 production in human monocytes.* Am J Physiol Lung Cell Mol Physiol, 2000. **279**(6): p. L1129-36.
18. Fujishima, S., et al., *Regulation of neutrophil interleukin 8 gene expression and protein secretion by LPS, TNF-alpha, and IL-1 beta.* J Cell Physiol, 1993. **154**(3): p. 478-85.
19. Gerszten, R.E., et al., *MCP-1 and IL-8 trigger firm adhesion of monocytes to vascular endothelium under flow conditions.* Nature, 1999. **398**(6729): p. 718-23.
20. Chousterman, B.G., F.K. Swirski, and G.F. Weber, *Cytokine storm and sepsis disease pathogenesis.* Semin Immunopathol, 2017. **39**(5): p. 517-528.
21. Hotchkiss, R.S., G. Monneret, and D. Payen, *Immunosuppression in sepsis: a novel understanding of the disorder and a new therapeutic approach.* Lancet Infect Dis, 2013. **13**(3): p. 260-8.
22. Hoffmann, E., et al., *Multiple control of interleukin-8 gene expression.* J Leukoc Biol, 2002. **72**(5): p. 847-55.
23. Johnson, G.L. and R. Lapadat, *Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases.* Science, 2002. **298**(5600): p. 1911-2.
24. Chen, L.Y., et al., *Synergistic induction of inflammation by bacterial products lipopolysaccharide and fMLP: an important microbial pathogenic mechanism.* J Immunol, 2009. **182**(4): p. 2518-24.
25. van Kessel, K.P., J. Bestebroer, and J.A. van Strijp, *Neutrophil-Mediated Phagocytosis of Staphylococcus aureus.* Front Immunol, 2014. **5**: p. 467.
26. Lee, W.L., R.E. Harrison, and S. Grinstein, *Phagocytosis by neutrophils.* Microbes Infect, 2003. **5**(14): p. 1299-306.
27. Soehnlein, O., et al., *Neutrophil primary granule proteins HBP and HNP1-3 boost bacterial phagocytosis by human and murine macrophages.* J Clin Invest, 2008. **118**(10): p. 3491-502.
28. Wan, M., et al., *Antimicrobial peptide LL-37 promotes bacterial phagocytosis by human macrophages.* J Leukoc Biol, 2014. **95**(6): p. 971-81.
29. Bazzoni, F., et al., *Phagocytosing neutrophils produce and release high amounts of the neutrophil-activating peptide 1/interleukin 8.* J Exp Med, 1991. **173**(3): p. 771-4.
30. Arnold, R. and W. Konig, *Interleukin-8 release from human neutrophils after phagocytosis of Listeria monocytogenes and Yersinia enterocolitica.* J Med Microbiol, 1998. **47**(1): p. 55-62.
31. Kang, H.J., et al., *The role of phagocytosis in IL-8 production by human monocytes in response to lipoproteins on Staphylococcus aureus.* Biochem Biophys Res Commun, 2011. **406**(3): p. 449-53.
32. Friedland, J.S., et al., *Regulation of interleukin-8 gene expression after phagocytosis of zymosan by human monocytic cells.* J Leukoc Biol, 2001. **70**(3): p. 447-54.
33. McDonald, P.P. and M.A. Cassatella, *Activation of transcription factor NF-kappa B by phagocytic stimuli in human neutrophils.* FEBS Lett, 1997. **412**(3): p. 583-6.
34. Spaan, A.N., et al., *Neutrophils versus Staphylococcus aureus: a biological tug of war.* Annu Rev Microbiol, 2013. **67**: p. 629-50.

35. DuMont, A.L., et al., *Staphylococcus aureus elaborates leukocidin AB to mediate escape from within human neutrophils.* Infect Immun, 2013. **81**(5): p. 1830-41.
36. Munzenmayer, L., et al., *Influence of Sae-regulated and Agr-regulated factors on the escape of Staphylococcus aureus from human macrophages.* Cell Microbiol, 2016. **18**(8): p. 1172-83.
37. Surewaard, B.G., et al., *Staphylococcal alpha-phenol soluble modulins contribute to neutrophil lysis after phagocytosis.* Cell Microbiol, 2013. **15**(8): p. 1427-37.
38. Greenlee-Wacker, M.C., et al., *Phagocytosis of Staphylococcus aureus by human neutrophils prevents macrophage efferocytosis and induces programmed necrosis.* J Immunol, 2014. **192**(10): p. 4709-17.
39. Gresham, H.D., et al., *Survival of Staphylococcus aureus inside neutrophils contributes to infection.* J Immunol, 2000. **164**(7): p. 3713-22.
40. Lehar, S.M., et al., *Novel antibody-antibiotic conjugate eliminates intracellular S. aureus.* Nature, 2015. **527**(7578): p. 323-8.
41. Thwaites, G.E. and V. Gant, *Are bloodstream leukocytes Trojan Horses for the metastasis of Staphylococcus aureus?* Nat Rev Microbiol, 2011. **9**(3): p. 215-22.
42. Velasco, E., et al., *Comparative study of clinical characteristics of neutropenic and non-neutropenic adult cancer patients with bloodstream infections.* Eur J Clin Microbiol Infect Dis, 2006. **25**(1): p. 1-7.
43. Venditti, M., et al., *Staphylococcus aureus bacteremia in patients with hematologic malignancies: a retrospective case-control study.* Haematologica, 2003. **88**(8): p. 923-30.

# Contributions to publications

Elisabeth Weiß and Dorothee Kretschmer:

**Formyl-Peptide Receptors in Infection, Inflammation, and Cancer**

The sections “Sensing Invading Bacterial Pathogens via FPRs”, “Role of FPRs in Viral Infections“, “Box 3. Formyl-Peptide Receptor Human Polymorphisms in Human Disease”, and part of the introduction, concluding remarks, and glossary were written by me. I generated all the figures of the review.

Elisabeth Weiss, Dennis Hanzelmann, Beate Fehlhaber, Andreas Klos, Friederike D. von Loewenich, Jan Liese, Andreas Peschel, and Dorothee Kretschmer

**Formyl-peptide receptor 2 governs leukocyte influx in local *Staphylococcus aureus* infections**

All experiments with RBL cells and Hoxb8 neutrophils were performed by me except for the peptide binding assay and the CD11b expression.

Elisabeth Weiß, Andreas Peschel, and Dorothee Kretschmer

**Costimulation of FPR1 and FPR2 leads to a synergistic release of chemokines in neutrophils**

All experiments were performed by me and I wrote the manuscript under the guidance of Dorothee Kretschmer and Andreas Peschel.

Elisabeth Weiß, Andreas Peschel, and Dorothee Kretschmer

**Always hungry: Formyl-peptide receptor activation augments phagocytosis of *Staphylococcus aureus* by human neutrophils**

All experiments were performed by me except for the cfu determination of the mice experiment, and I wrote the manuscript under the guidance of Dorothee Kretschmer and Andreas Peschel.

# Curriculum vitae

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