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Neutrophils amplify their own recruitment to group B streptococcus infection sites by means of chemokine production

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Contents

Abstract

Neutrophil recruitment to sites of infection is a critical first step in the innate immune response to bacterial pathogens. The mechanisms underlying this process are incompletely understood. Here we used a model of peritonitis induced by *Streptococcus agalactiae* (Group B streptococcus, GBS), a frequent gram-positive pathogen, to investigate *in vivo* neutrophil recruitment. We found that cell influx occurred concomitantly with the release of Cxcl1 (chemokine (C-X-C motif) ligand 1) and Cxcl2. GBS-induced peritoneal exudates had higher numbers of neutrophils and higher concentrations of Cxcl2, but not Cxcl1, than those induced by classical pro-inflammatory stimuli, such as lipopolysaccharide (LPS) and zymosan. Moreover, GBS-induced neutrophil recruitment was significantly reduced by the administration of either anti-Cxcl1 or anti-Cxcl2 neutralizing antibodies. Cell depletion experiments demonstrated that Cxcl1 production was predominantly dependent on macrophages, while both neutrophils and macrophages were required for optimal Cxcl2 release. *In vitro* studies indicated that bone-marrow derived macrophages could secrete high levels of Cxcl1/2 when stimulated with either GBS or LPS. Bone marrow-derived neutrophils, in contrast, produced low levels of either chemokine in response to LPS while releasing high amounts of Cxcl2 when stimulated with GBS. This first set of data suggested that inflammatory responses induced by live bacteria are characterized by the release of high levels of Cxcl2, which is at least in part produced by neutrophils and mediates further neutrophil influx in concert with Cxcl1. To study the mechanisms underlying high-level Cxcl2 production induced by live GBS in neutrophils, we examined the role of Toll-like receptors (TLRs), which were previously shown to mediate pro-inflammatory cytokine responses to these bacteria. In both neutrophils and macrophages, synthesis of Cxcl1/2 occurred *de novo* after GBS stimulation and was totally dependent on the Toll-like receptor (TLR) adaptor MyD88. Moreover, chemokine production was significantly reduced in the absence of functional UNC93B1, a chaperone protein involved in the recruitment of nucleic acid sensing TLRs to the endosomes. The phenotype of UNC93B1-defective cells could be recapitulated by the simultaneous absence of TLR 7, 9 and 13, but not by the absence of individual TLRs, indicating that optimal chemokine production requires the simultaneous activation of multiple endosomal TLRs. We observed that high-level Cxcl2 release by neutrophils in response to live, but not heat-killed, bacteria requires, in addition, signaling by formylated peptide receptors (FPRs), as suggested by significant inhibition of chemokine production in the presence of Boc-2, an antagonist of both FPR1 and FPR2. Conversely, an FPR1 agonist, as well as bacterial culture supernatants, was capable of strongly synergizing with heat-killed GBS in the induction of Cxcl2 production. Collectively our data show that both macrophages and neutrophils participate in inflammatory cell recruitment through *de novo* chemokine production after recognition of gram positive bacteria by means of multiple endosomal

TLRs and FPRs. Moreover, neutrophils amplify their own recruitment through high-level Cxcl2 production in response to live, but not killed, bacteria. Finally, these cells are apparently capable of discriminating between live and killed bacteria by detecting extracellular FPR ligands. These data may be useful to devise alternative strategies to treat bacterial infections by potentiating the recruitment and the functional activity of polymorphonuclear leukocytes.

Abbreviations

Absent in melanoma 2 (AIM2)-like receptors (ALRs)

Activator protein 1 (AP-1)

Adaptor protein complex 3 (AP-3)

Bacterial surface adhesin of GBS (BsaB)

Blood-brain barrier (BBB)

Boc-Met-Leu-Phe (Boc-MLF or Boc-1)

Boc-Phe-D-Leu-Phe-D-Leu-Phe (BocPLPLP)

Boc-Phe-Leu-Phe-Leu-Phe (Boc-FLFLF or Boc-2)

Bone marrow derived macrophages (BMDMs)

Capsular polysaccharide (CPS)

Chemokine (C-X-C motif) ligand (CXCL)

Chemokine receptors (CKRs)

c-Jun N-terminal kinases (JNKs)

Clonal complexes (CC)

Community-associated methicillin resistant Staphylococcus aureus (CA-MRSA)

Complement component 5a (C5a)

Control of virulence, sensor and regulator system (CovRS)

C-type lectin receptors (CLRs)

Cyclic AMP-Responsive Element-Binding Protein (CREB)

Cyclic undecapeptide cyclosporine H (CysH)

Damage-associated molecules patterns (DAMPs).

Deformed epidermal autoregulatory factor-1 (DEAF1)

Dendritic cells (DCs),

Diacylglycerol (DAG)

Diphtheria toxin (DT)

Early-onset disease (EOD)

Enzyme-linked immunosorbent assay (ELISA)

Epithelial-neutrophil activating peptide 78 (ENA78)

Evolution conserved signalling intermediate in Toll pathway (ECSIT)

Extracellular matrix components (ECM)

Extracellular signal-regulated kinases (ERKs)

Fetal calf serum (FCS)

Fibrinogen-binding proteins A, B, and C (FbsA, FbsB, and FbsC)

fMLF (N-formyl-Met-Leu-Phe)

fMMYALF (N-formyl-Met-Met-Tyr-Ala-Leu-Phe)

Formyl peptide receptor 2-lipoxin receptor (FPR2/ALX)

Formyl peptide receptors (FPRs)

Formylpeptide receptor (FPR)-like (FPRL)-2 ligand (F2L)

FPR like (FPRL)

FPR2/ALX-inhibitory protein (FLIPr)

G protein-coupled receptors (GPCRs)

GBS immunogenic bacterial adhesin (BibA)

Glutamic acid–leucine–arginine (ELR)

Granulocyte-colony stimulating factor (G-CSF)

Granulocyte-macrophage colony stimulating factor (GM-CSF)

Group B streptococcal C5a peptidase (ScpB)

Group B streptococcus (GBS)

Growth-regulated oncogene alpha (GROα)

Guanine-nucleotide exchange factors (GEFs)

Heat shock protein (Hsp)

Heat-killed bacteria (HK-GBS)

High-mobility group box 1 (HMGB1)

Hypervirulent GBS adhesin (HvgA)

Inositol 1,4,5-triphosphate (IP_3)

Intercellular adhesion molecule (ICAM)

Interferon (IFN)

Interferon regulatory factor (IRF)

Interleukin 1 alpha (IL-1α)

Interleukin 1 beta (IL-1β)

Interleukin receptor (IL-R)

Interleukin-1 receptor-associated kinase (IRAK)

Intrapartum antibiotic prophylaxis (IAP)

Intraperitoneal (i.p.)

Intravenous (i.v.)

IκB kinase (IKK)

Keratinocyte chemoattractant (KC)

Laminin-binding protein (Lmb)

Late-onset disease (LOD)

Leucine-rich repeats (LRRs)

Leukotriene B4 (LTB4)

Lipopolysaccharide (LPS)

Lipoteichoic acid (LTA)

Lymphocyte function-associated antigen 1 (LFA-1 or αLβ2)

Macrophage colony-stimulating factor (M-CSF)

Macrophage inflammatory protein 2 alpha (MIP2α)

Macrophage-1 antigen (Mac-1 or αMβ2)

Major histocompatibility complex (MHC)

Mitogen-activated protein kinase (MAPK)

Mitogen-activated protein kinase kinase kinase (MAPKKK)

Monoclonal antibodies (mAbs)

Multilocus sequence typing (MST)

Multiplicity of infection (MOI)

MyD88-adaptor-like/TIR-associated protein (MAL/TIRAP)

Myeloid differentiation factor 88 (MyD88)

N-[1-(2,3-dioleoy-loxy)propyl]-N,N,N-trimethylammonium methyl-sulfate (DOTAP)

NF-kappa-B essential modulator (NEMO)

N-formyl-Met-Ile-Phe-Leu (fMIFL)

N-formyl-Met-Ile-Val-Ile-Leu (fMIVIL)

N–terminal formylated methionine (fMet)

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)

Nucleotide oligomerization domain (NOD)-like receptors (NLRs)

Pathogen-associated molecular patterns (PAMPs)

Pattern-recognition receptors (PRRs)

Peritoneal lavage fluid (PLF)

Phenol-soluble modulins (PSM)

Phosphoinositol-3,4,5-trisphosphate (PIP_3)

Phosphoinositol-4,5-bisphosphate (PIP_2)

Phospholipase C beta (PLCβ)

Pilus tip adhesin (PilA)

Plasminogen binding surface Protein (PbsP)

Platelet activating factor (PAF)

Polymorphonuclear leukocytes (PMNs)

Prolipoprotein diacylglyceryl transferase (lgt)

Protein associated with TLR4 (PRAT4A)

Protein kinase B (PKB or Akt)

Protein kinase C (PKC)

P-selectin glycoprotein ligand-1 (PSGL-1)

Receptor for advanced glycation end products (RAGE)

Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs)

Sequence types (ST)

Serine/threonine-protein kinase 1 (RIP-1)

Serine-rich repeat (Srr)

Serum amyloid A (SAA)

Signal transduction systems (TCS)

Sodium deoxycholate-trichloro-acetic acid (DOC–TCA)

Staphylococcal chemotaxis-inhibitory proteins (CHIPS)

Sterile alpha and HEAT/Armadillo-motif-containing protein (SARM)

Streptococcal fibronectin-binding protein A (SfbA)

TAK1 binding proteins (TAB)

TANK Binding Kinase 1(TBK1)

tert-butyloxycarbonyl group (t-Boc)

Tetanus toxoid (TT)

Todd-Hewitt broth (THB)

Toll/IL-1R-like (TIR)

Toll-like receptors (TLRs)

Toll-receptor-associated activator of interferon/Toll Like Receptor Adaptor Molecule 1 (TRIF/ TICAM1)

Transforming growth factor beta-activated kinase 1 (TAK1)

TRIF-related adaptor molecule/Toll Like Receptor Adaptor Molecule 2 (TRAM/TICAM2)

Trp-Lys-Tyr-Met-Val-D-Met-NH_2 (WKYMVm)

Tumor necrosis factor (TNF)

Tumor necrosis factor receptor (TNFR)-associated factor 6 (TRAF6)

Vascular cell adhesion molecule 1 (VCAM-1)

Vasodilator-stimulated phosphoprotein (VASP)

Very Late Antigen-4 (VLA-4 or α4β1)

Wild-type (WT)

WRWWWW(WRW4)

β-hemolysin/cytolysin toxin (β-h/c)

List of additional papers, not included in the thesis

- "*The TLR4 adaptor TRAM controls the phagocytosis of Gram-negative bacteria by interacting with the Rab11-family interacting protein 2"*
  Submitted to PLOS Pathogens
  Skjesol A., Yurchenko M., Melsæther Grøvdal L., Bösl K., Agliano F., Patanè F., **Lentini G.**, Kim H., Teti G., Kandasamy R.K., Sporsheim B., Starheim K., Schink K.O., Golenbock D.T., Stenmark H., McCaffrey M., Espevik T., Husebye H.

- *"The plasminogen binding protein PbsP is required for brain invasion by hypervirulent CC17 Group B streptococci"*
  Scientific Reports – Nature, In Press
  Lentini G., Midiri A., Firon A., Galbo R., Mancuso G., Biondo C., Mazzon E., Passantino A., Romeo L., Trieu-Cuot P., Teti G., Beninati C.

- *"The Streptococcus agalactiae cell wall-anchored protein PbsP mediates adhesion to and invasion of epithelial cells by exploiting the host vitronectin/αv integrin axis"*
  Mol Microbiol. 2018 Jul 21.
  De Gaetano G. V., Pietrocola G., Romeo L., Galbo R., **Lentini G.**, Giardina M., Biondo C., Midiri A., Mancuso G., Venza M., Venza I., Firon A., Trieu-Cuot P., Teti G., Speziale P., Beninati C.

- *"Toll-Like Receptor 8 Is a Major Sensor of Group B Streptococcus But Not Escherichia coli in Human Primary Monocytes and Macrophages"*
  Front Immunol. 2017 Oct 3; 8:1243
  Ehrnström B., Beckwith K.S., Yurchenko M., Moen S.H., Kojen J.F., **Lentini G.**, Teti G., Damås C.J.K., Espevik T., Stenvik J.

Introduction

1. Neutrophils in the inflammatory response

Inflammation is generally defined as a complex sequence of events triggered by an injury such as invading pathogens or endogenous signals that results in tissue repair or sometimes pathology, when the response goes unchecked (Nathan *et al.*, 2002). The innate immune system mediates inflammatory responses, providing broad spectrum protection against tissue damage caused by pathogens, trauma or other endogenous and exogenous agents and orchestrates long-term adaptive immunity toward specific pathogens (Barton *et al.*, 2008; Newson *et al.*, 2014). The inflammatory response is coordinated by a large range of mediators that form complex regulatory networks. Dysfunction or dysregulation of these regulatory mechanisms may lead to various immune-related diseases, such as chronic inflammatory disease and autoimmune disorders. Chronic and uncontrolled inflammation can inflict more severe tissue damage than the original infection or trauma (Cochrane, 1968; Niwa *et al.*, 1982; Goldblatt and Thrasher, 2000; Nathan et al., 2002; Okin and Medzhitov, 2012; Chen and Xu, 2015). Neutrophils or polymorphonuclear leukocytes (PMNs) are key cells during the initial inflammatory response, as they provide the first line of defence against numerous infectious pathogens, including bacteria, fungi, and protozoa (Kobayashi *et al.*, 2005; Deniset and Kubesa, 2016). They originate and mature in the bone marrow and are subsequently released into the peripheral vasculature, representing the dominant leukocyte population in the circulation (Leiding, 2017). The neutrophil-mediated inflammatory response can be regarded as a multi-step process involving the initial adhesion of circulating neutrophils to activated vascular endothelium and the subsequent extravasation and migration of neutrophils towards inflammatory foci. These cells promote pathogen clearance through different mechanisms such as: (i) production of reactive oxygen and nitrogen species (ROS/RNS) in the pathogen-containing vacuole; (ii) fusion of neutrophil granules containing various antimicrobial mediators to the vacuole; (iii) neutrophil extracellular trap (NET) formation (Smith, 1994; Faurschou and Borregaard, 2003; Brinkmann and Zychlinsky, 2012). This repertoire of antimicrobial arsenal generates a highly lethal environment that is essential for efficient microbe killing and degradation (Segal, 2005).

Efficient neutrophil activation and migration are essential for a protective innate immune response and for maintaining host health. Neutrophil malfunction or an overall decrease in PMN abundance (neutropenia) are deleterious to human health and often result in severe and recurrent infections (Leliefeld *et al.*, 2016). Likewise, their excess infiltration and activation at a site of tissue damage can cause chronic inflammation, limit injury repair and lead to loss of organ function (Caielli *et al.*, 2012; Kolaczkowska and Kubes, 2013).

Although classically considered to be effector cells 'only', in recent years it has become evident that neutrophils not only have a pivotal role in the acute phase of inflammation when they actively eliminate pathogens but are also capable of modifying the overall immune response by interacting with other cells. This is accomplished by influencing, recruiting, and secreting signals for surrounding innate and adaptive immune cells (Smith, 1994; Mantovani *et al.*, 2011; Jaillon *et al.*, 2013).

2. Neutrophil migration and recruitment to infection sites

Acute tissue damage generates a wide variety of signals produced by complex networks that establish chemoattractant gradients throughout tissues. As 'leader' cells in host defence responses, neutrophils sense and integrate all of these chemotactic cues into a migration response towards damaged tissue and pathogens (Ley, 2011; Gambardella and Vermeren, 2013; de Oliveira *et al.*, 2016). Indeed, the initial early neutrophil recruitment to the infection site is largely initiated by pathogen-associated products, described as molecular patterns (PAMPs), released by invading microorganisms, and by damage-associated molecular patterns (DAMPs), derived from damaged and/or necrotic cells after tissue injury or in response to tissue and/or cellular stress, which are detected by neutrophils through their pattern recognition receptors (PRRs) (Medzhitov, 2008; de Oliveira *et al.*, 2016). Host DAMPs can be represented by damaged DNA, proteins such as high-mobility group box 1 (HMGB1), extracellular matrix (ECM) components, ATP and uric acid (Broggi *et al.*, 2015; Vénéreau *et al.*, 2015). Early neutrophil chemotaxis and activation is also triggered by N-formyl peptides derived from both bacterial proteins and from host mitochondria after tissue damage (Carp, 1982, Stålhammar *et al.*, 2017). These molecules are recognized by formyl peptide receptors (FPRs) belonging to the class of G-protein-coupled receptors (GPCRs). The importance of this signal as a short-range cue for proper neutrophil migration and localization to a site of injury has been demonstrated by *in vitro* studies using human FPR1-specific antibodies which blocked neutrophil migration to disrupted mitochondrial products (Raoof *et al.*, 2010) or necrotic cells (McDonald *et al.*, 2010). FPR1 blockade or deficiency also prevented the guidance of recruited neutrophils to necrotic cells in a localized hepatic injury model in mice (McDonald *et al.*, 2010).

The primary step in neutrophil migration process is the establishment of weak and transient adhesive interactions between neutrophils and endothelial cells of postcapillary venular walls in close vicinity to inflamed tissues. This step is indicated as "selectin-mediated rolling" because it is mediated by weak binding between the P-selectin glycoprotein ligand-1 (PSGL-1) of neutrophils and P-selectin/E-selectins present on endothelial cells (Chase *et al.*, 2012; Rigby *et al.*, 2012). P-selectin / PSGL-1 interactions are predominantly involved in the initial tethering (Greenlee-Wacker *et al.*, 2015),

whereas slow and more stable rolling is mediated by E-selectin (Chase *et al.*, 2012). Due to these transient and reversible interactions, neutrophils can roll along the endothelial cells.

After initial migration, the neutrophil recruitment becomes amplified by PRR binding to DAMPs or PAMPs which triggers the production of a variety of proinflammatory host cytokines, chemokines and lipid mediators (such as chemokine (C-X-C motif) ligand 8 (CXCL8) family of chemokines (in humans the interleukin-8 protein (IL-8)is encoded by the CXCL8 gene), Interleukin 1 alpha (IL-1α), Interleukin 1 beta (IL-β), chemokine (C-X-C motif) ligand 1 (CXCL1 also known as growth-regulated oncogene alpha (GROα) and keratinocyte chemoattractant (KC) in mice), chemokine (C-X-C motif) ligand 2(CXCL2 or macrophage inflammatory protein 2 alpha (MIP2α)), chemokine (C-X-C motif) ligand 5(CXCL5 or Epithelial-neutrophil activating peptide 78 (ENA78), leukotriene B4 (LTB4), tumor necrosis factor (TNF), granulocyte-colony stimulating factor (G-CSF), or granulocyte-macrophage colony stimulating factor (GM-CSF)). These molecules serve as chemoattractants and promote neutrophil recruitment and accumulation at sites of acute inflammation within a few hours (Springer, 1994¸ de Oliveira *et al.*, 2016).

During this chemokine-induced activation step, host-derived chemokines and chemoattractants (such as IL-8, Complement component 5a (C5a), LTB4, platelet activating factor (PAF) and microbial products (e.g. bacterial N-formyl peptides) induce rapid neutrophil adhesion by converting the low-affinity selectin-mediated interaction into a high-affinity, integrin-mediated firm adhesion (Rot *et al.*, 2004; Kobayashi, 2006; Ley *et al.*, 2007). These chemical mediators of inflammation can be produced by a wide variety of cells, including mast cells, macrophages, dendritic cells (DCs), endothelial and epithelial cells, platelets and parenchymal cells (Bagaitkar, 2014). In addition, antigens, largely through activation of resident memory T cells, can trigger PMNs recruitment via secretion of various primary inflammatory cytokines (Nourshargh and Alon, 2014). Neutrophil presence is also amplified and sustained via neutrophil recruitment, either by the active neutrophils themselves or the surrounding tissue-resident macrophages (Selders *et al.*, 2017).

Subsequently, neutrophil adhesion to endothelial cells is mediated by interactions between integrins present on neutrophil surface, such as Very Late Antigen-4 (VLA-4 or α4β1), Macrophage-1 antigen (Mac-1 or αMβ2) and Lymphocyte function-associated antigen 1 (LFA-1 or αLβ2) and members of the immunoglobulin superfamily present on endothelial cells, such as intercellular adhesion molecule 1 (ICAM-1), intercellular adhesion molecule 2 (ICAM-2), vascular cell adhesion molecule 1 (VCAM-1) or the receptor for advanced glycation end products (RAGE) (Yonekawa and Harlan, 2005). These endothelial counter-receptors are constitutively expressed (ICAM-1, ICAM-2, RAGE) or further up-regulated (ICAM-1, RAGE) or are induced (VCAM-1) (Chavakis *et al.*, 2003; Yonekawa and Harlan, 2005). This high affinity contact generates the adhesion necessary for

transendothelial migration or diapedesis (Muller, 2011). At this point, firmly attached neutrophils rapidly protrude and translocate through the endothelial barrier, predominately at paracellular endothelial cell junctions, or use their integrins to translocate (crawl) on the apical aspects of blood vessels in search for exit cues (Muller *et al.*, 1993; Phillipson *et al.*, 2006; Ley *et al.*, 2007; Rao *et al.*, 2007) (Fig. 1).

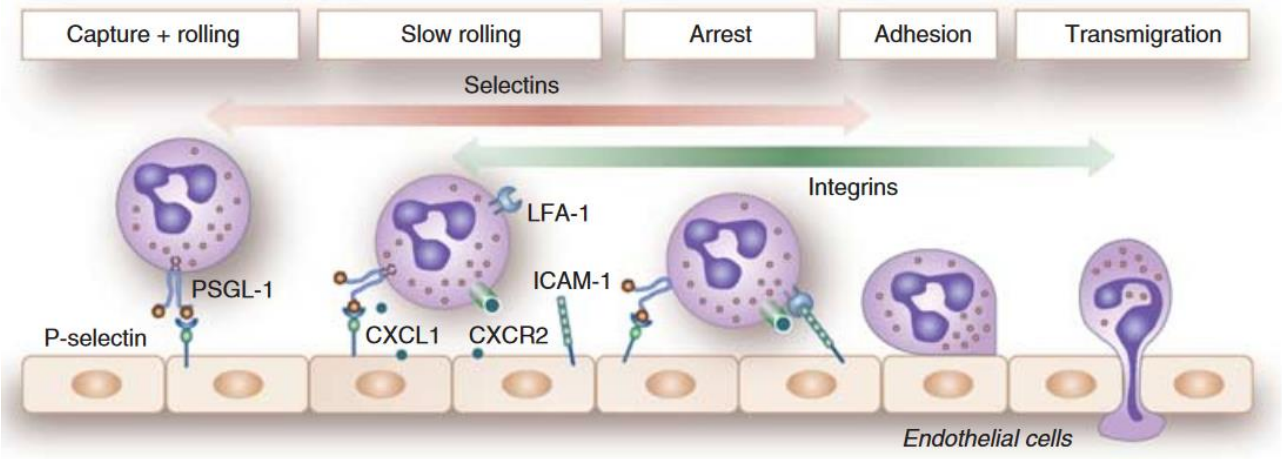

Figure 1: Neutrophil recruitment cascade (from Pindjakova and Griffin., 2011. Kidney Int. Sep;80(5):447-50).

The last phase is characterized by the removal of neutrophils from the area either by phagocytosis via macrophages, apoptosis or reverse migration. Reverse migration is described as a process by which neutrophils return to the vasculature in a phenomenon deemed “reverse transendothelial migration” (de Oliveira *et al.*, 2016).

CHAPTER 1: Toll-like receptors (TLRs) and their role in innate immunity

1.1. Recognition of pathogens by TLRs and generation of inflammatory immune response

The innate immune system employs germline-encoded pattern-recognition receptors (PRRs) for the initial detection of microbes. PRRs recognize microbe-specific molecular signatures known as pathogen-associated molecular patterns (PAMPs) and self-derived molecules derived from damaged host cells, referred to as damage-associated molecules patterns (DAMPs). PRRs activate downstream signalling pathways that lead to the induction of innate immune responses by producing inflammatory cytokines, type I interferon (IFN), and other mediators. These processes not only trigger immediate host defensive responses such as inflammation, but also prime and orchestrate antigen-specific adaptive immune responses (Janeway and Medzhitov, 2002). These responses are essential for the clearance of infecting microbes as well as crucial for the consequent instruction of antigen-specific adaptive immune responses (Kawasaki and Kawai, 2004). Mammalian PRRs are classified into different categories, including Nucleotide oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), Absent in melanoma 2 (AIM2)-like receptors (ALRs), G protein–coupled receptors (GPCRs), Toll-like receptors (TLRs), C-type lectin receptors (CLRs) and intracellular DNA sensors (Suresh and Mosser, 2013; Dempsey and Bowie, 2015). Among these, TLRs were the first to be identified, and are the best characterized. TLRs are germline-encoded type I transmembrane proteins derived from a toll gene family which play a crucial role in the detection of many microbial patterns and activating the innate immune system. Identification of TLR innate immune functions began in 1988 with the discovery that *Drosophila melanogaster* mutants in the Toll gene are highly susceptible to fungal infection (Lemaitre *et al.* 1996). This was soon followed by identification of a human Toll homolog, now known as TLR4 (Medzhitov *et al.* 1997). The TLR family currently includes 10 human TLRs (i.e. TLR1-TLR10) that function as pattern recognition receptors for a broad range of predominantly microbial stimuli and at least 13 active TLRs have been identified in laboratory mice (RIF.). Human and murine neutrophils appear to express all of these receptors, except for TLR3, at least at the mRNA level (Hayashi *et al.*, 2003). These receptors are located at the cell surface or into intracellular compartments (e. g. endosomes, lysosomes or endolysosomes) and recognize distinct or overlapping PAMPs derived from a unique class of microbial antigens. Cell-surface TLRs recognizes PAMPs on the pathogen's surface, including bacterial cell wall components and flagellin and fungal polysaccharides. These TLRs and their cognate ligands include: TLR2 that forms heterodimers with TLR1 or TLR6 and recognizes bacterial lipoproteins and lipopeptides; TLR4 recognizing bacterial lipopolysaccharides (LPS); TLR5 recognizing flagellin (Fig. 2). Intracellular TLRs recognize nucleic acids derived from bacterial,

fungal and viral pathogens and include: TLR3 recognizing double-stranded (ds) RNA; TLR7 and TLR8 recognizing single-stranded (ss) RNA; TLR9 recognizing DNA of bacterial and viral origin and unmethylated CpG dinucleotide-containing DNA; murine TLR13 recognizing a conserved motif present in the large subunit ribosomal RNA (23S rRNA) of bacteria (Fig. 3) (Akira *et al.*, 2006; Liu *et al.*, 2008; Li *et al.*, 2012; Oldenburg *et al.*, 2012).

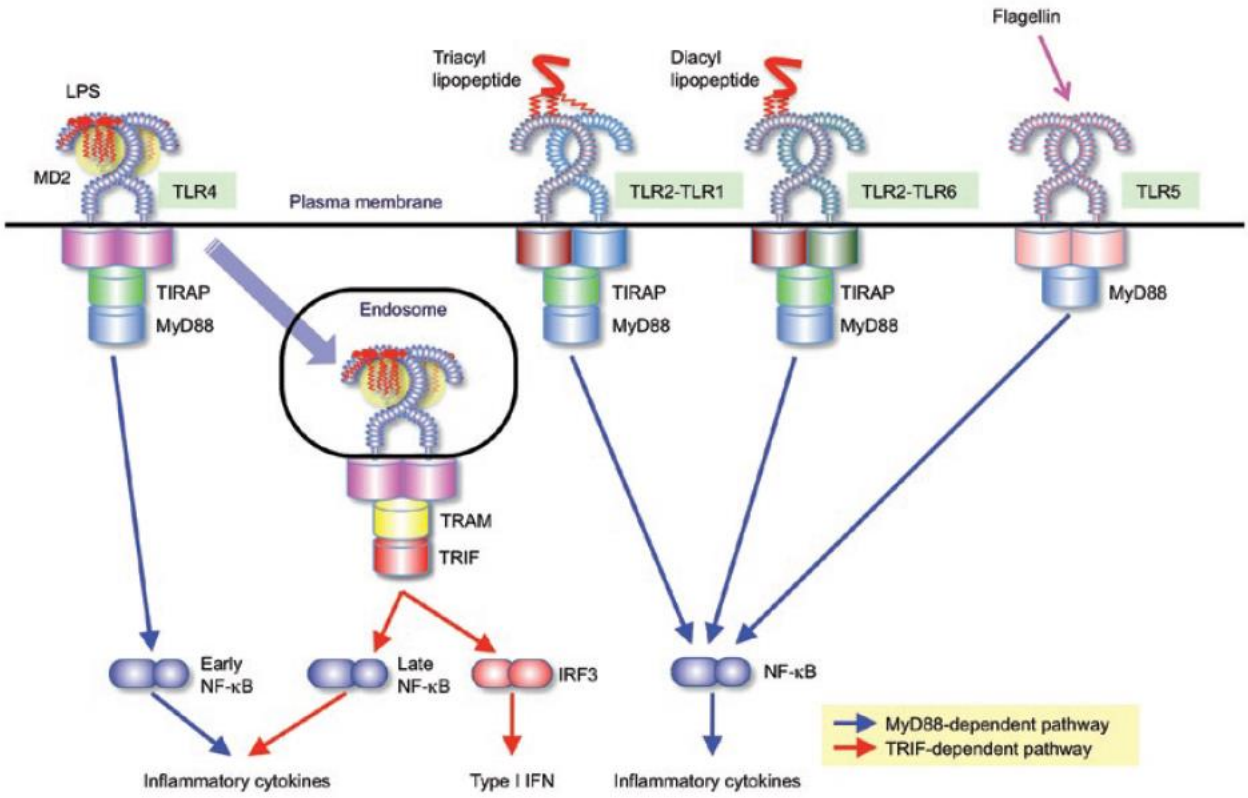

Figure 2: PAMP recognition by cell surface TLRs (from Kawai and Akira, 2010. Nat Immunol. May;11(5):373-84)

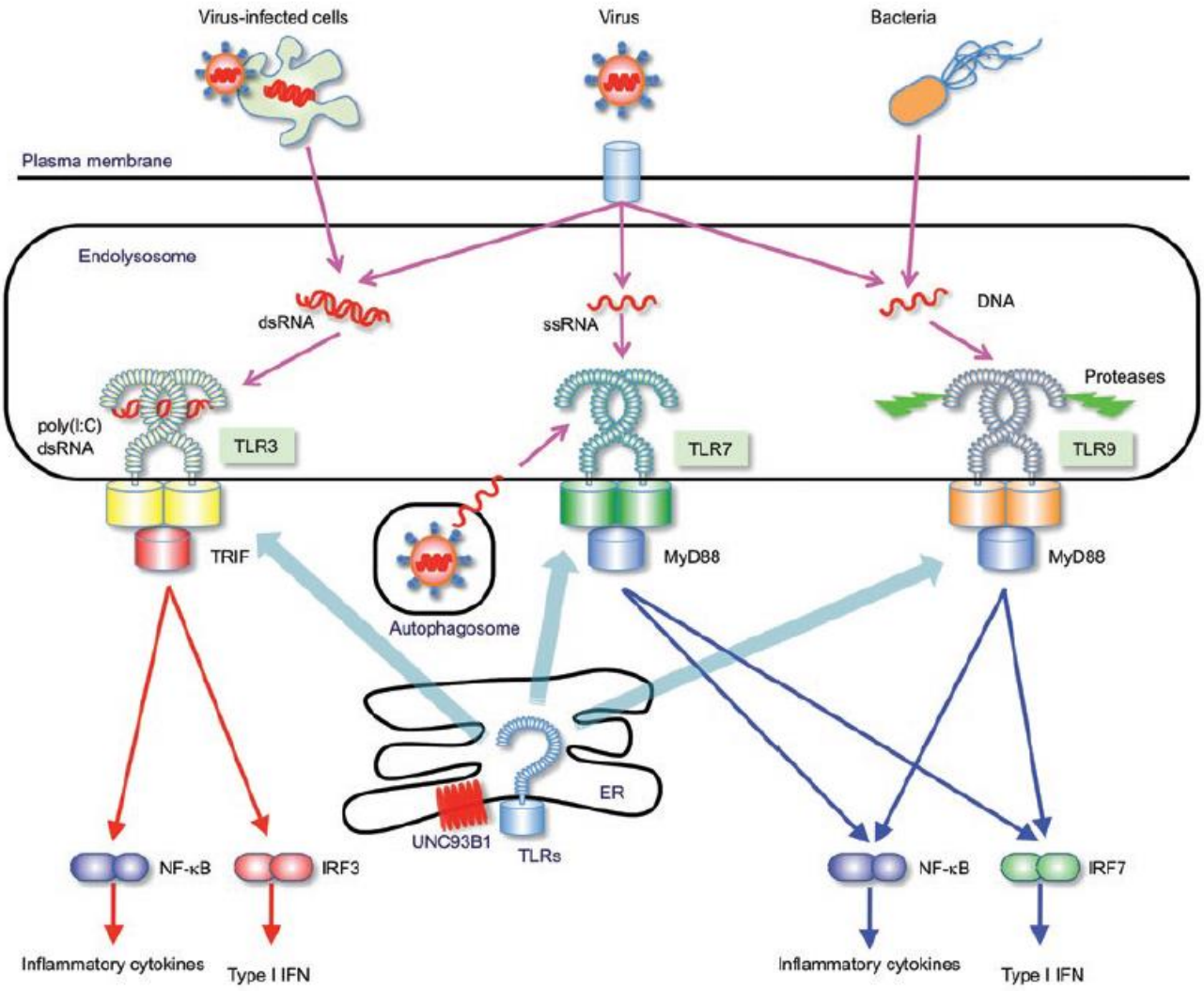

Figure 3: PAMP recognition by intracellular TLRs (from Kawai and Akira, 2010. Nat Immunol. May;11(5):373-84)

1.1.1. TLR localization and trafficking

The expression of TLRs differs widely between different cell types. These differences, together with differential intracellular TLR localization, deeply influence the type of pathogens recognized and the responses elicited (Biondo *et al.*, 2012). All TLRs are synthesized in the endoplasmic reticulum (ER), transported to Golgi complex and from there are transported to either the cell membrane or to various intracellular compartments (Kawasaki and Kawai, 2014). The trafficking of intracellular TLRs to endosomes is controlled and tightly regulated by different proteins that have been shown to act as chaperones. A multi-pass transmembrane protein localized to the ER, known as UNC93B1 (Unc-93 homolog B1), plays a pivotal role in facilitating this trafficking (Brinkmann *et al.*, 2007; Kim *et al.*, 2008; Lee *et al.*, 2013).

In mice, a missense mutation in the Unc93b1gene (encoding UNC93B1 with a H412R mutation) abrogates signalling via TLR3, TLR7 and TLR9 without compromising other TLRs (Tabeta *et al.*, 2006). These mutant mice (termed '3d' or 'triple D' for the combined signalling defects of TLR3, TLR7 and TLR9) show increased susceptibility to infection by various pathogens as well as major histocompatibility complex (MHC) class II presentation and cross-presentation defects (Tabeta *et al.*, 2006; Brinkmann *et al.*, 2007; Kim *et al.*, 2008). Other proteins are involved in trafficking of TLRs to the cell surface and to endosomes such as ER chaperones gp96 (also known as Heat shock protein 90kDa beta member 1, HSP90b1) and Protein associated with TLR4 (PRAT4A). gp96 (a member of Hsp90 family) acts as a general chaperone for most TLRs. In fact, macrophage deficient in this molecule do not respond to TLR1, TLR2, TLR4, TLR5, TLR7 and TLR9 ligands (Randow and Seed, 2001; Yang *et al.*, 2007). Protein associated with TLR4 (PRAT4A) is another ER resident protein molecule involved in the trafficking of both cell surface and intracellular TLRs (Takahashi *et al.*, 2007). TLR1, TLR2, TLR4, TLR7 and TLR9 responses to their ligands are compromised in PRAT-deficient cells (Takahashi *et al.*, 2007). Cells lacking PRAT4A showed impaired trafficking of TLR2 and TLR4 to the cell surface and TLR9 to the endo-lysosomal compartments (Takahashi *et al.*, 2007). Moreover, in DCs from PRAT4A-deficient mice, TLR7 and 9 responses were completely abolished, however, TLR3 responses were unaffected suggesting that in these cells not all of the nucleic acid sensing intracellular TLRs are regulated identically (Takahashi *et al.*, 2007). More recently, Adaptor protein complex 3 (AP-3), a 4-subunit (d, b3A, m3A, s3) clathrin-associated adaptor protein complex, was involved in TLR7 or TLR9 activation and required for trafficking of TLR9 and UNC93B1 to late, but not to early endosomes (Sasai *et al.*, 2010).

1.1.2. TLR-mediated signalling pathways

TLRs have complex expression patterns in different cell types and differ from one another in their ligand specificity, the signaling adaptors they utilize and the cellular responses they induce (Iwasaki and Medzhitov, 2004). Each TLR is essentially characterized by an ectodomain with leucine-rich repeats (LRRs) that mediates recognition of PAMPs, a transmembrane region and a cytosolic or intracellular Toll/IL-1R-like (TIR) domain that initiates downstream signaling pathways. Although there are differences in the details of the mechanisms of ligand binding by these TLRs, in all cases the ligands bridge two TLR molecules forming homo- or heterodimers between ectodomains that have a similar overall architecture and serve to dimerize the cytoplasmic TIR domains (Chaturvedi and Pierce, 2009).

Upon PAMPs and DAMPs recognition, PRRs present at the cell surface or intracellularly signal to the host the presence of infection and trigger proinflammatory and antimicrobial responses by

recruiting a distinct set of TIR-containing adaptor molecules. Four adaptor proteins — MyD88 (myeloid differentiation factor 88), MAL/TIRAP (MyD88-adaptor-like/TIR-associated protein), TRIF/ TICAM1 (Toll-receptor-associated activator of interferon/Toll Like Receptor Adaptor Molecule 1) and TRAM/TICAM2 (TRIF-related adaptor molecule/Toll Like Receptor Adaptor Molecule 2) — transduce signals from all of the TIR domains, activating a multitude of intracellular signaling transduction pathways that lead to cytokines, chemokines, and type I IFNs expression and ultimately protect the host from microbial infection (Fig. 4) (Akira and Takeda, 2004; O'Neill and Bowie, 2007).

MyD88 is involved in transmitting signals from TLR and interleukin 1 receptor (IL-1R) family members, with the exception of TLR3, and activates NF-κB and MAPKs for the induction of inflammatory cytokine genes (O'Neill *et al.*, 2003; Takeda *et al.*, 2003; Beutler, 2004). TIRAP is a sorting adaptor that recruits MyD88 to cell surface TLRs such as TLR2 and TLR4 and endosomal TLRs such as TLR9. TRIF is recruited to TLR3 and TLR4 and promotes an alternative pathway that leads to the activation of IRF3, NF-κB, and MAPKs for induction of type I IFN and inflammatory cytokine genes. TRAM is selectively recruited to TLR4 but not TLR3 to link between TRIF and TLR4. A fifth adaptor, known as SARM (sterile alpha and HEAT/Armadillo-motif-containing protein) has now been shown to interact with TRIF and thereby interfere with TRIF function (Carty *et al.*, 2006).

Collectively, depending on the adaptor usage, TLR signaling is largely divided into two pathways: the MyD88-dependent and TRIF-dependent pathways.

1.1.2.1. MyD88-Dependent Pathway

After TLR engagement, MyD88 forms a complex with Interleukin-1 receptor-associated kinase (IRAK) family members, referred to as the Myddosome (Lin *et al.*, 2010). During Myddosome formation, IRAK4 activates IRAK1, which is then autophosphorylated at several sites (Kollewe *et al.*, 2004) and released from MyD88 (Jiang et al., 2002). IRAK1 associates with E3 ubiquitin ligase Tumor necrosis factor receptor (TNFR)-associated factor 6 (TRAF6) that promotes K63-linked polyubiquitination of both TRAF6 itself and the Transforming growth factor beta-activated kinase 1 (TAK1) protein kinase complex. TRAF6 is also translocated to mitochondria following bacterial infection, where it interacts with Evolution conserved signalling intermediate in Toll pathway (ECSIT). TRAF6 promotes ECSIT ubiquitination, resulting in increased mitochondrial and cellular ROS generation (West *et al.*, 2010) TAK1 is a member of the Mitogen-activated protein kinase kinase kinase (MAPKKK) family and forms a complex with the regulatory subunits TAK1 binding proteins

(TAB1, TAB2, and TAB3) which interact with polyubiquitin chains generated by TRAF6 to drive TAK1 activation (Chen, 2012; Ajibade *et al.*, 2013). TAK1 then activates two different pathways that lead to activation of the IκB kinase (IKK) complex- nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway and - mitogen-activated protein kinase (MAPK) pathway. The IKK complex is composed of the catalytic subunits IκB kinase alfa and beta (IKKα and IKKβ) and the regulatory subunit NF-kappa-B essential modulator (NEMO, also called inhibitor of nuclear factor kappa-B kinase subunit gamma, IKKγ). TAK1 binds to the IKK complex through ubiquitin chains, which allows it to phosphorylate and activate IKKβ. The IKK complex phosphorylates the NF-κB inhibitory protein IκBα, which undergoes proteasome degradation, allowing NF-κB to translocate into the nucleus to induce proinflammatory gene expression. TAK1 activation also results in activation of MAPK family members such as extracellular signal-regulated kinase 1/2 (ERK1/2), p38 and c-Jun N-terminal kinases (JNKs), which then mediate various transcription factors, including Activator protein 1 (AP-1), Cyclic AMP-Responsive Element-Binding Protein (CREB) or stabilize mRNA to regulate inflammatory responses (Akira *et al.*, 2006; Kawai and Akira 2010; Kawasaki and Kawai,2014). CREB transcription factor also regulates diverse cellular responses, including proliferation, survival, and differentiation (Wen *et al.*, 2010) as well as regulate the differentiation of Th17 cells and survival of Treg cells (Wang *et al.*, 2017)

1.1.2.2. TRIF-Dependent Pathway

The TRIF-dependent pathway culminates in the activation of both IRF3 and NF-κB (Kawai and Akira, 2008). The adaptor TRIF interacts with TRAF6 and TRAF3. TRAF6 recruits the kinase Receptor-interacting serine/threonine-protein kinase 1 (RIP-1), which in turn interacts with and activates the TAK1 complex, leading to activation of NF-κB and MAPKs and induction of inflammatory cytokines. In contrast, TRAF3 recruits the noncanonical IKKs related kinases TANK Binding Kinase 1(TBK1) and IKKi (IKKε) along with NEMO for Interferon regulatory factor 3 (IRF3) phosphorylation (Hacker and Karin, 2006). TRAF3 also recruits IKK alfa (IKKα) for Interferon regulatory factor 7 (IRF7) phosphorylation. Subsequently, IRF3 forms a dimer (either a homodimer or a heterodimer with IRF7) and translocates into the nucleus from the cytoplasm, where it induces the expression of type I IFN genes (Akira *et al.*, 2006; Kawai and Akira 2008). The Pellino family E3 ubiquitin ligases are also implicated in TLR signaling (Jiang and Chen, 2011). Recent data have shown that Pellino-1 mediates TRIF-dependent NF-κB activation by recruiting RIP-1. Furthermore, Pellino-1 regulates IRF3 activation by binding to Deformed epidermal autoregulatory

factor-1 (DEAF1), a transcription factor that facilitates binding of IRF3 to the IFNβ promoter (Jiang and Chen, 2011).

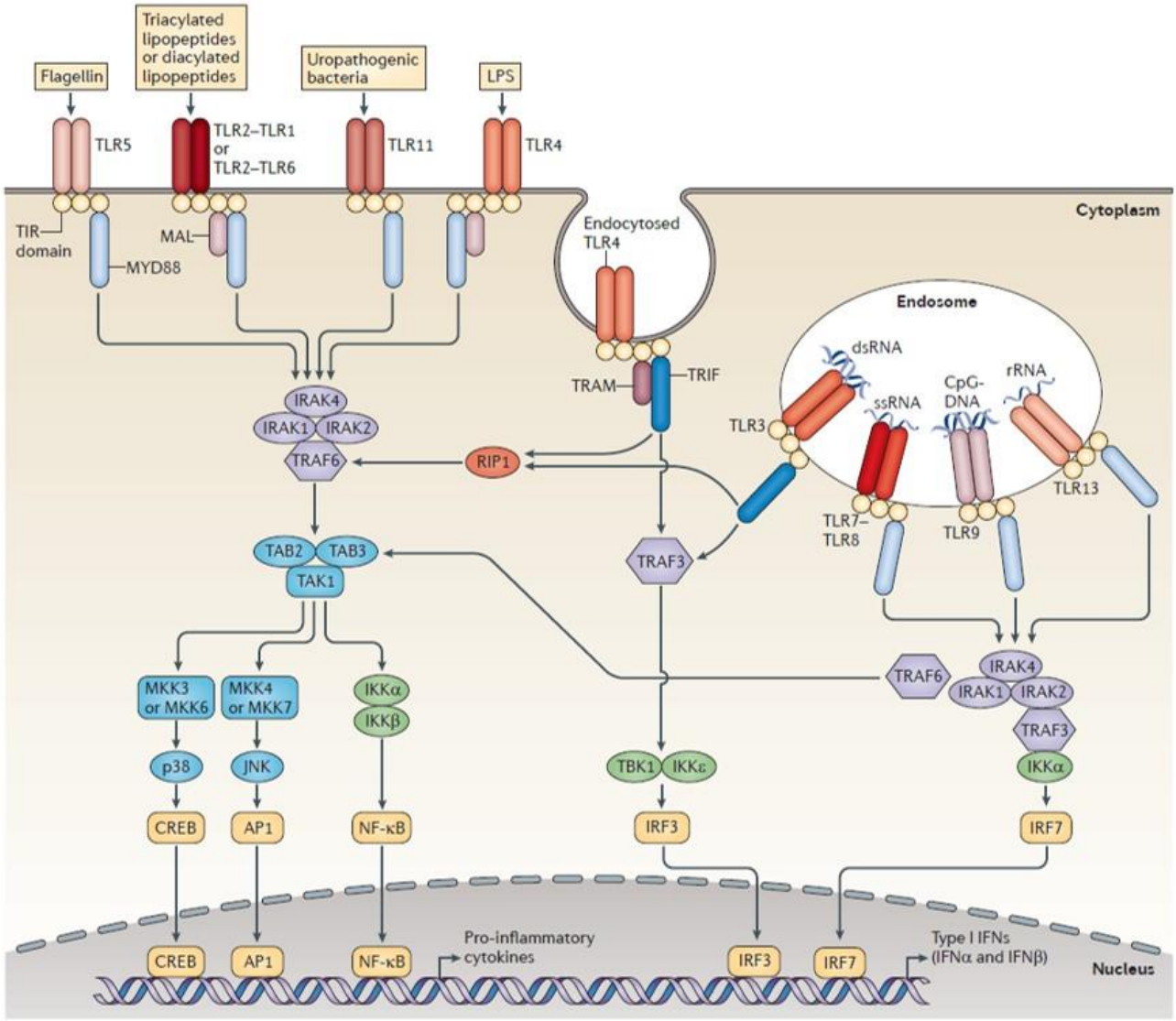

Figure 4: Mammalian TLR signalling pathways (from O'Neill *et al.*, 2013. Nat Rev Immunol. Jun;13(6):453-60)

All of these events initiate signal transduction pathways resulting in the activation of gene expression and synthesis of a broad range of molecules, including cytokines, chemokines, cell adhesion molecules, and immunoreceptors (Akira *et al.*, 2006), which together orchestrate the early host response to infection and at the same time represent an important link to the adaptive immune response (Areschoug and Gordon, 2008; Mogensen, 2009).

CHAPTER 2: The PRR properties of Formyl Peptide Receptors (FPRs) and their implications in host defenses

2.1. Biological Role of the N-Formyl Peptide Receptors

Formyl peptide receptors (FPRs) belong to a class of G-protein-coupled receptors (GPCRs) that play important roles in host defense and inflammation (Migeotte *et al.*, 2006). The best-known biological activity of FPR is induction of leukocyte chemotactic migration into areas of infection or injury (Le et al., 2001). In addition to chemotaxis, FPRs mediate phagocytosis, ROS generation, and production of diverse cytokine/chemokines (Boulay *et al.*, 1990; Ye *et al.*, 2009).

In the mid-1970's, Shiffmann et al. showed that synthetic peptides that contained an N–terminal formylated methionine (fMet) could constitute a ''molecular pattern" recognized by cells of our innate immune system acting as chemoattractants for phagocytic cells (Shiffmann *et al.*, 1975). The rational for this is that bacterial protein synthesis starts with fMet, a residue sequentially cleaved off by peptide deformylase to generate mature proteins during bacterial growth. These fMet-containing peptides were also generated and released by growing bacteria, so several natural formylated peptides have been purified from bacterial culture supernatants (Ye *et al.*, 2009), such as *Escherichia coli, Staphylococcus aureus, Mycobacterium avium,* and *Listeria monocytogenes* providing evidence that they are able to activate human phagocytes (Marasco *et al.*, 1984; Rot *et al.*, 1987; Rot *et al.*, 1989; Rabiet *et al.*, 2005; Gripentrog *et al.*, 2008; Southgate *et al.*, 2008; Kretschmer *et al.*, 2010; Bufe *et al.*, 2015). Mitochondrial proteins are also N-formylated and are chemotactic for neutrophils, representing a possible source of endogenous chemoattractants (Carp *et al.*, 1982).

Compared with other chemoattractant receptors, FPRs exhibit unique properties in the number of variants and the spectrum of ligands they interact with. Although FPRs have been named according to their capability to detect formylated peptides, these receptors can recognize structurally diverse agonists with no obvious common pattern in amino acid sequence or natural origin (Li *et al.*, 2011; Migeotte *et al.*, 2006). Such ligands include C-amidated and unmodified peptides from bacterial and viral pathogens as well as several non-peptide agonists such as annexin 1, resolvin D1 and lipoxin A4 (Yang *et al.*, 2011; Bäck *et al.*, 2014). Moreover, FPRs also detect a wide range of structurally diverse pro and anti-inflammatory ligands associated with important human diseases such as amyloidosis, Alzheimer's disease, HIV, and inflammatory pain (Lee *et al.*, 2008; Mollica *et al.*, 2012; Li *et al.*, 2013).

2.2. The family of formyl peptide receptors

2.2.1. Human Formyl Peptide Receptors

The prototype human FPR (the formyl peptide receptor 1; FPR1) was the first neutrophil receptor to be cloned by functional screening of a cDNA library constructed from differentiated HL-60 myeloid leukemia cells (Boulay *et al.*, 1990a; Boulay *et al.*, 1990b; Perez *et al.*, 1992). Soon after the cloning of FPR1, two additional genes, FPR like-1 (FPRL1) and FPR like-2 (FPRL2), were subsequently cloned from a promyelocyte cDNA library by low stringency hybridization using the FPR1 cDNA as a probe (Bao *et al.*, 1992; Murphy *et al.*, 1992; Ye *et al.*, 1992; Gao and Murphy, 1993). Both FPRL1 and FPRL2 are single copy genes with intronless open reading frames (ORFs), which are co-localized with FPR in a cluster located on chromosomal region 19q13.3 (Bao *et al.*, 1992; Alvarez *et al.*, 1994). Recently, these receptors were renamed as FPR1, FPR2/ALX (Formyl peptide receptor 2-lipoxin receptor, previously FPRL1), and FPR3 (previously FPRL2). FPR1 and FPR2/ALX share 69% identity at the amino acid level (Fig. 5), whereas FPR3 has 56% amino acid sequence identity to human FPR1 and 83% to FPR2 (Ye *et al.*, 2009).

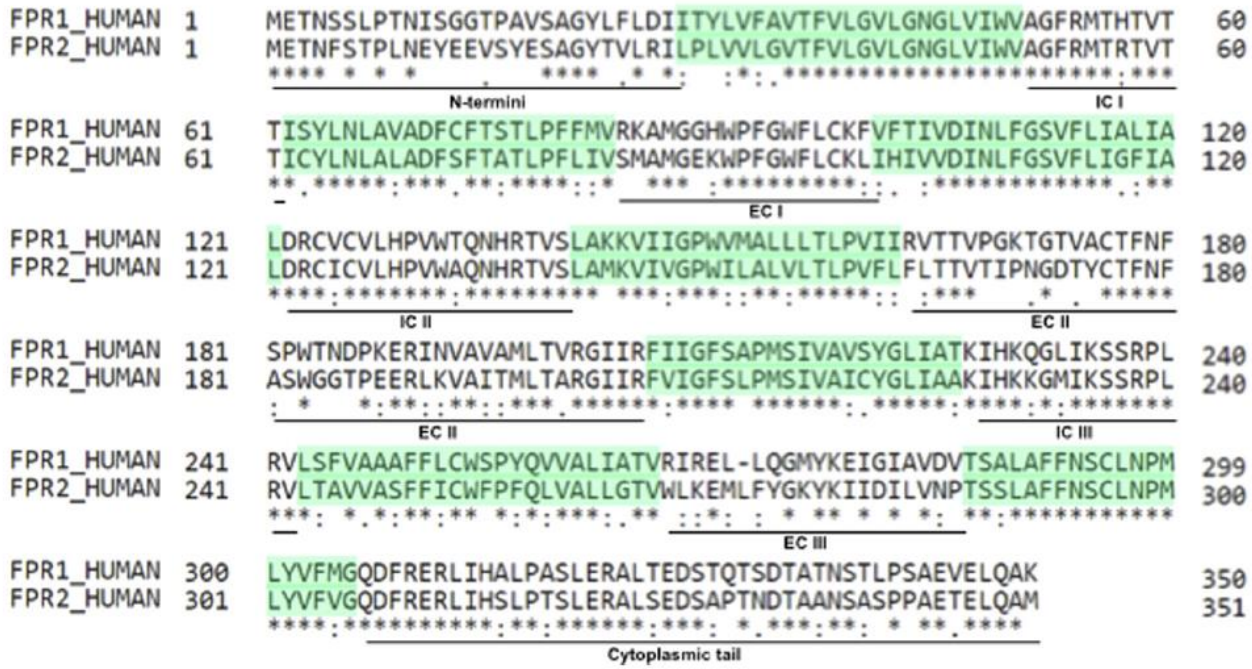

Figure 5: Sequence comparison between FPR1 and FPR2 (from Dahlgren *et al.*, 2016. Biochem Pharmacol. Aug 15(114):22-39)

2.2.1.1. Activating ligands for the human FPRs

The tripeptide fMLF (*N*-formyl-Met-Leu-Phe) derived from *Escherichia coli* is the shortest high-affinity agonist for FPR1 (He and Ye, 2017). On the other hand, FPR2/ALX is a low affinity receptor for fMLF and many potent formyl peptide agonists for FPR1, displaying relatively high affinity for N-formylated peptides of specific composition and longer length (He *et al.,* 2014) such as peptides carrying positive charges at the C-terminus and formyl peptides of microbial origin other than E. coli (e.g. N-formyl-Met-Ile-Phe-Leu (fMIFL) from *Staphylococcus aureus* and N-formyl-Met-Ile-Val-Ile-Leu (fMIVIL) from *Listeria monocytogenes*) (Southgate *et al.,* 2008; He and Ye, 2017). Moreover, other microbial products also activate FPRs such as community-associated methicillin resistant *Staphylococcus aureus* (CA-MRSA)- released PSM (phenol-soluble modulins). These are α-helical peptides composed of 20–25 amino acids secreted in formylated form which are more selective for FPR2 than for FPR1 (Kretschmer *et al.*, 2010; Mader *et al.*, 2010; Forsman *et al.*, 2012). Of note, FPR2/ALX can interact with agonists of various structures, including small proteins, peptides and synthetic molecules, such as serum amyloid A, lipoxin A4, and a substituted quinazolinone Quin-C1 (4-butoxy-*N*-(2-[4-methoxy-phenyl]-4-oxo-1,4-dihydro-2H–uinazolin-3-yl)-benzamide) (He and Ye, 2017). In contrast, FPR3 responds poorly to formyl peptides except fMMYALF (*N*-formyl-Met-Met-Tyr-Ala-Leu-Phe), a hexapeptide derived from mitochondrial NADH dehydrogenase subunits 4 (He and Ye, 2017) and has only a few high-affinity endogenous ligands such as (formylpeptide receptor (FPR)-like (FPRL)-2 ligand) F2L, an acetylated amino-terminal peptide derived from the cleavage of the human heme-binding protein (Migeotte *et al.*, 2005). Among all the synthetic peptide agonists for FPRs, the synthetic hexapeptide WKYMVm (Trp-Lys-Tyr-Met-Val-D-Met-NH_2) was identified to be a strong activator for FPR1, FPR2 and FPR3 (Le *et al.,* 1999; Kang *et al.,* 2005). WKYMVm conjugated with FITC in the second residue Lys was shown slightly more efficacious in binding to FPR2 than to FPR1 (He *et al.,* 2014). WKYMVm is by far the most potent peptide agonist for FPR2, being able to activate FPR2 at picomolar concentrations in chemotaxis assays. WKYMVM, a derivative of WKYMVm with the substitution of L-methionine at the carboxyl terminus, becomes highly selective for FPR2 and is weakly agonistic for FPR3 (Christophe *et al.*, 2001; He and Ye, 2017).

2.2.1.2. Inhibitory ligands for the human FPRs

Several molecules acting as antagonists for FPR have been described. The first FPR antagonists were obtained by modifying the N-formyl group of the classical tripeptide fMLF. Freer et al. substituted this group with tert-butyloxycarbonyl group (t-Boc) and found that the resulting peptide (t-Boc-MLF,

Boc-1) exhibited FPR1 antagonistic properties (Freer *et al.*, 1980). The t-butyloxycarbonyl analog of formylated peptide f-FLFLF (t-Boc-FLFLF, also known as Boc-2) is another antagonistic peptide obtained after the replacement of the MLF sequence with FLFLF (Stenfeldt *et al.*, 2007). These antagonistic peptides potently inhibit neutrophil NADPH-oxidase activity induced by fMLF. However, there is evidence that Boc1 and Boc2, are not receptor-selective when used at higher concentrations, as they then partially inhibit also FPR2 (Stenfeldt *et al.*, 2007). Thus, despite their fairly specific activity at low concentrations, these Boc peptides may be classified as pan-antagonists for FPRs (He and Yee, 2017). Other Boc-related compounds were tested for their human neutrophil FPR-selective agonist or antagonist activities by evaluating receptor binding, superoxide anion release, and cell adhesion (He and Yee, 2017). For example, the t-butyloxycarbonyl (t-Boc) peptide derivative t-Boc-Phe-D-Leu-Phe-D-Leu-Phe (BocPLPLP) is able to block the fMLP activation of, both human and rabbit phagocytes, through competitive binding of the antagonist to FPR (Wenzel-Seifert et al.,1998). Moreover, a series of amino-terminal carbamate analogues of the peptide Met-Leu-Phe (MLF) have been synthesized and their activity tested in human neutrophils. Among them, branched carbamates (iso-butyloxycarbonyl, tert-butyloxycarbonyl, and benzyloxycarbonyl) have been described act as FPRs antagonists.

With respect to FPR1 antagonists, the cyclic undecapeptide cyclosporine H (CysH), derived from peptide metabolites of the fungus *Tolypocladium inflatum*, is the most potent and selective (Wenzel-Seifert and Seifert, 1993; de Paulis *et al.*, 1996; Cevik-Aras *et al.*, 2012). It is a potent inhibitor of fMLP-induced superoxide anion formation in human neutrophils and is much more effective than other well-known FPR antagonists (Wenzel-Seifert and Seifert, 1993). CysH have been shown to be specific to FPR1 only at low concentrations, showing also a blocking effect on FPR2 signaling at high concentrations (>2.5 μM) (Stenfeldt *et al.*, 2007). In a ligand screen of hexapeptide libraries, several peptides were found to inhibit the binding of the FPR2 agonist WKYMVm. The peptide WRWWWW(WRW4) is the most potent of these peptides with FPR2-selective antagonistic activity (Bae *et al.*, 2004; Shin *et al.*, 2006). In addition, the WRW4 peptide blocked the activation of FPR3 by F2L in FPR3-transfected HEK293 cells (Shin *et al.*, 2006) and F2L-induced response in mature monocyte-derived dendritic cells, which express FPR3 but no other FPRs (Ye *et al.*, 2009; He and Ye, 2017). Recently other selective FPR inhibitors have been discovered (He and Ye, 2017). Among them, the rhodamine-conjugated, gelsolin-derived peptide (PBP10) and Pam-(Lys-βNSpe)6-NH2, a lipidated α-peptide/β peptoid oligomer, have been identified as a potent inhibitor of FPR2, being able to reduce the pro-inflammatory activity of human neutrophils by selective inhibition of signaling through this receptor (Forsman *et al.*, 2012; Skovbakke *et al.*, 2015; He and Ye, 2017). Quin-C1 (4-butoxy-N-[2-(4-methoxy-phenyl)-4-oxo-1, 4-dihydro-2H-quinazolin-3-yl]-benzamide) and Quin-C7

(4-butoxy-N-[2-(4-hydroxyphenyl)-4-oxo-1,2-dihydroquinazolin-3-yl]-benzamide) quinazolinone derivative compounds were found to be highly selective for FPR2 as opposed to FPR1 (Zhou *et al.*, 2007; He *et al.*, 2011). Moreover, bacteria also produce molecules that can block FPRs such as the staphylococcal chemotaxis-inhibitory protein of *S. aureus* (CHIPS) and the FPR2/ALX-inhibitory protein (FLIPr) (Dürr *et al.*, 2006). CHIPS blocks formylated peptide recognition by the human FPR1 (de Haas *et al.*, 2004), while FLIPr is a specific inhibitor for human neutrophil FPR2/ALX (Prat *et al.*, 2006).

2.2.1.3. Human FPR expression

Human FPRs are expressed in many cell types (Lee *et al.*, 2001). FPR1 and FPR2 are expressed both in myeloid cells including monocytes/macrophages, neutrophils and immature dendritic cells (DCs) and in non-immune cell types such as endothelial cells, hepatocytes, astrocytes, microglial cells, and fibroblasts (Ye *et al.*, 2009; Cattaneo *et al.*, 2010; Lee *et al.*, 2017). The expression of human FPR3 is more restricted, being detected in monocytes/macrophages and DCs, but not in neutrophils (Durstin *et al.*, 1994; Dorward *et al.*, 2015). FPR3 is also expressed in plasmacytoid DCs and in donor-specific NK cells (Kim *et al.*, 2009). Generally, FPRs are expressed on the cell surface, where they recognize soluble ligands (Venkatakrishnan *et al.*, 2013; Lee *et al.*, 2017). However, FPR3 has shown a unique, constitutive intracellular localization (Rabiet *et al.*, 2011). In addition, activation of FPR2 by WKYMVm induces receptor internalization (Christophe *et al.*, 2001), indicating that FPRs can be present not only on the cell membrane, but also in the cytosol or the nucleus (Lee *et al.*, 2017). The genes encoding for FPR members are comprised in a large family with a seemingly complex evolutionary history characterized by positive selection (Yang and Shi, 2010). Orthologs of human FPR genes have been identified in other mammalian species, including other primates, rabbits, and mice, but also in rats, guinea pigs, dogs, and horses. However, despite overall sequence homology, these genes vary considerably between humans and other species in their numbers, sequences and in their functional profiles of agonist/antagonist recognition (Muto *et al.*, 2015).

2.2.2. The Mouse Formyl Peptide Receptors

The mouse Fpr gene family contains at least eight members, including Fpr1, Fpr2, Fpr-rs1, Fpr-rs3, Fpr-rs4, Fpr-rs5, Fpr-rs6, Fpr-rs7, and Fpr-rs8 (Ye *et al.*, 2009; He *et al.*, 2013), clustered on mouse chromosome 17A3.2. The first three genes (mFpr1, mFpr2, and mFpr-rs1/mFpr3) are widely expressed in mouse phagocytic leukocytes and exhibit high sequence homology to the human FPRs. The sequence similarities between FPR1 and Fpr1 (72% identity, Fig. 6A and B) and the fact that the prototype peptide FPR1 ligand fMLF is an activating agonist for mFpr1 (He *et al.*, 2013), suggest that this receptor is the FPR1 ortholog. The murine FPR-rs1 (now named Fpr3) and FPR-rs2 (now named Fpr2) are structurally similar to FPR2 and overlap with the monocyte-expressed FPR3 (He and Ye, 2017; Winther *et al.*, 2018). Published reports indicate that other members of the murine mFpr family are not coding for stereotypic formyl peptide receptors such as mFpr-rs8 which has been characterized as a constitutively expressed gene associated with mouse longevity (Tiffany *et al.*, 2011) and mFpr-rs1, mFpr-rs3, mFpr-rs4, mFpr-rs6, and mFpr-rs7 gene products that are recently described as mouse vomeronasal sensory receptors (Liberles *et al.*, 2009; Riviere *et al.*, 2009; Takano *et al.*, 1997). Murine Fprs tissue distribution profile also resembles that of the human FPRs, showing high similarity to their human counterparts (Gao and Murphy, 1993; Hartt *et al.*, 1999; He *et al.*, 2013).

Figure 6: Sequence comparison between the human FPR1 and the mouse Fpr1 (A) and between the human FPR2 and the mouse Fpr2 (B) (modified from Dahlgren *et al.*, 2016. Biochem Pharmacol. Aug 15(114):22-39)

2.2.2.1. Activating ligands for the murine FPRs

mFrp1 is an ortholog of the human FPR1, and both are high affinity receptors for *N*-formyl peptides. Major differences between these two receptors are that mFpr1 displays relatively low affinity for the *E. coli*-derived fMLF, and that it responds well to the synthetic molecule Quin-C1, whereas the human FPR1 does not (Nanamori *et al.*, 2004). mFpr1 shares the latter property with human FPR2/ALX. This difference in affinity to fMLF is attributed to alterations in the folding of the

transmembrane and extracellular domains, as determined by the apposition of multiple noncontiguous residues (Gao and Murphy, 1993). mFPR1 responds well to several N-formyl peptides originating from other bacteria (i.e., *S. aureus*, fMIFL; *L. monocytogenes*, fMIVITLF) and mitochondria (i.e., fMMYALF). The second mouse receptor, mFpr2, is believed to be a low-affinity receptor for formyl peptides (Hartt *et al.*, 1999), suggesting that its native ligands may not be formylated peptides. Longer formyl peptides, are better agonists for this receptor, even if the sequence as well as side chains of C-terminal residues in these peptides are more crucial than their length for binding to mFpr2 (He *et al.*, 2013). This receptor responds to endogenous peptide agonists for FPR2, including the amyloidogenic proteins SAA (serum amyloid A) and Aβ42 (Liang *et al.*, 2000; Tiffany *et al.*, 2001). Small molecules like the nitrosylated pyrazolone derivative compound 43 and Quin-C1 and the synthetic hexapeptide WKYMVm also activate both mFpr1 and mFpr2 with similar potency, even if mFpr1 is the preferred receptor (Winther *et al.*, 2018) (Fig. 7). For agonist recognition, there are some similarities between FPR2 and Fpr2 sharing 76% sequence similarity (Fig. 6B). For this reason, the highly selective FPR2 agonist, WKYMVM, a derivative of WKYMVm with the substitution of L-methionine at the carboxyl terminus, activates also mFpr2. Moreover, *S. aureus*-derived PSMα peptides activate also murine neutrophils, and Fpr2 is the preferred receptor (He and Ye, 2017; Winther *et al.*, 2018).

No highly specific agonist has been described that is selectively recognized by the third mouse receptor, Fpr3/mFpr-rs1, expressed in phagocytes. mFpr-rs1 shares similar structural features with mFpr2 and mFpr1 but differs in its unique intracellular distribution profile. Unlike mFpr1 and mFpr2, which are found mostly on cell surface, this receptor has limited cell surface expression, a property shared with the human FPR3 (He *et al.*, 2013). Therefore, Fpr3/mFpr-rs1 may be an ortholog of human FPR3. A ligand for human FPR3, F2L, has been shown to activate mFpr2, suggesting an overlapping feature of mFpr2 and human FPR3 (Gao *et al.*, 2007).

	FPR1	FPR2	Fpr1	Fpr2
Agonist				
fMIFL	+++[3] (≈0.1[4])	–	++ (≈3)	–
fMLF	++ (≈50)	–	+ (≈2000)	–
H2-M3 peptides	+++ (≈5)	–	++ (≈1–25)	–
WKYMVM	–	++ (≈40)	–	+ (≈400)
WKYMVm	+	+++ (≈2)	+	++
MMK-1	–	+ (≈200)	–	–
PSMα2	–	++ (≈50)	–	++ (≈10)
$F2Pal_{16}$	–	+ (≈500)	–	++ (≈300)
$F2Pal_{10}$	–	+ (≈300)	–	+
Lau-[(S)-Aoc]-(Lys-βNphe)$_6$-NH_2	–	+ (≈150)	–	++ (≈100)
Antagonist				
CysH	+++	–	–	–
Boc2	++	–	+	–
WRWWWW	–	++	–	+
PBP_{10}	–	+++	–	–
$F1Pal_{16}$[5]	–	++	–	++
Lau-(Lys-βNSpe)$_6$-NH_2	–	+ +	–	++

Figure 7: Effects of most studied human FPR ligands in modulating (activating by agonist and inhibiting by antagonist) human and mouse neutrophil (modified from Winther *et al.*,2018. Basic Clin Pharmacol Toxicol. Feb;122(2):191-198)

2.2.2.2. Inhibitory ligands for the murine FPRs

Little is known about the inhibitory potency or profile of FPRs antagonists in relation to the murine receptors. Boc-1 and Boc-2 peptides have been suggested to inhibit primarily Fpr2, although Boc-1 is a poor inhibitor of Fpr-induced signaling in murine neutrophils and Boc-2 is primarily an Fpr1 antagonist (Stenfeldt *et al.*, 2007; Winther *et al.*, 2017). As in the human model, Boc-1 and Boc-2 peptides are not receptor-selective when used at high concentrations, as they then partially inhibit also Fpr2 (Stenfeldt *et al.*, 2007). In fact, Boc-2 peptide together with the lipidated peptidomimetic Lau-(Lys-βNSpe)$_6$-NH_2 ligand, were recently found to be selective inhibitors for Fpr1 and also for Fpr2 (Skovbakke *et al.*, 2016). In contrast to Lau-(Lys-βNSpe)$_6$-NH_2, the analogue displaying a four carbon longer fatty acid (i.e. Pam-(Lys-βNSpe)$_6$-NH_2), which is a strong FPR2 antagonist is not selective in inhibiting Fpr2 (Skovbakke *et al.*, 2016). The FPR2-selective peptide inhibitor WRW4 is active also against Fpr2 (Skovbakke *et al.*, 2016; Winther *et al.*, 2017). Moreover, the two most potent and selective inhibitors of FPR-mediated activities in human neutrophils (cyclosporin H and PBP10) do not possess any inhibitory effects on murine neutrophils when activated by the Fpr-selective peptides (Stenfeldt *et al.*, 2007) (Fig. 7).

2.3. FPR signaling and regulation in neutrophils

FPRs belong to the superfamily of G protein-coupled receptors (GPCRs), containing seven transmembrane (7TM) domains with an extracellular domain and an intracellular C-terminus tail joined together by three intracellular and three extracellular loops (Fig.8).

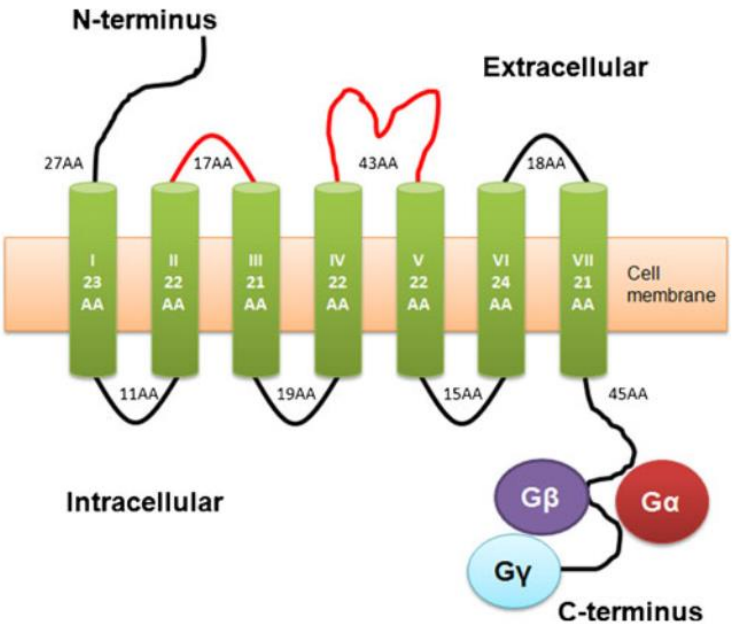

Figure 8: Schematic diagram of FPR1 (from Li and Ye, 2013. J Mol Med (Berl). Jul;91(7):781-9)

The study of FPRs signaling pathways revealed a highly complex and integrated chain of intracellular signaling events (Prossnitz and Ye, 1997; Rabiet *et al.*, 2007). These receptors signal through coupling to heterotrimeric guanine nucleotide-binding proteins (G-proteins) that are composed of an α-subunit and a heteromeric βγ-complex. There are four main α-subunits ($G_{\alpha S}$, $G_{\alpha 12}$, $G_{\alpha q}$ and $G_{\alpha i/0}$) and they can be combined with at least 5 different β-subunits and 12 different γ-subunits. Characterization of the FPRs signaling cascades has been conducted using human FPR1 as a receptor model and fMLF as its stereotypical ligand (Prossnitz and Ye, 1997) The activation of FPR1 induces a complex pattern of intracellular signals resulting in the regulation of several immune responses, such as chemotactic migration, ROS production and neutrophil degranulation (Dorward *et al.*, 2015) (Fig. 9). Agonist binding to FPR1 results in G_i-type G-protein activation, with the exchange of GDP for GTP inducing the dissociation of the α subunit from the βγ subunits. Alpha subunits, which are inactive in their GDP-bound state, become activated when they are separated from the βγ-complex in the GTP-bound form. Upon ligand binding, the conformational change of the agonist-occupied receptor initiates the GDP/GTP exchange, which results in dissociation of the βγ-complex from the α-subunit (Bokoch and Gilman, 1984; Gierschik *et al.*, 1989; Rabiet *et al.*, 2007). Once activated, the dissociated $G\alpha_i$-protein subunits, but also the βγ -complex, activate multiple downstream second messengers. Activation of phospholipase C beta (PLCβ) is an early signal initiated by the activated α and βγ complexes that secondarily generates further downstream messengers produced during hydrolysis of the membrane-bound phosphoinositol-4,5-bisphosphate (PIP_2), giving rise to diacylglycerol (DAG) and Inositol 1,4,5-triphosphate (IP_3). IP_3 mediate the release of intracellular

calcium stores, principally from the endoplasmic reticulum and DAG activates protein kinase C (PKC), a kinase associated with the activation of superoxide-generating NADPH-oxidase in neutrophils (Flaherty *et al.*, 1990; Dorward *et al.*, 2015). Meanwhile, the phosphoinositide 3-kinase gamma (PI3Kγ)-mediated conversion of PIP2 to phosphoinositol-3,4,5-trisphosphate (PIP_3) acts as the principal regulator of neutrophil cytoskeletal reorganization and respiratory burst after FPR1 activation and influences the chemotactic response (Rabiet *et al.*, 2007; Heit et al., 2008). Moreover, PI3K activation phosphorylates and activates Protein kinase B (PKB, also known as Akt), a serine/threonine protein kinase that functions as a critical regulator of cell survival and proliferation. This protein is also involved in the signaling pathways in response to growth factors and other extracellular stimuli to regulate several cellular functions including nutrient metabolism, cell growth, apoptosis and survival (Song *et al.*, 2005; Yu and Cui, 2016). As the βγ -complex activates PI3Kγ and pulls it toward the plasma membrane, the activity of Src-like tyrosine kinases increases with the phosphorylation of the Src homology 2 domain-containing (Shc) adaptor protein, which, in turn, increases its association with growth factor receptor-bound protein 2 and subsequently activates mitogen-activated protein kinase (MAPK) signaling pathways. Two groups of MAPK cascades, the extracellular signal-regulated kinases (ERKs) and p38 kinases are stimulated in polymorphonuclear cells (PMN) by FPRs agonists (Worthen *et al.*, 1994; Avdi *et al.*, 1996; Pillinger *et al.*, 1996; Krump *et al.*, 1997). ERKs participate in PMN adherence and oxidative metabolism whereas p38 kinases are involved in PMN adherence, chemotaxis and respiratory burst activation (Avdi *et al.*, 1996; Pillinger *et al.*, 1996). On the other hand, the activation of guanine-nucleotide exchange factors (GEFs) activate small G-proteins of the Rho family (Rho, Rac, Cdc42), which are key regulators of several cellular functions such leukocyte adhesion and transmigration, actin polymerization, phagocytosis and NADPH-oxidase-dependent ROS production (Ye *et al.*, 2009; Morris *et al.*, 2011).

FPRs can be activated by distinct peptide ligands, leading to differential cellular signaling pathways and different functional consequences (Amatruda *et al.*, 1995; Cattaneo *et al.*, 2013; Dorward *et al.*, 2015). For example, the specific FPR2 agonist WKYMVm (Trp-Lys-Tyr-Met- Val-D-Met) induces in eosinophils ERKs phosphorylation and superoxide production via a PI3K-mediated ERKs pathway thereby activating chemotaxis, mobilization of complement receptor-3, cytokine release and activation of NADPH oxidase (Shin *et al.*, 2005). For WKYMVm and its analogs, cytosolic calcium increase is induced by the hydrolysis of phosphatidylinositol through PLCβ activation, whereas ERK and Akt phosphorylation is mediated by the activation of MEK and PI3K, respectively (Bae *et al.*, 2003). Differently, other FPRs agonists trigger phagocyte functions through the activation of complex alternative FPRs signalling pathways (Amatruda *et al.*, 1995; Cattaneo *et al.*, 2013; Dorward *et al.*, 2015).

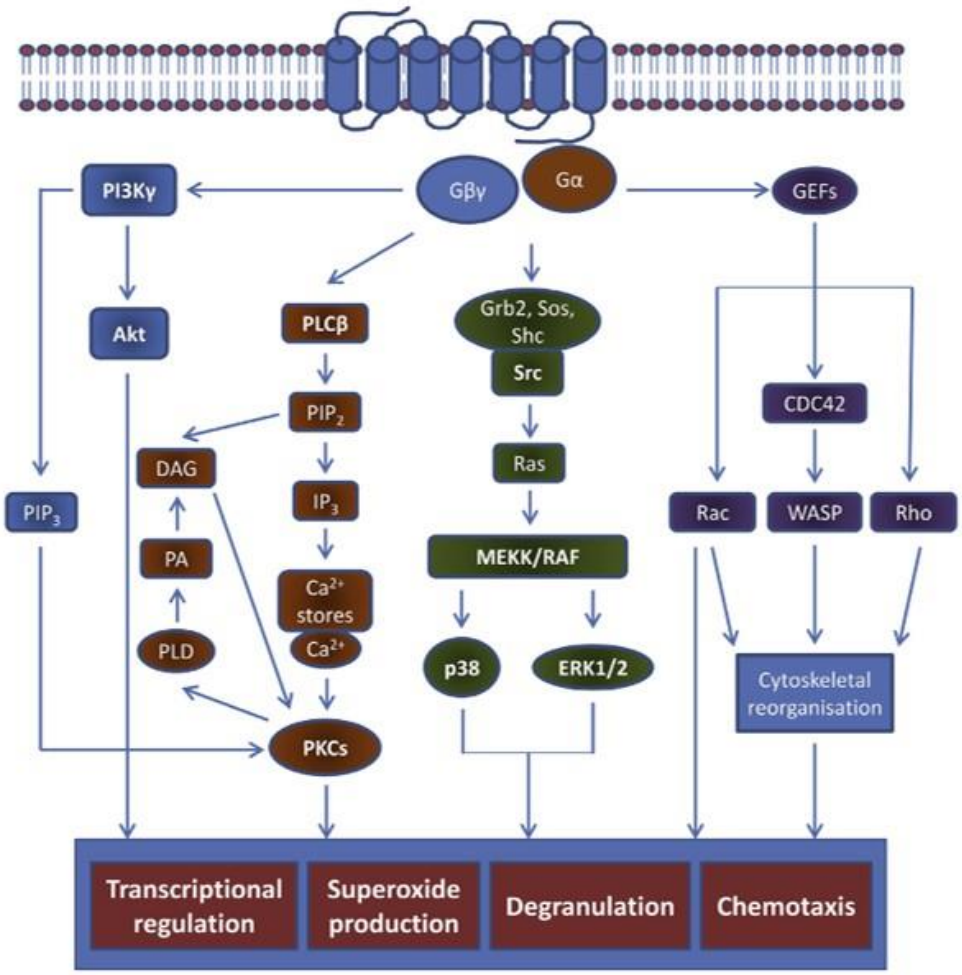

Figure 9: Intracellular signaling events after FPR1 receptor activation (from Dorward *et al.*, 2015. Am J Pathol. May; 185(5): 1172–1184)

CHAPTER 3: Neutrophils as a source of chemokines

3.1. Characteristics and biological activities of chemokines

Leukocyte recruitment at the sites of infection is a crucial step in the progression and resolution of inflammation. This process, as previously described, is tightly regulated and is dependent on the presence of chemotactic factor gradients. Pivotal to the process of leukocyte chemoattraction are the chemokines, a large family of chemotactic small proteins, typically ranging from 8 to 17 kDa. These proteins represent a very ancient system of cellular communication that has undergone extensive refinement over evolutionary time. In phylogenetic terms, directed cellular movement in response to external stimuli is, in fact, an ancient biologic response. In primitive single-cell organisms it was frequently used as a foraging mechanism, while in multicellular organisms chemotaxis is an essential property of cells at different stages of development and plays an essential role in processes such as coordination of both innate and adaptative immune system responses, in regulating B- and T- cell development and in modulating angiogenesis and wound healing (Rossi and Zlotnik, 2000; Mantovani *et al.*, 2011).

The superfamily of chemokines is subclassified on the basis of the number and arrangement of cysteine residues located in the N-terminal region of these molecules. These are designated C, CC, CXC, and CXXXC, where C represents the number of N-terminal region cysteine residues and X represents the number of intervening amino acids. The largest family is composed by 28 CC chemokine family members possessing two adjacent cysteines in the vicinity of the N-terminus of the mature peptide. The 16 CXC family members, instead, share two cysteine residues with an interposed amino acid. In both families, the first cysteine forms a disulfide bond with the third, and the second with the fourth cysteine. Due to the conserved disulfide bonds, tertiary structures of chemokines are similar (Yung and Farber, 2013). CXC chemokines are characteristically chemotactic for neutrophils and lymphocytes, whereas CC chemokines act upon the majority of leukocytes, but generally have little activity on neutrophils (Yung and Farber, 2013). CXC chemokines can be further subdivided into ELR and non-ELR types based on the presence/absence of a triplet amino acid motif (Glu-Leu-Arg) that precedes the first cysteine amino acid residue of the primary structure of these chemokines. The presence of this motif imparts angiogenic function to this class of chemokines (e.g. CXCL1, CXCL8, and CXCL7), while the ELR-negative chemokines (e.g. CXCL9, CXCL10, and CXCL11) are typically inducible by IFNγ and have angiostatic properties (Keane *et al.* 1998; Strieter *et al.*, 2002).

3.2. Chemokine receptors

Chemokine receptors (CKRs) are G protein-coupled receptors containing seven transmembrane hydrophobic domains with three intracellular and three extracellular hydrophilic loops. A potentially glycosylated extracellular amino-terminal region is involved in chemokine binding, while the intracellular carboxy-terminal region is involved in G-protein linking and is subject to regulatory phosphorylation (Huskens *et al.*, 2007). Upon ligand binding, chemokine receptors undergo a conformational change that enables activation of the heterotrimeric G protein by GDP to GTP exchange, resulting in the dissociation into Gα-GTP and a Gβγ dimer. As previously described for FPRs, this event leads to a cascade of phosphorylation events involving a series of kinases and small GTPases (e.g., Ras and Rho) that ultimately effect cellular functions such as adhesion, chemotaxis, degranulation, and respiratory burst (Luttrell *et al.*, 1997). After ligation, chemokine receptors may be internalized and then degraded or recycled, leaving the membrane temporarily unresponsive to further ligand stimulation (Bunemann and Hosey, 1999; Ferguson *et al.*, 1998). The subfamily of chemokine receptors consists of about twenty GPCRs, most of which display canonical signalling via the activation of heterotrimeric G-proteins, although some of them, referred to as atypical chemokine receptors, are devoid of G-protein-dependent signalling (Bonecchi and Graham, 2016). Chemokines are redundant in their action on target cells and promiscuous in receptor usage, with few receptors only binding a single ligand and several chemokines binding to more than one receptor (Fig. 10). However, closer inspection of the receptors and their ligands suggests that they can be broadly categorized into constitutive (developmentally regulated) or inducible (inflammatory) CKRs. This division should not be regarded as absolute but rather as a rule with some exceptions. For example, CCR6 is constitutively expressed in immature dendritic cells and in T cells, but it is downregulated as dendritic cells mature. However, it is upregulated in skin lesions (Proudfoot, 2002). CCR8 is constitutively expressed in the thymus but is upregulated during T-cell activation and is a marker of TH2 cells. Lastly, CCR7 is upregulated during the antigen recognition process, a requisite for immune surveillance, and it is upregulated as dendritic cells mature (Proudfoot, 2002).

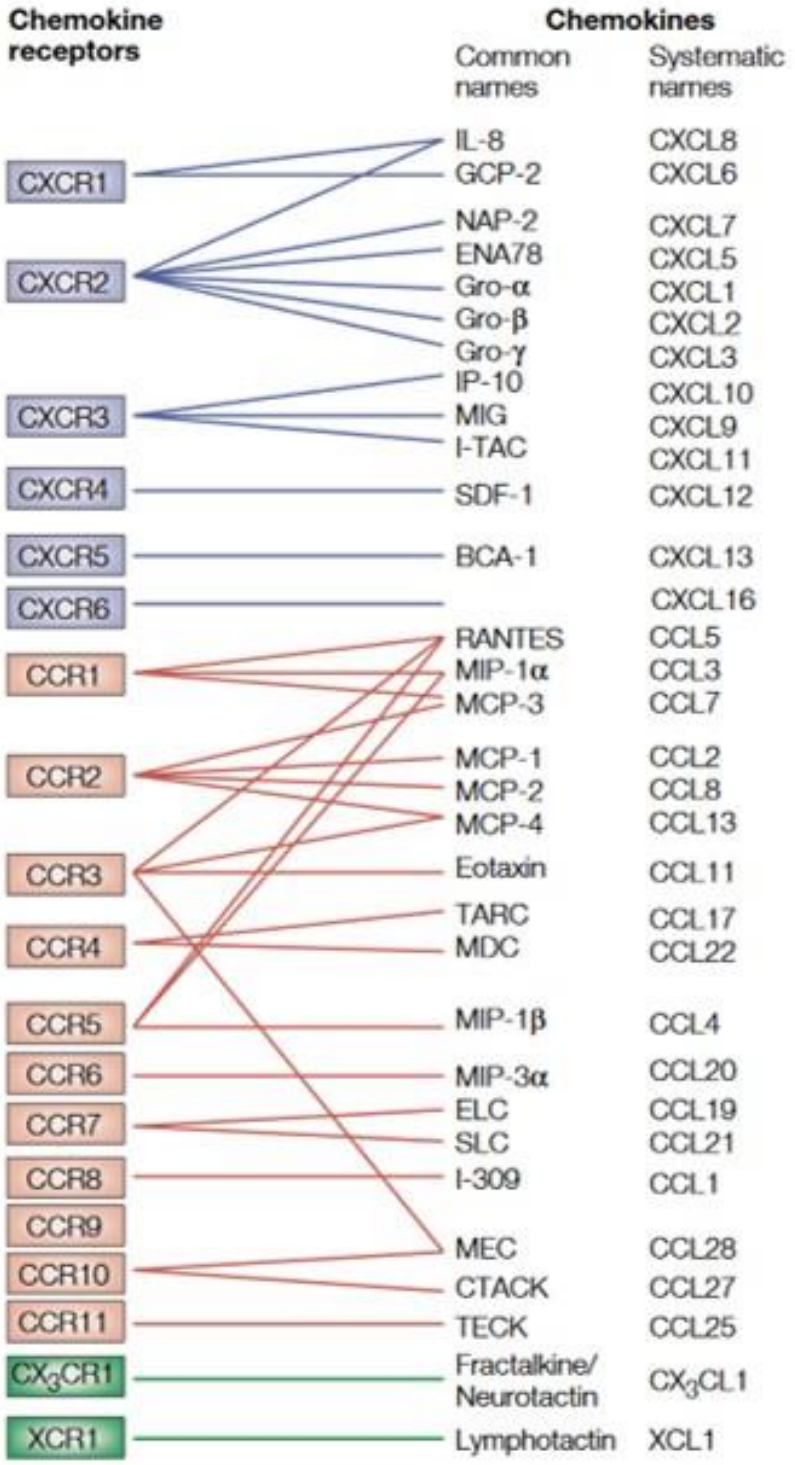

Figure 10: Chemokine receptors and their ligands (from Proudfoot, 2002. Nat Rev Immunol. Feb;2(2):106-15)

Chemokine receptors are expressed in many cell types, including epithelial cells of the skin, digestive and reproductive tracts, and neuronal and glial cells of the central nervous system (Chensue, 2001), suggesting that some chemokines have organ-specific functions. Moreover, analysis of chemokine receptor expression by leukocytes has revealed that receptor subtypes are expressed to differing degrees by different cell types, thereby dictating their responsiveness to the various chemokines. For example, neutrophils strongly express CXCR1 and CXCR2, making them most responsive to ELR_ CXC ligands, whereas eosinophils appear more responsive to CCR3 ligands (Chensue, 2001).

3.3. Chemokine control of neutrophil innate responses during acute inflammation

Neutrophils are the most abundant nucleated cells in the blood and are the main body's guardians against bacterial and fungal infections due to their ability to kill microorganisms intracellularly, by means of phagocytosis, or extracellularly after the release of an arsenal of antimicrobial products. Although they mostly receive attention for their phagocytic and killing abilities, neutrophils can perform a variety of other important functions, including tissue remodeling, antigen presentation, recruitment of other leukocytes and polarization of T cell responses (Tvinnereim *et al.*, 2004; Megiovanni *et al.*, 2006; Nathan, 2006; Appelberg, 2007; Beauvillain *et al.*, 2007; Pesce *et al.*, 2008). Neutrophils are produced in the bone marrow and are rapidly recruited from the blood into tissues in response to a variety of chemoattractants released from damaged cells or actively produced by resident cells during inflammation (Baggiolini,1998; Scapini *et al.*, 2000). Neutrophils persist in the periphery for only about 6 h. Therefore, increased release of neutrophils from the bone marrow as well as increased myelopoiesis are necessary to sustain normal neutrophil numbers during an infection. During inflammation, systemic levels of granulocyte-colony stimulating factor (G-CSF) lead to decreased CXCL12 production by the bone marrow and to decreased CXCR4 expression by developing neutrophils (Semerad *et al.*, 2002). In addition to G-CSF, LPS also induces neutrophilia, which is caused by down-regulation of CXCR4 expression on neutrophils (Kim *et al.*, 2007). Loss of CXCR4-mediated retention and the presence of CXCR2-mediated exit signals promote the release of neutrophils into the blood (Kim *et al.*, 2006; Eash *et al.*, 2010). Once in the peripheral blood, neutrophils migrate to inflammation sites by first interacting with and then transmigrating across the activated endothelium. Endothelial cells are activated by local production of inflammatory cytokines, such as TNF, IL-1, and IL-17, and this activation leads to activation-induced expression of P-selectin, E-selectin, and integrins. Neutrophil rolls along the endothelium and adhere on it when they bind to CXCL1, CXCL2, or CXCL8 on the luminal surface of the endothelium (Sokol and Luster, 2015). These chemokines can be directly produced from cytokine-activated endothelial cells or distantly

produced chemokines can reach the basal surface of the endothelium and transcytose to the luminal surface. Once they have transcytosed, neutrophils follow multiple chemoattractant gradients as they migrate through the interstitium to the sites of infection. The different classes of chemoattractants can amplify immune cell recruitment to the site of tissue injury by acting sequentially in time as well as in space (Griffith *et al.*, 2014). Although chemoattractant molecules can promote and amplify cellular migration, different forms of chemokines can also play distinct roles in migration. A major role in this process is played by chemokines of the CXCL8 family, which all bind to the CXCR1 and CXCR2 receptors and share a common ELR+ (glutamic acid–leucine–arginine) motif in their structure (Russo *et al.*, 2014). In humans, CXCL8 (IL-8) is believed to play a preeminent role in neutrophil recruitment. A murine ortholog of CXCL8 does not exist, although many members of the CXCL family have been identified. The murine chemokines CXCL1 (formerly named KC) and CXCL2 (or MIP-2) are highly homologous, bind to the same receptor (Cxcr2) and are believed to be functionally similar to human CXCL8 (Bozic *et al.*, 1994).

Activation of Cxcr2, a G-protein coupled receptor (or GPCR), by CXCL1/2 leads to downstream signaling through the vasodilator-stimulated phosphoprotein (VASP), phosphoinositide 3-kinase (PI3K) and Rac, which localize asymmetrically along the cell and orchestrate the directed migration of neutrophils (Neel *et al.*, 2009; de Oliveira *et al.*, 2016). Both resident cells (including macrophages, epithelial and endothelial cells) and inflammatory leukocytes can produce CXCL1/2 in in the presence of injury and/or infection. The administration of CXCL1/2 induce a dose- and time-dependent increase in neutrophil rolling, which is significantly inhibited by antibodies against P-selectin, suggesting that these chemokines induce P-selectin-dependent rolling (Zhang *et al.*, 2001). Interestingly, these chemokines have the ability to bind glycosaminoglycans in the extracellular matrix, thus generating a chemo-attractant gradient over distances of hundreds of µm along tissues which amplify the initial acute inflammatory response by promoting the influx of additional immune cells (Tanino *et al.*, 2010; Sawant *et al.*, 2016).

CHAPTER 4: *Streptococcus agalactiae* (Group B streptococcus, GBS)

4.1. Group B Streptococcus: a common commensal capable of causing severe infections

Streptococcus agalactiae (also known as group B streptococcus [GBS]) is an encapsulated Gram-positive bacterium typically found in the human gastrointestinal or lower genital tract of up to 30% of asymptomatic individuals (Verani *et al.*, 2010). Due to its ability to invade host tissues, GBS is a major causative agent of systemic infections such as pneumonia, septicaemia, and meningitis in susceptible hosts including neonates and parturient women (Edmond *et al.*, 2012; Le Doare and Heath, 2013). Indeed, this bacterium is a leading cause of sepsis and meningitis in the neonate, with a reported incidence of 0.4-0.6 per 1,000 live births (Edmond *et al.*, 2012). Neonatal disease occurring during the first 6 days of life (referred to as early-onset disease or EOD) is likely due to transmission of the bacterium from the pregnant mother to the neonate secondary to aspiration of infected amniotic fluid or vaginal secretions during delivery, followed by bacterial translocation across the respiratory epithelium and subsequent systemic infection (Edwards and Baker, 2005; Maisey *et al.*, 2008; Rajagopal, 2009; Verani *et al.*, 2010) (Fig. 11). Most cases of EOD are, in fact, characterized by pneumonia followed by septicaemia (Lin and Troendle, 2006). Late-onset disease (LOD), occurring from age 7 days to 3 months, also consists of septicemia, but displays a higher rate of meningitis, compared with EOD (Luan *et al.*, 2005; Bohnsack *et al.*, 2008; Manning *et al.*, 2009). For LOD, the mode of transmission and the infection route are poorly understood. A plausible scenario would involve early intestinal colonization by GBS that would lead in the first days of life to its intraluminal intestinal multiplication, translocation across the intestinal epithelium, and access to the bloodstream. Once translocated in the bloodstream, GBS has the ability to cross the blood–brain barrier (BBB) and cause meningitis. LOD may develop also through vertical transmission from mother to neonate, nosocomial transmission, contaminated breast milk or prematurity (Rajagopal, 2009; Le Doare and Kampmann, 2014; Zimmermann *et al.*, 2017). GBS has also been associated with high rates of invasive diseases which include arthritis, endocarditis, pneumonia, bacteremia and urinary tract infections, as well as soft tissue, skin, and bone infections in nonpregnant adults especially in elderly and immunocompromised individuals (Schuchat, 1998; Farley, 2001; Phares *et al.*, 2008; Sendi *et al.*, 2008; Skoff *et al.*, 2009).

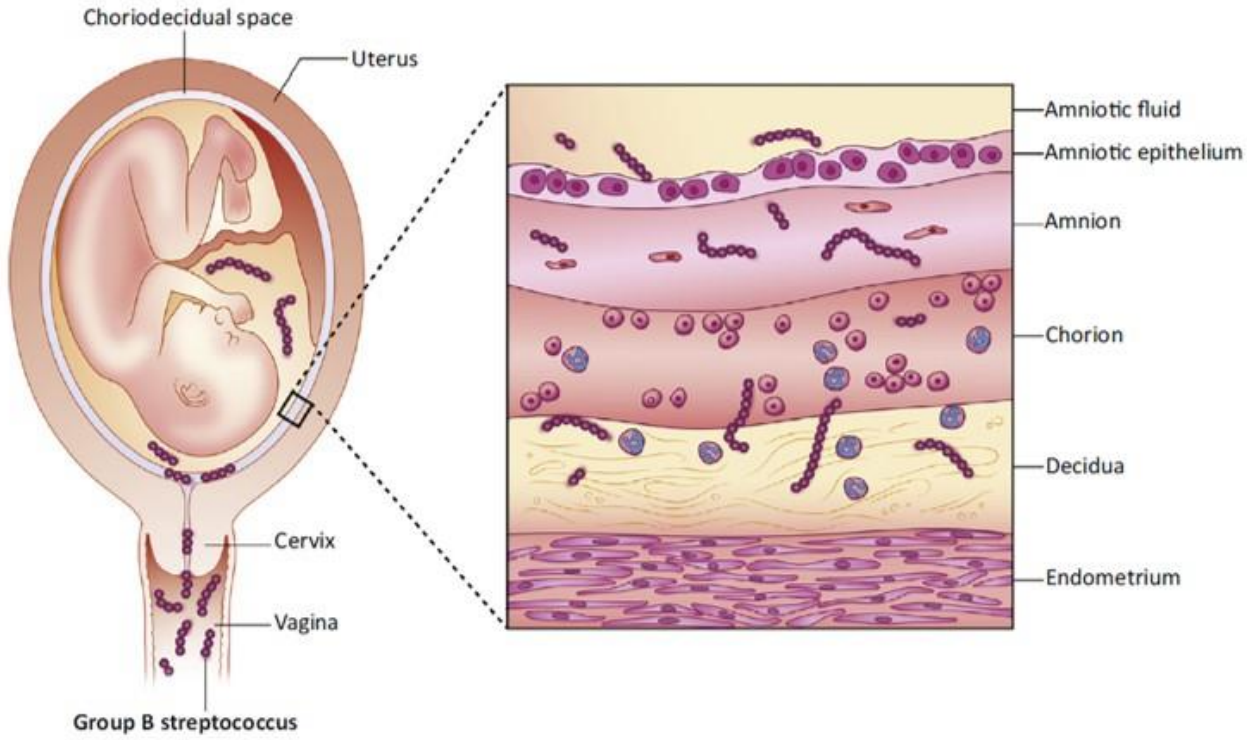

Figure 11: Ascending Group B Streptococcus (GBS) Infection (from Vornhagen *et al.*, 2017. Trends Microbiol. 2017 Nov; 25(11):919-931)

4.2. GBS colonization and virulence factors

On the basis of the antigenic and structural properties of the capsular polysaccharide (CPS), which the microorganism can express at high levels on its surface, Group B Streptococci can be classified into 10 different serotypes (Ia, Ib, II, III, IV, V, VI, VII, VIII, and IX) (Hickman *et al.*, 1999; Zaleznik *et al.*, 2000; Kong *et al.*, 2002; Kong *et al.*, 2005; Slotved *et al.*, 2007). The capsule represents the major virulence factor, which helps GBS evade host defence mechanisms by protecting bacteria from opsonization thereby interfering with phagocytic clearance (Avci and Kasper, 2010; Chen *et al.*, 2013). Epidemiological data collected worldwide have shown that the majority of invasive neonatal diseases are associated with the serotype III (Musser *et al.*, 1989; Poyart *et al.*, 2008; Edmond *et al.*, 2012). In addition to serotyping, GBS can be further classified by multilocus sequence typing (MST), with more than 700 identified sequence types (ST). GBS strains with the same ST may have different serotypes (Jones *et al.*, 2003). Several STs are then grouped into clonal complexes (CC) when sharing six or seven matching alleles (Jones *et al.*, 2003). Among these, one highly homogenous clone belonging to the CC17 clonal complex is isolated in the majority of LODs and is referred to as the "hypervirulent" GBS (Musser *et al.*, 1989; Lamy *et al.*, 2006; Phares *et al.*, 2008; Poyart *et al.*, 2008; Edmond *et al.*, 2012).

This “hypervirulence” is related to the acquisition of an arsenal of virulence factors that enable bacteria to colonize, penetrate placental or epithelial barriers and prevent innate immune system clearance from the bloodstream (Bellais *et al.*, 2012; Almeida *et al.*,2017). Invasion of host barriers is largely dependent on bacterial adherence abilities to host cells and extracellular matrix components (ECM) (Singh *et al.*, 2012; Landwehr-Kenzel and Henneke, 2014). Several GBS surface-proteins have been shown to mediate vaginal and cervical epithelial cell adherence and/or invasion such as fibrinogen-binding proteins A, B, and C (FbsA, FbsB, and FbsC) and the serine-rich repeat glycoproteins Srr1 and Srr2, the laminin-binding protein (Lmb), pilus tip adhesin (PilA), group B streptococcal C5a peptidase (ScpB), Streptococcal fibronectin-binding protein A (SfbA), Plasminogen binding surface Protein (PbsP), the C-17-specific hypervirulent GBS adhesin (HvgA) and the GBS immunogenic bacterial adhesin (BibA) (Fig. 12). In addition, other identified virulence factors contribute to the pathogenesis of GBS disease, including the β-hemolysin/cytolysin (β-h/c) toxin and lipoteichoic acid (LTA) (Doran *et al.*, 2002; Doran *et al.*, 2003; Doran *et al.*, 2005; Kulkarni *et al.*, 2013; Rosa-Fraile *et al.*, 2014).

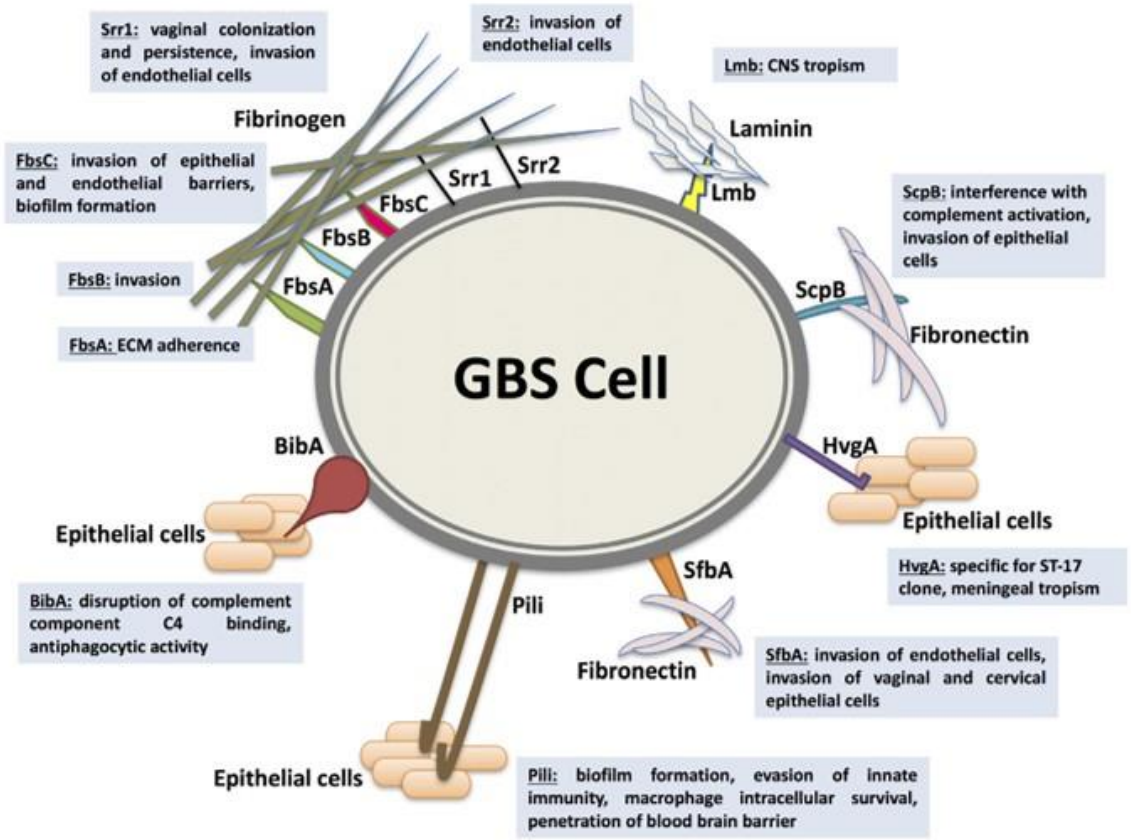

Figure 12: Major adhesins mediating GBS interaction with host cells
(from Shabayek and Spellerberg, 2018. Front Microbiol. 2018 Mar 14; 9:437)

FbsA was mainly shown to promote adherence (Schubert *et al.*, 2004), while FbsB and FbsC (or bacterial surface adhesin of GBS, BsaB) were shown to promote invasion of epithelial and endothelial barriers (Gutekunst *et al.*, 2004; Pietrocola *et al.*, 2005; Buscetta *et al.*, 2014; Jiang and Wessels, 2014). These proteins indirectly bind also to Plg (Gutekunst *et al.*, 2004; Pietrocola *et al.*, 2005; Buscetta *et al.*, 2014). Similarly, Srr1 and Srr2 were reported to mediate invasion of microvascular endothelial cells (Seo *et al.*, 2012; Seo *et al.*, 2013). Comparative genome analyses revealed that Srr2 is a cell wall-anchored protein specific for ST-17 strains, while the non-ST-17 isolates express only Srr1 (Six *et al.*, 2015). Both proteins have a key role in the development of GBS disease: Srr1 was demonstrated to promote vaginal colonization and persistence and Srr2 was shown to increase bacterial survival to phagocytic killing and bacterial persistence in a murine model of meningitis (Sheen *et al.*, 2011; Six *et al.*, 2015).

Lmb mediates the attachment of S. agalactiae to human laminin while ScpB is a fibronectin-binding protein. Another fibronectin-binding protein is SfbA. This protein was shown to bind immobilized and cell surface fibronectin, contributing to invasion of brain endothelium and development of meningitis (Mu *et al.*, 2014). Both proteins are essential for the bacterial colonization of damaged epithelium and translocation of bacteria into the bloodstream (Spellerberg *et al.*, 1999; Cheng *et al.*, 2002). PbsP is a recently identified cell wall protein, which efficiently binds plasminogen (Plg) but not other matrix components (Buscetta *et al.*, 2016). The role of this protein as a virulence factor was demonstrated both *in vitro* and *in vivo* (Buscetta *et al.*, 2016). Absence of PbsP resulted in decreased bacterial adherence and transmigration across brain endothelial cells and impaired virulence in an intravenous mouse infection model (Buscetta *et al.*, 2016). Despite PbsP was firstly characterized in the CC23 strain NEM316 (Buscetta *et al.*, 2016), Lentini and colleagues demonstrated that this protein also plays a major role in the context of the "hypervirurlent" clonotype. Moreover, PbsP is markedly upregulated *in vivo* during mouse infection and *in vitro* upon contact with cultured human endothelial cells (Lentini *et al.*, submitted for publication).

HvgA is an important CC17-specific surface-anchored protein required for GBS hypervirulence. GBS strains that express HvgA adhere more efficiently to several cell lines than do strains lacking this protein, demonstrating its critical role in GBS intestinal colonization and translocation across the intestinal barrier and the BBB, leading to meningitis (Tazi *et al.*, 2010) BibA is an immunogenic adhesin with anti-phagocytic activity due to its capability to bind to human C4-binding protein, a regulator of the classic complement pathway (Santi *et al.*, 2007). The role of this protein in GBS adhesion was demonstrated by the impaired ability of a bibA knockout mutant strain to adhere to both human cervical and lung epithelial cells and to survive in human blood resisting from opsonophagocytic killing by human neutrophils (Santi *et al.*, 2007).

In addition, surface-protruding structures like the pilus adhesin PilA were demonstrated to be essential adhesins in promoting GBS colonization, persistence, biofilm production, and central nervous system invasion (Maisey et al., 2007; Banerjee *et al.*, 2011). Using a mouse model of GBS hematogenous meningitis Banerjee *et al.* demonstrated that PilA contributes to GBS adherence to blood-brain barrier (BBB) endothelium, binding collagen and promoting bacterial interaction with the α2β1 integrin (Banerjee *et al.*, 2011). Surface-associated β-h/c toxin, encoded by the cyl gene cluster, is another protein which plays a key role in GBS pathogenesis. It induces cytolysis of eukaryotic cells and promote bacterial invasion across epithelial and endothelial walls (Rosa-Fraile *et al.*, 2014). Hyperproduction of this haemolysin is associated with fulminant disease in clinical GBS cases as well as severe cases of infection in various animal models (Doran *et al.*, 2002; Doran *et al.*, 2003; Liu *et al.*, 2004; Hensler *et al.*, 2005; Randis *et al.*, 2014).

4.3. Regulation of GBS virulence

GBS transition from a commensal to pathogenic lifestyle is related to adaptive regulated changes in expression of specific bacterial genes in response to diverse host environments. Expression of genes which encode most of GBS virulence factors is tightly regulated by two-component signal transduction systems (TCS) (Poyart *et al.*, 2001; Glaser *et al.*, 2002; Spellerberg *et al.*, 2002; Tettelin *et al.*, 2002; Tettelin *et al.*, 2005; Quach *et al.*, 2009; Faralla *et al.*, 2014). Among them, the two-component regulatory system CovRS (Cov =control of virulence) is the most studied TCS used by pathogenic streptococci to adapt to host conditions (Lamy *et al.*, 2004; Jiang *et al.*, 2005; Jiang *et al.*, 2008). It consists of a membrane-associated histidine kinase sensor (CovS) and a cytoplasmic transcriptional regulator (CovR). CovS senses host environment variations such as pH (Santi *et al.*, 2009) or glucose levels (Di Palo *et al.*, 2013), modulating the activation of the regulatory domain CovR by transphosphorylation at the aspartate residue D53 (Landwehr-Kenzel and Henneke, 2014). CovRS system has largely inhibitory effects on virulence gene expression, including downregulation of the expression of genes which encode virulence factors, such as β-H/C toxin (Whidbey *et al.*, 2013) pili, capsule and surface proteins, such as HvgA (Tazi *et al.*, 2010), FbsA (Schubert *et al.*, 2004), FbsB (Gutekunst *et al.*, 2004) and BsaB/FbsC (Buscetta *et al.*, 2014; Jiang and Wessels, 2014) and PbsP (Buscetta *et al.*, 2016; Lentini *et al.*, 2017 submitted for publication).

4.4. GBS prevention strategies: chemoprophylaxis and vaccination

During the 1990s, the American College of Obstetricians and Gynecologists, Centers for Disease Control and Prevention and the American Academy of Pediatrics issued the first consensus guidelines for the prevention of perinatal GBS disease promoting an intrapartum antibiotic prophylaxis (IAP) strategy (Hankins and Chalas, 1993). This was followed by revised guidelines for the prevention of GBS disease issued in 2002 and the updated guidelines in 2010 (Verani et al., 2010; Verani and Schrag, 2010) which are currently in use. These recommendations include a universal culture-based screening for GBS vaginal and rectal colonization for all pregnant women between 35 and 37 weeks' gestation in order to limit IAP to certain risk groups (Bidgani et al., 2016; Wollheim et al., 2017). However, although IAP of colonized women has decreased the incidence of GBS EOD, the incidence of neonatal LOD was left unchanged (Van Dyke et al., 2009). In addition, GBS morbidity outside of the perinatal risk period has been steadily increasing and now accounts for more than 90% of all cases of invasive GBS disease in the U.S.A. (Centers for Disease Control and Prevention, 2015). Thus, GBS disease persists as a major health problem for which additional or alternative control measures are needed.

Vaccination represents the most attractive strategy for GBS disease prevention. Most cases of EOD and young infant disease occur within the first 24 hours. Therefore, maternal immunization rather than direct vaccination of newborns is required to prevent neonatal and young infant disease. Effective vaccines would stimulate the production of functionally active antibodies that could cross the placenta and provide protection to the foetus against GBS infection. The GBS capsular polysaccharide (CPS) has been the primary target for vaccine development. In the 1930s, Rebecca Lancefield demonstrated that protection against lethal GBS infection in mice could be achieved using capsular polysaccharide (CPS)-specific rabbit sera (Lancefield, 1938) thus paving the way to the development of a potentially protective vaccines. The first purified type III CPS vaccine underwent phase I testing in healthy adults in 1978, and subsequently type Ia and II CPS vaccines were tested (Baker *et al.*, 1978). However, candidate vaccines using CPS alone as immunoprophylactic antigens have shown poor immunogenicity (Baker and Edwards, 2003). In newer candidate vaccines, polysaccharide immunogenicity is enhanced by covalent conjugation with carrier proteins. The development of GBS vaccines for maternal immunization has been identified as a priority for World Health Organization (WHO) Initiative for Vaccine Research, which organized in 2014 the first meeting of the Product Development for Vaccines Advisory Committee for consultation regarding the development of GBS vaccines (Lin *et al.*, 2018). In this meeting, they agreed that native CPS vaccine is ineffective due to its poor immunogenicity, proposing the GBS polysaccharide conjugate vaccine (PCV) as a stronger inducer of functional CPS-specific IgG responses (Chen *et al.*, 2013;

Leroux *et al.*, 2016). Several proteins have been tested as CPS conjugates, e.g. tetanus toxoid or CRM197, a non-toxic diphtheroidal protein. The first GBS glycoconjugate vaccine trial conducted in humans involved a GBS III CPS-tetanus toxoid (III-TT) glycoconjugate (Kasper *et al.*, 1996). 100 healthy non-pregnant women were recruited and randomized to receive III-TT, unconjugated type III CPS vaccine, or placebo. Results showed that the highest dose of III-TT produced higher levels of type III CPS-specific antibody measured two weeks after vaccination, and that conjugated CPS was able to induce a more robust immune response compared to polysaccharide-only one (Kasper *et al.*, 1996). CRM_{197}, a nontoxic mutant of diphtheria toxin (DT), is another carrier protein, which has been used for the formulation of a vaccine against GBS. Novartis Vaccines and Diagnostics division (now part of the GlaxoSmithKline group of companies) has developed a trivalent (Ia, Ib, and III) CRM197 conjugate vaccine which has completed Phase I and II clinical trials in non-pregnant and pregnant women (clinicaltrials.gov NCT01150123, NCT01193920, NCT01446289) (Heyderman *et al.*, 2016; Kobayashi *et al.*, 2016; Leroux-Roels *et al.*, 2016; Madhi *et al.*, 2016; Donders *et al.*, 2016; Fabbrini *et al.*, 2018). Maternal immunization led to higher CPS-specific titers (measured out to 90 days) in infants born to vaccinated mothers compared with a placebo group, without impacting antibody responses to diphtheria and pneumococcal vaccination (Madhi *et al.*, 2017). These antibodies are transferred across the placenta from the pregnant women to infants protecting them against GBS infection (Madhi *et al.*, 2017; Lin *et al.*, 2018). Trivalent conjugate vaccine containing serotype Ia, Ib, and III was found to be safe and immunogenic in phase 1 and 2 clinical trials. However, despite their immunogenic properties, the use of TT and CRM_{197} may interfere with responses to routine infant vaccines that use these molecules as the carrier proteins (Nuccitelli *et al.*, 2015; Kobayashi e*t al.*, 2016). In 2017, Pfizer started to evaluate a pentavalent GBS PCV targeting Ia, Ib, II, III, and V in a phase 1 trial on healthy nonpregnant volunteer (clinicaltrials.gov number NCT03170609). Despite the promising result from the clinical trial, recent change of serotype distribution worldwide requires the replacement of old serotypes, or the addition of new serotypes in the GBS PCV (Lin *et al.*, 2018). GBS surface proteins have been also used successfully as conjugates with CPS in mice and have an obvious advantage as they confer immunity on their own (Paoletti and Madoff, 2002; Paoletti and Kasper, 2003). Polysaccharide-based vaccines typically only provide protection against CPS types included in the vaccine or closely related serotypes and may be vulnerable to serotype replacement/switching. Therefore, efforts have been made to overcome serotype-specificity and to identify antigens common to all GBS as the basis of a globally effective vaccine against this bacterium. Some surface proteins of GBS have been identified as conserved antigens capable of inducing protective responses in animal studies, such as Rib and alpha proteins (Stålhammar-Carlemalm *et al.*, 1993; Larsson *et al.*, 1996; Larsson *et al.*, 1999; Larsson *et al.*, 2006), Sip (Brodeur

et al., 2000; Martin *et al.*, 2002) and C5a peptidase (Santillan *et al.*, 2011). Some of these are being investigated, at the pre-clinical stage, as broad-spectrum vaccines (Nuccitelli *et al.*, 2015; Kobayashi *et al.*, 2016; Lin *et al.*, 2018). Recently, MinervaX, a privately held Danish biotech company, has initiated phase I clinical trials with a protein vaccine based on the fusion of highly immunogenic N-terminal domains of two GBS surface proteins, Alpha C and Rib (GBS-NN) (ClinicalTrials.gov Identifier: NCT02459262*)* (Kobayashi *et al.*, 2016). In the meantime, reverse vaccinology has been successfully applied by GlaxoSmith-Kline to identify a conserved sequence encoding components of GBS pili, which induced an immune response directed against different GBS serotypes in preclinical studies (Margarit *et al.*, 2009; Nuccitelli *et al.*, 2011; Nuccitelli *et al.*, 2015). Despite these interventions, however, the global incidence of invasive GBS disease remains high, with this bacterium remaining the leading infectious cause of neonatal morbidity and mortality in most industrialized and developing countries.

Aims of the thesis

Since little is known about the mechanisms underlying the production of neutrophil attracting chemokines in the context of *in vivo* infection with group B streptococcus, the first objective of the studies presented in this thesis concerns the role of Cxcl1 and 2 in the induction of inflammatory responses to this pathogen. A second objective was to analyze the signal transduction pathways leading to Cxcl1/2 responses in macrophages and neutrophils using both *in vivo* and *in vitro* models. Having found that live GBS, but not killed bacteria or isolated microbial products, are capable of inducing high-level Cxcl2 production in neutrophils, we also aimed at studying the mechanisms by which these cells discriminate between live and killed bacteria. A better understanding these mechanisms is crucial for designing improved therapies to augment host defense and potentiate the recruitment and the functional activity of polymorphonuclear leukocytes.

Materials and methods

Mice

C57BL/6 wild-type (WT) mice, used as controls, and IL-1R$^{-/-}$ mice were purchased from Charles River Laboratories. Mice lacking single TLRs (TLR 3, 7 or 9) or TLR adaptors (MyD88, TRAM, TRIF or MAL) were obtained from Shizuo Akira (Osaka University, Japan).

Heterozygous TLR13$^{-/+}$ mice were provided by the KOMP Repository (www.komp.org) and the Mouse Biology Program (www.mousebiology.org) at the University of California Davis. Subsequently, TLR13$^{-/-}$ mice were bred in the Animal Facility of the Department of Pathology of the University of Messina, Messina, Italy, as described (Mohammadi *et al.*, 2016). TLR7, 9, 13$^{-/-}$ triple-knockout mice were generated in the above animal facility by crossing TLR9$^{-/-}$ with TLR7$^{-/-}$ and TLR13$^{-/-}$ mice. All KO mice, bred on a C57BL/6J background, were born and developed normally. All mice used in the present study were housed under specific pathogen-free conditions in individually ventilated cages.

GBS strains and culture

GBS WT strain H36B serotype Ib was used for most experiments. In selected experiments we also used the NEM316 strain and its isogenic *lgt* mutant strain (Δ*lgt*) lacking the prolipoprotein diacylglyceryl transferase gene (Henneke *et al.*, 2005). GBS were grown at 37°C with 5% CO_2 to the mid-log phase in Todd-Hewitt broth (THB, Oxoid), washed twice in nonpyrogenic PBS (0.01 M phosphate, 0.15 M NaCl [pH 7.4]; Euroclone), and resuspended to the desired concentration in PBS. To obtain preparations of heat-killed bacteria (HK-GBS), GBS strains were grown in Carey's chemically defined medium (Carey *et al.*, 1980) to the late log phase, washed three times, and resuspended in nonpyrogenic PBS. Bacteria were killed by heating at 80°C for 45 min, followed by extensive washing with distilled water and lyophilization.

To produce GBS culture supernatants, GBS strains were grown in chemically defined medium to the late log phase and cells were separated from the medium by centrifugation at 4,000 ×g for 30 min at 4°C. The supernatants were then filtered using 0.22 μm pore size filters (Merck Millipore) to remove residual bacterial cells, concentrated to 20 ml by ultrafiltration with a Pellicon XL cassette (10,000-Da NMWCO, Merck Millipore) and dialyzed extensively against DPBS (Dulbecco's Phosphate Buffer Saline w/o Calcium w/o Magnesium, Euroclone). Peptides larger than the pore size were concentrated in the retentate, while those lower than 10 kDa were excluded into the permeate. In order to use them as stimulus, proteins from an aliquot (15ml) of both permeate and bacterial cell-free culture supernatant were precipitated by sodium deoxycholate-trichloro-acetic acid (DOC–TCA) precipitation method. Briefly, DOC was added to reach a final concentration of 0.03%, followed by

incubation at room temperature for 5 min. Subsequently, TCA was added to a final concentration of 7.5% and, after 1 h, the pellets were collected by centrifugation at 10,000 × g at 4 °C for 30 min, washed twice with ice-cold acetone, and resuspended in DPBS. Samples were kept frozen (−80 °C) until use. Protein yield was determined by the Bradford method using bovine serum albumin as a standard (Protein Assay, Biorad). The endotoxin levels of all bacterial preparations were 0.06 endotoxin unit (EU)/mg, as determined by a Limulus amebocyte lysate assay (Pyrotell; ACC).

Extraction and purification of bacterial nucleic acids

Bacterial pellets (GBS grown to mid-log phase in THB) were suspended in 350 µl of Tris-HCl (pH 8, 10 mM) in 1.5 ml microcentrifuge tubes, to which 25 µg of glass beads (106 µm, Sigma-Aldrich) were added. The tubes were placed in a RETSH MM30 homogenizer and shaken at 30 Hz for 20 minutes. To isolate RNA in the homogenized samples, RNA purification columns (RNeasy minikit, Qiagen) were used, according to the manufacturer's protocols. Purified mRNA was obtained from total RNA by sequential removal of rRNA and small RNA with a Microbe Express kit and a Megaclear kit, respectively, according to the manufacturer's protocols (Ambion). Electrophoresis on agarose gels, followed by slicing of 23S and 16S bands and electroelution, was used to isolate rRNA. Small RNA (under 200 nucleotides; enriched in tRNA and 5S rRNA) was obtained from total RNA by use of a microRNA purification kit according to the manufacturer's protocol (Norgen). Purification of GBS genomic DNA were performed with the DNeasy Blood and Tissue kit (Qiagen). The quantity and purity of all preparations were determined by Nanodrop 2000 spectrophotometry (ThermoFisher Scientific) using the manufacturer's instructions and by electrophoresis on agarose gels. DNA and RNA preparations were ''complexed'' with DOTAP {N-[1-(2,3-dioleoy-loxy)propyl]-N,N,N-trimethylammonium methyl-sulfate; Sigma-Aldrich} as described previously (Mancuso *et al.*, 2009) before cell stimulation. Control cultures received DOTAP alone.

Murine infection model

Six-week-old female mice were injected intraperitoneally (i.p.) with the indicated bacterial doses (in 0.2 ml of PBS) of live H36B GBS grown to the mid-log phase and treated 1 h later with penicillin (500 IU i.p.) to prevent bacterial overgrowth. In each experiment the actual number of injected bacteria was determined by colony counts. In other experiments, *Escherichia coli* K12 ultrapure LPS (InvivoGen) or zymosan (InvivoGen) were injected i.p. at the indicated doses_in 0.2 ml of PBS respectively. When indicated, mice were injected i.v. at 3 h before GBS challenge with 100 µg rat monoclonal antibodies (mAbs) specific for Cxcl1, Cxcl2, or IgG2a and IgG2b isotype controls (R&D). To ablate macrophages, mice were injected i.p. with a suspension of clodronate liposomes (1

mg clodronate in 0.2 ml of PBS) or control PBS liposomes (Van Rooijen and Sander, 1994). Briefly, phosphatidylcholine (86 mg) and cholesterol (19 mg; both from Sigma-Aldrich) were dissolved in chloroform (5.0 ml) in a 1-liter flask. The organic phase was removed under a flow of nitrogen. The lipid film formed at the bottom of the flask was dispersed at room temperature for 15 minutes with 10 ml of plain PBS or PBS containing 0.7 mol/liter clodronate (Roche). The resulting suspension was incubated for 1 hour at room temperature and then sonicated for 10 minutes in a water bath. After incubation at room temperature for an additional hour, liposomes were diluted in 50 ml PBS and centrifuged twice at 100,000 g for 30 minutes at 20°C. The final pellet was washed once and resuspended in 20 ml of PBS. In selected experiments, WT mice were pretreated i.v. with 100 μg of rat monoclonal anti-mouse Ly6G antibody (clone 1A8) or rat IgG2a control (isotype control; both from BD Pharmingen) 24 h before i.p. inoculation with 2×10^5 CFU of live GBS. Peritoneal lavage fluids were collected at various times to measure cell numbers by flow cytometry and cytokine concentrations by ELISA.

Isolation and stimulation of bone marrow-derived neutrophils and macrophages

Neutrophils were obtained from the bone marrow of WT and KO mice using Percoll density gradient centrifugation, as previously described by Mócsai *et al.*, 2003 with some modifications. Briefly, after removing the femurs and the tibias and cutting off the epiphyses of the bones, bone marrow cells were recovered by two centrifugation steps (spins), resuspended in DPBS carefully with a Pasteur pipette and flushed onto a 50 ml conical tube through a 70 μm cell strainer (Euroclone) to remove any remaining bone pieces. Cells were collected by centrifugation at 400 × g for 15 min and resuspended into 5ml of DPBS. For isolation of neutrophils, bone marrow cells were carefully layered on the top of a two-layer (0/62.5%) Percoll (GE Healthcare Life Sciences) gradient. After centrifugation at 1060 × g for 30 min at 4°C, pellet (neutrophils + red blood cells) was washed twice with DPS and erythrocytes were hypotonically lysed by 0.2% NaCl. The osmolarity was restored with 1.6% NaCl. After two washing steps with DPBS, neutrophils were suspended in RPMI 1640 containing 10% (v/v) fetal calf serum (FCS). The viability of cells obtained via this procedure was routinely >90% as assessed by trypan blue exclusion assay. Isolated cells ($15\text{-}20 \pm 0.6 \times 10^6$ cells per mouse) were stained with May/Grunwald/Giemsa (Hiramoto *et al.*, 2013), and approximately 90% of them were morphologically mature neutrophils (bands and segmented). Purity of neutrophil populations was also determined by flow cytometry using the neutrophil-specific marker Ly6G (see below). In selected experiments Percoll-isolated cells were further treated with the anti-CD115

MACS bead separation kit (Miltenyi Biotec) to remove contaminating macrophages, as described (Mayer-Barber *et al.*, 2010).

To obtain bone marrow derived macrophages (BMDMs), marrow cells were cultured for 6 to 7 days in RPMI 1640 supplemented with 10% FCS, penicillin (50 IU/ml), and streptomycin (50 µg/ml). Medium was supplemented with either 100 ng/ml macrophage colony-stimulating factor (M-CSF) or 20 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) (both from PeproTech) to obtain BMDMs. On day 3, fresh cytokine-supplemented culture medium was added to each petri dish. Isolated bone marrow derived neutrophils and macrophages (5×10^5 per well in 0.2 ml of RPMI supplemented with 10% FCS) were seeded in microtiter plates and stimulated with HK or live GBS grown to the mid-log phase at the indicated concentrations or multiplicities of infection (MOI) respectively. All infections were carried out by centrifuging cell suspensions for 10 min at 400 × g in order to facilitate bacteria/neutrophil interactions. After incubation for 1 h at 37°C with 5% CO_2, penicillin (250 IU/ml) and streptomycin (250 µg/ml) were added to kill extracellular bacteria. In preliminary experiments we verified that this antibiotic treatment did not affect viability of intracellular bacteria, as shown by counting colony forming units in cell lysates. Control wells were stimulated with *Escherichia coli* K12 ultrapure LPS (InvivoGen). In some experiments, neutrophils and BMDMs were treated with actinomycin D (5 µg/ml, Sigma-Aldrich) or cycloheximide (5 µg/ml), both from Sigma-Aldrich.

For the inhibitor studies, neutrophils (5×10^5/well) were preincubated for 1 h at 37°C with 5% CO_2 with the selective Fpr2 antagonist WRW4 (Abcam) and the pan-FPR inhibitor Boc-2 (GenScript) at the indicated concentrations before stimulation with live GBS. In selected experiments, neutrophil stimulation with live H36b was preceded by a preincubation of 1 h at 37°C with 5% CO_2 with the indicated concentrations of the selective formyl peptide receptor agonist fMIFL (GenScript). Cell culture supernatants were collected at 24h and stored at -80°C for cytokine measurements.

Flow Cytometry

Flow cytometry analysis of leukocyte subsets in peritoneal lavage fluid was performed on a FACS Canto II flow cytometer (BD Biosciences) as previously described (Cardaci *et al.*, 2012; Biondo *et al.*, 2014). Briefly, all cells were incubated with 0.5 µg Fc Block (BD Biosciences) for 20 minutes before staining for 20 minutes with antibodies directed against F4/80 (macrophages), Ly-6G (clone 1A8, which is highly specific for neutrophils (Daley *et al.*, 2008) or CD11c (dendritic cells) using the respective isotype Abs as controls. Cells count were determined in peritoneal lavage fluids using BD TruCount tubes (BD Biosciences).

Cytokine measurement

Keratinocyte-derived chemokine (KC; CXCL1), macrophage inflammatory protein 2 (MIP-2; CXCL2) and Tumor necrosis factor alpha (TNF-α) concentrations were determined in duplicate using the murine enzyme-linked immunosorbent assay (ELISA) kits CXCL1/KC Quantikine, CXCL2/MIP-2 DuoSet and TNF-α DuoSet, according to the manufacturer's recommendations (R&D Systems). The lower detection limits of these assays were 15.6 pg/ml and 31.3 pg/ml respectively.

Statistical analyses

Differences in cytokine levels and cell counts were assessed by Student's t test or one-way analysis of variance (ANOVA) and Bonferroni multiple-comparison post-test. Differences were considered statistically significant when P values were lower than 0.05 *(P* <0.05). Statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA).

Ethics statement

All studies were performed in strict accordance with the European Union guidelines for the use of laboratory animals. The procedures were approved by the Animal Welfare Committee of the University of Messina (OPBA) and by the Ministero della Salute of Italy.

Results

Neutrophil recruitment to sites of GBS infection is mediated by Cxcl1/2

In order to study the mechanisms underlying neutrophil influx into GBS infection sites, we developed a peritonitis model using live bacteria. In initial experiments we injected mice with graded doses of GBS and measured the numbers of inflammatory cells and bacterial CFUs in peritoneal lavage fluid (PLF) samples at different times after challenge. Cell influx increased in both extent and duration with increasing bacterial challenge doses (Fig. 13A). We found a direct relationship between the number of inflammatory cells and that of bacteria in the exudates when plotting together all the data shown in Fig. 13A, without taking into account the time of collection or the bacterial dose used for challenge ($P<0.0001$; Fig. 13B) Since we noted the presence of bacterial overgrowth in some mice at 3 or 6h after challenge, we sought to reduce this potential source of variability by administering penicillin at 1 h after challenge. Under these conditions, there was a lower degree of individual variability in GBS-induced cell influx, which consistently reached peak values at 6 h post challenge (Fig. 13C). Eighty to 90% of inflammatory cells were represented by neutrophils at 1 to 12 h post-challenge, with a moderate influx of macrophages and dendritic cells occurring at 24h (Fig 13C).

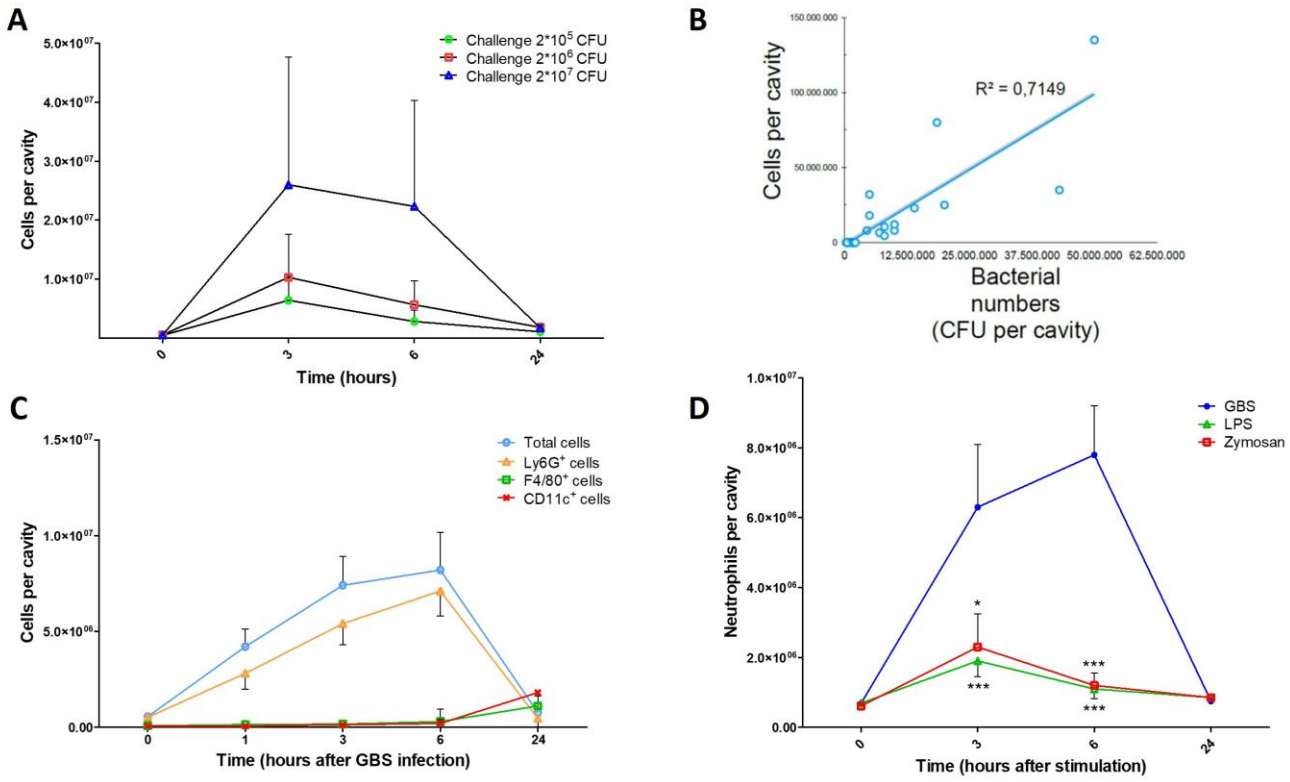

Figure 13: Neutrophil recruitment during GBS-induced peritonitis. (A) Kinetics of total cell influx in peritoneal lavage fluid samples after i.p. challenge with the indicated doses of live bacteria. (B) Correlation between the numbers of inflammatory cells and of bacteria (CFU) per cavity in peritoneal lavage fluid samples. Data were taken from the experiments shown in Fig. 13A. (C) Kinetics of recruitment of cells positive for Ly6G (neutrophils), F4/80 (macrophages) and CD11c (dendritic cells) in mice inoculated with GBS ($2x10^7$CFU) followed 60 min later by penicillin administration (500 IU). (D) Kinetics of neutrophil influx in peritoneal lavage fluid samples after i.p. challenge with GBS plus penicillin, as detailed in C, as compared to challenge with LPS (10 ng) or with zymosan (0.2 mg). Data are expressed as the means + SDs of three determinations, each conducted in a different animal, during the course of one of two experiments producing similar results. *P < 0.05, ***P < 0.001 *vs* GBS, as determined by Student's t-test.

When we compared this newly developed live bacteria model with classical peritonitis models, such as LPS- or zymosan-induced peritonitis, we found that GBS were considerably more efficient in recruiting neutrophils than optimal doses of LPS or zymosan (Fig. 13D). To gain insights into the roles of Cxcl1/2, the main neutrophil-attracting chemokines, we measured their concentrations in GBS-induced peritoneal exudates. Cxcl1/2 elevations were already detectable at 1 h after bacterial challenge and peaked at 2 to 3 h, thus associating in timing with neutrophil influx, at least at these early time points (Fig. 14A and B). Notably, Cxcl2, but not Cxcl1, peak levels were significantly higher in GBS-induced exudates relative to those induced by LPS or zymosan (Fig. 14B). Therefore, this first set of data indicated that GBS induced a more pronounced neutrophil influx, which was associated with the release of higher amounts of Cxcl2, in comparison with classically

proinflammatory stimuli, such as LPS and zymosan. In addition, neutrophil recruitment was proportional to the number of bacteria present at the infection sites.

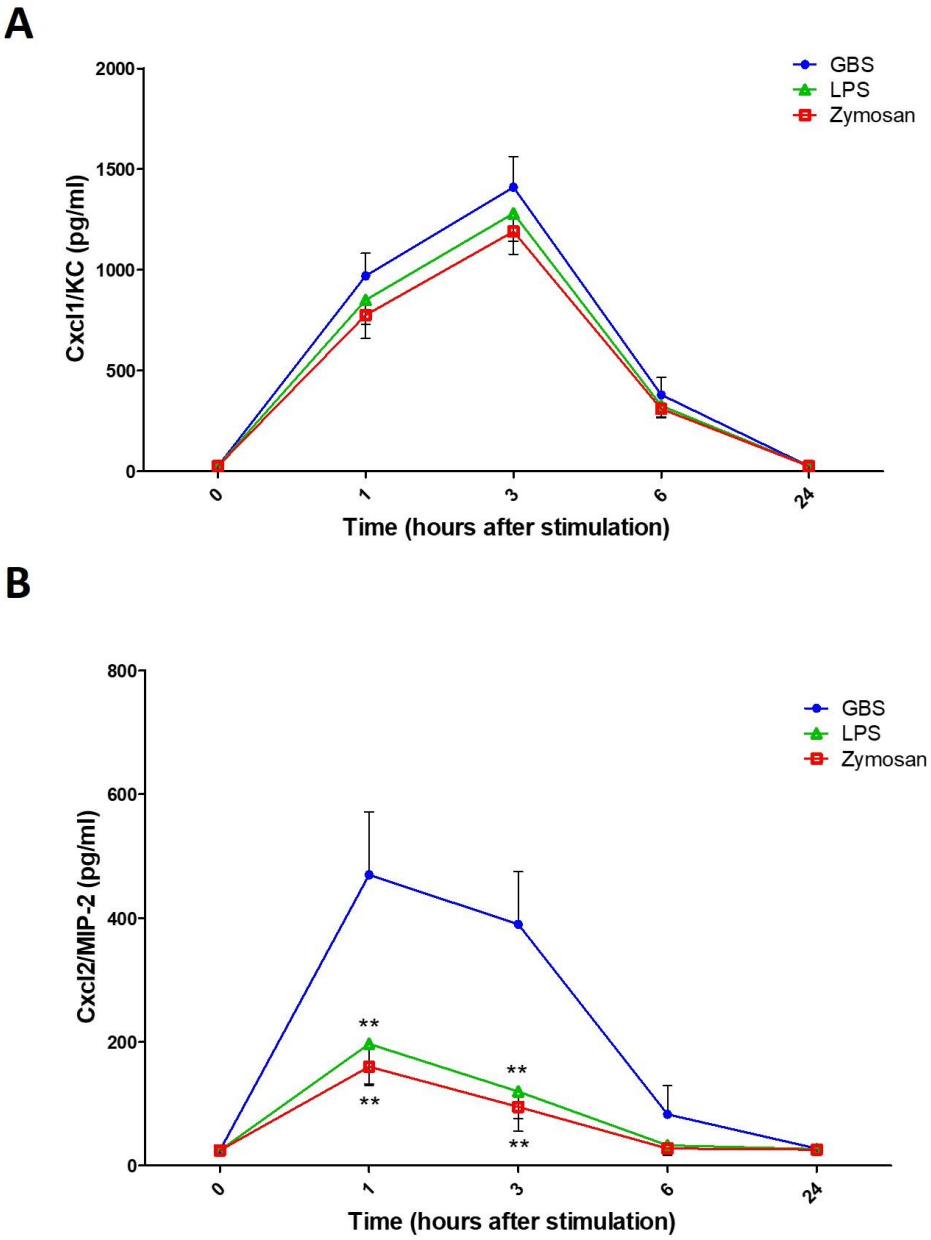

Figure 14: Kinetics of chemokine production in peritoneal lavage fluid after challenge with live GBS. Cxcl1 (A) and Cxcl2 (B) protein levels in peritoneal lavage fluid samples at the indicated times after i.p. injection with GBS + penicillin, LPS or zymosan. GBS and penicillin were administered as described in Fig.13C. Data are expressed as the means and SDs of three observations, each conducted on a different animal. **P < 0.01 *vs* GBS, as determined by Student's t-test.

To explore the role of these chemokines in neutrophil recruitment, we injected mice intra-peritoneally with neutralizing anti-Cxcl1, anti-Cxcl2 or isotype control mAbs before GBS challenge. Neutrophil recruitment into the peritoneal cavity was significantly reduced by either anti-chemokine antibody (Fig. 15A). Combined administration of anti-Cxcl1 and anti-Cxcl2 further reduced neutrophil influx

(Fig. 15B), suggesting that the two chemokines cooperate functionally and act in an additive fashion. Of note, none of the mAb treatments affected the number of circulating neutrophils, excluding depletion of these cells as a cause of reduced influx into the peritoneal cavity (data not shown). Collectively these data indicated a major *in vivo* role of both Cxc1 and 2 in GBS-induced neutrophil recruitment.

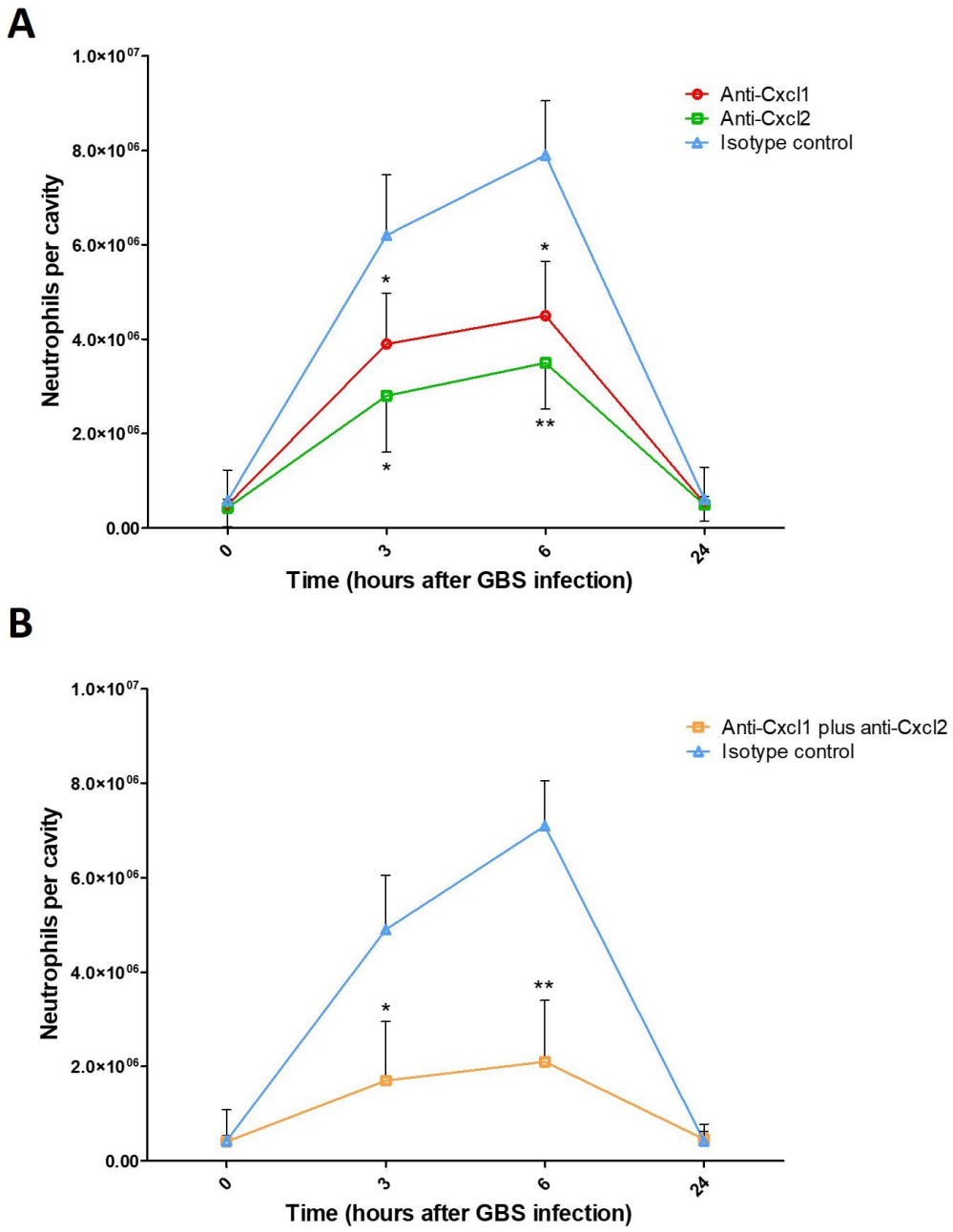

Figure 15: Effect of chemokine neutralization on neutrophil numbers in peritoneal exudates of GBS-challenged mice. Neutralizing antibodies directed against Cxcl1 and Cxcl2 or isotype control mAb were administered at 3 h before GBS challenge. Data are expressed as the means + SDs of three observations, each conducted on a different animal. *P <0.05, **P < 0.01 *vs* isotype control, as determined by Student's t-test.

Both macrophages and neutrophils contribute to *in vivo* production of Cxcl2 in response to GBS

Since macrophages are strong chemokine producers in response to a number of stimuli (De Filippo *et al.*, 2013; Duque and Descoteaux, 2014), we investigated the *in vivo* impact of these cells on Cxcl1/2 production during GBS infection using a macrophage ablation strategy. To this end, mice were treated with clodronate liposomes (Biewenga *et al.*, 1995), which resulted in a 95% reduction in macrophage numbers in the peritoneal cavity relative to control liposome-treated mice (not shown). Clodronate liposome treatment was associated with markedly decreased Cxcl1 and Cxcl2 levels in PLF samples (Fig. 16B and C), concomitantly with a significant reduction in GBS-induced neutrophil influx (Fig. 16A), while control liposomes had no effect, suggesting that macrophages are key to GBS-induced chemokine production and neutrophil recruitment.

Since neutrophils can also produce Cxcl1/2 (Biondo *et al.*, 2014a; Li *et al.*, 2016), it could not be discerned from our data whether the observed clodronate-induced reduction in chemokine levels resulted from macrophage ablation *per se* or from the reduced neutrophil numbers in the exudates of clodronate-treated mice. To gain further insights, we observed the effects of *in vivo* neutrophil depletion on GBS-induced Cxcl1/2 release. Animals were injected with anti-Ly-6G clone 1A8 monoclonal antibody, which is highly specific for neutrophils (Daley *et al.*, 2008), or with an equal amount of isotype control Ig. Anti-Ly6G treatment was sufficient to reduce neutrophil blood counts to < 2% by 24 h and these low numbers persisted for at least 72 h after treatment (data not shown). Next, anti-Ly6G- or control Ig-treated mice were inoculated i.p. with GBS and cell influx and chemokine concentrations were measured in PLF over time. As expected, neutrophils were almost completely absent in peritoneal exudates from anti-Ly6G-treated, but not in those from control Ig-treated, mice after GBS challenge (Fig 17A). Despite this, little reduction was observed in the levels of Cxcl1 in the neutrophil-depleted mice, relative to Ig-treated controls (Fig. 17B). In contrast, Cxcl2 concentrations were significantly decreased in the neutrophil-depleted animals (Fig. 17C). Collectively these data suggest that, neutrophils significantly contribute to Cxcl2 production at sites of GBS infection, although they are apparently dispensable for the secretion of Cxcl1.

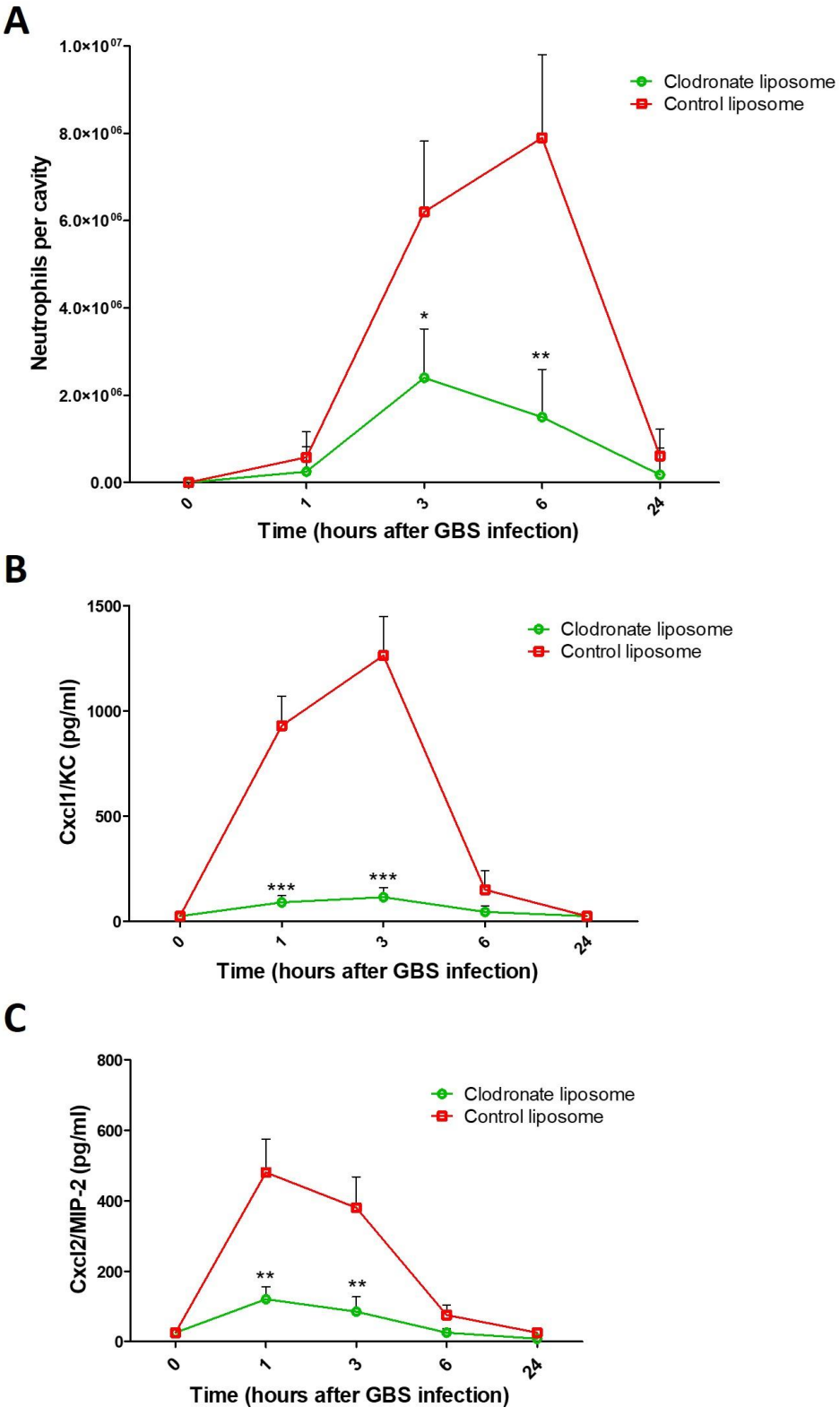

Figure 16: Effect of macrophage depletion on neutrophil recruitment and chemokine production. Kinetics of neutrophil influx (A) and Cxcl1/2 production (B and C) in peritoneal lavage fluid samples after clodronate-liposome treatment and subsequent i.p. challenge with GBS plus penicillin. Data are expressed as the means + SDs of three observations, each conducted on a different animal. *P < 0.05, **P < 0.01, ***P < 0.001 *vs* control liposome, determined by Student's t-test.

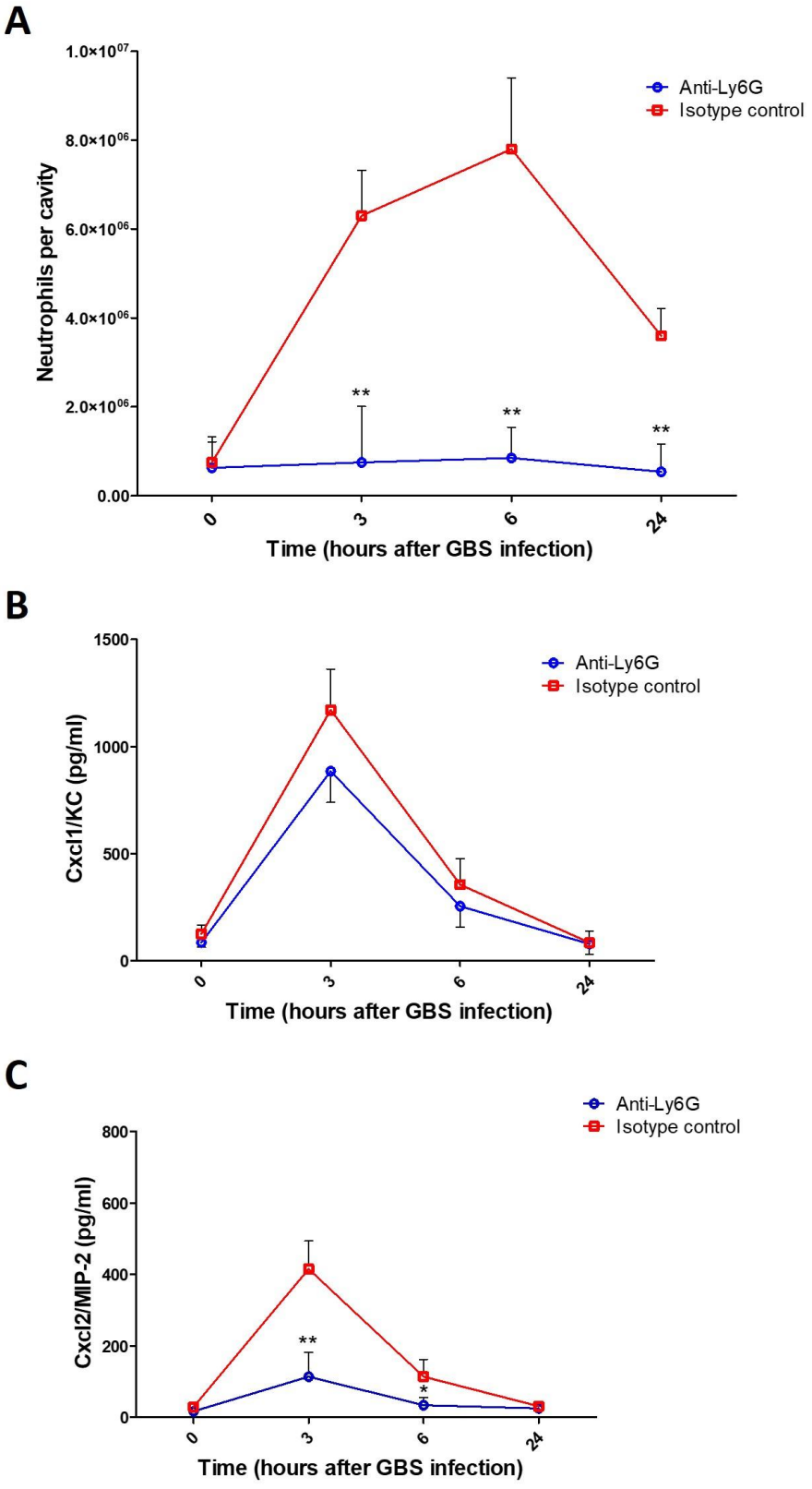

Figure 17: Effect of neutrophil depletion on chemokine production. Kinetics of neutrophil influx (A) and Cxcl1/2 production (B and C, respectively) in peritoneal lavage fluid samples after administration of anti-Ly6G or isotype control mAbs and subsequent i.p. challenge with GBS. Data are expressed as the means SDs of three observations, each conducted on a different animal. *P < 0.05, **P < 0.01 *vs* isotype control, determined by Student's t-test.

Cxcl1/2 are synthesized *de novo* in neutrophils following GBS recognition

The above *in vivo* data could not discern whether neutrophils are directly or indirectly involved in Cxcl2 production during GBS infection. For these reasons, we analyzed chemokine release in bone marrow derived neutrophils and, for comparison, in macrophages, after *in vitro* stimulation with GBS. We preliminarly investigated whether Cxcl1/2 are constitutively stored inside neutrophils, given that these cells mostly function by releasing a large number of preformed proinflammatory and antimicrobial products in response to activating stimuli. However, cell lysates from peritoneal macrophages or bone-marrow derived neutrophils contained undetectable levels of chemokines, as measured by ELISA (data not shown). In further experiments bone-marrow-derived neutrophils and macrophages were exposed *in vitro* to various doses of GBS and chemokine concentrations were measured in supernatants collected at 24 h after stimulation. As a positive control, we used LPS, a known inducer of Cxcl1/2 in macrophages (De Filippo *et al.*, 2013). Both GBS and LPS induced dose-dependent, high-level production of Cxcl1 and 2 in macrophages (Fig. 18A and B). Although neutrophils were relatively weak producers of Cxcl1, they could mount robust Cxcl2 responses to GBS. Moreover, the GBS-induced Cxcl2 response was 20- to 30-fold higher than that induced by LPS (Fig. 18B).

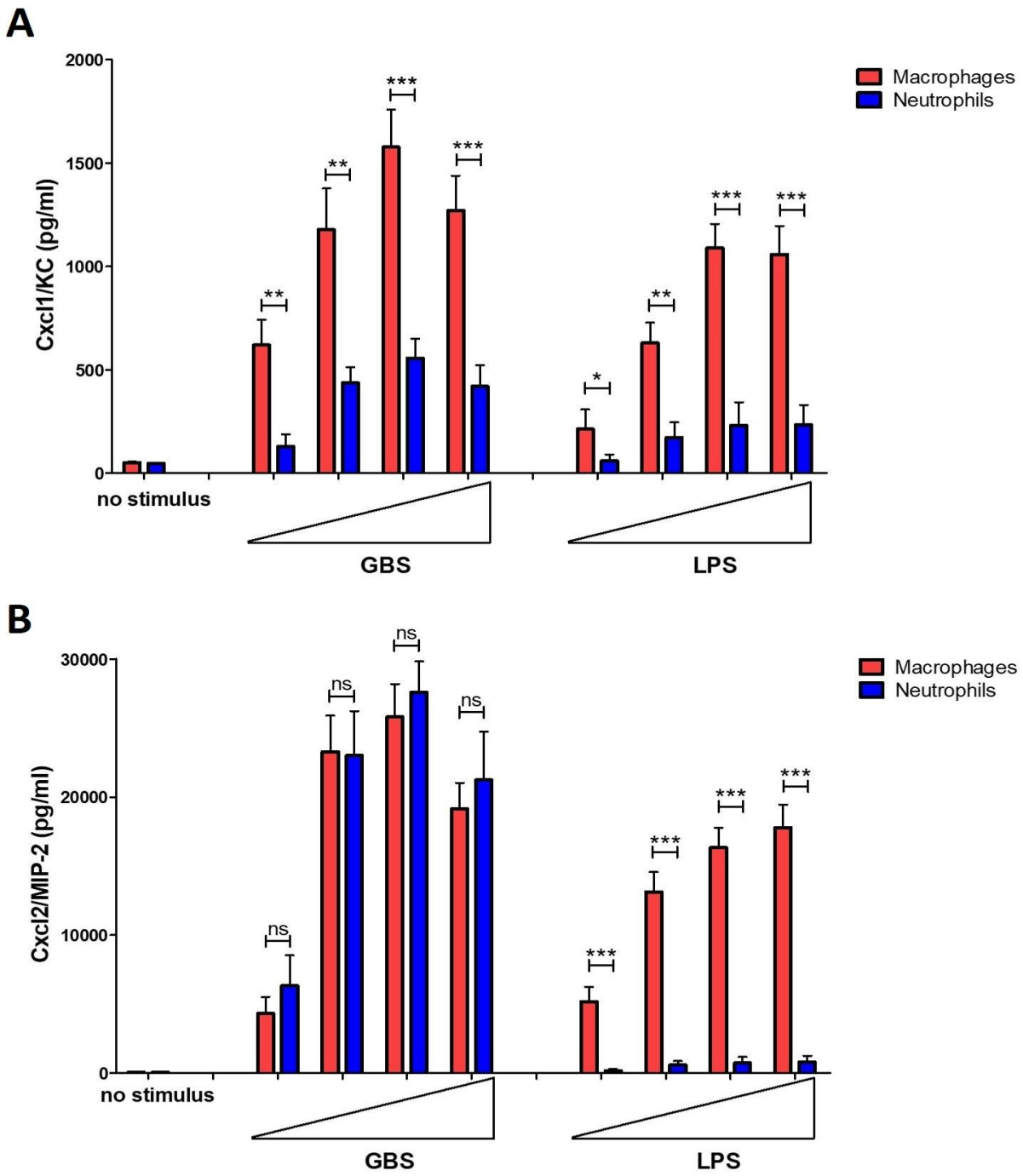

Figure 18: Cxcl1 (A) and Cxcl2 (B) production in bone-marrow-derived neutrophils and macrophages stimulated with GBS or LPS. Cytokines were measured at 24 h after treatment with increasing MOIs (2, 5, 10 and 20) of live GBS or increasing doses of LPS (1, 10, 100 and 1,000 ng/ml). Data are expressed as means + SD of results from three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, as determined by Student's t-test.

Chemokine production by neutrophils could not be accounted for by contamination of cell preparations with macrophages since removal of these cells with anti-CD115 antibodies coupled to magnetic beads did not affect chemokine production (Fig. 19A and B).

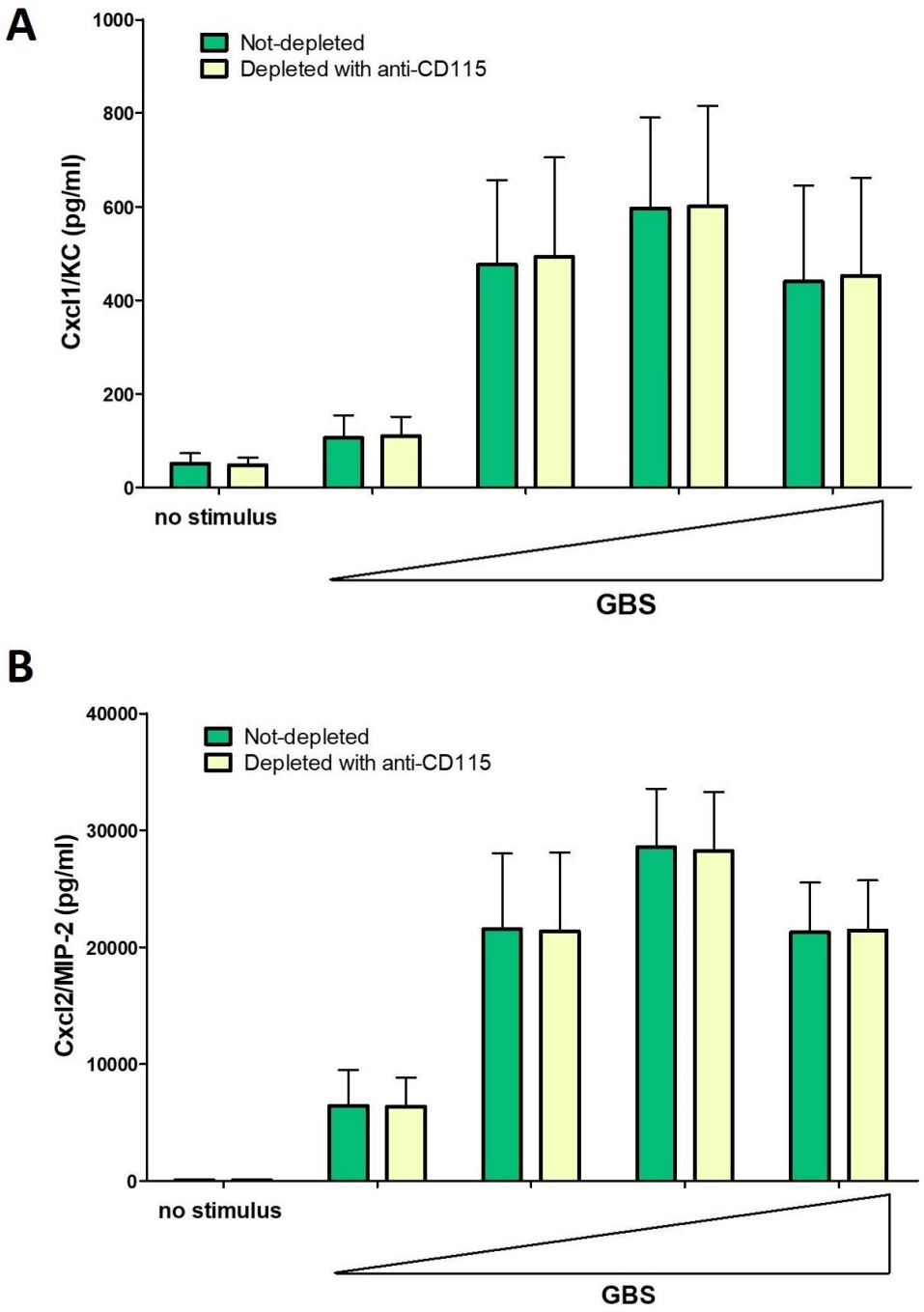

Figure 19: Cxcl1 (A) and Cxcl2 (B) production in bone-marrow-derived neutrophils before and after depletion of macrophages with anti-CD115 mAb-coated beads. Cytokines were measured at 24 h after treatment with increasing MOIs (2, 5, 10 and 20) of live GBS. Data are expressed as means + SD of results from three independent experiments.

To confirm that chemokine release required their *de novo* production, phagocytes were pretreated with cycloheximide, an inhibitor of protein synthesis. As shown in Fig. 20, Cxcl1/2 release was markedly reduced in both macrophages and neutrophils by cycloheximide treatment.

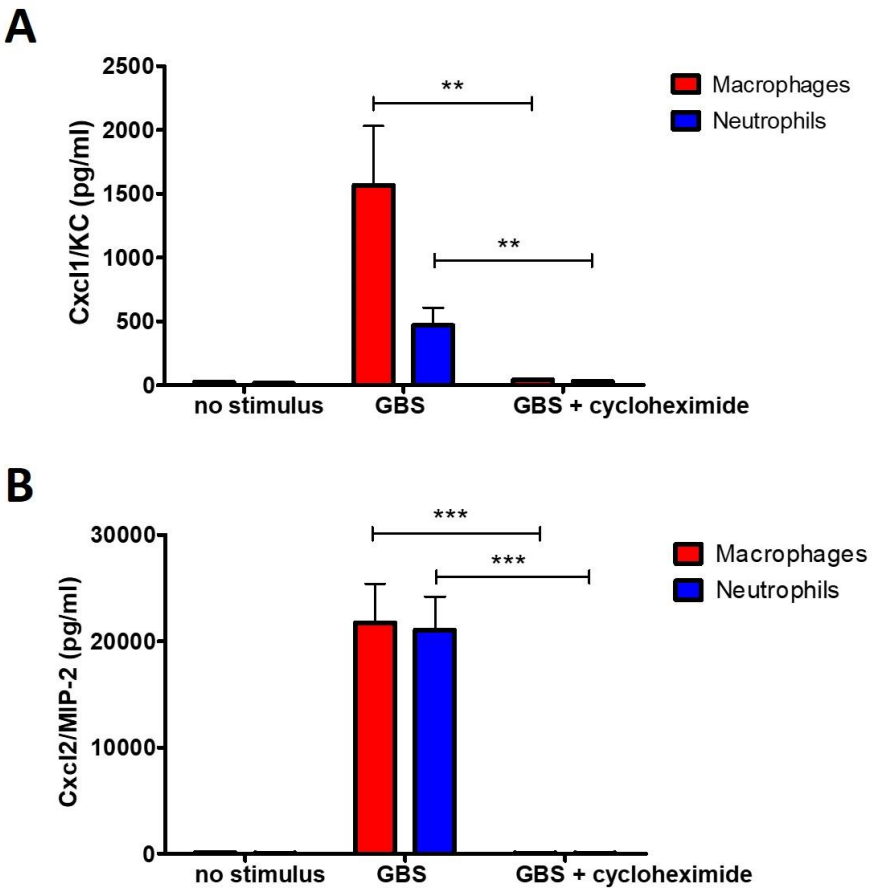

Figure 20: Effect of cycloheximide on Cxcl1 (A) and Cxcl2 (B) production by phagocytes. Bone-marrow-derived neutrophils and macrophages were stimulated with GBS (MOI 5) in the presence or absence of cycloheximide (5µg/ml). Chemokine levels in supernatants were measured after 24 hours of culture. Data are expressed as means + SD of three independent experiments. **P < 0.01, ***P < 0.001 *vs* cycloeximide-treated cells, as determined by Student's t-test.

Moreover, blocking transcription with actinomycin D during the first hour of stimulation delayed production of both chemokines (Fig. 21A and B). Collectively, these data indicated that GBS can directly induce gene activation and *de novo* production of Cxcl1/2 in myeloid cells and that neutrophils are as efficient as macrophages in mounting Cxcl2 responses to these bacteria, despite their inability to produce the chemokine at high levels when stimulated with LPS.

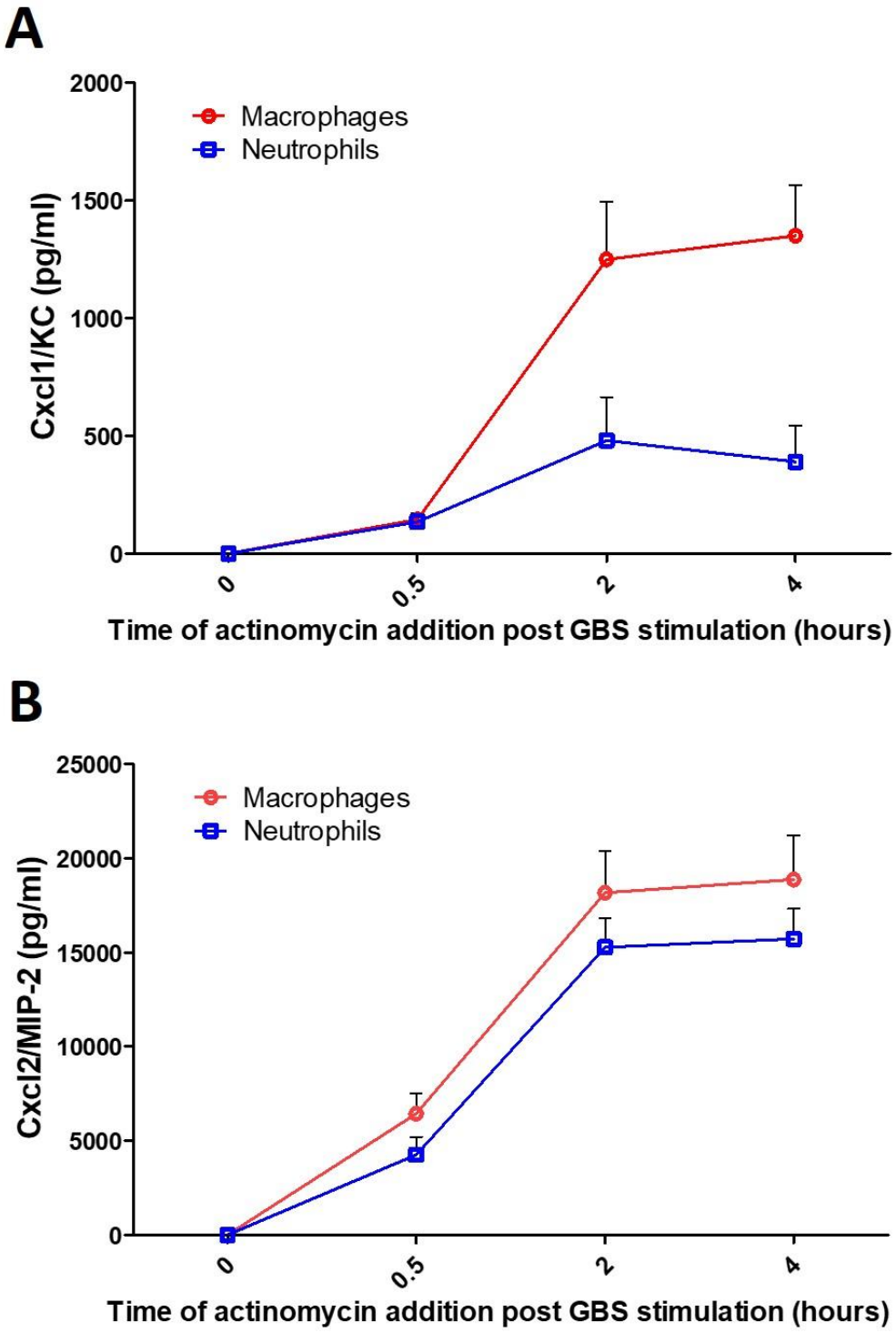

Figure 21: Cxcl1 (A) and Cxcl2 (B) production by bone-marrow-derived neutrophils and macrophages treated with actinomycin D (5 µg/ml) either at the same time as stimulation with GBS at a MOI of 5 (0 h), or 0.5, 1 and 2h afterwards. Data are expressed as means ± SD of three independent experiments.

GBS-induced chemokine production requires endosomal TLRs

Since previous studies have demonstrated a prominent role of TLRs in the recognition of GBS-by phagocytes (Costa *et al.*, 2012; Mohammadi *et al.*, 2016), we sought to investigate whether these receptors are required for GBS-induced Cxcl1/2 production. To this end, we used cells isolated from the bone marrow of mice with genetic defects in TLR adaptor/chaperone proteins or in single TLRs. In both macrophages and neutrophils, the release of Cxcl1 and Cxcl2 in response to GBS absolutely required MyD88, a TLR adaptor protein, but not other TLR adaptors, such as MAL, TRIF or TRAM (Fig. 22A and B).

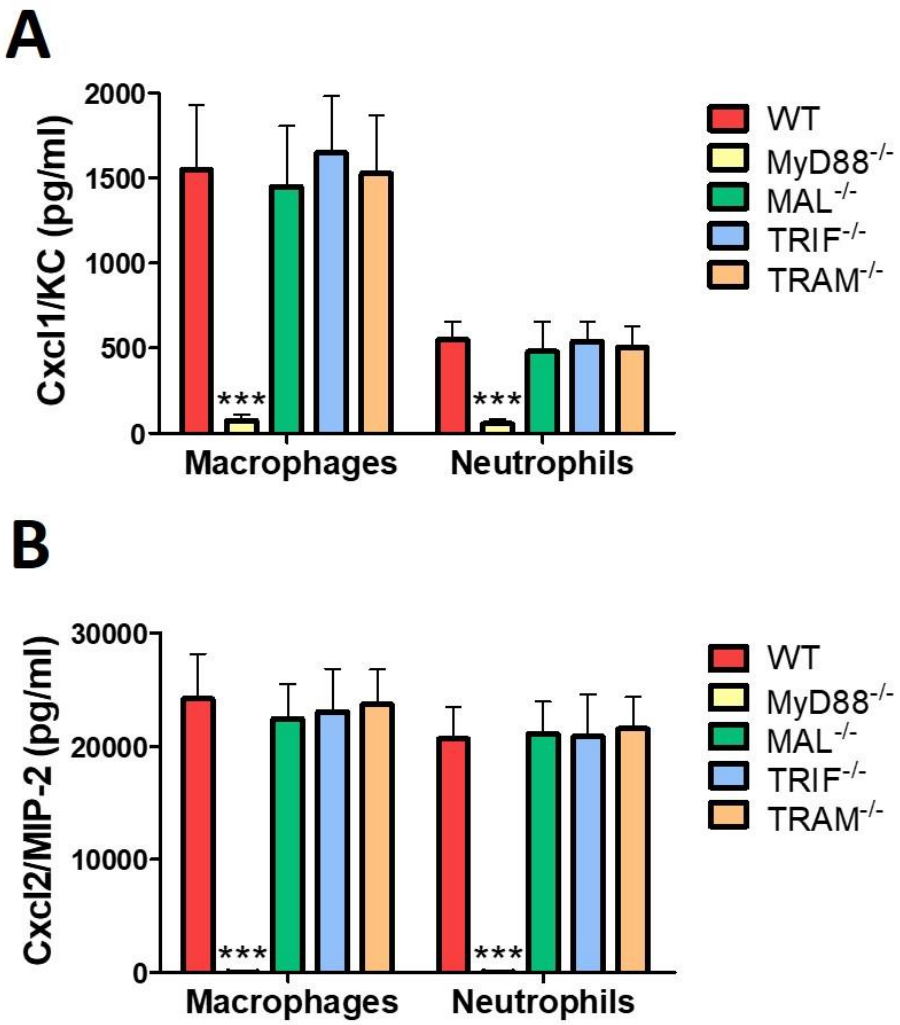

Figure 22: Concentrations of Cxcl1 (A) and Cxcl2 (B) in supernatants of bone marrow-derived macrophage and neutrophil cultures after GBS stimulation. Cells were obtained from wild type (WT) mice or mice lacking the TLR adaptors MyD88, MAL, TRIF or TRAM. Supernatants were collected at 24 h after infection with GBS (MOI 5). Data are expressed as means + SD of three independent observations, each conducted with cells from a different animal. ***P < 0.001 versus WT mice, as determined by one-way ANOVA and Bonferroni post-test.

Since MyD88 transduces signals originating not only from TLRs, but also from interleukin 1 and 18 receptors (IL-1R and IL-18R), in further experiments we used cells lacking the latter receptors to ascertain whether they might be involved in GBS-induced chemokine production. Macrophages, but not neutrophils, lacking IL-1R produced moderately lower chemokine levels relative to wild-type cells. Moreover, absence of IL-18R in either cell type had no effect on chemokine production (Fig. 23A and B). Taken together, these data indicate that lack of IL-1/18Rs cannot account for the total abrogation of Cxcl1/2 production observed in MyD88-deficient phagocytes and suggest a role for TLR in these responses.

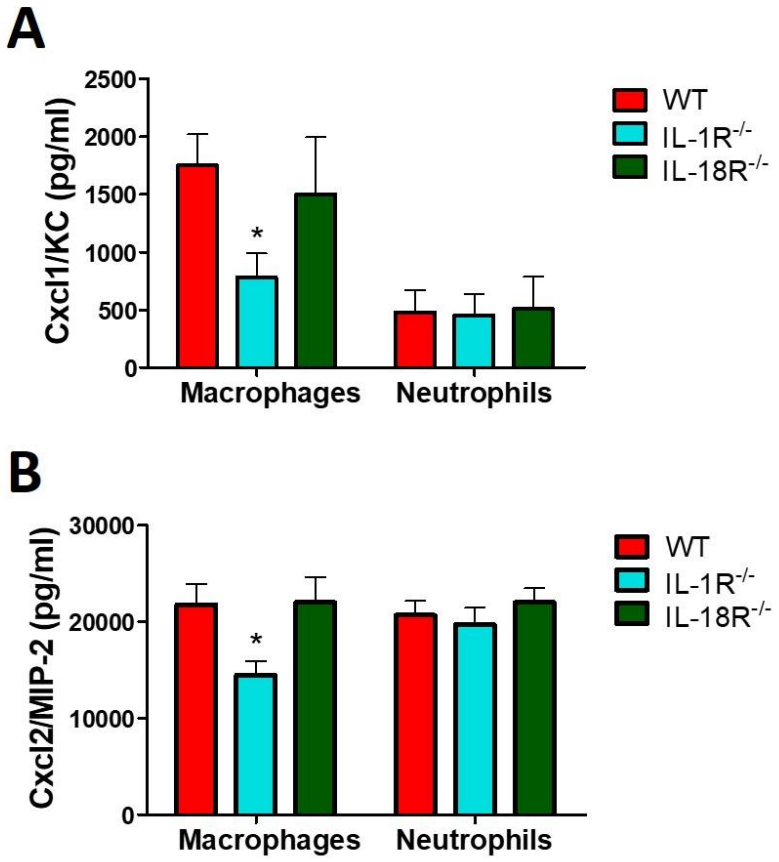

Figure 23: Concentrations of Cxcl1 (A) and Cxcl2 (B) in supernatants of bone marrow-derived neutrophils obtained from wild type (WT) mice and mice lacking interleukin-1 (IL-1R) or interleukin 18 (IL-18R) receptors. Supernatants were collected at 24 h after infection with GBS (MOI 5). Data are expressed as means + SD of three independent observations, each conducted with cells from a different animal. *P < 0.05 versus WT mice, as determined by one-way ANOVA and Bonferroni post-test.

Therefore, we next tested cells lacking single TLRs, including TLR 3/7/9/13. In addition, we used cells from 3d mice, which have defective signaling from all endosomal TLRs, such as TLR 3/7/9/13, due to a mutation in UNC93B1, a chaperone protein required for the localization of TLRs to

endosomal compartments (Tabeta *et al.*, 2006; Mohammadi *et al.*, 2016). It was found that cells from 3d mice were significantly impaired in their ability to produce Cxcl1/2 (Fig. 24A and B). In contrast, lack of single endosomal TLRs did not impair chemokine release (Fig. 24A and B). Notably, the simultaneous absence of TLR 7, 9 and 13 reconstituted the phenotype observed in the 3d mice. All together, these data suggest that Cxcl1/2 production in neutrophils requires MyD88-dependent transduction of signals originating from the simultaneous activation of TLR 7, 9 and 13. Moreover, lack of single TLRs can be compensated for by the other TLRs.

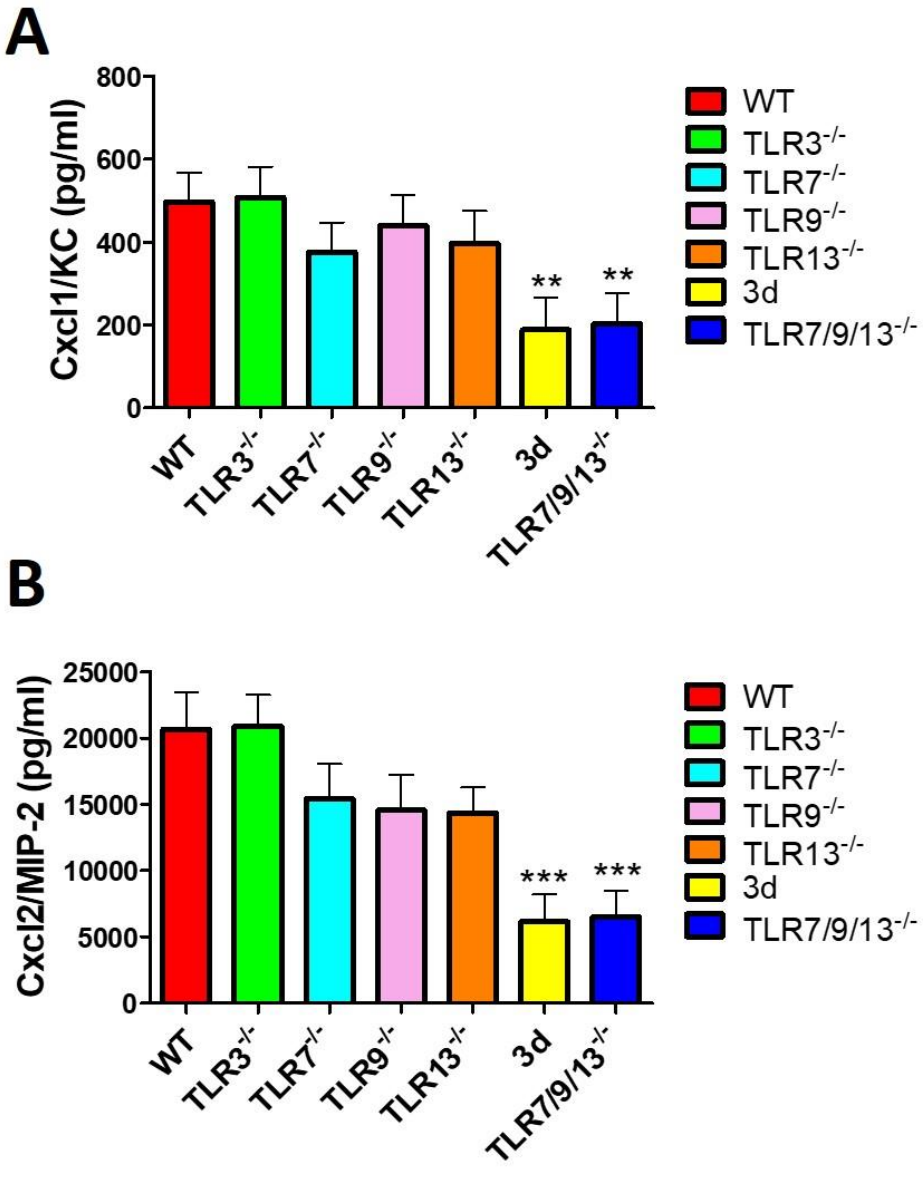

Figure 24: Concentrations of Cxcl1 (A) and Cxcl2 (B) in supernatants of bone marrow-derived neutrophils from mice with genetic defects in single or multiple endosomal TLRs. Supernatants were collected at 24 h after infection with GBS (MOI 5). Data are expressed as means + SD of three independent observations, each conducted with cells from a different animal. **P < 0.01, ***P < 0.001 versus WT mice, determined by one-way ANOVA and Bonferroni post-test.

Killed bacteria or purified nucleic acids cannot recapitulate the high-level Cxcl2 responses induced by live bacteria

The above data demonstrated that chemokine responses to whole bacteria required functional endosomal TLRs, which are known to recognize bacterial nucleic acids. Therefore, in further studies we investigated whether purified nucleic acids from GBS can induce Cxcl1/2 production. To this end, RNA and DNA were extracted from GBS and used to stimulate phagocytes after complexation with DOTAP, a liposome transfection reagent. Both nucleic acids were capable of stimulating chemokine responses in neutrophils, although RNA was more effective than DNA in these activities (Fig. 25A and B). Similar data were obtained using macrophages (data not shown). To identify the RNA type that was predominantly responsible for chemokine induction, we next purified mRNA, rRNA and small RNA (composed mostly of tRNA and 5S rRNA). Both rRNA and mRNA induced chemokine release in neutrophils (Fig. 25C and D), while small RNA was only weakly stimulatory. In further experiments we found that mRNA- and rRNA-induced chemokine responses were, respectively, TLR7- and TLR13-dependent, as found using neutrophils lacking these receptors (Fig. 25E and F). Moreover, as expected, cells from 3d mice were totally unable to respond to any of the tested RNA preparations (Fig. 25E and F). These data indicated that GBS nucleic acids, particularly rRNA and mRNA, are effective at inducing the production of Cxcl1/2 in neutrophils by TLR-dependent mechanisms. However, the Cxcl2 levels observed after stimulation with any of the tested preparations of nucleic acids were considerably lower than those observed after stimulation with live bacteria (confront, for example, Fig. 25 and Fig. 18). Therefore, in further experiments we investigated whether a combination of RNA and DNA could recapitulate the high-level stimulation induced by live bacteria. In these experiments we also tested, for comparison, live and heat-killed bacteria, which can function as potent inducers of pro-inflammatory cytokines (Signorino *et al.*, 2014).

All of the tested stimuli induced a dose-dependent production of Cxcl1, Cxcl2 and TNF-α. Similar levels of TNF-α were induced by live bacteria, compared with heat-killed bacteria (HK-GBS), a DNA/RNA combination or LPS (Fig. 26C). In contrast, 10- to 20-fold greater elevations in Cxcl2 release were observed using live GBS (Fig. 26B). Approximately 2-fold higher Cxcl1 levels were induced by live bacteria as compared with the levels observed after stimulation with HK-GBS, LPS or the DNA/RNA combination (Fig. 26A). Thus, GBS nucleic acids were able to recapitulate Cxcl1/2 responses induced by heat-killed, but not by live GBS (Fig. 26A and B). These data indicate that additional microbial components –which are different from DNA or RNA and are present in live, but not dead bacteria– also participate in the high-level chemokine responses observed in neutrophils stimulated with live GBS.

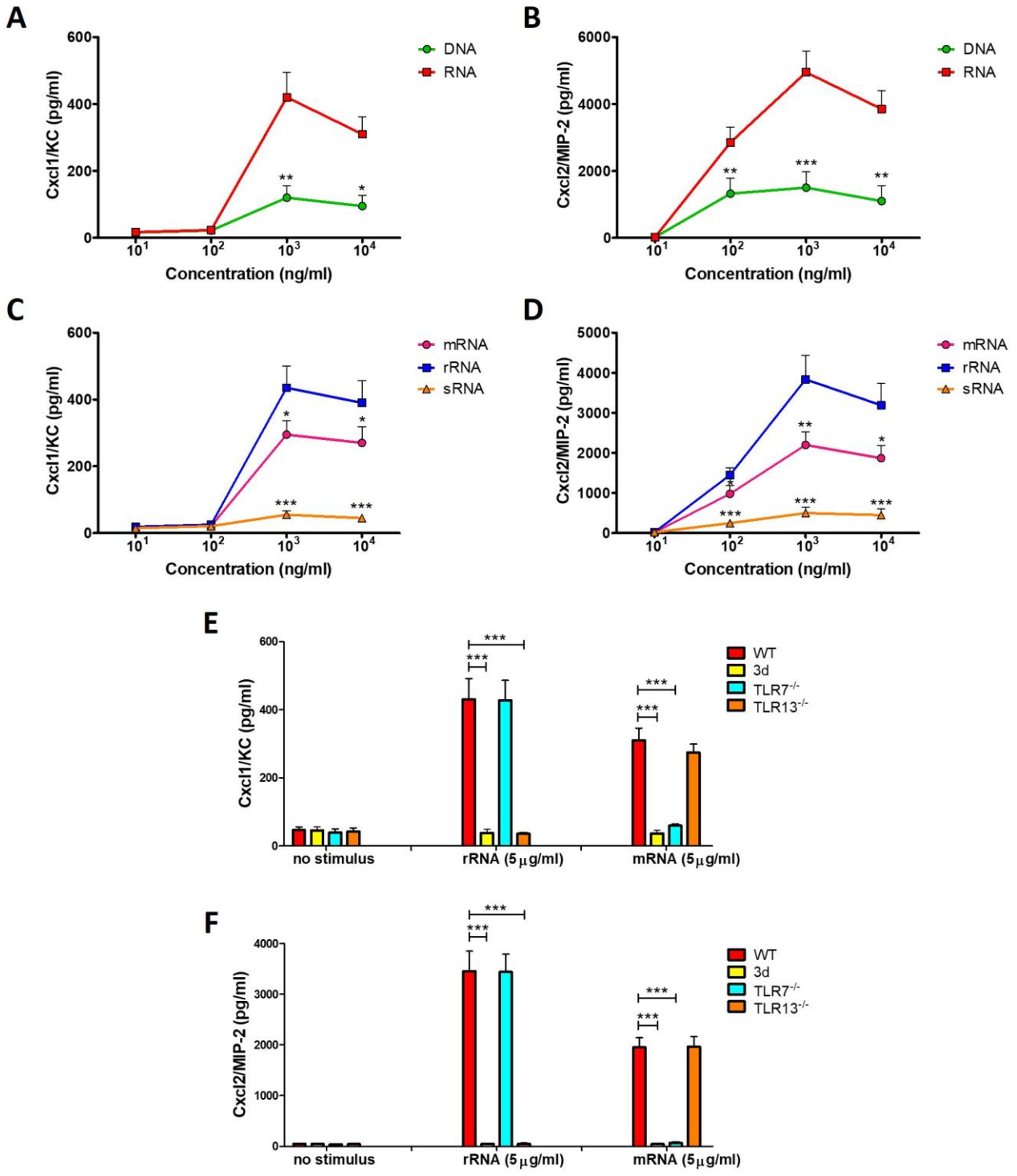

Figure 25: Concentrations of Cxcl1 (A and C) and Cxcl2 (B and D) in supernatants of neutrophils stimulated with increasing concentrations (10^1, 10^2,10^3 and 10^4 ng/ml) of the indicated nucleic acids. Cxcl1 (E) and Cxcl2 (F) concentrations in supernatants of bone marrow-derived neutrophils from mice with genetic defects in TLR7, TLR13 or in the chaperon protein UNC93B1 (3d) after stimulation with rRNA or mRNA. Chemokine levels in supernatants were measured at 24 hours after stimulation. Data are expressed as means + SD of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 by Student t test as compared with RNA (A and B) or rRNA (C and D). E and F, ***P < 0.001 versus WT mice, determined by one-way ANOVA and Bonferroni post-test.

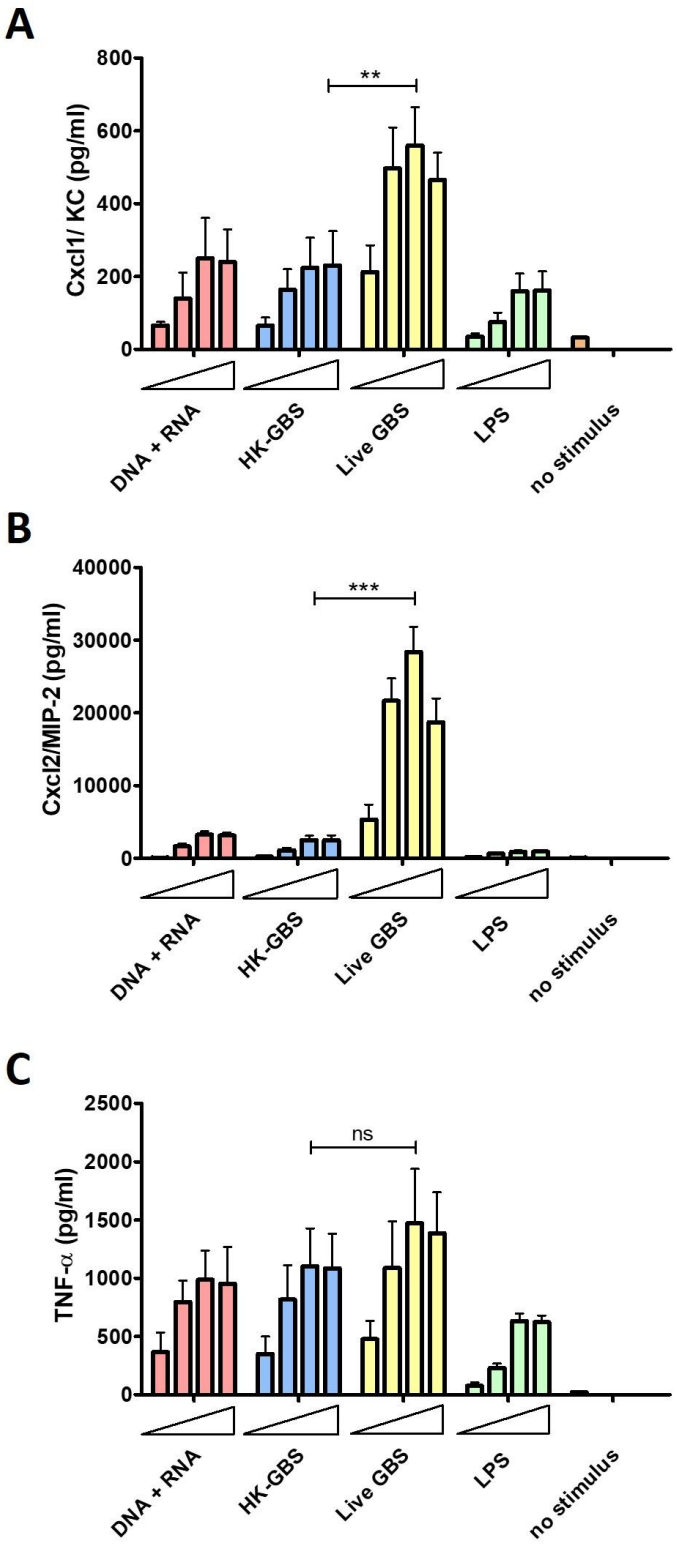

Figure 26: Release of Cxcl1 (A) Cxcl2 (B) and TNF-α (C) in neutrophil cultures stimulated with GBS. Bone-marrow-derived neutrophils were exposed to increasing concentrations of nucleic acids (1, 5, 10 and 20 µg of equal proportions of DNA and RNA), HK-GBS (1, 5, 10 or 20 µg/ml) or live GBS (MOIs of 2, 5, 10 and 20). Chemokine levels in supernatants were measured at 24 hours after stimulation. LPS (1, 10, 100 and 1000 ng/ml) was used as a positive control stimulus. Data are expressed as means + SD of three independent experiments. **P < 0.01, ***P < 0.001 *vs* live GBS, determined by Student's t-test.

High-level Cxcl2 release in response to live bacteria requires formylated peptide receptor (FPR) signalling

A major difference between live and dead bacteria is that the latter are unable to release extracellularly any of the components that are normally secreted during growth. Therefore, we hypothesized a role for secreted bacterial products in the observed induction of high Cxcl2 levels by live GBS. To test our hypothesis, we cultured GBS in a chemically defined medium and separated the culture supernatants into dialyzable and non-dialyzable components by filtration through a 10,000-molecular-weight-cutoff membrane. The whole supernatant, the retentate and the permeate were precipitated and re-dissolved to the same protein concentration. Since lipoproteins are potent TLR2 agonists, we used in these experiments a GBS strain (designated Δlgt) lacking lipoproteins because of a mutation in the Lgt enzyme which is required to lipidate pro-lipoproteins. Combined stimulation with HK-GBS and with whole bacterial supernatants or with the permeate fraction significantly increased Cxcl2 production compared to the levels observed after stimulation with HK-GBS or supernatant/permeate alone (Fig. 27). In contrast, only weak stimulatory activities were detected in the retentate. Studies are underway to purify active components present in the permeate fraction of streptococcal supernatants.

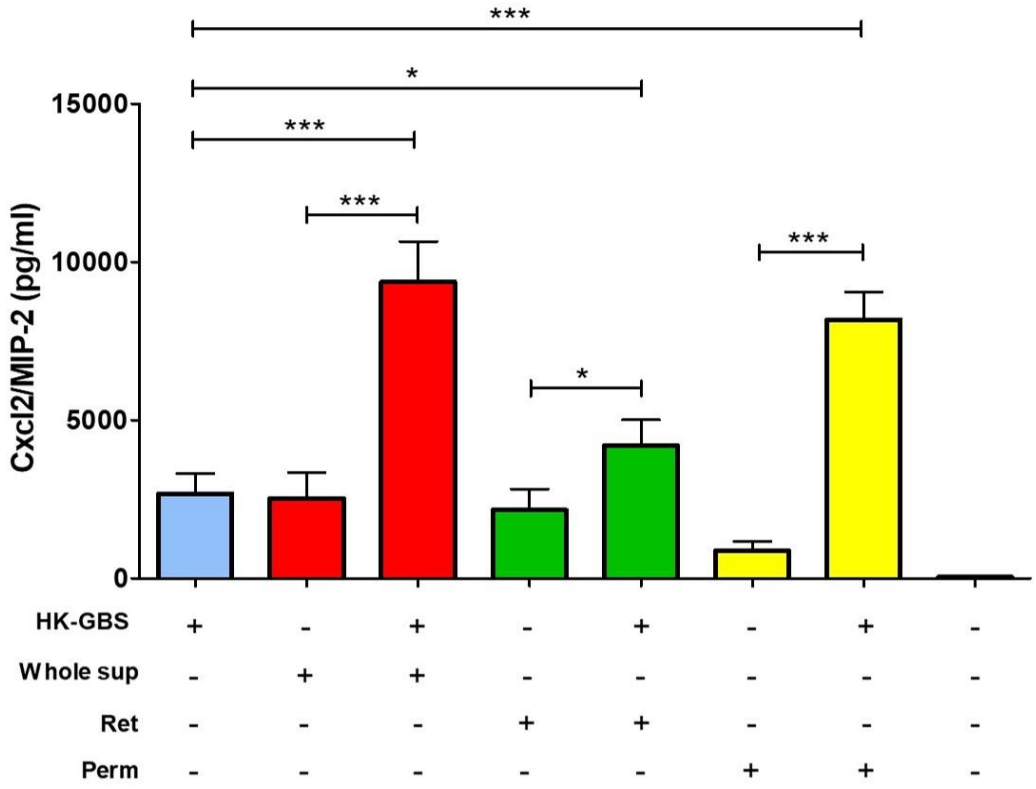

Figure 27: Cxcl2 release in neutrophil cultures stimulated with HK-GBS and/or GBS culture supernatants. Supernatants were concentrated using a tangential flow filtration apparatus with a 10,000-Mw porosity and separated into permeate (Perm), and retentate (Ret) fractions. Whole supernatants and fractions were precipitated and re-dissolved to the desired

protein concentration. Bone-marrow-derived neutrophils were exposed whole supernatant, permeate and retentate (1 µg/ml) for 1 h before the addition of HK-GBS (10 µg/ml). Chemokine levels in supernatants were measured at 24 hours after stimulation. Data are expressed as means + SD of three independent experiments. *P < 0.05, ***P < 0.001, as determined by Student's t-test.

However, in the mean time, we hypothesized that the co-stimulatory activity present in the permeate fraction may be linked to small molecular weight compounds, such as formylated peptides, which are capable to activate formylated peptide receptors (FPRs). These receptors are capable of sensing a plethora of pathogen- and host-derived chemotactic and activating molecules (Le *et al.*, 2002; He and Ye, 2017). In order to determine if FPR signalling could be involved in Cxcl1/2 production, neutrophils were pre-treated with different concentration of t-Boc-FLFLF (also termed Boc-2), a specific pan-FPR antagonist, before stimulation with live GBS. Treatment with 50 µM concentrations of Boc-2 dramatically reduced Cxcl2 production induced by live, but not killed, bacteria (Fig. 28B). Only moderate effects on GBS-induced Cxcl2 release were observed when Boc-2 was used at a 10 µM concentration, while the inhibitor was totally ineffective when used at 5 or lower µM concentrations (Fig. 28B). Moreover, 50 µM Boc-2 reduced by more than 60% the Cxcl1, but not the TNF-α, elevations induced by live GBS (Fig. 28A and C).

These data indicate that pre-treatment of neutrophils with the FPR antagonist Boc-2 at a 50 µM concentration reduces the Cxcl1/2 responses stimulated by live bacteria to the levels observed with killed bacteria. This effect is Cxcl1/2-specific since it was not observed in the case of TNF-responses. Collectively these data suggest that FPR signalling might participate in the high-level Cxcl1/2 release observed after stimulation with live bacteria.

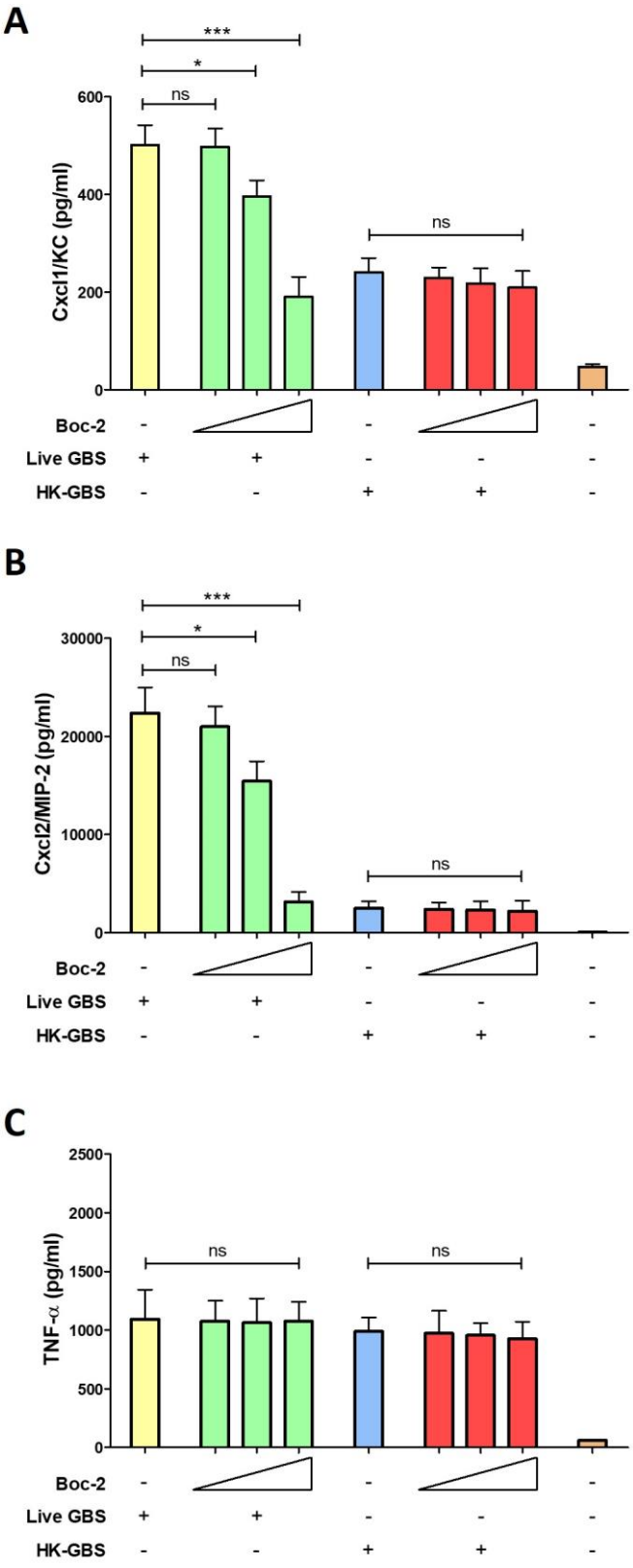

Figure 28: Release of Cxcl1 (A), Cxcl2 (B) and TNF-α (C) in neutrophil cultures stimulated with GBS in the presence or absence of the FPR antagonist Boc-2. Bone-marrow-derived neutrophils were exposed to different concentrations of Boc-2 (1, 10 and 50µM) for 1 hour and then stimulated with HK-GBS (10 µg/ml) or live GBS (MOI of 5). Chemokine levels in supernatants were measured at 24h after stimulation. Data are expressed as means + SD of three independent experiments. *P < 0.05, ***P < 0.001, as determined by Student's t-test.

Both Fpr1 and 2 participate in chemokine responses to live GBS

As outlined in the introduction, several types of FPR can be found in mammals. Neutrophils express both Fpr1 and Fpr2, which can be both inhibited by Boc-2 at the concentration (50μM) used in the study reported above (Stenfeldt *et al.*, 2007). To clarify which FPR is involved in this process, we first tested the effect of a Fpr2-specific antagonist, the synthetic hexapeptide WRW4 (Bae *et al.*, 2004). When neutrophils were exposed to different concentrations of WRW4, we found a significant reduction of Cxcl2 levels after stimulation with live, but not heat-killed bacteria (Fig. 29), indicating that Fpr2 might be involved in the high-level Cxcl2 production observed with live GBS.

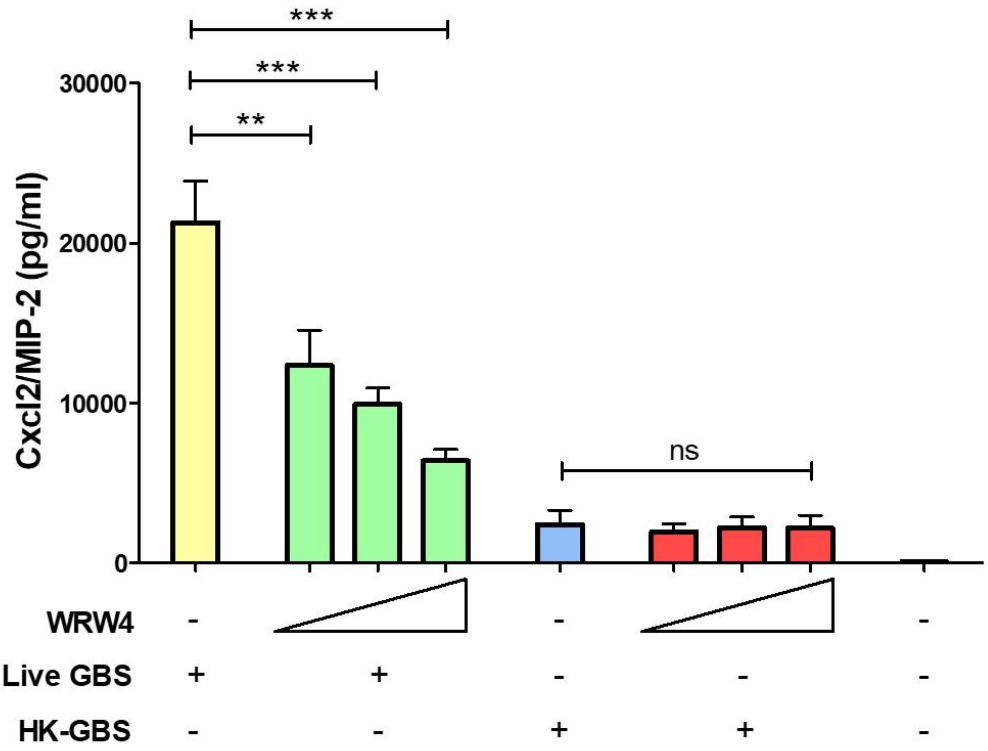

Figure 29: Cxcl2 release in neutrophil cultures stimulated with GBS in the presence or absence of the Fpr2-specific antagonist WRW4. Bone-marrow-derived neutrophils were exposed to different concentrations of WRW4 (1 to 10μM) for 1 hour and then stimulated with of HK-GBS (10 μg/ml) or live GBS (MOIs of 5). Cxcl2 levels in supernatants were measured at 24 hours after stimulation. Data are expressed as means + SD of three independent experiments. **P < 0.01, ***P < 0.001, as determined by Student's t-test.

To gain further insights into the role of FPRs involved in this process, we tested the effect of a well-known Fpr1 agonist, the tetraeptide fMIFL (N-formyl-Met-Ile-Phe-Leu), derived from *Staphylococcus aureus* (Southgate *et al.*, 2008). Combined stimulation with HK-GBS and fMIFL resulted in significant increases of Cxcl2 production relative to the levels observed with HK-GBS alone (Fig. 30). The Fpr1 agonist by itself was unable to induce any significant chemokine responses

when used over a wide dose range (Fig. 30). However, combined stimulation with FPR agonist and HK-GBS induced Cxcl2 levels that were considerably lower than those observed with live bacteria.

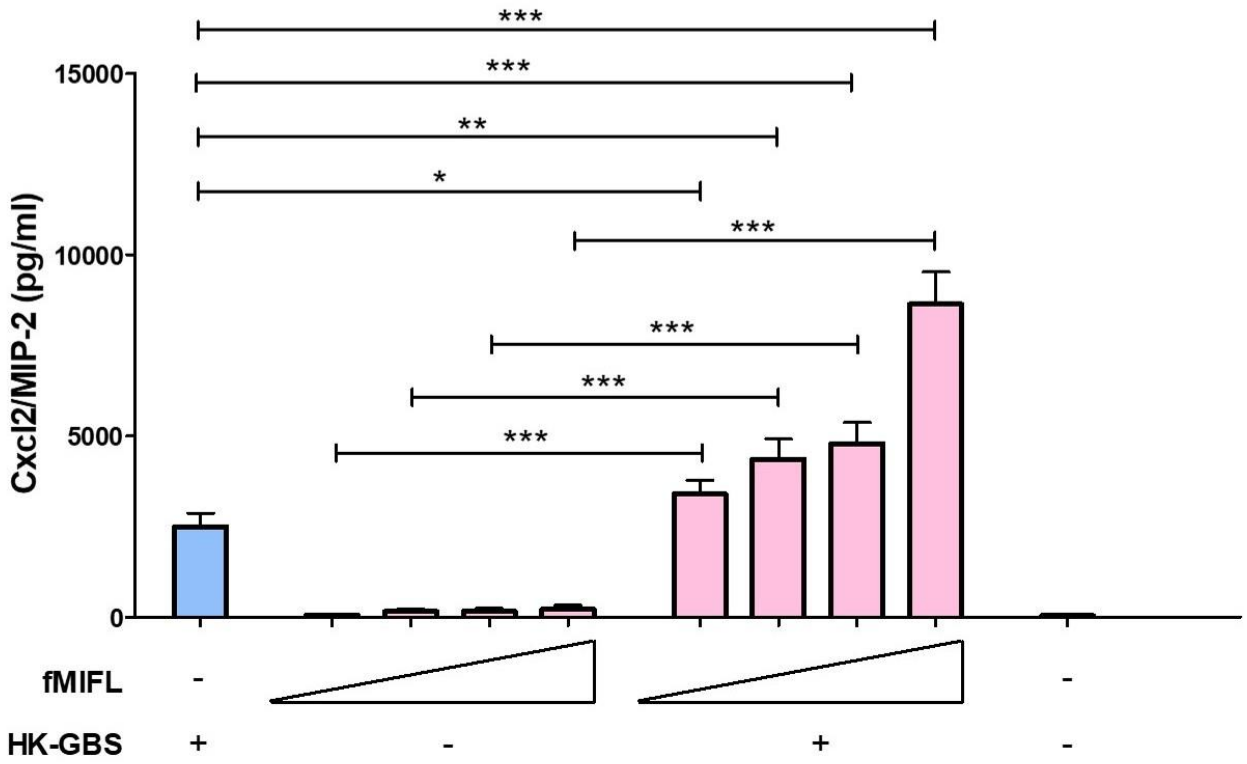

Figure 30: Cxcl2 release in neutrophil cultures stimulated with combinations of HK-GBS and the Fpr1 agonist fMIFL. Bone-marrow-derived neutrophils were exposed to increasing concentrations of fMIFL (12.5, 25, 50 and 100 µM) for 1 h before the addition of HK-GBS (10 µg/ml). Chemokine levels in supernatants were measured at 24 hours after stimulation. Data are expressed as means + SD of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, as determined by Student's t-test.

Collectively these data suggest that neutrophils might be capable of discriminating between live and dead bacteria at least in part by sensing formylated peptides or other FPR ligands, which synergize with endosomal TLRs in production of Cxcl1/2.

Discussion

The past two decades have witnessed progress in understanding the mechanisms whereby host cells recognize pathogens and initiate inflammatory responses. The presence of microorganisms or of damaged cells is detected by specialized molecular sensors (commonly defined as innate immunity recognition receptors) present at critical sites in the body, including extracellular fluids and various compartments of resident cells. Cells of the innate immune system integrate these signals and release mediators capable of recruiting inflammatory cells to sites of infection or damage. In the present study, we studied the mechanisms underlying inflammatory responses induced by the gram-positive pathogen group B streptococcus (GBS), with special reference to the neutrophil attracting chemokines Cxcl1 and 2. We focused on GBS because it is a frequent pathogen and major cause of neonatal infections, despite advances in intensive care and huge efforts at prevention in high-income countries. Elucidation of the molecular mechanisms that underlie GBS disease pathogenesis and its interaction with the innate immunity system is pivotal for the development of alternative control measures. In addition, over the years, this gram- positive organism has been frequently used to model the innate immune responses against bacteria that are devoid of LPS, the prototypical quasi-infectious stimulus used for over a century to study inflammation. In the last two decades much has been learned on the functional role of pro-inflammatory cytokines in infections by gram positive bacteria and, more specifically by GBS, as well as on the identity of the main PAMPS of these bacteria and of their cognate receptors (Teti *et al.*, 1992; Mancuso *et al.*, 1994; Mancuso *et al.*, 1997; Biondo *et al.*, 2012; Costa *et al.*, 2012; Biondo *et al.*, 2014a; Biondo *et al.*, 2014b; Signorino *et al.*, 2014; Mohammadi *et al.*, 2016). Innate immune responses to GBS ultimately result in potentiation of phagocytic killing and clearance of bacteria (Teti *et al.*, 1993; Cusumano *et al.*, 1996; Mancuso *et al.*, 2004; Henneke *et al.*, 2005; Mancuso *et al.*, 2007; Mancuso *et al.*, 2009). However excessive production of proinflammatory mediators is responsible for the pathophysiological manifestation of neonatal infection, which often results in sepsis and septic shock (Mancuso *et al.*, 2004).

Little is known of the mechanisms underlying recruitment of neutrophils to GBS infection sites or of their function during GBS disease, despite clinical observations that neonates with decreased numbers of these cells are more susceptible to GBS sepsis (Engle *et al.*, 1988; Verdrengh and Tarkowski, 1997). It was recently shown in a murine model that neutrophil depletion results in overwhelming septicemia and death under conditions whereby control animals restrict bacterial growth and show almost no signs of illness (Biondo *et al.*, 2014a). These findings point to a crucial role of neutrophils during GBS disease and suggest that the macrophages are unable, by themselves, to control the infection. Chemokines are important mediators of tissue homeostasis and inflammation and have been traditionally associated with their property to directly recruit specific subsets of immune cells. Chemokines containing an ERL amino acid motif, such as Cxcl1/2/5, have been shown to promote

neutrophil mobilization from the bone marrow and/or extravasation in experimental models involving cell influx into the peritoneal or pleural cavities and to the lung (Ajuebor *et al.*, 1999; García-Ramallo *et al.*, 2002; Beck-Schimmer *et al.*, 2005; Cailhier *et al.*, 2005; Cailhier *et al.*, 2006). Moreover, previous studies on the role of IL1R signaling during GBS infection indicated an association between impaired Cxcl1/2 production and reduced neutrophil numbers in tissues and body fluids (Biondo *et al.*, 2014a; Biondo *et al.*, 2014b). Therefore, in the first part of the present studies we directly investigated the role of Cxcl1/2 in neutrophil recruitment by looking at the effect of neutralizing antibodies in a model of GBS-induced peritonitis. In particular we were interested in learning whether these chemokines have distinctive or similar functions in neutrophil recruitment.

Although previous studies in other inflammation models have indicated a predominant role of either Cxcl1 or Cxcl2 (Mehrad *et al.*, 1998; Keane *et al.*, 1999; Tsai *et al.*, 1999; Zwijnenburg *et al.*, 1999; McColl and Clark-Lewis, 2003), it was found here that blockade of either chemokine at least partially reduced neutrophil influx. Therefore, both chemokines are apparently needed for robust neutrophil recruitment to GBS infection sites. It is possible that Cxcl1 and Cxcl2 have distinctive roles, although both signal through the same cell surface receptor (CxcR2). For example, Cxcl2 more avidly binds to glycosaminoglycans than Cxcl1 (Tanino *et al.*, 2010), likely playing a more important role in the formation of gradients spanning relatively long distances across tissues. Of course, our data do not exclude the possibility that other factors, such as lipid mediators and formylated peptides (of bacterial or mitochondrial origin) also participate in direct neutrophil mobilization to GBS infection sites. This is actually suggested by our observation that combined blockade of Cxcl1 and Cxcl2 did not result in a complete abrogation of neutrophil influx. Neutrophils follow a great variety of signals, varying in potency, location within tissues and length of formed gradients, to reach infection sites, including those generated by well characterized attractants such as lipid mediators, formylated peptide receptor (FPR) agonists and complement activation products (Soehnlein and Lindbom, 2010). Among lipid mediators, leukotriene B4 has attracted considerable attention. Although apparently dispensable in the early phase of chemotaxis toward a pro-inflammatory stimulus such as dead cells or bacteria, it plays a non-redundant role in the subsequent amplification phase ("swarming") of neutrophil recruitment, by facilitating the formation of dense aggregations of activated neutrophils or "swarms" (Lammermann, 2016). Leukotriene B4 might functionally overlap with neutrophil attracting chemokines, since, similar to them, functions by activating G protein-coupled transmembrane receptors (Soehnlein and Lindbom, 2010). Elucidation of the role and different functions of lipid mediators, FPR agonists and complement activation products in neutrophil recruitment to sites of GBS infection will require future studies. Here we identify tissue macrophages as a major source of Cxcl1/2 in response to *in vivo* GBS infection. Macrophage ablation with chlodronate liposomes

almost completely abrogated the production of these chemokine and neutrophil infiltration in the GBS peritonitis model. These findings are in agreement with previous studies in which Cxcl1/2 production and neutrophil influx were substantially reduced by ablation of resident macrophages in thioglycolate- or LPS-induced peritonitis models (Cailhier *et al.*, 2005; De Filippo *et al.*, 2013). Morover, chemokine and proinflammatory cytokine release, as well as neutrophil infiltration, were markedly reduced in models of pleuritis induced by killed S*taphylococcus aureus* or carrageenan (Cailhier *et al.*, 2006). In the present studies, macrophages stimulated *in vitro* with these bacteria produced Cxcl1/2 at levels that were equal or higher than those induced by LPS, used as a positive control, indicating that macrophages are a direct source of neutrophil attracting chemokines.

Chemokines are synthetized and released by host cells in different ways. They can be present as preformed proteins stored in vesicles that are released upon stimulation, as shown for endothelial cells (Chertov *et al.*, 2000; Øynebråten *et al.*, 2005). Alternatively, a variety of diverse stimuli, including pro-inflammatory cytokines, LPS and ROS can induce activation of chemokine genes with *de novo* synthesis of these mediators (Introna *et al.*, 1987; Tessier *et al.*, 1997; Yamamoto *et al.*, 2008). Some cell, such as mast cells, both release preformed granules and produce *de novo* Cxcl1/2 (De Filippo *et al.*, 2013). Although a multitude of neutrophil mediators and effector molecules are stored in granules, in the present study we show that this is not the case for Cxcl1/2 since lysates from unstimulated neutrophils do not contain appreciable quantities of preformed chemokines. Instead, Cxcl1/2 production occurs *de novo* and depends on mRNA and protein synthesis following stimulation with whole GBS. Both *in vivo* and *in vitro*, these chemokines are quickly synthesized and released during the first few hours of GBS stimulation. *In vivo*, these kinetics were temporally associated with neutrophil recruitment. The mechanisms by which bacteria induce *de novo* Cxcl1/2 synthesis during infection are only partially understood. A variety of stimuli including proinflammatory cytokines (e.g. IL-1α, TNF-α, IL17A and F), growth factors (e.g. G-CSF) and ROS can activate Cxcl1/genes (Soehnlein and Lindbom, 2010). Moreover, LPS and gram- negative bacteria can induce *de novo* chemokine synthesis through the activation of TLR4 (De Filippo *et al.*, 2008). Little is known of the ability of gram positive bacteria, particularly streptococci, to induce Cxcl1/2. In keratinocytes, *S. aureus* infection can induce transcription of the gene encoding CXCL8 (the human ortologue of Cxcl1/2) via an autocrinous mechanism involving IL-1 alpha release (Olaru and Jensen, 2010). In models of *S. aureus* dermatitis involving subcutaneous, rather than superficial infection of epithelial tissues, IL-1 β plays an essential role in inducing neutrophil recruitment, although it is unclear whether such IL-1 β-dependent recruitment is actually mediated by Cxcl1/2 production (Miller and Cho, 2011). Recently, it was found that Il-1β production and IL1-R signaling potentiate Cxcl1 production in macrophages stimulated with GBS (Biondo *et al.*, 2014a; Biondo *et*

al., 2014b). Interestingly, during *in vivo* GBS infection, Il-β was produced in part by neutrophils, initiating a positive feedback cytokine circuit whereby these cells promoted their own recruitment by potentiating macrophage-derived Cxcl1/2 production (Biondo *et al.*, 2014a; Mohammadi *et al.*, 2016). However, whether neutrophils can produce chemokines in response to GBS stimulation was not previously reported, with the exception of an early study showing IL-8 release from human neutrophils stimulated with heat-killed GBS (Albanyan *et al.*, 2000).

A major finding of the present study is that Cxcl1 and 2 are produced downstream of TLRs in both macrophages and neutrophils directly in response to GBS stimulation. This was evidenced by complete abrogation of chemokine production in the absence of the TLR adaptor Myd88, but not in the absence of other TLR adaptors such as MAL, TRIF and TRAM. Although Myd88 also transduces signals originating from ILR1 and ILR18, chemokine production was only partially reduced in the absence of ILR1 in macrophages, while it was unaffected in neutrophils. Moreover, lack of IL-18R had no effects on chemokine production. Since cytokine-containing GBS-stimulated supernatants from macrophage cultures induced little Cxcl1/2 mRNA (data not shown), collectively our data indicate that Cxcl1 and 2 are primary mediators (such as TNF-α, IL1β, IL-12, IL-18 and IFN-β) that are directly produced by immune cells after TLR-mediated GBS recognition.

Further experiments indicated that chemokine production was significantly reduced in the absence of functional UNC93B, which is necessary for signaling through nucleic acid sensing, endosomal TLR. For these reasons, we next focused on these receptors by using cells lacking TLR3, 7, 9 or 13. Although none of these receptors was absolutely required for chemokine production, the combined absence of TLR7, 9 and 13 fully reconstituted the phenotype of UNC93B1-defective cells. Moreover, both RNA and DNA were capable of inducing chemokine synthesis. Cxcl1/2 responses induced by rRNA and mRNA were totally dependent on, respectively, TLR13 and 7. These data suggest that TLR7, 9 and 13 all participate in GBS recognition, although none is absolutely required for this function. It is possible that signaling from any of the above TLRs upon recognition of a specific target can compensate for the lack of other TLRs. Together with previous evidence (Biondo *et al.*, 2014a) our data highlight the presence of a multimodal GBS detection system, whereby innate immune cells are exposed to various types of conserved bacterial products, each capable of engaging a different TLR family member. The defective production of Cxcl1/2 in mice with the 3d UNC93B1 mutation indicates that activation of multiple TLRs may be crucial for mounting optimal chemokine responses to GBS. An integrated multireceptor system, such as that operating in GBS sensing, is perhaps important to prevent pathogens from easily escaping immune recognition by mutating or deleting a single critical TLR ligand.

In the present study, we compared inflammatory responses induced by live GBS with those elicited

by isolated microbial products, including classical stimuli such as LPS and zymosan. We noted that live bacteria induced a more intense and persistent neutrophil infiltration in association with higher concentrations of Clcl2, but not Cxcl1, in peritoneal exudates. Further studies suggested that neutrophils, rather than macrophages, accounted for increased Cxcl2 production in response to live bacteria. Two lines of evidence support this conclusion. Firstly, neutrophil ablation resulted in reduced *in vivo* production of Cxcl2, but not Cxcl1, after GBS challenge. Secondly, neutrophils could produce high levels of Cxcl2, but not Cxcl1, when stimulated *in vitro* with live bacteria. For example, neutrophils did not differ from macrophages in the amount of Cxcl2 produced after stimulation with live GBS, while producing much lower amounts of Cxcl1. Interestingly, neutrophils were unable to release high Cxcl2 levels when stimulated with killed bacteria, purified bacterial nucleic acids, LPS or zymosan, although these stimuli were capable of inducing robust TNF-α production in macrophages and neutrophils. These findings prompted us to investigate the mechanisms underlying high-level Cxcl2 production in neutrophils after recognition of live, but not killed, bacteria. We first noted that GBS secreted products, as found in supernatants of bacterial cultures, by themselves induced only low Cxcl2 levels. These supernatants, however, strongly synergized with heat-killed bacteria in Cxl2 induction and this activity mostly resided in culture filtrates, rather than retentates, suggesting that bacterial products with a molecular mass lower than 10 kilodaltons predominantly mediated the synergistic effects. Since neutrophils express FPR receptors, such as Fpr1 and 2, at high densities and most of the known FPR agonists have low molecular weights, we hypothesized that high-level Cxcl2 production in neutrophils might originate from the simultaneous activation of FPRs and TLRs. Further studies suggested that this might be indeed the case. In the first place, pretreatment with the FPR antagonist Boc-2, at concentrations that block both Fpr1 and 2 signaling, decreased Cxcl2 release in neutrophils stimulated with live GBS to the levels observed after stimulation with killed bacteria. This effect could be partially mimicked by pretreatment with selective Fpr2 inhibitors, such as the synthetic hexapeptide WRW4. Secondly, pretreatment with a well-known Fpr1 agonist (fMIFL from *Staphylococcus aureus*) recapitulated the synergistic effects of bacterial culture supernatants. Collectively these data suggest that FPR agonists produced by live, but not killed bacteria, synergize with TLR agonists (particularly bacterial RNA) in the induction of robust Cxcl2 responses in neutrophils. Bacterial FPR agonists, such as formylated peptides, have long been known for their potent chemotactic properties in neutrophil recruitment. Our data suggest that these products might also be involved in an amplification loop of neutrophil recruitment and activation characterized by high-level Cxcl2 production in neutrophils. Since FPR agonists are released not only by bacteria but also by dying or damaged cells, such a feed forward mechanism may apply to inflammatory conditions caused not only by infectious agents but also by sterile insults.

Concluding remarks and future perspectives

The main findings of this thesis can be summarized as follows:

- Neutrophil recruitment to GBS infection sites is mediated at least partially by Cxcl1 and 2;
- Both macrophages and neutrophils contribute to *in vivo* production of Cxcl1/2 in response to GBS: macrophages are important for the production of both Cxcl1 and 2, while neutrophils make a significant contribution to the production of Cxcl2;
- Cxcl1/2 are synthetized *de novo* directly in response to GBS;
- GBS-induced Cxcl1/2 production requires MyD88-dependent transduction of signals originating from the simultaneous activation of multiple endosomal TLRs and particularly TLR7, 9 and 13 by GBS nucleic acids;
- Extracellular microbial components –which are released by live, but not dead bacteria– participate in the high-level Cxcl2 responses observed in neutrophils stimulated with live GBS;
- These extracellular components are likely to function as formylated peptide receptor (FPR) agonists, since high-level Cxcl2 release in response to live bacteria requires FPR signalling;
- Neutrophils might be capable of discriminating between live and dead bacteria by sensing FPR ligands by means of both FPRs, which synergize with endosomal TLRs in production of Cxcl1/2.

Our findings point out to the essential role of neutrophils attracting chemokines during bacterial infection. Neutrophils can recognize bacteria as efficiently as mononuclear phagocytes using a range of endosomal TLRs and FPRs and are an important source of Cxcl2, which amplifies neutrophil recruitment to infection sites. These data may lead to new strategies to treat bacterial infections by potentiating the recruitment and the functional activities of polymorphonuclear leukocytes.

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