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First line of defense: viral RNA-induced cell death mechanisms in macrophages and neutrophil responses to live bacteria.

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ABSTRACT

Inflammation is a fundamental protective response that relies on the coordinated action of innate immune cells to eliminate pathogens, remove damaged tissues, and restore homeostasis. This work addresses two complementary aspects of inflammatory regulation, focusing on (i) the mechanisms of macrophage cell death induced by viral RNA mimetics, and (ii) the ability of human neutrophils to discriminate live from dead bacteria, with particular attention to Group B Streptococcus (GBS).

First, we investigated how human macrophages respond to cytosolic Poly(I:C), a synthetic analogue of viral dsRNA. Our findings demonstrate that Poly(I:C) triggers a mixed lytic cell death program involving pyroptosis secondary to apoptosis and necroptosis. This response is mediated by TLR3 and requires TRIF-dependent activation of caspase-8, caspase-3, and GSDME, while GSDMD remains inactive. Importantly, Poly(I:C)-induced macrophage death is also caused by RIPK3, which leads to necroptosis, and occurs independently of NLRP3 inflammasome activation. Although Poly(I:C) did not prime or activate inflammasome pathways in unprimed macrophages, it acted as a secondary signal in LPS-primed cells; however, transfection reagents influenced these outcomes, prompting ongoing optimization. Overall, our data identify a TLR3–TRIF–caspase-8/3–GSDME axis as the central driver of dsRNA-induced lytic death in human macrophages.

Second, we examined how neutrophils detect and respond to GBS. We show that human neutrophils preferentially sense live GBS through the combined activity of formyl peptide receptors (FPR1 and FPR2) and TLR8. TLR8-mediated recognition of bacterial RNA emerged as the dominant pathway for cytokines and ROS production, while FPR1 and FPR2 selectively engaged distinct formylated GBS peptides, acting as crucial determinants of bacterial viability sensing. Neutrophil activation was markedly reduced when either TLR8 or FPR signaling was inhibited, indicating that robust inflammatory responses require concurrent engagement of both receptor systems. This multisensory mechanism likely functions as a safeguard to ensure that potent neutrophil effector functions are deployed only in the presence of live, potentially dangerous bacteria.

Together, these findings highlight key innate immune mechanisms governing inflammatory responses to viral and bacterial stimuli, revealing how macrophages integrate dsRNA-driven death pathways and how neutrophils combine FPRs and TLR8 signaling to discriminate live from dead microbes and calibrate inflammation accordingly.

ABBREVIATIONS

ALX Lipoxin A4 receptor

AP-1 Activator Protein 1

APAF1 Apoptotic Protease Activating Factor 1

ASC Apoptosis Associated Speck-like protein containing CARD

ATP Adenosine Triphosphate

BAX BCL-2 associated X protein

BAK BCL-2 antagonist/killer

BCL-2 B-cell lymphoma 2

BH3 BCL-2 Homology 3 domain

BID / tBID BH3-interacting domain death agonist / truncated BID

Boc/t-Boc tert-butoxycarbonyl peptide antagonist of FPRs

Btk Bruton's Tyrosine Kinase

CA-MRSA Community Associated Methicillin Resistant Staphylococcus aureus

CARD Caspase Recruitment Domain

CD40 Cluster of Differentiation 40

CLRs C-type Lectin Receptors

CpG Cytosine-phosphate-Guanine dinucleotide

CXCL C-X-C motif chemokine ligand

cyt C Cytochrome c

DAMPs Damage Associated Molecular Patterns

DD Death Domain

DED Death Effector Domain

DEAF-1 Deformed Epidermal Autoregulatory Factor 1

DFNA5 Deafness autosomal dominant 5

dsRNA Double stranded RNA

ECSIT Evolutionarily Conserved Signaling Intermediate in Toll pathways

ERK1/2 Extracellular Signal Regulated Kinases 1 and 2

F2L Heme-binding protein–derived peptide (FPR3 ligand)

FADD Fas Associated protein with Death Domain

FITC Fluorescein Isothiocyanate

fMLF N-formyl-Met-Leu-Phe

fMMYALF Mitochondrial hexapeptide ligand for FPR3

FPRs Formyl Peptide Receptors

GBS Group B Streptococcus

G-CSF Granulocyte Colony Stimulating Factor

GM-CSF Granulocyte Macrophage Colony Stimulating Factor

GPCRs G protein–coupled receptors

GSDMs Gasdermins

hMDMs Human Monocyte-Derived Macrophages

HIV-1 Human Immunodeficiency Virus type 1

Hp(2-20) Helicobacter pylori–derived peptide

HSV-1 Herpes Simplex Virus 1

IAV Influenza A Virus

IFNs Interferons

IKK IκB kinase complex

IL-1α Interleukin 1 alpha

IL-1β Interleukin 1 beta

IKBα Inhibitor of NF-κB alpha

IRAKs IL-1 Receptor Associated Kinases

IRF3 Interferon Regulatory Factor 3

JNK c-Jun N-terminal Kinase

LPS Lipopolysaccharide

LRR Leucine Rich Repeat

MAL / TIRAP MyD88 Adaptor-Like protein

MAPKs Mitogen Activated Protein Kinases

MAPKKK Mitogen Activated Protein Kinase Kinase Kinase

MAVS Mitochondrial Antiviral Signaling protein

MDA-5 Melanoma Differentiation-Associated gene 5

MEFs Mouse Embryonic Fibroblasts

MHC II Major Histocompatibility Complex II

MLKL Mixed Lineage Kinase Like protein

MOMP Mitochondrial Outer Membrane Permeabilization

MCP-1 Monocyte Chemoattractant Protein 1

MyD88 Myeloid Differentiation Primary Response 88

MRSA Methicillin Resistant Staphylococcus aureus

NEMO NF-κB Essential Modulator

NETs Neutrophil Extracellular Traps

NF-κB Nuclear Factor kappa B

NLRs NOD-like Receptors

NLRP3 NOD-, LRR-, and Pyrin domain–containing protein 3

NOD Nucleotide-binding Oligomerization Domain

NRDP-1 Neuregulin Receptor Degradation Protein 1

PAMPs Pathogen Associated Molecular Patterns

PCD Programmed Cell Death

PI(3)P Phosphatidylinositol 3-phosphate

PI(4,5)P_2 Phosphatidylinositol 4,5-bisphosphate

PIKfyve Phosphoinositide Kinase FYVE-type

pMLKL Phospho Mixed Lineage Kinase Like protein

PRRs Pattern Recognition Receptors

PSMs Phenol Soluble Modulins

PYD Pyrin Domain

RHOIM RIP Homotypic Interaction Motif

RIG-I Retinoic acid Inducible Gene I

RIP-1 / RIPK1 Receptor Interacting Protein Kinase 1

RIPK3 Receptor Interacting Protein Kinase 3

RING Really Interesting New Gene domain

RLRs / RLHs RIG-I-like Receptors / Helicases

ROS Reactive Oxygen Species

SARS-CoV-2 Severe Acute Respiratory Syndrome Coronavirus 2

TAB TAK1 Binding Protein

TAK1 Transforming growth factor β Activated Kinase 1

TBK1 TANK Binding Kinase 1

Th1 T helper 1 cells

TIR Toll IL-1 receptor domain

TLRs Toll-Like Receptors

TNF Tumor Necrosis Factor

TNFR TNF Receptor

TRAF3 / TRAF6 TNF Receptor Associated Factors

TRAM TRIF Related Adaptor Molecule

TRIF TIR-domain-containing Adaptor Inducing IFN-β

t-Boc tert-Butoxycarbonyl group

vita-PAMPs Vitality-associated PAMPs

WRW4 WRWWWW

INTRODUCTION

1 INFLAMMATION AND ROLE OF NEUTROPHILS AND MONOCYTES/MACROPHAGES

Inflammation is a complex biological response that can be initiated by invading pathogens or tissue injury-associated danger signals, known as Pathogens associated molecular pattern (PAMPs) and Damage associated molecular pattern (DAMPs), respectively. This response aims to remove pathogens, promote tissue repair and restore homeostasis. However, inflammation can lead to pathology when left unchecked[1]. The innate immune system plays a central role in orchestrating the inflammatory response, providing broad-spectrum defense against infections, trauma, and other harmful stimuli, while also shaping the long-term adaptive immune response to specific pathogens[2,3]. A wide array of mediators regulates the inflammatory process through intricate signaling networks. However, dysfunction or dysregulation of these mechanisms can give rise to immune-related pathologies, including chronic inflammatory diseases and autoimmune disorders. In such cases, persistent and uncontrolled inflammation may cause more extensive tissue damage than the initial insult. The main innate immune cells responsible for triggering and activating first-line immune responses are neutrophils and monocytes/macrophages. The relationship between neutrophils and monocytes/macrophages is complex and together, these two cell types orchestrate effective first line immune response by regulating other immune cells as well as each other [4].

Neutrophils, due to their potent and rapid reactivity against pathogens, are typically not resident within tissues or body cavities. Instead, they are produced in the bone marrow and stored in large reserves, ready for rapid mobilization into the circulation upon demand[5]. In fact, neutrophils represent the most abundant leukocyte population in human blood and possess strong microbicidal capabilities[6]. Monocytes also arise from the bone marrow; however, when they migrate into tissues, they can differentiate into macrophages or dendritic cells (DCs) depending on the tissue context[7].

In contrast to neutrophils, tissue-resident macrophages are less immunoreactive under steady-state conditions, an essential characteristic that allows them to patrol tissues and detect microbial invasion without triggering unnecessary inflammation[8]. Upon sensing pathogens, macrophages become activated and secrete various neutrophil chemoattractants, such as C-X-C motif chemokine ligand 1 (CXCL1), CXCL2, interleukin-1α (IL-1α), and monocyte chemoattractant protein-1 (MCP-1), thereby initiating neutrophil recruitment to the site of infection [9,10].

The rapid influx of neutrophils forms a critical first wave of the innate immune response. Traditionally, recruited neutrophils have been considered short-lived cells, programmed to die rapidly in order to minimize collateral tissue damage and prevent excessive inflammation[11]. However, macrophages can prolong neutrophil survival by releasing growth and survival factors such as

granulocyte–macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), and tumor necrosis factor-alpha (TNF-α), effectively sustaining the inflammatory response [12].

Overall, neutrophils and macrophages function in a coordinated manner to mount an effective immune response against pathogens. Nevertheless, this interaction must be tightly regulated, as dysregulation can lead to excessive inflammation and contribute to tissue pathology [13].

2 PATTERN RECOGNITION RECEPTORS

The innate immune system cells can recognize PAMPs or DAMPs through Pattern-Recognition Receptors (PRRs). PRRs are distributed across various subcellular compartments, including the cellular and endosomal membranes, the cytosol, and also exist in secreted forms found in the bloodstream and interstitial fluids[14]. PRRs are classified into four major subfamilies: Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-Leucine Rich Repeat (LRR)-containing receptors (NLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs, also known as RIG-I-like helicases—RLHs), and C-type lectin receptors (CLRs)[15]. Upon activation, PRRs initiate downstream signalling cascades that induce innate immune responses through the production of inflammatory cytokines, type I interferons (IFNs), and other immune mediators. These responses not only provide immediate host defense mechanisms, such as inflammation and programmed cell death, but also play a crucial role in priming and coordinating antigen-specific adaptive immune responses[16].

2.1 Toll-like receptors and main signalling pathways

Among PRRs, Toll-like receptors (TLRs) were the first to be identified and remain the best characterized to date. These germline-encoded type I transmembrane proteins, derived from the Toll gene family, play a pivotal role in recognizing a wide range of microbial patterns and activating the innate immune response. The innate immune functions of TLRs were first uncovered in 1988, when *Drosophila melanogaster* mutants lacking a functional Toll gene were found to be highly susceptible to fungal infections[17]. Shortly thereafter, a human homolog, now known as TLR4, was identified[18]. 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. 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 (Figure 1). 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 (Figure 5)[19–22] .

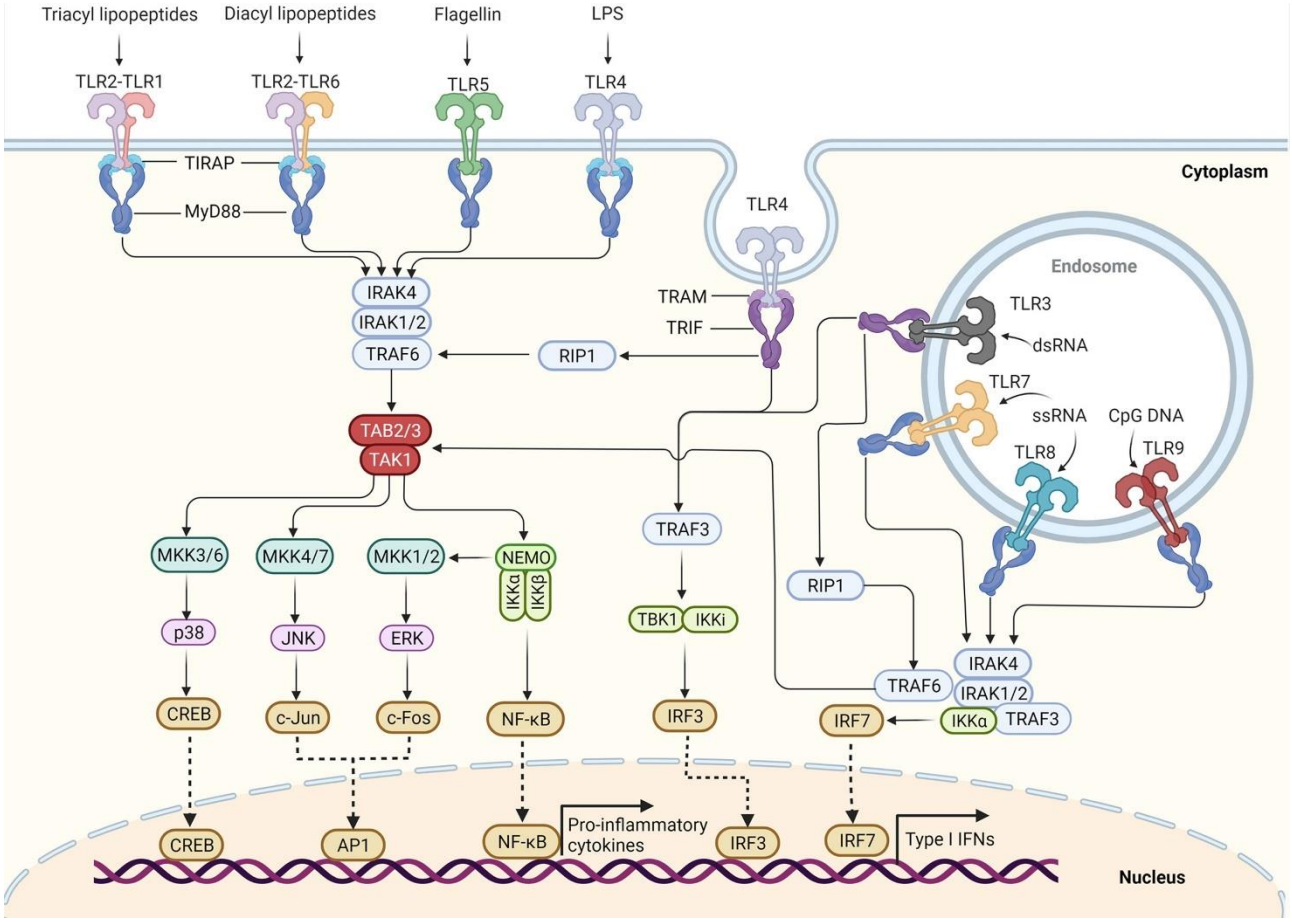


Figure 1: TRLs signaling pathway (from Duan T *et al.* 2022. *Front. Immunol.* 13:812774.)

Individual TLRs differentially recruit members of a set of Toll IL-1 receptor (TIR) domain-containing adaptors such as MyD88, TIR-domain-containing Adaptor Inducing IFN-β (TRIF), MyD88 Adaptor-Like protein (MAL/TIRAP), or TRIF Related Adaptor Molecule (TRAM). MyD88 is utilized by all TLRs and activates Mitogen Activated Protein Kinases (MAPKs) and Nuclear Factor kappa B (NF-κB) for the induction of inflammatory cytokine genes. TIRAP is a sorting adaptor that recruits MyD88 to cell surface TLRs such as TLR2 and TLR4. However, it was demonstrated that TIRAP also participates in signaling through endosomal TLRs such as TLR9[23]. The lipid-binding domain of

TIRAP binds to PI(4,5)P_2 at the plasma membrane and to PI(3)P on endosomes, which mediates the formation of functional TLR4 and TLR9 signaling complexes at their respective sites. Thus, TIRAP associates with both cell surface and endosomal TLRs by binding to different lipids[23]. However, a high concentration of TLR9 agonists can promote receptor activation in the absence of TIRAP, suggesting that TIRAP is required for TLR9 signaling in natural situations such as HSV-1 infection[24]. TRIF is recruited to TLR3 and TLR4 and promotes an alternative pathway that leads to the activation of Interferon Regulatory Factor 3 (IRF3), NF-κB and MAPKs for induction of type I Interferon (IFN) and inflammatory cytokine genes. TRAM is selectively recruited to TLR4 but not TLR3 to link TRIF and TLR4. TLR3 directly interacts with TRIF, and this interaction requires phosphorylation of the two tyrosine residues in the cytoplasmic domain of TLR3 by the epidermal growth factor ErbB1 and Btk[25,26]. Collectively, depending on the adaptor usage, TLR signaling is largely divided into two main signaling cascades: the MyD88-dependent and TRIF-dependent cascade.

2.2 Myd88 signalling pathway

Upon engagement of Toll-like receptors (TLRs), the adaptor protein MyD88 recruits members of the IL-1 receptor-associated kinase (IRAK) family, forming a multiprotein complex known as the Myddosome (Figure 2)[27]. During this assembly, IRAK4 activates IRAK1, which undergoes autophosphorylation at multiple sites[28] and is then released from MyD88[29].

Activated IRAK1 subsequently interacts with TRAF6, a RING-domain E3 ubiquitin ligase. In cooperation with the ubiquitin-conjugating enzymes UBC13 and UEV1A, TRAF6 catalyzes K63-linked polyubiquitination of both itself and the Transforming growth factor β Activated Kinase 1 (TAK1) kinase complex.

TAK1, a MAPKKK family member, forms a complex with regulatory subunits TAB1, TAB2, and TAB3, which recognize and bind the polyubiquitin chains generated by TRAF6, leading to TAK1 activation[30,31]. Although the precise mechanism remains unclear, TAK1 may be activated via K63-linked ubiquitination or proximity-induced transphosphorylation.

Once activated, TAK1 initiates two critical signaling cascades: The IκB kinase (IKK) complex pathway and the MAPK pathway.

In the IKK pathway, TAK1 binds the IKK complex, composed of catalytic subunits IKKα, IKKβ, and the regulatory subunit NEMO/IKKγ, via ubiquitin chains. TAK1 phosphorylates and activates IKKβ, which then phosphorylates the NF-κB inhibitor IκBα. IκBα is subsequently degraded by the

proteasome, allowing NF-κB to translocate to the nucleus and initiate transcription of proinflammatory genes.

Simultaneously, TAK1 activation triggers the MAPK signaling cascade, activating kinases such as ERK1/2, p38, and JNK. These, in turn, activate AP-1 transcription factors and promote mRNA stabilization to regulate inflammatory responses[32,33].

The role of TAK1 appears to be cell type-specific. In mouse embryonic fibroblasts (MEFs), TAK1 deficiency impairs phosphorylation of IKKs, p38, and c-Jun N-terminal Kinase (JNK) following LPS stimulation. However, in neutrophils from TAK1-deficient mice, TLR4-mediated activation of IKK, p38, and JNK, as well as cytokine production, is paradoxically enhanced, suggesting alternative regulatory mechanisms in different immune cell types[34].

Similarly, the physiological roles of the TAB proteins remain unclear. Mice deficient in TAB1 or TAB2 do not exhibit obvious defects in TLR signaling[35], and even double knockout mice lacking both TAB2 and TAB3 show normal cytokine responses to TLR stimulation in MEFs and macrophages[36]. These findings suggest functional redundancy or compensation among TAB family members.

Finally, TLR2 and TLR4 activation in macrophages also promotes mitochondrial recruitment to phagosomes and enhances production of mitochondrial reactive oxygen species (ROS) for bactericidal activity[36]. During bacterial infection, TRAF6 translocates to the mitochondria where it interacts with ECSIT, promoting its ubiquitination and leading to increased ROS generation both within mitochondria and throughout the cell.

2.3 TRIF signalling pathway

The adaptor protein TRIF plays a central role in the MyD88-independent TLR signaling pathway by engaging two distinct downstream signaling branches via interactions with TRAF6 and TRAF3 (Figure 2).

TRAF6 recruits the kinase RIP-1, which then interacts with and activates the TAK1 complex. This leads to the activation of NF-κB and MAPKs, ultimately inducing the production of inflammatory cytokines such as TNF-α and IL-1β (Figure 2). In contrast, TRAF3 recruits the IKK-related kinases TBK1 and IKKi, along with the regulatory subunit NEMO, to mediate phosphorylation of IRF3. Phosphorylated IRF3 then dimerizes and translocates from the cytoplasm to the nucleus, where it drives the transcription of type I interferon (IFN) genes[32,33].

Additional regulation of TRIF-dependent signaling involves the Pellino family of E3 ubiquitin ligases. In particular, Pellino-1 has been shown to be crucial in this pathway:

- Mice lacking Pellino-1 exhibit impaired TRIF-mediated NF-κB activation and reduced cytokine production[37].
- Pellino-1 is phosphorylated by TBK1/IKKi, which enables it to ubiquitinate RIP-1, suggesting that Pellino-1 promotes NF-κB activation by facilitating RIP-1 recruitment[38].
- Moreover, Pellino-1 also contributes to IRF3 activation by binding to DEAF-1, a transcription factor that enhances the binding of IRF3 to the IFNβ promoter[38].

Recent findings have identified a novel regulatory element in this pathway: the inositol lipid PtdIns5P. This lipid binds both IRF3 and TBK1, thereby promoting their interaction and facilitating proximal phosphorylation of IRF3. During viral infection, the kinase PIKfyve is responsible for generating PtdIns5P, linking inositol lipid metabolism to antiviral signaling[39].

2.4 Balance between MyD88 and TRIF

TLR4 activates both the MyD88-dependent and TRIF-dependent signaling pathways, enabling a balanced induction of inflammatory cytokines and type I interferons (IFNs), which is critical for controlling tumor growth and autoimmune diseases:

- TRAF3 is involved in both pathways. In the MyD88 complex, TRAF3 is degraded, leading to TAK1 activation, while in the TRIF pathway, TRAF3 promotes type I IFN production. This dual role allows TRAF3 to suppress inflammation while supporting antiviral responses[40].
- The E3 ubiquitin ligase NRDP-1 ubiquitinates MyD88 and TBK1, promoting MyD88 degradation and enhancing TBK1 activation. This shift favors type I IFN production over proinflammatory cytokine release[40].
- In antigen-presenting cells, MHC class II molecules in endosomes interact with the tyrosine kinase Btk via CD40 signaling. Activated Btk engages with both MyD88 and TRIF, enhancing activation of both pathways and promoting the production of inflammatory cytokines and type I IFNs[41].

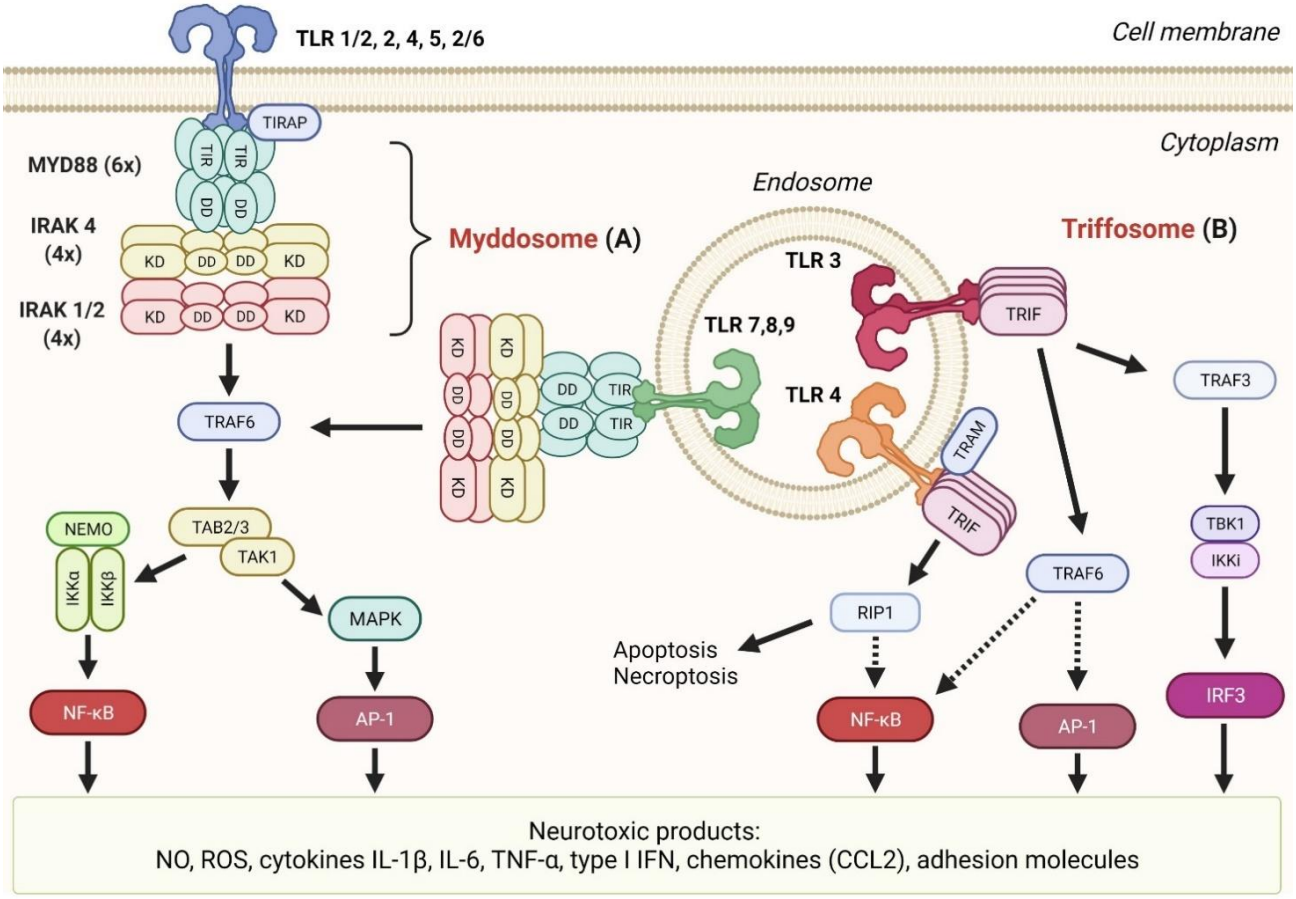


Figure 2: MyD88 and TRIF signaling pathway (from Sušjan-Leite P *et al.* 2022. *Front. Immunol.* 13:940969.)

2.5 Formyl peptide receptors (FPRs)

Phagocytic leukocytes also express another class of innate immune receptors, named *N*-formyl peptide receptors (FPRs), which play a key role in host defense and inflammation. To date, three subtypes (FPR1, FPR2, and FPR3) have been identified in humans though the role of FPR2 in inflammation is much debated. They are members of the seven transmembrane receptors, also known as G protein-coupled receptors (GPCRs), which are responsible for transducing a wide range of signals across the plasma membrane[42].

In the mid-1970s, Schiffmann *et al.* discovered that synthetic peptides with N-terminal formylated methionine (fMet) act as chemoattractants for phagocytic cells, serving as molecular patterns recognized by the innate immune system[43]. This is due to the fact that bacterial protein synthesis initiates with fMet, which is later cleaved during protein maturation. As a result, fMet-containing peptides are released by growing bacteria and have been isolated from cultures of species such as *E. coli*, *S. aureus*, *M. avium*, and *L. monocytogenes*, all shown to activate human phagocytes[44–47].

Additionally, mitochondrial proteins, which are also N-formylated, can serve as endogenous chemoattractants for neutrophils[48].

In humans, the N-formyl peptide receptor family consists of three isoforms, named FPR1, FPR2, and FPR3. FPRs are traditionally recognized as key G protein-coupled receptors (GPCRs) involved in inflammatory and immune responses to pathogen infection and cellular damage. Structurally, they belong to the seven-transmembrane domain receptor family, characterized by an extracellular N-terminus, seven transmembrane helices, and an intracellular C-terminus. FPR1 and FPR2 share 69% identity at the amino acid level (Figure 3), whereas FPR3 has 56% amino acid sequence identity to human FPR1 and 83% to FPR2 [46].

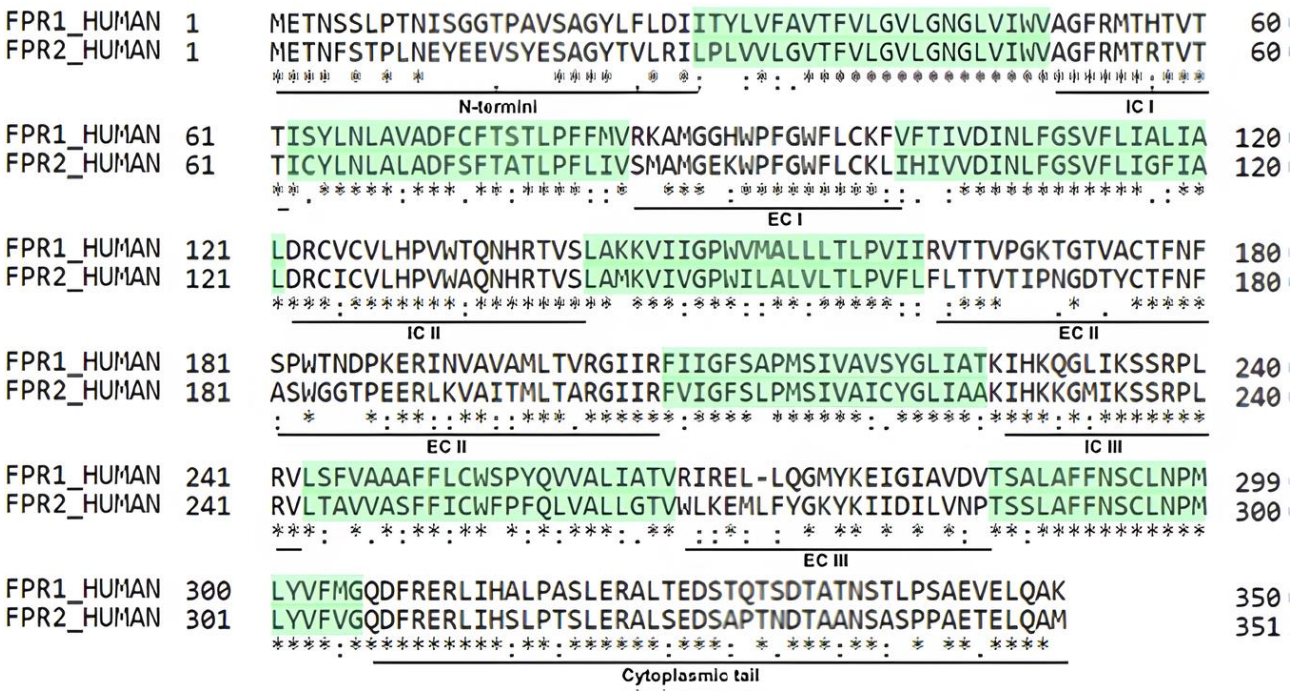


Figure 3: Sequence comparison between FPR1 and FPR2 (from Dahlgren *et al.*, 2016. Biochem Pharmacol. Aug 15(114):22-39).

Recent studies have revealed a more complex picture, highlighting the heterogeneity and ligand promiscuity of FPRs. Beyond their classical role in recognizing formylated peptides, FPRs are now known to participate in diverse pathophysiological processes, including tissue repair and chronic inflammation, independent of formyl peptide binding[49–51].

Additionally, while FPRs are conventionally localized to the plasma membrane, emerging evidence shows their presence in noncanonical subcellular locations. For instance, FPR2 has been detected in the nucleus of lung carcinoma and gastric adenocarcinoma cells, where it is involved in intranuclear

signaling [52]. Similarly, FPR1 has been found in primary granules and secretory vesicles of circulating neutrophils, as demonstrated by subcellular fractionation studies [53].

2.6 Ligands and inhibitors for human FPRs

The tripeptide fMLF (N-formyl-Met-Leu-Phe), derived from *Escherichia coli*, is the shortest and most potent high-affinity agonist for FPR1[54]. In contrast, FPR2/ALX has low affinity for fMLF and other potent FPR1 agonists but shows relatively high affinity for longer N-formylated peptides with specific sequences, particularly those with C-terminal positive charges or microbial origin other than *E. coli*, such as fMIFL from *Staphylococcus aureus* and fMIVIL from *Listeria monocytogenes*[54–56]. Additionally, FPRs are activated by other microbial peptides like phenol-soluble modulins (PSMs) from CA-MRSA, which are 20–25 amino acid α-helical formylated peptides that preferentially activate FPR2 over FPR1[57,58].

FPR2/ALX recognizes a broad range of structurally diverse agonists, including small proteins, peptides, and synthetic compounds like serum amyloid A, lipoxin A4, and the synthetic molecule Quin-C1. In contrast, FPR3 shows weak responses to most formyl peptides, with the exception of fMMYALF, a mitochondrial hexapeptide. FPR3 also has limited high-affinity endogenous ligands, such as F2L, derived from human heme-binding protein[54].

Among synthetic peptide agonists, WKYMVm (Trp-Lys-Tyr-Met-Val-D-Met-NH_2) is a potent activator of FPR1, FPR2, and FPR3, with strongest activity on FPR2, effective at picomolar concentrations[59]. When conjugated with FITC at lysine, WKYMVm shows slightly higher affinity for FPR2 than FPR1[55]. A variant, WKYMVM (with L-methionine at the C-terminus), is highly selective for FPR2 and only weakly activates FPR3[54].

Several antagonists of formyl peptide receptors (FPRs) have been identified, with the earliest examples derived from modifications of the classical FPR1 agonist fMLF. In a pioneering study, Freer *et al*. (1980) replaced the N-formyl group of fMLF with a tert-butyloxycarbonyl (t-Boc) group, resulting in t-Boc-MLF (Boc-1), a peptide that exhibited FPR1 antagonistic activity[60].

Building on this approach, another modified peptide was developed by replacing the MLF sequence with FLFLF, producing t-Boc-FLFLF (also known as Boc-2), which also functions as an FPR1 antagonist. However, there is evidence that Boc1 and Boc2, are not receptor-selective when used at higher concentrations, as they then partially inhibit also FPR2 [61]. 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 (Table 1)[62].

	FPR1	FPR2
Agonist		
fMIFL	+++ (≈0.1)	−
fMLF	++ (≈50)	−
WKYMVM	−	++ (≈40)
WKYMVm	+	+++ (≈2)
Antagonist		
Boc(1/2)	++	−
WRWWWW	−	++

Table 1: The most sudied agonist and antagonist for human FPR1 and FPR2 (modified from Winther *et al.*,2018. Basic Clin Pharmacol Toxicol. Feb;122(2):191-198)

Recently, other compounds were discovered as activators or inhibitors of FPRs.

In addition to the classic *E. coli*-derived fMLF, among the other ligands recognized by FPR1 there are other formylated bacterial peptides, such as fMAMKKL from *Salmonella*, fMFIYYCK from *Staphylococcus*, and fMKKIML from *Listeria*. Non-formylated microbial ligands, mostly of viral origin, also interact with FPR1, though further research is needed. Notably, HIV envelope proteins like gp41 T20/DP178 and T21/DP107 have been proposed as FPR1 ligands, although chemotactic peptides from HIV-1 gp41 mainly signal through FPR2 [63]. Additionally, C5a peptide is an activator of FPR2 as well as Hp(2-20) derived from *Helicobacter pylori*[64].

On the other hand, Quin-C1 (4-butoxy-N-[2-(4-methoxy-phenyl)-4-oxo-1, 4-dihydro-2H-quinazolin-3-yl]-benzamide) and Quin-C7 31 (4-butoxy-N-[2-(4-hydroxyphenyl)-4-oxo-1,2-dihydroquinazolin-3-yl]-benzamide) quinazolinone derivative compounds were found to be inhibitors highly selective for FPR2 as opposed to FPR1 [65].

3 PROGRAMMED CELL DEATH

Besides cytokines and chemokines production, programmed cell death (PCD) executed by innate immune cells can sustain and amplify the inflammatory response, leading to pathogen killing, and promoting the recruitment of additional immune cells. However, when dysregulated or abnormally activated, PCD may potentially drive chronic inflammation with tissue damage and impaired organ function[66]. The best described types of PCD are apoptosis, pyroptosis and necroptosis, although new types of PCD are continuously being discovered.

3.1 Apoptosis

Apoptosis was the first PCD to be described and was morphologically characterized in 1972[67]. Apoptosis is now understood to occur via two main pathways: the intrinsic (mitochondria-dependent) and the extrinsic (death receptor-mediated) pathways.

The intrinsic pathway was defined by the discovery of APAF1, which acts as a cytosolic sensor of cytochrome c released from damaged mitochondria[68]. Mitochondrial outer membrane permeabilization (MOMP), triggered by cellular stress or damage, results in the release of cytochrome c and other pro-apoptotic factors into the cytosol. Once in the cytoplasm, cytochrome c binds to APAF1, leading to the formation of the apoptosome complex along with pro-caspase-9, the initiator caspase of this pathway[69]. The assembly of the apoptosome, driven by ATP hydrolysis, facilitates the cleavage and activation of caspase-9, which in turn activates downstream executioner caspases such as caspase-3[70].

The regulation of mitochondrial membrane permeability is tightly controlled by members of the BCL-2 protein family. The first BCL-2 protein was identified in 1984 in studies on B-cell lymphoma, and later, a homolog in *C. elegans* (ced-9, homologous to BCL-XL) was shown to inhibit apoptosis in genetic screens[71]. The role of BCL-2 family proteins in apoptosis regulation has since been extensively characterized. BCL-2 is a member of the anti-apoptotic BCL-2 family located on the outer mitochondrial membrane. This factor binds and inhibits BAX and BAK pro-apoptotic proteins, by preventing mitochondrial outer membrane permeabilization (MOMP) and cytochrome c release. As soon as BCL-2 is inactivated and caspase-8 cleaves bid into tbid, this activates BAX and BAK which oligomerize in the mitochondrial outer membrane by causing permeabilization and releasing cytochrome c into the cytoplasm[72].

.

Shortly after the intrinsic pathway was described, the extrinsic pathway of apoptosis was identified. This pathway is initiated by the activation of death receptors such as Fas and the tumor necrosis factor-alpha receptor (TNFR). Engagement of these receptors leads to recruitment of the adaptor protein FADD (Fas-associated protein with death domain) and pro-caspase-8[73–77]. FADD binds to the death domain (DD) of the receptor and recruits caspase-8 via homotypic interactions between their death effector domains (DED)[76,77]. Once assembled, pro-caspase-8 undergoes autoproteolytic activation into mature caspase-8, which then activates executioner caspases, primarily caspase-3 and -7. Activation of caspase-3/-7 leads to nuclear condensation and fragmentation, and mitochondrial membrane depolarization which are followed by the formation of apoptotic bodies that are rapidly engulfed by phagocytes [78–81]

In summary, caspase-9 and caspase-8 serve as the initiator caspases of the intrinsic and extrinsic apoptotic pathways, respectively. Despite originating from distinct triggers and upstream signaling mechanisms, both pathways converge towards a common set of executioner caspases, namely caspase-3 and caspase-7, responsible for carrying out the apoptotic program. Recent evidence has shown that caspase-3 is able to cleave and activate the pore-forming protein Gasdermin E (GSDME) and this may turn apoptosis into pyroptosis (Figure 4)[82,83]. Indeed, in normal tissues, GSDME-mediated cell death can enhance immune responses to apoptotic cells. Additionally, in cancer cells, GSDME expression is often silenced by promoter methylation, suggesting a tumor suppressor role and mutations in GSDME are associated with hereditary hearing loss (DFNA5)[84] .

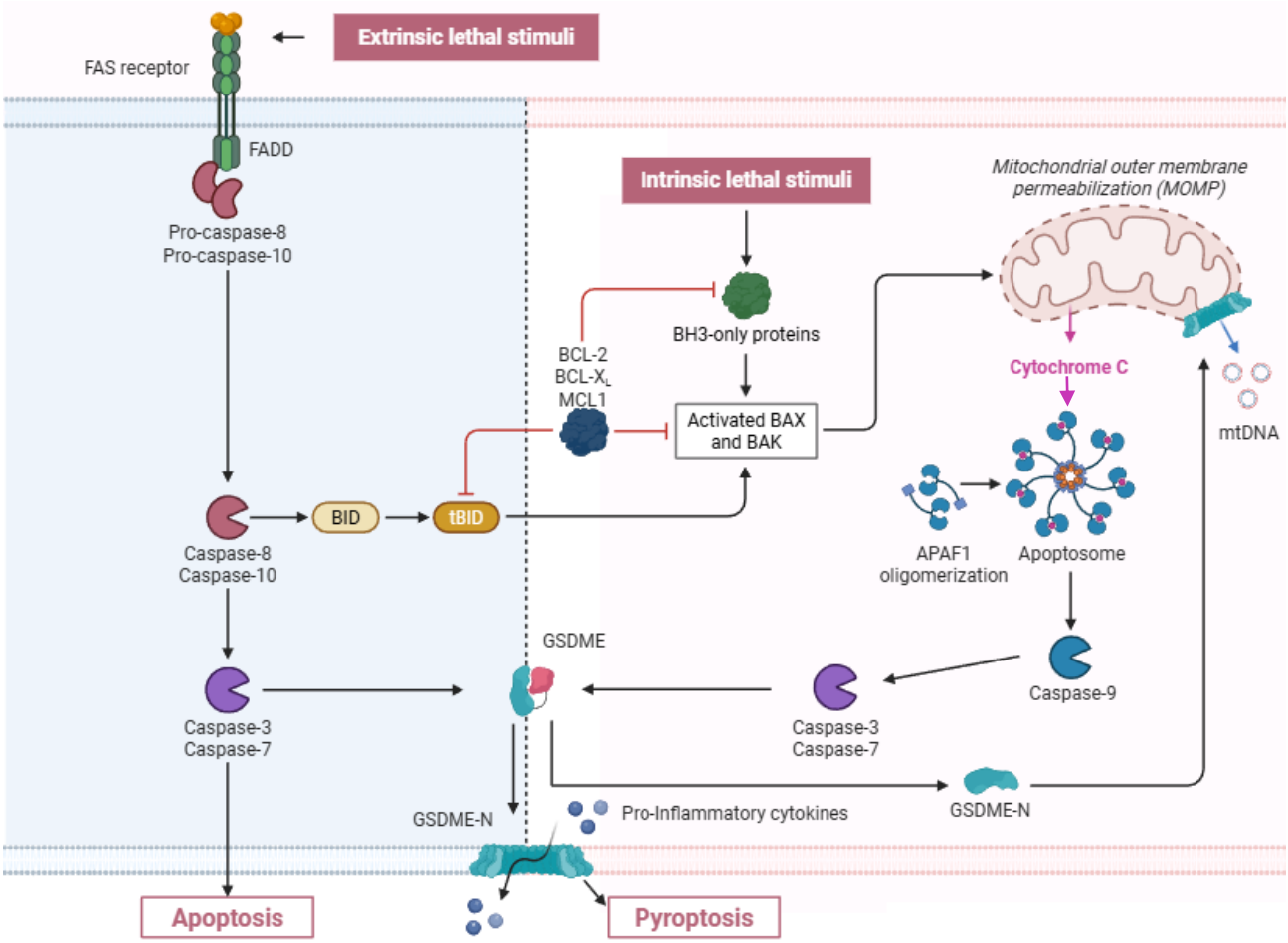


Figure 4: Extrinsic and intrinsic apoptotic pathway (from Biorender Template, creator: Samara Ona, modifed)

3.2 Pyroptosis

Pyroptosis has been classically described as a pro-inflammatory form of cell death occurring downstream of inflammasome activation and gasdermin cleavage [82,83]. Gasdermins (GSDMs) are a family of pore-forming proteins and effectors of pyroptosis. Upon cleavage by inflammatory caspases or other proteases, gasdermin N-terminal domain is released and moves into cellular membranes forming pores, leading to release of IL-1β/IL18 and small DAMPs and eventually promoting membrane rupture with cytosolic content release. The main gasdermin members family are GSDMA, GSDMB, GSDMC, GSDMD and GSDME [85–87], but the two most studied, highly expressed in myeloid cells and involved in inflammatory processes are GSDMD and GSDME.

Among inflammasomes, NOD-, LRR-, and Pyrin domain–containing protein 3 (NLRP3) has been the best described so far. Its activation can occur via two main pathways, the canonical and non-canonical pathway (Figure 5). In the canonical pathway, the activation of NLRP3 inflammasome involves two steps. The first step requires TLR-mediated transcriptional priming for NLRP3

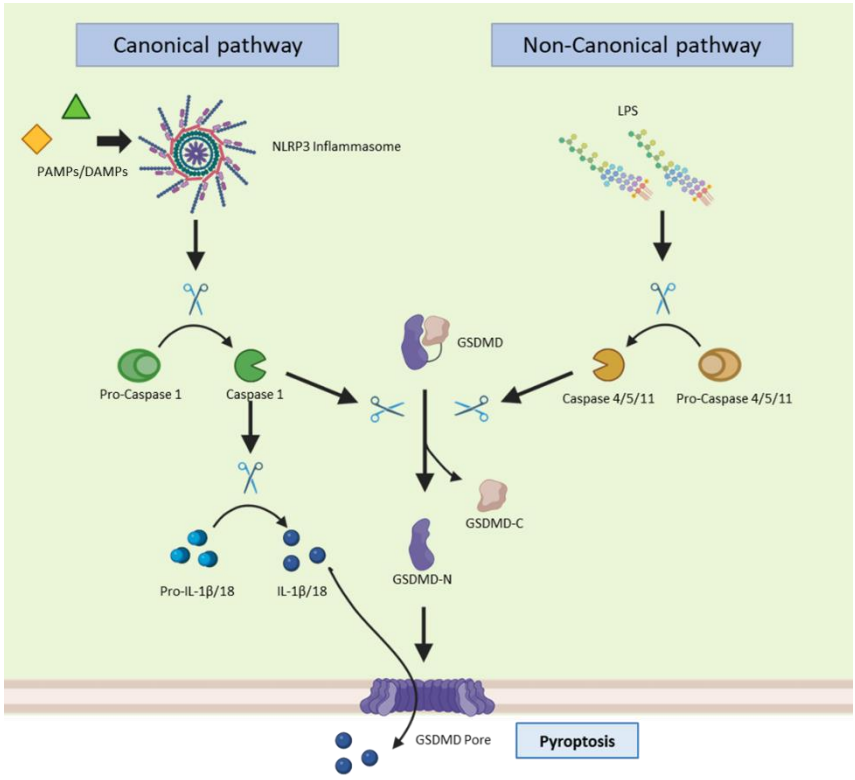


Figure 5: Canonical and non-canonical pathway of GSDMD cleavage (generated by BioRender)

expression. During the second step, upon sensing an apt stimulus, NLRP3 complexes with pro-caspase-1 and the adaptor molecule apoptosis-associated speck-like protein containing a CARD (ASC). Sensor proteins containing PYD or CARD domains initiate inflammasome assembly, with ASC bridging these sensors to caspase-1 via homotypic interactions, leading to caspase-1 activation. Activated caspase-1 cleaves pro–IL-1β and pro–IL-18 into their active cytokine forms and also cleaves GSDMD, which forms membrane pores, triggering pyroptosis[88]. It has been reported that RNA viruses such as Influenza A virus (IAV) and SARS-CoV-2 can induce lytic cell death mediated by NLRP3 inflammasome and caspase-1 activation[89,90]. Additionally, non-canonical inflammasome activation via murine caspase-11 or human caspase-4/-5 in response to intracellular lipopolysaccharide (LPS) also leads to GSDMD cleavage, pore formation and consequent activation of the NLRP3 inflammasome and caspase-1 (Figure 5)[91,92]. Some CARD-containing sensors (e.g., NLRC4, NLRP1b) can directly activate caspase-1 without ASC, though the physiological relevance is still unclear.

3.3 Necroptosis

Necroptosis is another form of regulated cell death that occurs when the apoptotic pathway is inhibited or compromised. Unlike apoptosis, which is a non-inflammatory and controlled process, necroptosis leads to plasma membrane rupture and the release of intracellular contents, promoting inflammation[93]. It plays crucial roles in host defense, especially against viral infections[94], as well as inflammatory diseases, neurodegeneration, and cancer[95–97]. The main upstream signaling pathway

necroptosis' factor is RIPK1, which, activated by death receptors like TNFR1, acts as activator of RIPK3 through RIP homotypic interaction motif (RHIM) domains. The phosphorylated form of RIPK3 is essential to induce the activation of necroptosis' executor known as MLKL that, once phosphorylated, oligomerizes and translocates to the plasma membrane, where it disrupts membrane integrity, leading to cell lysis[93].

Necroptosis is typically initiated when death receptors such as TNF receptor 1 (TNFR1) are activated in the absence of active caspase-8, which normally inhibits RIPK1/RIPK3-mediated necrosis. The formation of the necrosome complex (RIPK1-RIPK3-MLKL) is the hallmark of necroptosis signaling (Figure 6). In addition, Toll-like receptors (TLRs) can also initiate necroptosis via RIPK3 and MLKL phosphorylation independent of RIPK1 kinase activity[98,99].

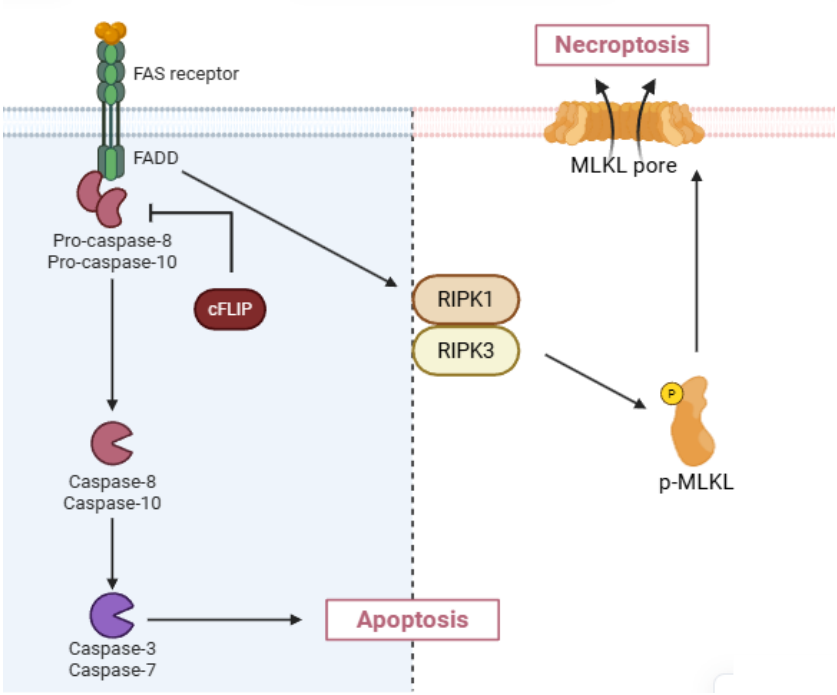


Figure 6: Necroptotic pathway (generated by BioRender)

In summary, the necroptosis and pyroptosis are lytic forms of cell death that can contribute to propagate inflammation by releasing DAMPs and pro-inflammatory cytokines. However, apoptosis has recently been considered also as potentially inflammatory form of cell death due to its link with pyroptosis.

4 NEUTROPHILS AND THEIR ROLES DURING BACTERIAL INFECTIONS.

Neutrophils are the most abundant type of white blood cells in humans, accounting for approximately 50–70% of all leukocytes. Produced in the bone marrow, they have a short lifespan, typically circulating in the bloodstream for about 5 to 90 hours, before migrating to tissues where they are needed. Their primary function is to respond to infections or injuries [100]. Thanks to their high motility, neutrophils can rapidly reach sites of inflammation through a process known as chemotaxis[101].

Once at the site of infection, neutrophils perform several key immune functions:

- **Phagocytosis**: Neutrophils engulf and digest pathogens like bacteria and fungi by forming a phagosome, which fuses with lysosomes containing enzymes and reactive oxygen species (ROS) to destroy the invaders. They also release antimicrobial peptides to assist in this process[101,102].
- **Release of Antimicrobial Substances**: Neutrophil granules are packed with enzymes and proteins that, once released, act extracellularly to kill pathogens. This targeted antimicrobial delivery is critical, particularly in viral elimination[103].
- **Neutrophil Extracellular Traps (NET) Formation**: Under certain conditions, neutrophils expel their DNA and proteins to form web-like structures (NETs) that trap and kill microbes[104]. However, NETs are non-specific and can promote tissue damage and chronic inflammation, contributing to autoimmune diseases like lupus, psoriasis, and rheumatoid arthritis[105].
- **Regulation of Inflammation**: Neutrophils secrete cytokines and chemokines that attract and activate other immune cells. While they can drive all stages of atherosclerosis, they may also have reparative roles in cardiovascular inflammation, illustrating their dual nature in immune regulation[106].

Recently, it has been demonstrated that neutrophils can activate several kinds of response and in different ways according to the presence of dead or live bacteria. This recognizing is possible through the activation of FPRs, which can bind formylated peptides, present exclusively in live bacteria. Indeed, these kinds of ligands are known as vita-PAMPs[107]. Lentini *et al.* have shown that murine neutrophils are able to discriminate the presence of live from dead bacteria, by triggering several types of responses like CXCL8 (also known as IL-8) release, transmigration and ROS production. The intensity of these responses increased with the synergic activation of FPRs and TLRs. In particular, the authors have demonstrated that in the presence of live GBS, it has been higher levels of Cxcl1 and 2 than heat killed bacteria in murine neutrophils. Additionally, it has been shown that

blocking Fpr1 and 2 with selective antagonist, it was possible to know which of these receptors depended on specific responses[108]. However, the mechanism underlying these kinds of response in human neutrophils remain unclear. Specifically, it is poorly known whether the live bacteria induce responses exclusively dependent by FPRs activation or there is synergize with TLRs and, if so, which them. For example, it has recently been found that TLR8, which recognizes single stranded bacterial RNA, is able to distinguish live bacteria from dead ones[109]. TLR8 has recently emerged as a central bacterial sensor, due to its capacity to detect pathogen RNA[110,111] and drive robust T-helper 1 (Th1) responses through the induction of a distinctive cytokine profile[112]. Whether neutrophil responses to live bacteria are due to the synergize between FPRs and TLR8 is still not defined.

5 MACROPHAGE RESPONSES TO VIRAL INFECTIONS

Macrophages are an important component of first-line defense against pathogens, including viruses. Macrophages primarily function in host defense and tissue maintenance through processes like phagocytosis, antigen presentation to activate adaptive immunity, and the secretion of inflammatory mediators (cytokines and anti-viral factors). They also play crucial roles in tissue repair, wound healing, tissue remodeling, iron recycling from old red blood cells, and homeostasis maintenance. These functions are achieved through a dynamic and plastic nature, allowing them to sense environmental cues and adapt their behavior to the specific needs of the moment[113]. In the context of viral infection, the complicated roles of macrophages in antiviral immunity and the pathogenesis of viral infections warrant further studies. In response to respiratory virus infection, lung tissue residents play a key role in orchestrating early responses. It has been shown that Lung macrophages can be actively infected by viruses, allowing their replication into the host cells[114,115]. Timely and effective signaling by macrophages helps to rapidly recruit other inflammatory cells, ensuring the prompt clearance of pathogens[116]. However, viral pathogens can cause excessive death of macrophages accompanied by cytokines release and propagating inflammation and tissue damage[117,118].

In particular, RNA viruses induce antiviral Type I Interferon (IFN) response in infected cells [119]. Following their internalization, viruses release their genomic RNA into the cytoplasm. This starts the replication process, during which double-stranded RNA (dsRNA) is generated as an intermediate product[120]. Viral dsRNA is sensed by intracellular receptors such as Toll-like receptor 3 (TLR3) [121], and ATP-dependent DExD/H box RNA helicases, including retinoic acid-inducible gene I (RIG-I) [122] and melanoma differentiation-associated protein 5 (MDA-5) [123]. The binding of these receptors with dsRNA triggers the activation of TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent and mitochondrial antiviral signaling (MAVS)-dependent signaling cascades,

respectively[124–128]. All these signaling cascades converge towards the activation of the transcriptional factor IRF-3 [124–128] , leading to the upregulation of Type I IFN genes (IFN-α/β)[119]. Furthermore, the activation of endosomal TLR3 leads to the expression of NF-kB-regulated genes, including IL-1β and TNF, via TRIF [129,130]

While these antivirals signaling pathways are well characterized, how dsRNA sensing leads to cell death and the relationship with inflammation remains to define.

In addition to mount Type I IFN responses, infected cells may undergo programmed cell death (PCD) as a defence mechanism to limit viral replication[131]. However, the molecular pathways determining which type of death is engaged following dsRNA detection remain unresolved.

Notably, TRIF is well known for its role in IRF3 and NF-κB activation. Moreover, recent evidence suggests that TRIF might also act as a pro-death adaptor, capable of engaging caspase-8[132]. What remains to be established is whether TRIF-dependent caspase-8 activation truly occurs in response to dsRNA in human macrophages and what kind of cell death it initiates. Additionally, whether GSDME acts downstream of TRIF–caspase-8 signaling during antiviral responses has not been determined yet [133–135].

There are many studies that describe several pathway and form of cell death which macrophages undergo to contrast viral replication. For example, it is known that RNA viruses such as Influenza or SARS-CoV-2 replicate into macrophages by leading to NLRP3-dependent pyroptosis[89,90]. However, it is unclear whether NLRP3 inflammasome can be involved and activated by a single PAMP such as double stranded RNA [136–138].

AIMS OF THE THESIS

The following PhD thesis has two main aims focusing on the inflammatory responses of macrophages and neutrophils to viral and bacterial insults, respectively. The first is to investigate the specific effects of dsRNA, independently of other viral components, on macrophage cell death and inflammasome activation, clarifying how this single PAMP can orchestrate complex antiviral responses. The second aim is to elucidate the ability of human polymorphonuclear leukocytes to discriminate live from dead bacteria, focusing on the responses to GBS and the role of FPRs and TLRs.

As for the first objective, viruses are recognized by macrophages through a variety of pathogen-associated molecular patterns (PAMPs), which together trigger strong immune responses, including the production of pro-inflammatory cytokines and different forms of programmed cell death, by impairing their replication into host cells[114,115]. However, it remains unclear which specific viral PAMPs are individually responsible for activating these macrophage responses.

This PhD project focuses on dissecting how human macrophages respond specifically to double-stranded RNA (dsRNA), a viral PAMP common to many RNA viruses. The goal is to define the types of cell death induced by dsRNA and to elucidate the molecular signaling pathways that connect dsRNA sensing to cell death and inflammatory response. Particular attention is given to understanding how TLR3 activation can lead to the activation of apoptotic caspases and the potential roles of Gasdermin D (GSDMD) and Gasdermin E (GSDME) in mediating membrane permeabilization and cell lysis.

Moreover, although it is well established that respiratory viruses such as SARS-CoV-2 and influenza A virus (IAV) can activate the NLRP3 inflammasome, it is still unclear whether dsRNA alone is sufficient to trigger this activation, or if an additional priming signal (e.g., through NF-κB activation) is required to license inflammasome assembly and function. To this purpose, we aimed at investigating whether dsRNA alone triggered the NLRP3-signaling pathway or it was necessary a priming step.

To achieve Objective 1, human monocyte-derived macrophages (hMDMs) derived from peripheral blood of healthy donors were used and stimulated with Polyinosinic:polycytidylic acid (Poly(I:C)), a dsRNA mimetic.

As for the second objective, the ability of neutrophils to discriminate between live and dead bacteria was investigated mostly in vivo, by evaluating the inflammatory responses through the FPRs activation alone or in synergize with TLRs. However, this ability to recognize vita-PAMPs is still not defined in human neutrophils. Notably, the second part of PhD project focuses on the responses

actuated by human neutrophils mainly to *Streptococcus agalactiae* (also known as group B streptococcus [GBS]), an encapsulated Gram-positive bacterium typically found in the human gastrointestinal or lower genital tract of up to 30% of asymptomatic individuals[139]. 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[140,141]. However, to investigate whether the enhanced ability of live bacteria to induce pro-inflammatory response is specific to GBS in human neutrophils, other two types of common bacterial pathogens such as *Klebsiella pneumoniae* and *Staphylococcus aureus* were used in our experimental model. On the other hand, the role of FPRs, alone or in synergize with TLRs, was investigated in human neutrophils stimulated by GBS or *Klebsiella pneumoniae* and *Staphylococcus aureus,* by focusing on the role of TLR8, whose activity could be involved in live bacteria-induced response[109].

For both objectives, a better understanding of these mechanisms is crucial for designing improved therapies to augment host defense and potentiate the recruitment and the functional activity of innate immune cells.

MATERIALS AND METHODS

1 1st OBJECTIVE

1.1 Reagents and antibodies

RPMI 1640 medium (ECB9006L), l-glutamine (ECB3000D), fetal bovine serum (FBS) (ECS0180L), sodium pyruvate (ECM0542D), HEPES (ECM0180D), and penicillin–streptomycin (ECB3001D) were purchased from Euroclone, Milan, Italy. Human M-CSF (130-096-492) was purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). The following chemicals were obtained from Sigma-Aldrich (Missouri, USA): LPS from Escherichia coli 0111:B4 (L3012), Nigericin sodium salt (N7143), Z-VAD-fmk Pan-Caspase Inhibitor I (627610), GSK872 RIPK3 Inhibitor (Hy-101872),Necrostatin-1 (Nec-1) RIPK1 Inhibitor (N9037), CP-456773 sodium salt NLRP3 Ihnibitor (MCC950, PZ0280) and Triton X-100 (9002-93- 1). Pepinh-TRIF Inhibitor (tlrl-pitrif) was purchased from InvivoGen (San Diego, California, USA). The following primary antibodies were used for western blot: actin (sc-81178, Santa Cruz Biotechnology, Dallas, TX, USA); b-tubulin (2146, Cell Signalling Technology, Massachusetts, USA); p-MLKL (91689S, Cell Signaling Technology,Massachusetts, USA); MLKL (14993, Cell Signaling Technology,Massachusetts, USA) full-length caspase 3 (9662S, Cell Signaling Technology,Massachusetts, USA); caspase-1 (AG 20B-0048- C100, San Diego, USA); caspase-8 (ALX-804-242-C100, Enzo Life Sciences; cleaved caspase-3 (9661S, Cell Signaling Technology); GSDME (ab215191, Abcam); GSDMD (97558, Cell Signaling Technology,Massachusetts, USA) and antibody against human IL-1β (AF-201-NA, R&D Systems, Minneapolis, MN). Primary antibodies were used at the following dilutions: anti-actin 1:10 000, anti-b-tubulin 1:10000, anti-p-MLKL 1:1000, anti-MLKL 1:1000, anti-caspase-1 1:1000, anti-caspase-8 1:100, anti-full-length caspase 3 1:1000, anti-cleaved caspase-3 1:500, anti-GSDME 1:1000, anti-GSDMD 1:1000 and anti-human IL-1β 1:1000. The following secondary antibodies for western blot assay were purchased from LI-COR (Lincoln, Nebraska USA): Goat Anti-Mouse IRDye 680RD (926-68070), Donkey Anti-Rabbit IRDye 800CW (926-32213) and Donkey Anti-Goat IRDye (926-68074). Secondary antibodies were used at the following dilutions: 1:2000 for anti-mouse and anti-goat and 1:5000 for anti-rabbit. Primary antibody ASC (sc-271 054), Goat Anti-Mouse IgG (Alexa FluorR 594) (ab150116) and DAPI (ab228549) for immunofluorescence staining were purchased from Abcam. Hoechst fluorescent nucleic acid stain (33342) was obtained from ImmunoChemistry Technology, Bloomington, MN, USA. Poly(I:C) (HMW) (tlrl-pic) was purchased from InvivoGen (San Diego, United States). JC-1 Dye (Mitochondrial Membrane Potential Probe) (T3168) was purchased from Invitrogen (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Lipofectamine 3000 Transfection Reagent (L3000-008) was purchased from Invitrogen (Waltham, MA, USA). Fugene Transfection reagent was purchased from PROMEGA (E2311).

1.2 human Monocyte-derived Macrophages (hMDMs)

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats derived from healthy subjects and received by ARNAS "Civico, Di Cristina, Benfratelli" (Palermo, Italy) according to a Material Transfer Agreement signed on 6/11/2019. Human macrophages were obtained by culturing PBMCs for 7 days in a complete RPMI 1640 medium supplemented with 10% FBS and 50 ng/ml of human M-CSF. The medium was replaced after 3 days of culture. The day before each experiment, human monocyte-derived macrophages (hMDMs) were treated with trypsin–EDTA for 5 min, scraped, plated in complete medium without M-CSF into 96-well plates (5×10^4 cells/well), 12-well plates (7×10^5 cells/well), or 6-well plates (1.5×10^6 cells/well), and incubated at 37°C, with 5% CO2.

The day of the experiment, culture medium was changed to 1% FBS and cells were stimulated for 24h, unless otherwise indicated, as follows: 1 μg/mL LPS, in presence or not of 1 μg/mL intracellular/extracellular Poly(I:C) after 3 h of LPS priming. Where indicated, Poly(I:C) was transfected with Fugene or Lipofectamine 3000 according to the manufacturer's protocol. Where indicated, 25 μM of the Pepinh-TRIF Inhibitor, 1 μM of the NLRP3 inhibitor MCC950, 20 μM of Z-VAD-fmk Pan-Caspase Inhibitor, in combination or not with 50 μM of RIPK1 inhibitor Nec-1 or with 10 μM of RIPK3 inhibitor GSK872, were added 1 hour before LPS/Poly(I:C) stimulation. Where indicated, after 3 h of LPS priming, 10 μM nigericin was added in the culture medium for 1 h.

1.3 Isolation of total RNA and Real-Time Quantitative PCR (RT-qPCR)

For RT-PCR analysis, total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA,) following the manufacturers' instruction. For reverse transcription, the equivalent volume to 1 μg RNA was reverse-transcribed to cDNA, using the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA). Realtime quantitative PCR was carried out on Step One Plus Real-time PCR System (Applied Biosystems, Foster City, CA, USA) using specific FAM-labeled probe and primers (prevalidated TaqMan Gene expression assay for IFN-β (Hs01077958-s1), NLRP3 (Hs00918082-m1), IL-1β (Hs01555410-m1) and TNF-α (Hs99999043-m1), Applied Biosystems). Gene expression was normalized to GAPDH (prevalidated TaqMan Gene expression assay for GAPDH, Hs03929097g1, Applied Biosystems) endogenous control gene. Relative quantification of mRNA was carried out with the comparative CT method ($2^{-\Delta\Delta CT}$) and was plotted as a relative fold-change compared to untreated cells that were chosen as the reference samples.

1.4 Lactate dehydrogenase (LDH) assay

At the indicated time, culture supernatants were transferred into a new 96-well plate and the LDH released from cells was measured using the CytoTox 96R Non-Radioactive Cytotoxicity Assay LDH Cytotoxicity Assay Kit (G1780, Promega) according to the manufacturer's protocol. Absorbance was measured using a Spark (Tecan) plate reader at wavelengths of 490 nm.

1.5 Western blot

Cells were lysed using a lysis buffer made as follows: 10 mM Tris–HCl, 50 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, protease inhibitor (P8340, Sigma-Aldrich), and phosphatase inhibitor (P0044, Sigma-Aldrich). Total protein content was determined using the Bradford protein assay kit (Thermo Fisher Scientific). A total of 40 μg protein was loaded, resolved by SDS-PAGE, and blotted onto nitrocellulose membranes (Bio-Rad). For supernatant precipitation, cells were stimulated in the medium with 1% FBS, and samples were processed as previously described [142]. Blots were incubated overnight with primary antibodies at 4°C. The blots were analyzed using the Odyssey Imaging System (LI-COR).

1.6 ELISA assay

IFN-β, IL-1β, TNFα and S100A8/A9 were measured in culture supernatants using ELISA kit (R&D Systems, Minneapolis, MN) following the manufacturers' instructions.

1.7 Fluorescence microscopy

Cells were seeded in 96-well plates (PhenoPlate 96-well, black, optically clear flat-bottom, Perkin Elmer, Waltham, Massachusetts, USA). After stimulation, cells were incubated with 10 μM of JC1 dye + 1.5 μg/ml of Hoechst 33342 in PBS for 15' at 37°C, and then gently washed with PBS. Image acquisition was performed using Operetta CLS (Perkin Elmer) with a 63X objective. Nuclear DNA condensation was measured by Harmony Software (Perkin Elmer) as mean fluorescence intensity (MFI) and nuclear area (μm^2) of the nucleus stain Mitochondrial membrane potential, indicated by the red (485/590nm)/ green (485/529nm) fluorescence intensity ratio was measured by TECAN plate reader. For quantification of ASC-speck, after stimulation, cells were fixed in paraformaldehyde/phosphate-buffered saline (PBS) 4% for 10′ at room temperature (RT), permeabilized with 0.2% Triton X-100 for 5′ at RT, blocked with PBS/bovine serum albumin 3% for 30′ at RT and incubated with anti-ASC primary antibody (1:150, 1 h at RT) followed by secondary

goat anti-mouse Alexa 594 antibody (1:500, 45′ at RT) and DAPI staining solution (1:5000, 10′ at RT). Image acquisition was performed using Operetta CLS (Perkin Elmer) with a 40× objective.

1.8 Statistical analysis

Statistical analysis was performed using the GraphPad Prism 9 software. Unless otherwise stated, data were expressed as mean ± SD. Differences were identified using one-way repeated measures ANOVA with Tukey post hoc test. Differences with $p < 0.05$ were considered significant.

2 2nd OBJECTIVE

2.1 Bacterial strains

GBS WT strain H36B serotype Ib was used for most experiments. Bacteria were grown at 37°C with 5% CO2 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. *K. pneumoniae* AC133 was used throughout the present investigation. Bacteria were grown to the mid-log phase in Luria Bertani broth, washed three times in nonpyrogenic phosphate-buffered saline (PBS) and resuspended to the appropriate concentration in PBS. *S. aureus* strains (USA300) were used in the present studies. Bacteria were grown to the midlog phase in Tryptic Soy Broth (Oxoid), washed three times in nonpyrogenic PBS, and resuspended to the appropriate concentration in PBS.

To obtain preparations of heat-killed bacteria (HK-GBS), GBS 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. 1 μg of the heat-killed bacteria preparation corresponds to approximately 1×10^6 bacteria.

2.2 GBS signal peptides

Fifteen GBS signal peptide sequences were identified in the Signal Peptide Database (http://www.signalpeptide.de). Custom-made peptides were synthesized by Genscript Corp. with a purity of ≥95%. Details on sequence and source are given in Table 1 as described in Lentini *et al.*2022[143].

2.3 Isolation and stimulation of human neutrophils

Human neutrophils were isolated from the whole blood of healthy donors using Ficoll-Paque Premium density gradient centrifugation as previously described[144]. Staining with May/Grunwald/Giemsa showed that ~90% of isolated cells (1,5–2 ± 0.6 × 10^6 cells per ml of blood) were morphologically mature neutrophils (bands and segmented). Isolated human neutrophils (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 bacteria grown to the midlog phase at the indicated concentrations or multiplicities of infection (MOI), respectively. All stimulations were carried out by centrifuging cell suspensions for 10 min at 1800 × rpm to facilitate bacteria/neutrophil interactions. After incubation for 1 h at 37°C with 5% CO2, penicillin (250 IU/ml), and streptomycin (250 µg/ml) were added to kill extracellular bacteria. Control wells were stimulated with *Escherichia coli* K12 ultrapure LPS (InvivoGen). In selected experiments, neutrophils (5 × 10^5/well) were preincubated for 1 h at 37°C with 5% CO2 with the selective Fpr2 antagonist WRW4 (Abcam) and the pan-FPR inhibitor Boc-2 (GenScript) at the indicated concentrations before stimulation with HK or live GBS; or with actionin before stimulation with live GBS. In another set of experiments, neutrophil stimulation with indicated concentrations of the selective FPR agonists f-MIFL (GenScript) or WKYMVM (Abcam) or with different concentrations of formylated peptides (fPep8 and fPep10) from GBS, was preceded by a 1 h preincubation at 37°C in 5% CO2 with the selective Fpr2 antagonist WRW4 (Abcam) and the pan-FPR inhibitor Boc-2 (GenScript) at the indicated concentrations. In another set of experiment, neutrophils (5 × 10^5/well) were preincubated for 1 h at 37°C with 5% CO2 with the selective TLR8 inhibitor CU-CPT9a (MedChemExpress) or with TLR2 inhibitor C29 (MedChemExpress) at the indicated concentrations before stimulation with HK or live GBS, LPS, Live-*Kp*, Live *S.aureus* and bacteria DNA/RNA. For cytokine measurement, cell culture supernatants were collected at 24 h and stored at -80°C.

2.4 Reactive oxygen species measurement

Bacteria-induced reactive oxygen species (ROS) production by neutrophils was measured using the CellROX Deep Red Flow Cytometry Assay Kit (Thermo Fisher Scientific), according to the manufacturer's instructions. Briefly, neutrophils (5 × 10^5 per well in 0.2 ml of RPMI supplemented with 10% FCS) were seeded in microtiter plates and stimulated for 30 min with live GBS, with TLR agonists (HK-GBS or UV-killed GBS) or with the indicated fMet peptides. In another set of experiments, neutrophils were stimulated with live *K. pneumoniae* and *S. aureus* at the indicated MOIs, with HK-*Kp* or HK-*S.aureus*. After stimulation, samples then stained with the CellROX

fluorescent reagent at a final concentration of 5 μM for 30 min at 37°C. Cells were washed, fixed with 3.7% formaldehyde for 15 min, and analyzed with a BD FACSCanto II instrument.

2.5 Neutrophil migration assay

Neutrophil chemotaxis was determined using ChemoTX multiwell chambers (NeuroProbe) with 3 μm pore size filters according to the manufacturer's protocol. Briefly, peptides or vehicle were diluted to the desired concentration in RPMI medium supplemented with 0.1% BSA (wt/vol) and 320 μl were added to the lower chamber of each well. Neutrophil suspensions (1×10^5 per well in 57 μl of RPMI/0.1% BSA) were placed on top of the polycarbonate filter above each well and allowed to migrate for 90 min at 37°C with 5% CO2. The assay was terminated by detaching and wiping the filter to remove non-migrated cells from the filter top. Thereafter, the number of transmigrated cells was determined using BD TruCount tubes (BD Biosciences).

2.6 Cytokine measurement

Interleukin 8 (IL-8 or chemokine (CXC motif) ligand 8, CXCL8), tumor necrosis factor alpha (TNF-α), and interleukin 6 (IL-6) concentrations were determined in duplicate using the human enzyme-linked immunosorbent assay (ELISA) kits CXCL8/IL-8 Quantikine, TNF-a DuoSet and IL-6 DuoSet, according to the manufacturer's recommendations (R&D Systems).

2.7 RNA extraction and gene expression

To evaluate gene expression of CXCL8, neutrophils were seed in 24 well plates (3×10^6 cells per well) and were stimulated with heat killed or live bacteria for 2 h of incubation at 37°C in 5% CO2. RNA extraction was conducted by using RNeasy Mini Kit (QIAGEN). For reverse transcription, RNA was reverse-transcribed to cDNA, using the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA). Realtime quantitative PCR was carried out on Step One Plus Real-time PCR System (Applied Biosystems, Foster City, CA, USA) using specific FAM-labeled probe and primers (prevalidated TaqMan Gene expression assay for CXCL8 (Hs00174103_m1) and ACTB (hs01060665_g1), Applied Biosystem). Relative quantification of mRNA was carried out with the comparative CT method ($2^{-\Delta\Delta CT}$) and was plotted as a relative fold-change compared to untreated cells that were chosen as the reference samples.

2.8 Statistical analysis

Differences in cytokine and gene expression levels were assessed by the Mann-Whitney test. The same statistic test was used for the analysis of neutrophil counts and ROS production. Differences were considered statistically significant when P values were less than 0.05 ($P < 0.05$). Statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA).

RESULTS

1 Molecular Mechanisms of dsRNA-induced cell death in primary human macrophages (hMDMs).

1.1 Cytosolic, but not extracellular Poly(I:C), induces lytic cell death and DAMP release in hMDMs

First, we investigated the difference between extracellular and intracellular Poly (I:C) effects in hMDMs. Poly(I:C) is a mimetic of dsRNA, a common PAMP of RNA viruses that generates during viral replication in infected cells. dsRNA is recognized by intracellular receptors such as ATP-dependent DExD/H box RNA helicases (including MDA5 and RIG-I, which bind long and short dsRNA, respectively), or TRL3, which is also an extracellular receptor. MDA5/RIG-I and TLR3 lead to IRF3- and NF-kB-dependent gene expression through signaling cascades involving MAVS and TRIF, respectively[145–149].

In order to evaluate the impact of intracellular versus extracellular Poly(I:C) on the TNF-α and IFN-β production (which are induced by IRF3 and NF-kB, respectively[150–152]), gene expression (6 h) and release (24 h) were evaluated in hMDMs stimulated with extracellular dsRNA mimetic or transfected with Poly(I:C). Cytosolic, but not extracellular, Poly (I:C) induced significant increase of IFN-β and TNF-α gene expression (Figure 7A and B) and protein release (Figure 7C and D).

To investigate the impact of cytosolic Poly(I:C) on human macrophage cell death, LDH release was evaluated. The inflammatory response characterized by IFN-β and TNF-α expression and release, as associated with lytic cellular death induced by intracellular, but not extracellular, Poly (I:C) (Fig 7E).

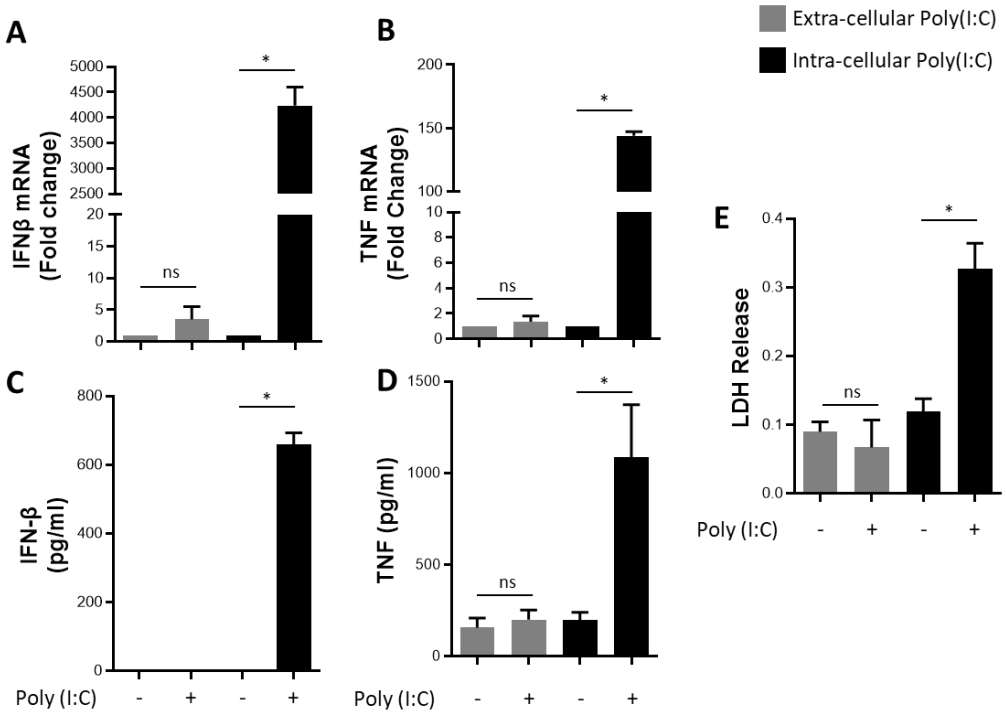


Figure 7: Impact of intracellular and extracellular Poly(I:C) on inflammatory response and cell death. hMDMs were treated with extracellular Poly(I:C) (1 µg/ml) or transfected with Poly(I:C) (1 µg/ml complexed with Fugene) and transcription and release of TNF-α (A and B) and IFN-β (C and D) were evaluated at 6 and 24 hours, respectively. hMDMs were treated with extracellular Poly(I:C) (1 µg/ml) or transfected with Poly(I:C) (1 µg/ml complexed with Fugene). After 24 h, cell death was assessed by LDH release (E). Data are presented as mean ± SD (N=3 independent donors).

To explore which pathways were involved in Poly(I:C)-induced cell death, we conducted a screening evaluating the action of specific inhibitors of apoptotic, necroptotic and pyroptotic pathways potentially involved (Fig.8A). Apoptotic and pyroptotic caspases were inhibited using Z-VAD-fmk, a pan-caspase inhibitor. Necroptosis was inhibited using RIPK1 inhibitor (Nec-1) or RIPK3 inhibitor (GSK872). MCC950 was used to inhibit NLRP3-dependent pyroptosis. LDH release remained unaffected after pre-treatment with pan-caspase inhibitor (Z-VAD-fmk), RIPK1 inhibitor (Nec-1) or NLRP3 inhibitor (MCC950) but not with Z-VAD-fmk combined with Nec-1. RIPK3 inhibitor (GSK872) significantly reduced cell death alone and furtherly in combination with Z-VAD-fmk or with Z-VAD-fmk/Nec-1. When Nec-1 was added in combination with GSK872, it did not further reduce cell death. NLRP3 did not appear to be involved in Poly(I:C)-induced cell death. Moreover, Z-VAD-fmk alone failed to reduce cell death, but its combination with GSK872 produced a stronger reduction than GSK872 pre-treatment alone. This pattern indicates that both apoptosis and necroptosis may contribute to Poly(I:C)-induced cell death.

Lytic cell death is usually associated with DAMPs release[153]. Among DAMPs, S100A8/A9 is emerging as key player during viral infections. S100A8/A9 is released by myeloid cells and exerts a critical role in modulating the inflammatory response by stimulating leukocyte recruitment and inducing cytokine secretion[154,155]. Thus, the release of S100A8/A9, was evaluated in hMDMs exposed to cytosolic Poly(I:C) (Figure 8B). We found that cytosolic Poly (I:C) induced DAMP release, which was reduced with Z-VAD-fmk/GSK872 pre-treatment.

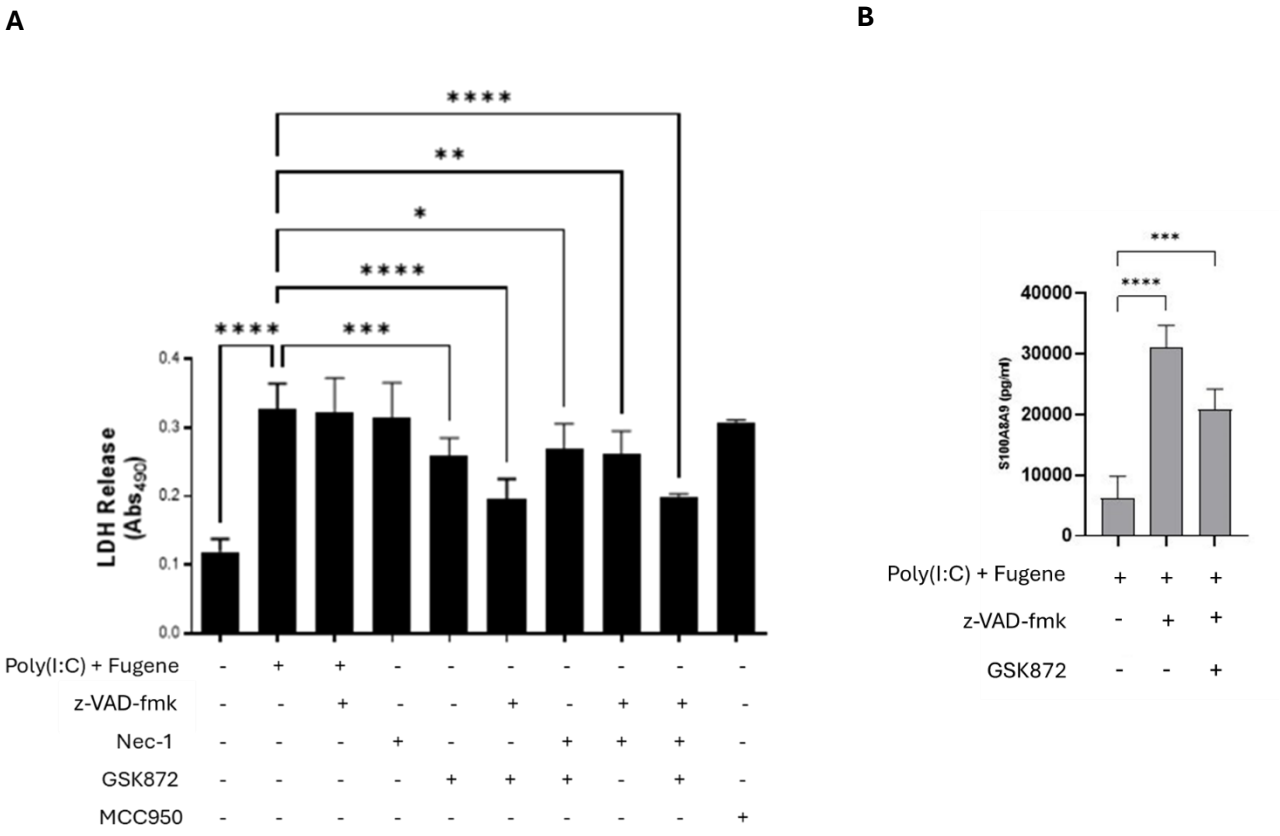


Figure 8: Impact of cytosolic Poly(I:C) on cell death and S100A8/A9 release in hMDMs pre-treated with inhibitors. Pre-treatment with cocktail inhibitors z-VAD-fmk and GSK782 reduced lytic cell death and S100A8/A9 (A and B). hMDMs were transfected with Poly(I:C) (1 µg/ml complexed with Fugene). Fugene alone was added as control in non-treated (NT) condition. Where indicated, cells were pre-treated for 1 h with 20 µM z-VAD-fmk Pan-Caspase inhibitor, 50 µM Nec-1 RIPK1 inhibitor, 10 µM GSK872 RIPK3 inhibitor and 1 µM MCC950 NLRP3 inhibitor, alone or in combination. (A) After 24 h, cell death was evaluated by LDH release. (B) S100A8/A9 release was evaluated by ELISA in cells stimulated for 24 hours. Data are presented as mean ± SD (N=3 independent donors).

1.2 Cytosolic Poly(I:C)-induced cell death exhibits apoptotic and necroptotic hallmarks

The screening using cell death inhibitors suggested that both the apoptotic and necroptotic pathways could be involved in Poly(I:C)-induced cell death. In order to confirm this finding, key hallmarks of apoptosis and necroptosis were evaluated.

During apoptosis, nuclear chromatin becomes highly condensed, followed by DNA fragmentation and packaging into apoptotic bodies [78,80]. Additionally, mitochondrial membrane depolarization and loss of the electrochemical gradient occur[79,80]. We assessed nuclear DNA condensation, fragmentation and mitochondrial membrane potential (MMP) in hMDM stimulated with intracellular Poly(I:C).

As shown in Figure 9, intracellular Poly(I:C) promoted nuclear condensation (as indicated by an increase of the fluorescence intensity of the DAPI), decreased nuclear area, and enhanced nuclear fragmentation (Figure 9A, B and C). Morphological alterations of nuclei were accompanied by loss of the MMP, as shown in Figure 9D and E.

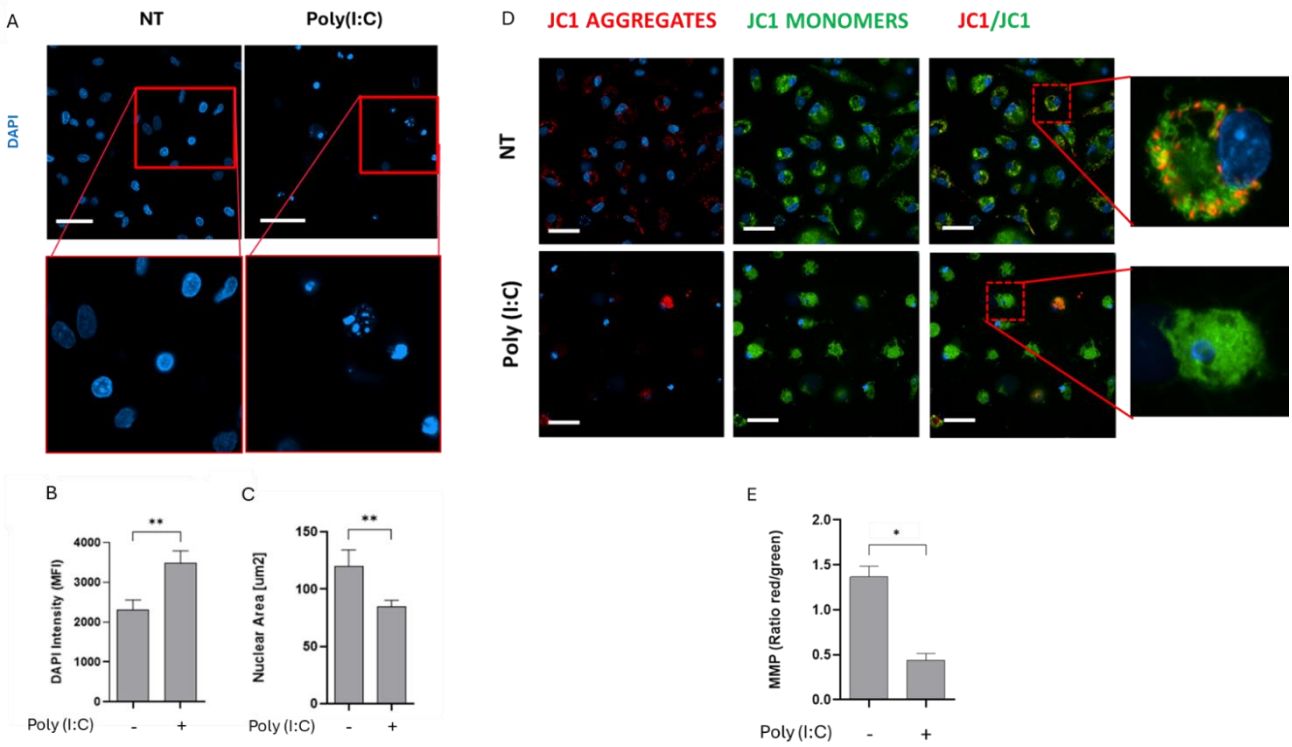


Figure 9: hMDMs exposed to cytosolic Poly(I:C) display apoptotic hallmarks. hMDMs were transfected with Poly(I:C) (1 µg/ml complexed with Fugene). Fugene alone was added as control in non-treated (NT) condition. Cells were stained with Hoechst 33342 for nuclear morphology evaluation. (A) Representative images (of 3 independent donors) were acquired using Operetta CLS (Perkin Elmer) with a 63X objective; (B) Quantification of the nuclear area and (C) Hoechst 33342 intensity. MFI, mean fluorescent intensity. After 24 h, cells were stained with JC-1 dye for MMP assessment. (D) Representative images (of 3 independent donors) acquired using Operetta CLS (Perkin Elmer) with a 63X objective. (E) Quantification of the red /green fluorescence intensity ratio after JC-1 staining. Data are presented as mean ± SD (N = 3 independent donors). Scale bar: 50 µm. MMP, mitochondrial membrane potential.

Additionally, we explored the phosphorylation status of MLKL which is indicative of necroptosis signalling pathway [93]. As shown in figure 10, Poly(I:C) induced phosphorylation of MLKL, which was reduced with GSK872 pre-treatment.

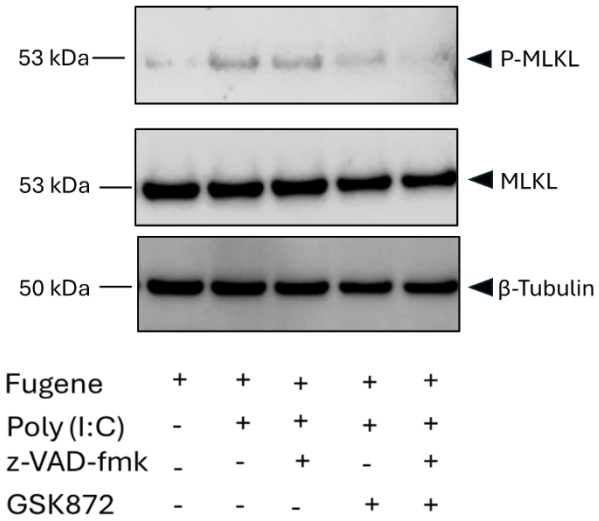


Figure 10: Poly(I:C) induces phosphorylation of MLKL. hMDMs were transfected with Poly(I:C) (1 μg/ml complexed with Fugene). Fugene alone was added as control. Where indicated, cells were pre-treated for 1 h with 20 μM Z-VAD-fmk Pan-Caspase inhibitor and 10 μM GSK872 RIPK3 inhibitor. Total proteins from cell lysates were analyzed by western blot. β-tubulin was used as loading control.

1.3 Cytosolic Poly(I:C) promotes TRIF-dependent activation of Caspase-8, -3 and GSDME.

To further confirm the activation of the apoptotic cascade in response to intracellular Poly(I:C), we assessed the cleavage of caspases-8 and -3. In figure 11A and B, we show that cytosolic Poly (I:C) induced the cleavage of caspase-8 and -3, whose activation was inhibited by pan-caspase inhibitor Z-VAD-fmk. Then, we explore what up-stream factors were involved. As described by Buscetta *et al.*, the activation of TLR4 by LPS stimulation can lead to caspase-8 activation by recruitment of TRIF under specific circumstances [133]. Since it is known that TLR3 can also recruit TRIF[133], we wondered whether in our experimental model the activation of caspases-8 and -3 could depend by TRIF after activation of TLR3 by cytosolic Poly(I:C). We found that treatment with TRIF inhibitor inhibited the activation of caspase-8 and -3, by suggesting that Poly(I:C) promoted the activation of these caspases via TRIF (Figure 11A and B). In addition, we noticed that the cleavage of caspase-8 and -3 was also inhibited by GSK872, by suggesting a correlation between apoptotic caspases activation with RIPK3.

Caspase 3 can cleave the pyroptotic effectors such as GSDME and GSDMD. Until now, the role of GSDMs in human macrophages stimulated with Poly(I:C) has never been explored. GSDME can be cleaved by caspase 3 at Asp270 to generate the GSDME-NT fragment. This fragment, when forming pores in the plasma membrane, triggers pyroptosis secondary to apoptosis [156–158]. We found that cytosolic Poly(I:C) induced GSDME cleavage and this effect was reverted by Z-VAD-fmk/GSK872 treatment (Figure 11C).

Caspase-3 also cleaves GSDMD at D87 in the N-terminal domain that inactivates its pyroptotic function, by generating a fragment at 43 kDa[159]. Consistently, we found that cytosolic Poly (I:C) induced the formation of GSDMD inactive form. Cleavage of GSDMD was inhibited by Z-VAD-fmk pre-treatment. (Figure 11D).

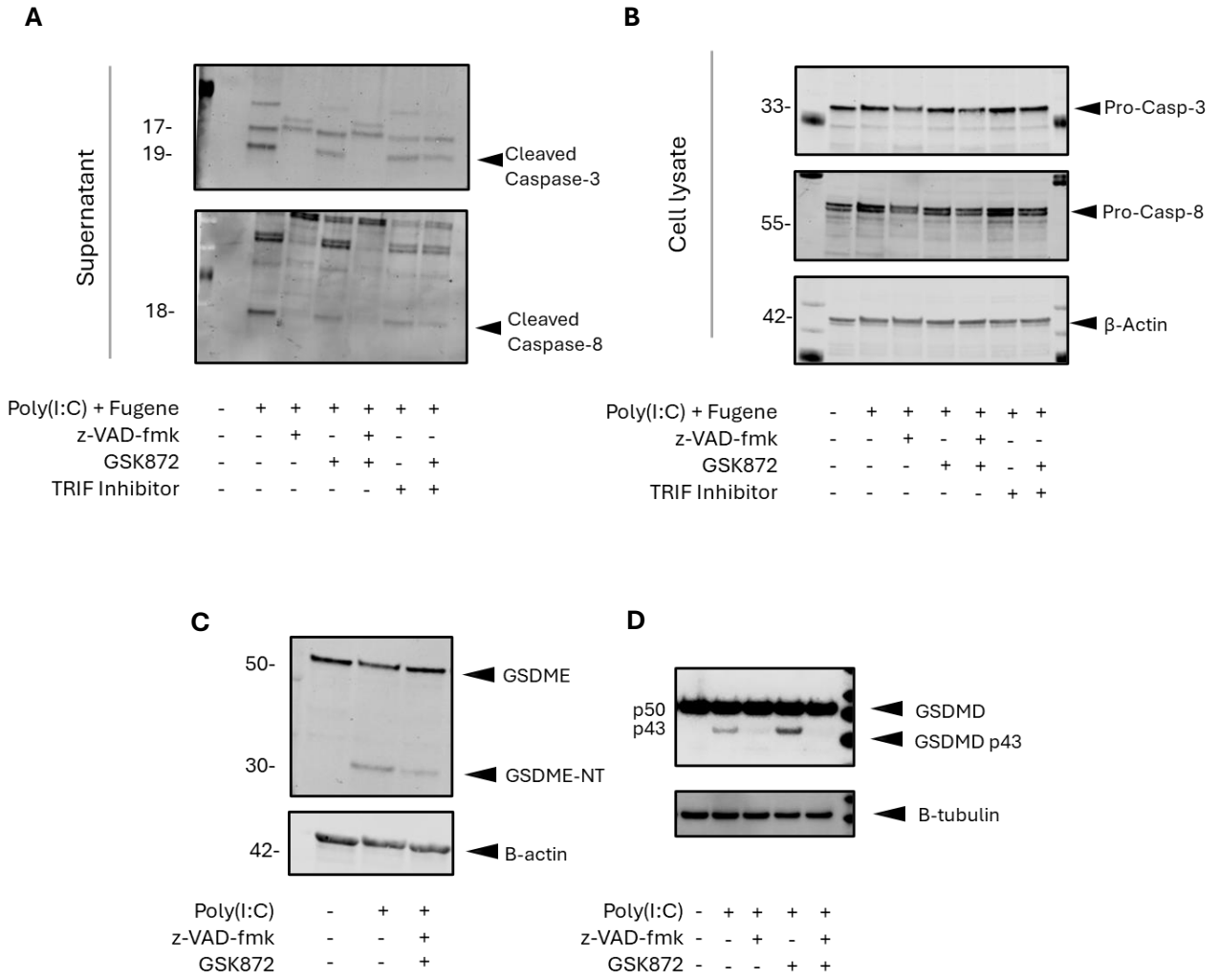


Figure 11: Cytosolic Poly(I:C) induces Caspase-8/-3/GSDME axis activation and inactive GSDMD. hMDMs were transfected with Poly(I:C) (1 µg/ml complexed with Fugene). Fugene alone was added as control. Where indicated, cells were pre-treated for 1 h with 20 µM z-VAD-fmk Pan-Caspase inhibitor, 10 µM GSK872 RIPK3 inhibitor and 25 µM TRIF Inhibitor. Total proteins from cell lysates and supernatant precipitates were analyzed by western blot. β-actin and β-tubulin were used as loading control.

1.4 Cytosolic Poly (I:C) does not activate NLRP3 Inflammasome

The NLRP3 inflammasome has been previously indicated as key inducer of macrophage pyroptosis following viral infections[89,90]. However, whether dsRNA is the trigger for NLRP3 activation in human macrophages remains unclear[136,137]. We therefore investigated whether lytic cell death caused by cytosolic Poly(I:C) was associated with NLRP3 inflammasome activation.

First, we evaluated the impact of Poly(I:C) on transcriptional activation of NLRP3 and pro-IL-1β. In this set of experiments conducted at the beginning of the doctoral period, the Poly(I:C) was transfected with Lipofectamine3000 and the LPS primed with lipofectamine condition was used as a control. As reported in Figure 12, LPS alone, as expected, caused significant upregulation of both genes. Cytosolic Poly(I:C) alone did not induce transcriptional upregulation of NLRP3 or pro-IL-1β and when added in combination with LPS did not further modulate gene expression compared to LPS alone. This indicated that Poly(I:C) did not prime hMDMs for NLRP3 inflammasome activation and confirmed that priming with LPS was required to explore a potential role of Poly(I:C) as second signal for NLRP3 inflammasome activation.

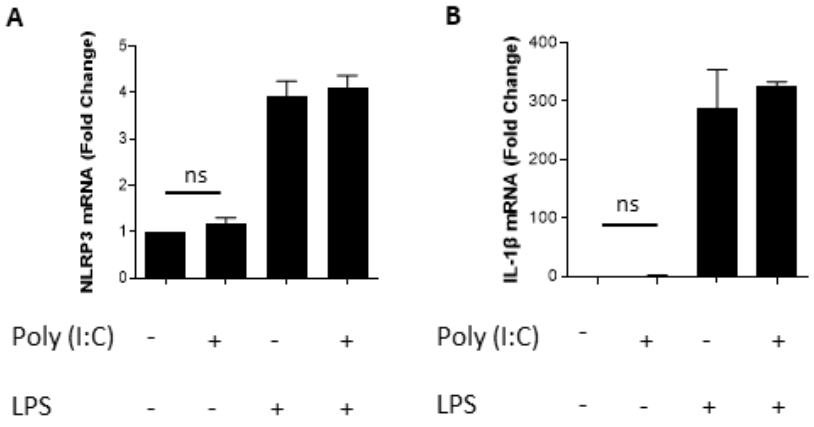


Figure 12: Cytosolic Poly(I:C) does not induce NLRP3 and Pro-IL-1β expression in hMDMs. Poly(I:C) did not prime hMDMs for NLRP3 inflammasome activation. hMDMs were transfected with Poly(I:C) (1 μg/ml complexed with Lipofectamine 3000). Where indicated, 1 μg/ml LPS was added 3 h before Poly(I:C) stimulation. Lipofectamine alone was added as control. After 6 h, gene expression of NLRP3 (A) and pro-IL-1β (B) was evaluated. Data are presented as mean ± SD (N = 3 independent donors).

To explore this possibility, we evaluated caspase-1 cleavage and IL-1β release in hMDMs primed with LPS and transfected with Poly(I:C) (Figure 13).

In the absence of priming, no caspase-1 cleavage and pro-IL-1β processing/release were observed in response to Poly (I:C) compared to macrophages primed with LPS. Notably, addition of Lipofectamin to LPS primed cell induced activation of caspase-1 and processing/release of IL-1β. The addition of cytosolic Poly(I:C) after LPS priming did not further modulate caspase-1 activation nor IL-1β processing/release. The NLRP3 inhibitor MCC950 blocked the cleavage of caspase-1 and IL-1β processing/release induced by treatment with LPS/Poly(I:C). LPS-primed with nigericin condition was used as positive control (Figure 13).

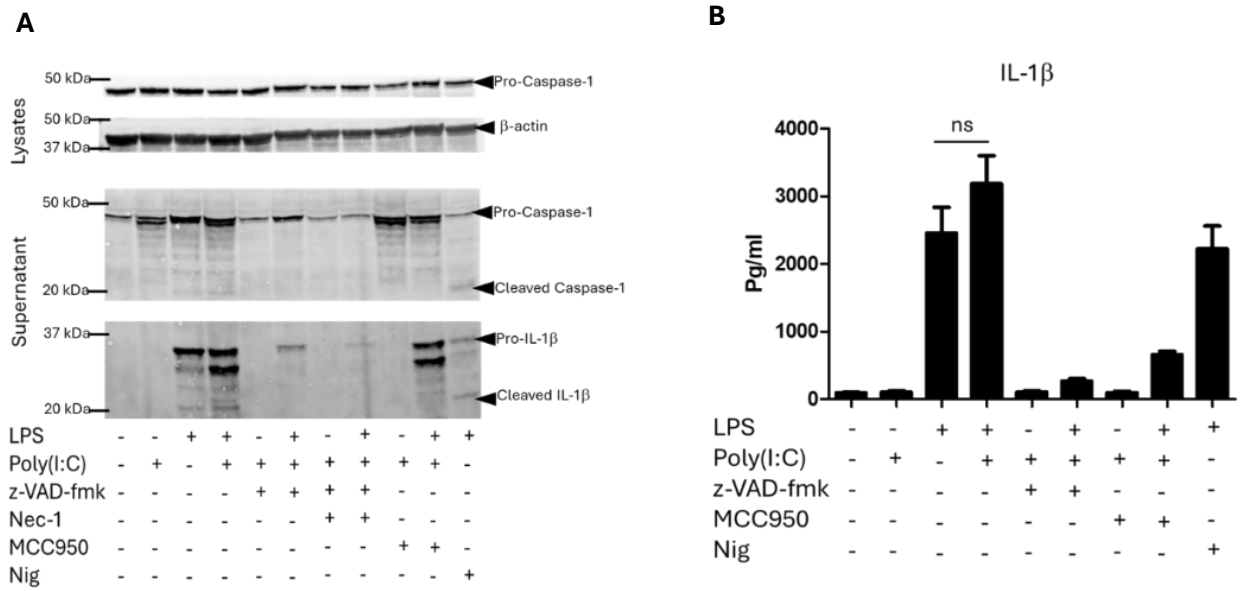


Figure 13: Poly(I:C) does not induce caspase-1 activation and IL-1β processing/release. hMDMs were transfected with Poly(I:C) (1 μg/ml complexed with Lipofectamine 3000). Where indicated, 1 μg/ml LPS was added 3 h before Poly(I:C) stimulation. Lipofectamine alone was added as control. LPS+Nigericin was used as positive control. Where indicated, cells were pre-treated for 1 h 20 μM Z-VAD-fmk, 50 μM NEC-1 and 1 μM MCC950. (A) Total proteins from cell lysates and supernatant precipitates were used for western blot analysis to investigate caspase-1 and pro-IL-1β processing. β-actin was used as loading control. (B) Release of IL-1β assessed by ELISA. Data are presented as mean ± SD (N = 3 independent donors).

Inflammasome activation is associated with the formation of adapter protein apoptosis associated speck-like protein containing a CARD (ASC) speck [160]. We therefore evaluated the presence of ASC-speck in hMDMs stimulated with cytosolic Poly(I:C) by immunofluorescence. No ASC specks were detected in hMDMs transfected with Poly(I:C). Addition of cytosolic Poly(I:C) to LPS-primed cells induced the formation of ASC speck, whose was reverted by the treatment with MCC950 (Figure 14). Of note, also Lipofectamin alone induced ASC speck formation in LPS-primed cells. These data suggest that cytosolic Poly(I:C) in not primed cells did not induce the inflammasome activation. However, in the presence of LPS, Lipofectamin may trigger the activation of the NLRP3 inflammasome.

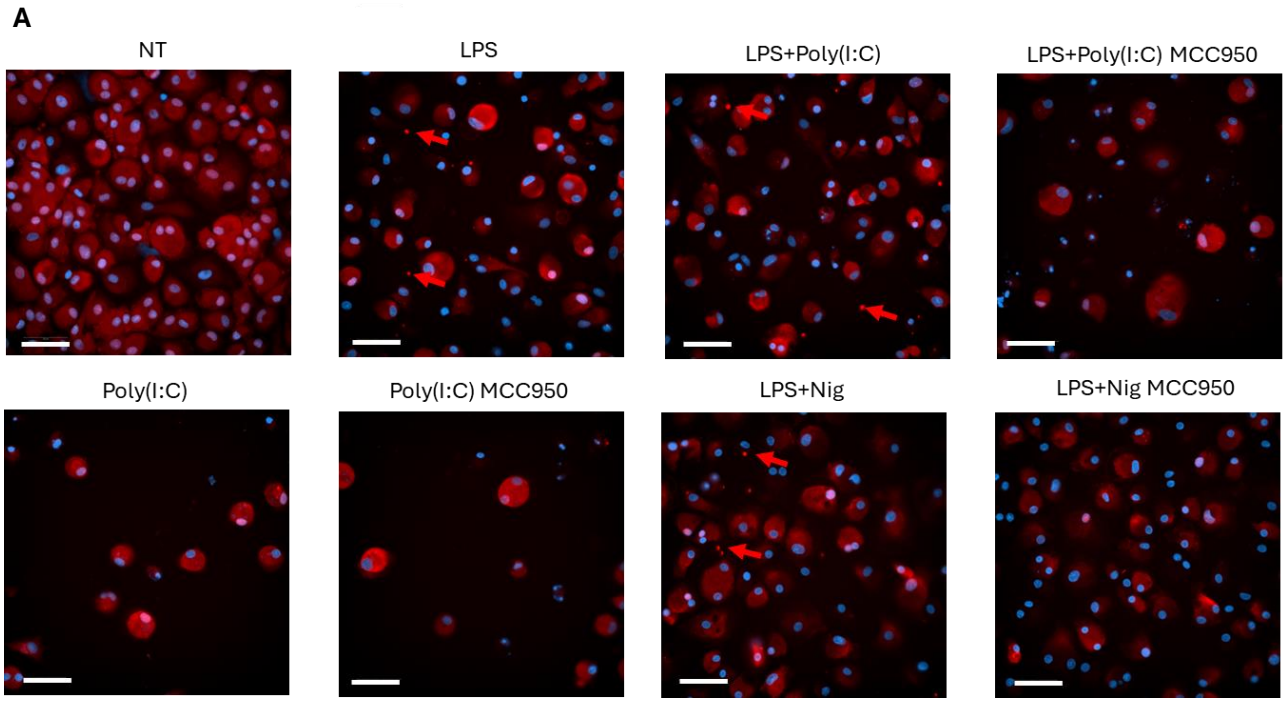


Figure 14: Poly(I:C) does not activate ASC-speck formation. hMDMs were transfected with Poly(I:C) (1 µg/ml complexed with Lipofectamine 3000). Where indicated, 1 µg/ml LPS was added 3 h before Poly(I:C) stimulation. Lipofectamine alone was added as control in non-treated (NT) and LPS-primed condition. Where indicated, cells were pre-treated for 1 h with the NLRP3 inhibitor MCC950. After treatment, cells were stained with anti-ASC antibody. Representative images (of 3 independent donors) were acquired using Operetta CLS (Perkin Elmer) with a 63X objective. Scale bar: 50 µm. LPS+Nigericin was used as positive control.

To remove these artifacts, we are currently investigating impact of Poly(I:C) as second signal after LPS priming using Fugene as transfection reagent. Preliminary data have shown that Fugene in LPS-primed cells did not induce ASC speck formation compared to the use of Lipofectamin (Figure 15A). Regarding IL-1β release, in the presence of Fugene we observed a difference between LPS and LPS+Poly(I:C), which was not present when Lipofectamin was used (Figure 15B).

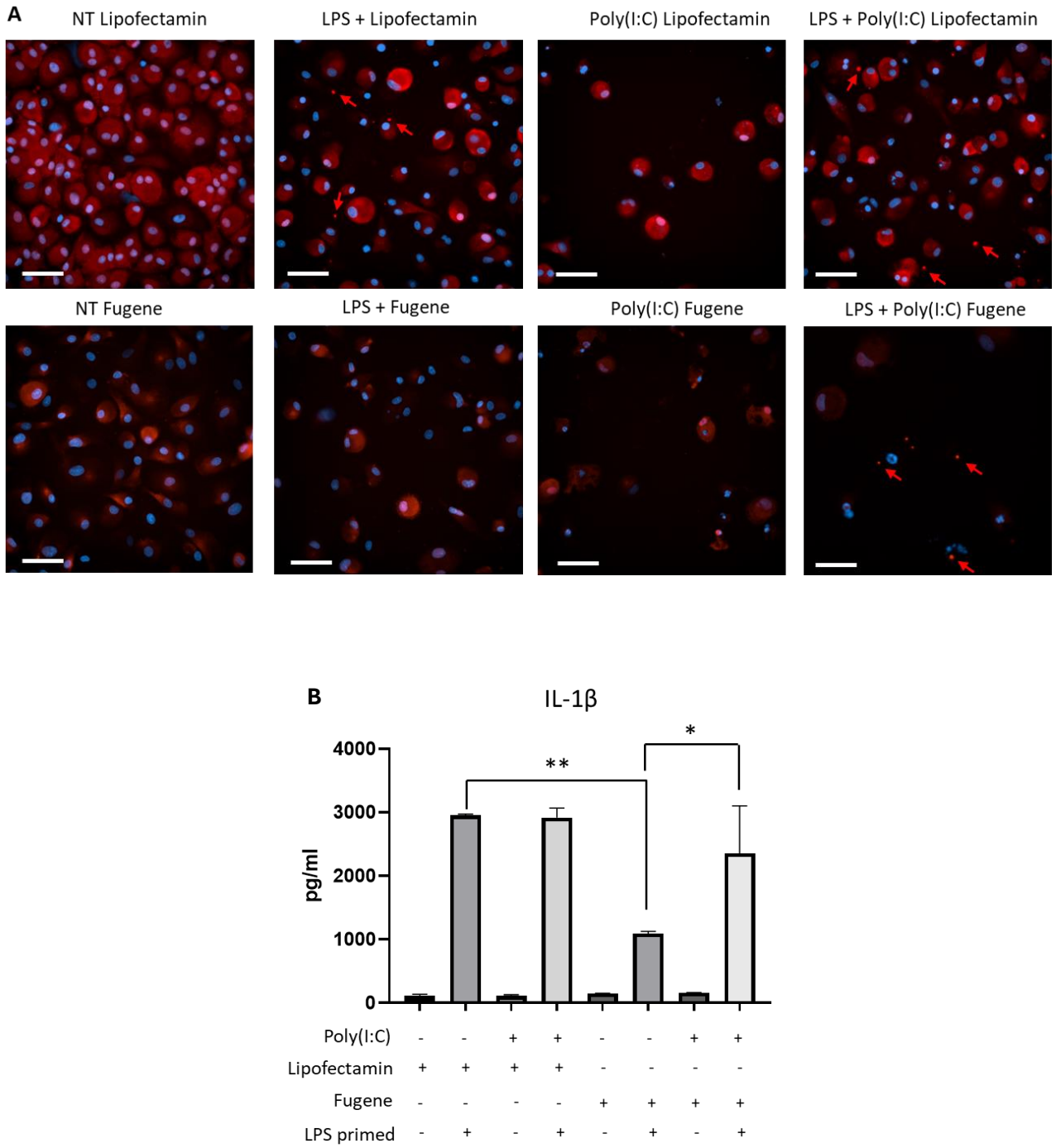
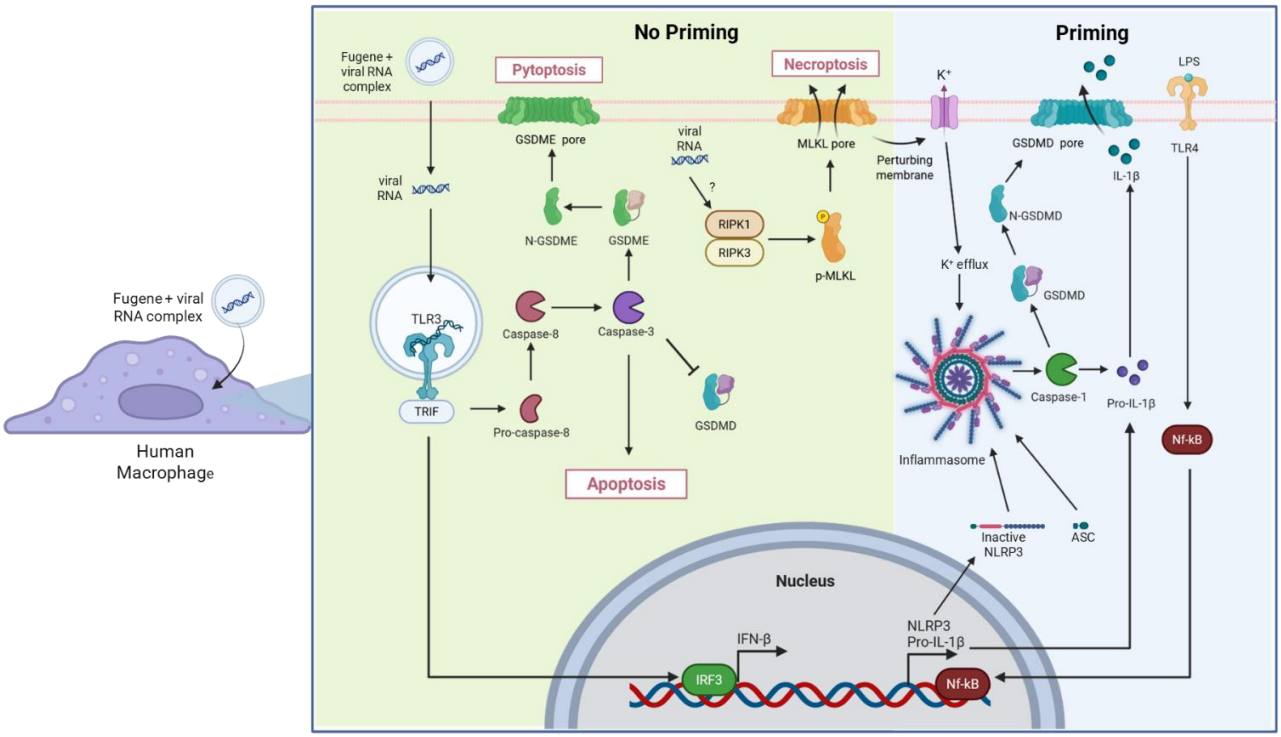


Figure 15: Fugene does not induce ASC speck formation no IL-1β release in LPS-primed human macrophages. hMDMs were transfected with Poly(I:C) (1 µg/ml complexed with Lipofectamine 3000 or Fugene). Where indicated, 1 µg/ml LPS was added 3 h before Poly(I:C) stimulation. Lipofectamine and Fugene alone were added as control in non-treated (NT) condition. (A) After treatment, cells were stained with anti-ASC antibody. Representative images (of 3 independent donors) were acquired using Operetta CLS (Perkin Elmer) with a 63X objective. Scale bar: 50 µm. (B) Release of IL-1β assessed by ELISA. Data are presented as mean ± SD (N = 2 independent donors).

Main findings:

The first objective explored aspects that are not yet fully understood in the context of dsRNA-induced cell death in human macrophages, particularly those related to the role of TRIF as upstream factor of apoptotic caspases and GSDME activation. Furthermore, until now it had not been determined whether the involvement of NLRP3 was due to the exclusive presence of dsRNA or whether a priming signal was necessary. Firstly, Poly(I:C)-induced cell death is characterized by cell lysis, caused by the activation of necroptosis and pyroptosis effectors, such as p-MLKL and GSDME, respectively. Despite this, the presence of apoptotic hallmarks such as nuclear fragmentation, mitochondrial dysfunction, and activation of apoptotic caspases, including caspase-8 and -3, responsible for the cleavage of GSDME, was observed, suggesting the occurrence of a pyroptotic cell death secondary to apoptosis. In addition, it has been verified that caspase-8 activation is mediated by TRIF, the main factor recruited by TLR3. Finally, cytosolic Poly(I:C) alone is not sufficient to induce activation of the NLRP3-mediated pathway, which requires a priming signal. However, due to the toxicity of lipofectamine in LPS-primed cells, these data will be confirmed using Fugene.



2 Live and dead bacteria recognition by human neutrophils

2.1 Live GBS induces high levels of IL-8 and ROS in an FPR-dependent manner

As mentioned in the objectives section, the project also focused on studying the ability of neutrophils to distinguish between live and dead bacteria, triggering different responses.

Previous work in murine models demonstrated that neutrophils selectively respond to live, but not heat-killed, bacteria by producing high levels of the chemokine Cxcl2, thereby amplifying neutrophil recruitment through a positive feedback mechanism [161]. This pathway is essential for detecting viable microbes and mounting an effective antimicrobial response [161,162]. In this study, we assessed whether human neutrophils display an analogous pattern, focusing on IL-8, the functional homolog of murine Cxcl1 and Cxcl2 [163].

Human neutrophils exposed to live GBS showed markedly elevated IL-8 mRNA expression (Fig. 16A) and protein secretion (Fig. 16B) compared with cells stimulated with heat-killed GBS (80°C, 45 min) or with *Escherichia coli* LPS, used as a positive control. Live GBS also induced substantially higher levels of reactive oxygen species (ROS), a key antimicrobial effector, than killed bacteria (Fig. 16C). In contrast, the production of other cytokines, including TNF-α (Fig. 16D) and IL-6 (Fig. 16E), was comparable between live and killed bacterial stimuli.

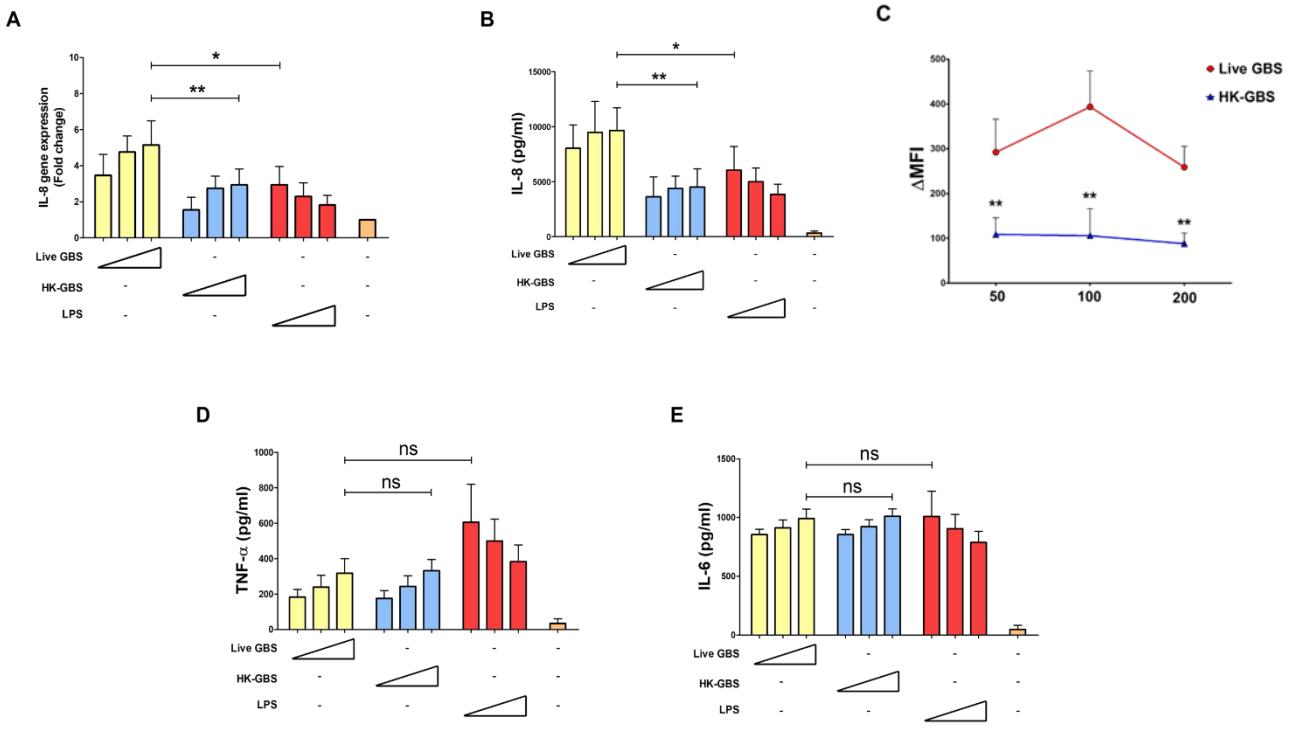


Figure 16: Live, but not killed, GBS induce IL-8 production and ROS release. The figure presents IL-8 gene expression (**A**) and protein secretion (**B**) in a dose-dependent manner following stimulation with live or heat killed (HK)-Group B Streptococcus (GBS, H36b). Increasing MOIs (5, 10, and 20) or concentrations (5, 10 or 20 µg/mL) were, respectively, used for live or HK bacteria in RT-PCR and ELISA tests. *Escherichia coli* lipopolysaccharide (LPS; 10, 100, and 1000 ng/mL) was included as a positive control, while unstimulated neutrophils served as a negative control. Panel (**C**) shows the Δ median fluorescence intensities (ΔMFI) relative to reactive oxygen species (ROS) released by neutrophils stimulated with increasing quantities of live (MOIs 50, 100, and 200) or HK-GBS (5, 10 or 20 µg/mL). Cytokine secretion of TNF-α (**D**) and IL-6 (**E**) was measured after stimulation of neutrophils with increasing concentrations of live, HK-GBS or LPS, used at the above-mentioned concentrations. Data are presented as means ± standard deviations from five independent experiments, each performed in duplicate. *p < 0.05 and **p < 0.01, as determined by the Mann-Whitney test;

To determine whether this preferential induction of IL-8 and ROS is specific to GBS, we evaluated responses to two additional clinically relevant pathogens: *Klebsiella pneumoniae* and *Staphylococcus aureus*. As shown in Figure 17, live *K. pneumoniae* and *S. aureus* similarly elicited significantly greater ROS generation and IL-8 release than their heat-killed counterparts. Collectively, these findings indicate that human neutrophils selectively recognize and respond to live bacteria, which act as potent inducers of IL-8 and ROS.

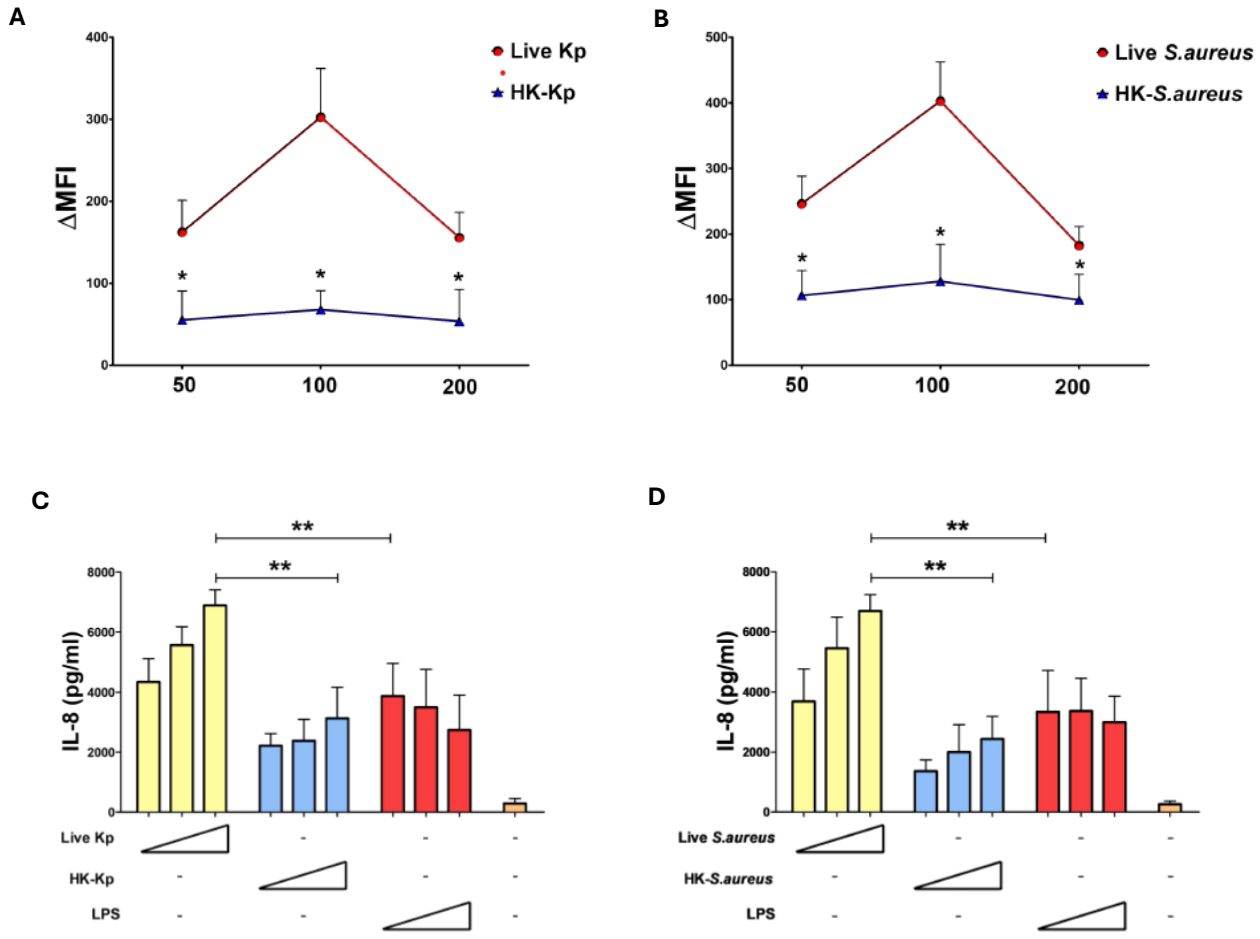


Figure 17: IL-8 and ROS production are a shared response of neutrophils upon stimulation with both Gram-negative and Gram-positive bacteria. Panel (**A**) and (**B**) display ROS release expressed as ΔMFI of neutrophils stimulated with increasing MOIs (50, 100, and 200) of live or HK-*Klebsiella pneumoniae* (*Kp*, AC133) and - *Staphylococcus aureus* (*S. aureus* USA300), respectively. IL-8 is secreted in a dose-dependent manner following stimulation with live or heat killed (HK)-*K. pneumoniae (Kp)* (**C**) or *Staphylococcus aureus* (*S. aureus*) (**D**). Increasing MOIs (5, 10, and 20) or concentrations (5, 10 or 20 µg/mL) were, respectively, used for live or HK bacteria in ELISA test. *Escherichia coli* lipopolysaccharide (LPS; 10, 100, and 1000 ng/mL) was included as a positive control, while unstimulated neutrophils served as a negative control. Data are presented as means ± standard deviations from five independent experiments, each performed in duplicate. *p < 0.05 and **p < 0.01, as determined by the Mann-Whitney test; ns, not significant.

Next, we hypothesized that the robust induction of IL-8 and ROS by live bacteria might be driven by formylated peptides generated during ongoing bacterial protein synthesis, a mechanism previously implicated in Cxcl2 induction in murine neutrophils [162]. To test this, we inhibited the receptors responsible for sensing formylated peptides: formyl peptide receptor 1 (FPR1) and formyl peptide receptor 2 (FPR2). Pretreatment of human neutrophils with 50 µM Boc2, a dual FPR1/FPR2 antagonist [164], significantly attenuated IL-8 (Fig. 18A) and ROS (Fig. 18B) responses to live GBS, while having no effect on responses to killed GBS. As expected, TNF-α and IL-6 levels were unaffected regardless of bacterial viability (Fig. 18C, D).

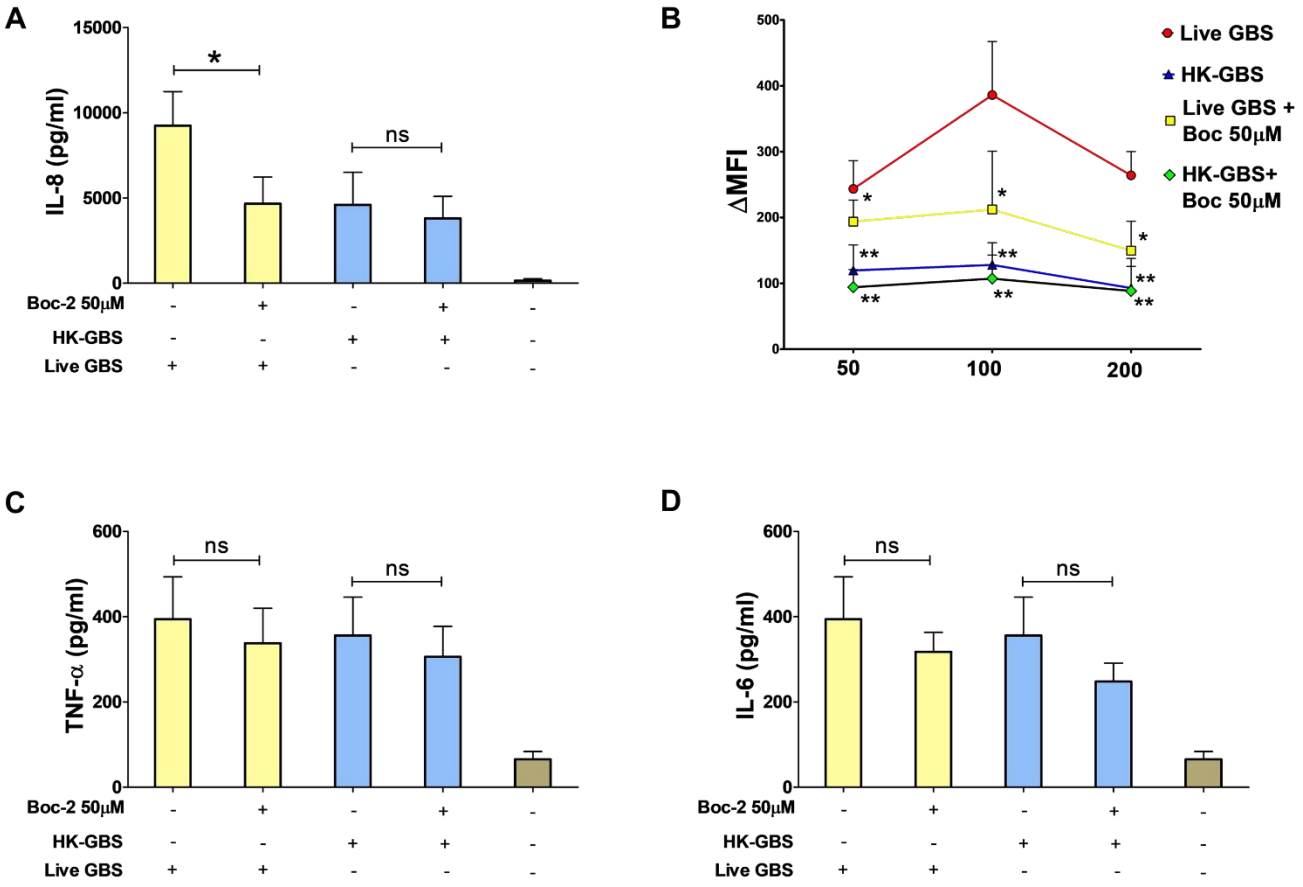


Figure 18: Cooperative Role of formyl peptide receptors (FPRs) in GBS-induced IL-8 production. (**A**, **C** and **D**) Effect of FPR antagonist Boc-2 on GBS-induced IL-8, TNF-α and IL-6 production, respectively. Neutrophils were exposed to Boc-2 (50μM) for 1 h and then stimulated with live GBS (MOI 10) or HK-GBS (10 μg/mL). (**B**) Effect of Boc-2 (50μM) on reactive oxygen species (ROS) after neutrophils stimulation with increasing concentrations of live (MOIs 50, 100, and 200) or HK-GBS (5, 10 or 20 μg/mL). Data are presented as means ± standard deviations from five independent experiments, each performed in duplicate. *$p < 0.05$ and **$p < 0.01$, as determined by the Mann-Whitney test; ns, not significant.

To delineate the specific contribution of each receptor, we next employed 10 μM Boc2, a concentration that selectively blocks FPR1. This treatment again resulted in a marked reduction in IL-8 (Fig. 19A) and ROS (Fig. 19B) production in response to live, but not killed, GBS. Similarly, pretreatment with WRW4, a selective FPR2 antagonist [165], partially yet significantly reduced IL-8 and ROS induction by live GBS, without altering responses to killed bacteria or affecting TNF-α or IL-6 production (Fig. 19A–D). Together, these observations indicate that FPR1 and FPR2 function in a non-redundant manner to mediate the heightened IL-8 and ROS responses elicited specifically by live bacteria, whereas the induction of other cytokines such as TNF-α and IL-6 occurs independently of this pathway.

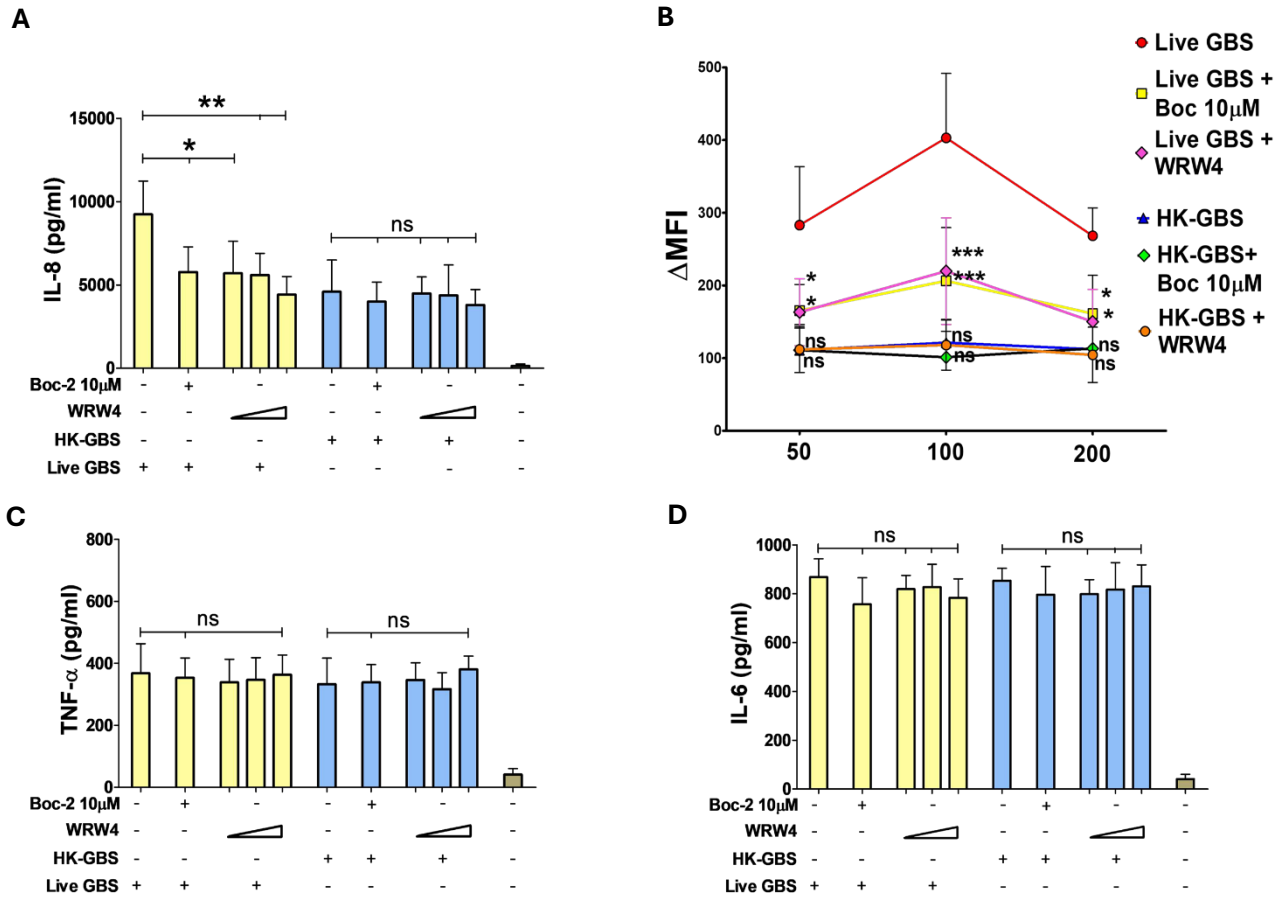


Figure 19: Both FPR1 and FPR2 lead to IL-8 and ROS release after GBS recognition. The figure shows IL-8 (**A**) and reactive oxygen species (ROS) (**B**) released by neutrophils after treatment with formyl peptides receptors (FPRs) antagonists and after stimulation with live (MOI 10 for ELISA and 50,100,200 for ROS measurement) or HK-GBS (10 μg/ml for ELISA and 5,10 and 20 μg/ml for ROS measurement). Neutrophils were exposed to Boc-2 (10 μM) or WRW4 (1, 5, and 10 μM) for 1 h to selectively inhibit FPR1 and FPR2, respectively. The same evaluations were performed for TNF-α (**C**) and IL-6 (**D**) cytokine measurement. Data are presented as means ± standard deviations from five independent experiments, each performed in duplicate. *p < 0.05 and **p < 0.01, as determined by the Mann-Whitney test; ns, not significant.

FPR1 and FPR2 are promiscuous receptors which are capable of recognizing not only formylated peptides but also compounds with other chemical structures. Therefore, it was of interest to assess whether formylated peptides are actually involved in promoting FPR-dependent stimulation of IL-8 and ROS release by live GBS. We sought to determine whether formylated peptides themselves underlie the FPR-dependent activation by live GBS. To directly address this question, GBS was grown in the presence of sub-inhibitory concentrations of actinonin, an inhibitor of peptide deformylase that promotes the intracellular accumulation of N-formylated peptides[166]. Strikingly, actinonin-treated GBS elicited significantly higher IL-8 and ROS production in neutrophils compared with bacteria grown in the absence of actinonin (Fig. 20A, B). By contrast, TNF-α induction was unaffected by actinonin exposure (Fig. 20C). Collectively, these findings suggest that the interaction between bacterial formylated peptides and FPR1/2 is crucial for the ability of live bacteria to stimulate high levels of IL-8 and ROS production in human neutrophils.

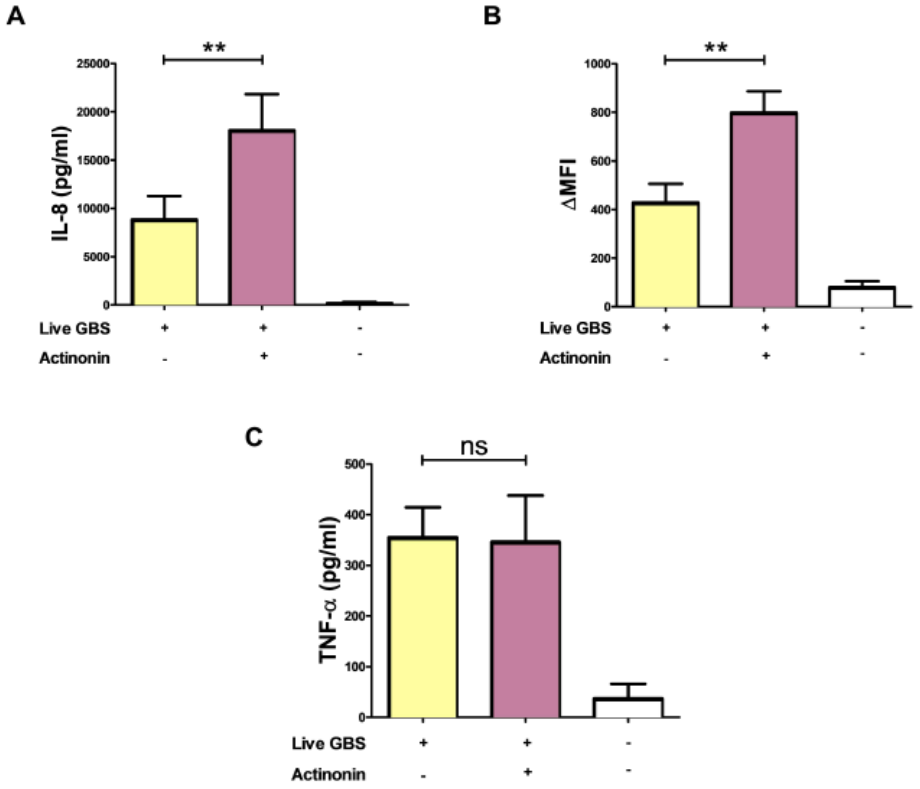


Figure 20: Formylation plays a role in IL-8 and ROS release upon GBS stimulation. Effect of actinonin on IL-8 (**A**) and TNF-α (**C**) production by neutrophils stimulated with live GBS. Neutrophils were stimulated with live GBS (MOI 10) in the presence or absence of the formyl peptidase inhibitor actinonin (32 μg/ml). Panel (**B**) shows the Δ median fluorescence intensities (ΔMFI) relative to reactive oxygen species (ROS) released by neutrophils stimulated with live GBS (MOI 100). Data are presented as means ± standard deviations from five independent experiments, each performed in duplicate. **$p < 0.01$, as determined by the Mann-Whitney test; ns, not significant.

2.2 Identification of Immunostimulatory Formylated Peptides in GBS

To identify the specific formylated peptides mediating the observed augmentation of IL-8 and ROS responses to GBS, we evaluated a panel of synthetic hexapeptides. These peptides were based on secretory signal sequences known to possess N-terminal formylation, a motif recognized by Formyl Peptide Receptors (FPRs). Previous work established the FPR activating capacity of selected GBS-derived formylated signal peptides in murine neutrophils [162].

Here, we assessed the ability of these peptides to stimulate human neutrophils, utilizing fMILF, FPR1 agonist and WKYMVM, FPR2 agonist, as positive controls. Among the fifteen peptides tested, two, fPep8 and fPep10, induced robust in vitro chemotactic responses (Fig. 21A) and significantly stimulated reactive oxygen species (ROS) production (Fig. 21B) in primary human neutrophils.

We subsequently characterized the receptor specificity of these two agonists using pharmacological antagonists. ROS production and chemotaxis triggered by fPep8 were effectively inhibited by 10 μM Boc-2, a concentration that selectively blocks FPR1, but remained insensitive to the FPR2 specific antagonist WRW4 (Fig. 21C). Conversely, the bioactivity of fPep10 was abolished by WRW4 but was not affected by Boc-2 (Fig. 21D). These results confirm that fPep8 and fPep10 are potent agonists of human neutrophils, acting through distinct FPRs subtypes: fPep8 selectively activates FPR1, while fPep10 selectively activates FPR2.

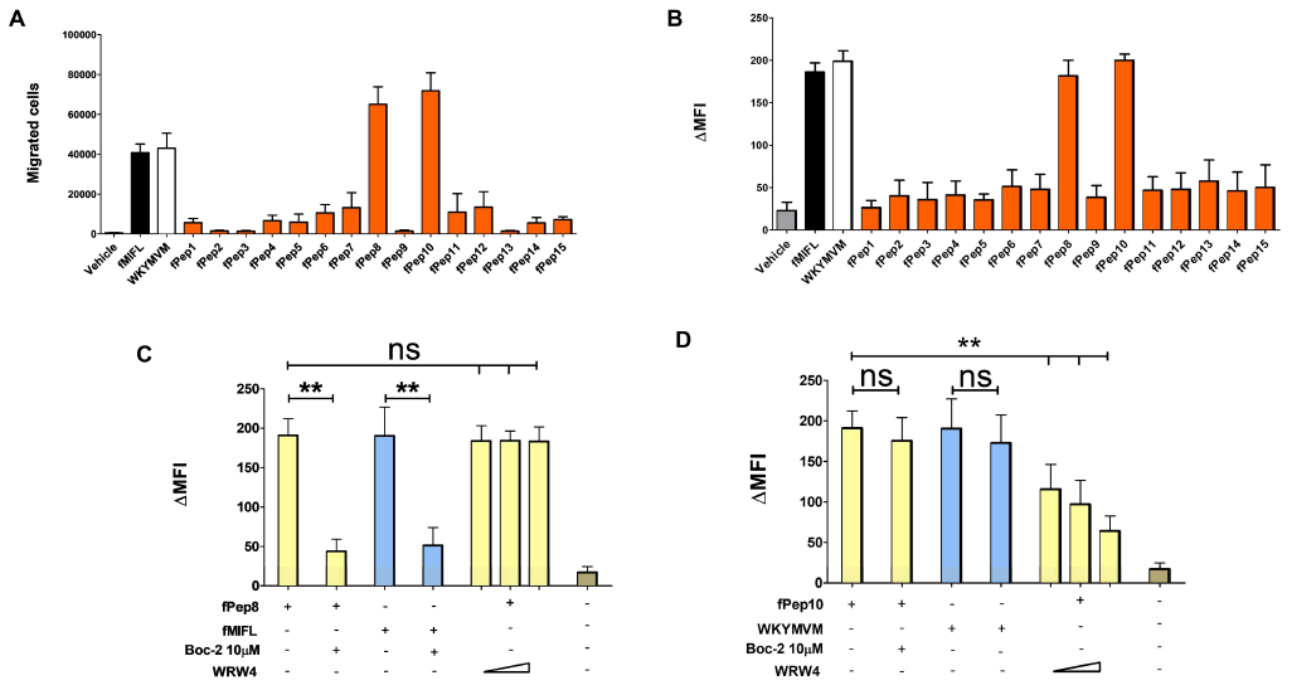


Figure 21: Neutrophil activation is enhanced by a selective group of GBS-derived signal peptides. (A) Neutrophils were allowed to migrate towards GBS signal peptides sequences, named as formylated peptides (fPep1-15, 1μM), and fMIFL or WKYMVM (both at 1μM) as positive control peptides. DMSO at 0.2% concentration was used as vehicle. (**B**) Reactive oxygen species (ROS) released by neutrophils stimulated with signal peptides sequences were measured and expressed as Δ median fluorescence intensities (ΔMFI). FPR1 and FPR2 were revealed as cellular receptor for fPep8 (**C**) and fPep10 (**D**) as shown by the reduced levels of mean fluorescence intensities (MFI) in presence of Boc-2 10μM and increasing concentration of WRW4, respectively. Data are presented as means ± standard deviations from five independent experiments, each performed in duplicate. $^{**}p < 0.01$, as determined by the Mann-Whitney test; ns, not significant.

2.3 TLR8 is involved in cytokine induction by both live and dead bacteria

As described previously, Toll-like Receptor 8 (TLR8) mediates recognition and antimicrobial responses to bacteria in innate immune cells. TLR8 is an endosomal pattern recognition receptor highly expressed in myeloid cells, including neutrophils, capable of sensing RNA derived from a broad spectrum of pathogens (viruses, bacteria, and protozoa)[167,168]. TLR8 has recently emerged as a dominant sensor for bacterial presence, including Group B Streptococcus GBS in human macrophages [169]. Furthermore, TLR8 has been proposed to mediate the ability of antigen-presenting cells to discriminate between viable and dead bacteria [112]. Given the limited knowledge regarding the functions of TLR8 in neutrophils, we assessed its role in these cells using CU-CPT9a, a recently developed, highly specific inhibitor that prevents TLR8 activation by stabilizing its dimeric resting state [170]. For comparative analysis, we also employed C29, a specific inhibitor of TLR2, which is known to be involved in cytokine induction by Gram-positive bacteria in macrophages [171].

Pretreatment of human neutrophils with CU-CPT9a, but not the TLR2 inhibitor C29, resulted in a marked, dose-dependent reduction in the production of IL-8 and TNF-α in response to both live GBS (Fig. 22A and B) and heat-killed GBS bacteria (Fig. 22C and D). Critically, this inhibitory effect was specific, as CU-CPT9a did not affect responses to LPS (used as a control, which activates TLR4). Furthermore, CU-CPT9a significantly reduced ROS induction triggered by live GBS (Fig. 22E). Similar TLR8-dependent reductions in cytokine and ROS production were observed when using *K. pneumoniae* and *S. aureus* as bacterial stimuli (Fig. 22F and G).

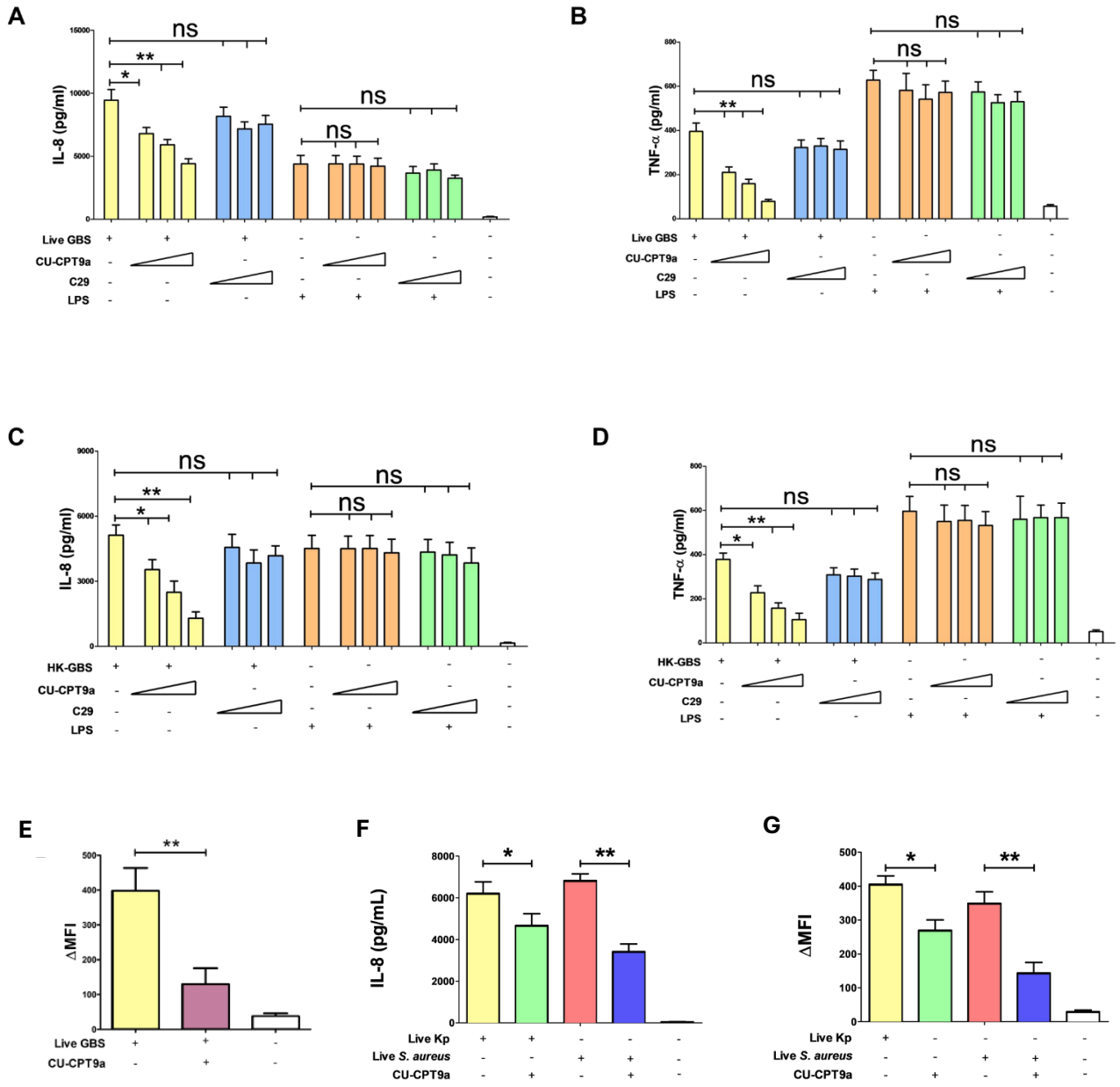


Figure 22: TLR8 is required for cytokine production following neutrophil stimulation by GBS. Treatment of neutrophils with the TLR8 inhibitor (CU-CPT9a, 1,3,6 µM), but not the TLR2 inhibitor (C29, 15,25,35 µM) significantly reduced IL-8 and TNF-α release upon stimulation with live (MOI 10) (**A** and **B**) or HK-GBS (10 µg/mL) (**C** and **D**). *Escherichia coli* lipopolysaccharide (LPS; 100 ng/mL) was included as a positive control, while unstimulated neutrophils served as a negative control. Panel (**E**) shows the Δ median fluorescence intensities (ΔMFI) relative to reactive oxygen species (ROS) released by neutrophils stimulated with live GBS alone or in presence of CU-CPT9a. Panels (F) and (G) show IL-8 and ROS production, respectively, in presence or absence of the TLR8 inhibitor (CU-CPT9a, 1,3,6 µM) after *K. pneumoniae* (*Kp*) or *Staphylococcus aureus* (*S. aureus*) stimulation (MOI 10 for ELISA and MOI 100 for ROS release). ROS release was expressed as Δ median fluorescence intensities (ΔMFI). Data are presented as means ± standard deviations from five independent experiments, each performed in duplicate. *p < 0.05 and **p < 0.01, as determined by the Mann-Whitney test; ns, not significant.

Since TLR8 is known to recognize RNA, we compared the ability of GBS RNA to stimulate human neutrophils with that of DNA, which activates TLR9, another endosomal receptor[171]. We found that RNA was significantly more potent than DNA in inducing cytokine production, and that RNA-induced cytokine release was strongly inhibited by the TLR8-specific inhibitor CU-CPT9a (Fig. 23A and B). Taken together, these results indicate that TLR8 plays a critical role in bacterial recognition and the promotion of antimicrobial responses in human neutrophils through its ability to recognize bacterial RNA. Notably, TLR8 is involved in responses to both live and heat-killed GBS, whereas FPR receptors are specifically required for the high-level IL-8 and ROS production induced by live, but not killed, bacteria.

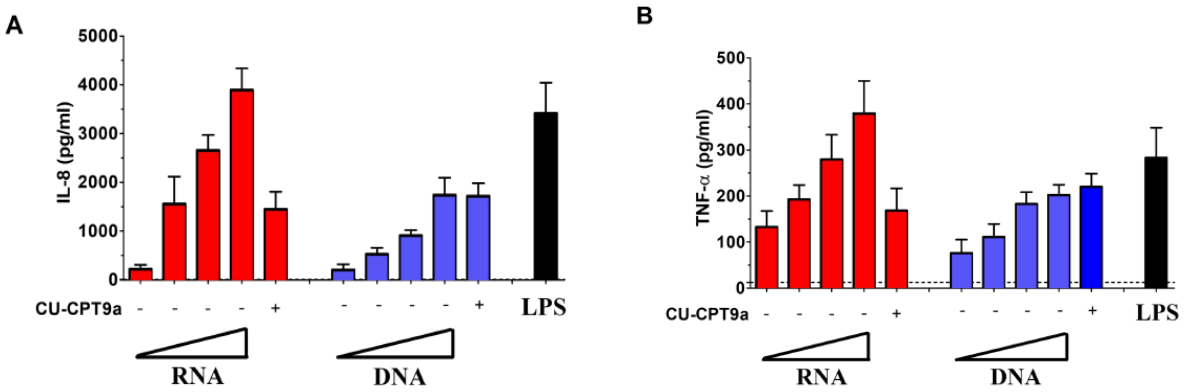


Figure 23: RNA form GBS induces cytokine production by activating TLR8 in neutrophils. Production of IL-8 (**A**) and TNF-α (**B**) in neutrophils stimulated with graded doses (0.01, 0.1, 1 and 10 µg/ml) of RNA or DNA from GBS. The last column indicates the inhibitory effect of the TLR8 inhibitor (CU-CPT9a, 3 µM) on RNA or DNA-dependent cytokines production. Data are presented as means ± standard deviations from three independent experiments, each performed in duplicate.

2.4 Fpep8 and fpep10 synergize with TLR agonists in the induction of CXCL8

Next, we investigated whether GBS formylated peptides could synergize with TLR8 or other TLR agonists to enhance IL-8 production in neutrophils. None of the tested formylated peptides, including the GBS-derived peptides as well as the established FPR agonists f-MIFL and WKYMVM, were able to induce IL-8 production on their own (Fig. 24). However, both f-MIFL and WKYMVM enhanced IL-8 production in response to TLR agonists such as LPS or heat-killed bacteria (Fig. 24). Similarly, the GBS-derived peptides fpep8 and fpep10 potentiated IL-8 induction in the presence of TLR stimulation, indicating a synergistic effect. Collectively, these results demonstrate that activation of FPR1 or FPR2 alone is insufficient to trigger IL-8 production, whereas their co-activation with TLRs synergistically drives robust IL-8 release in human neutrophils.

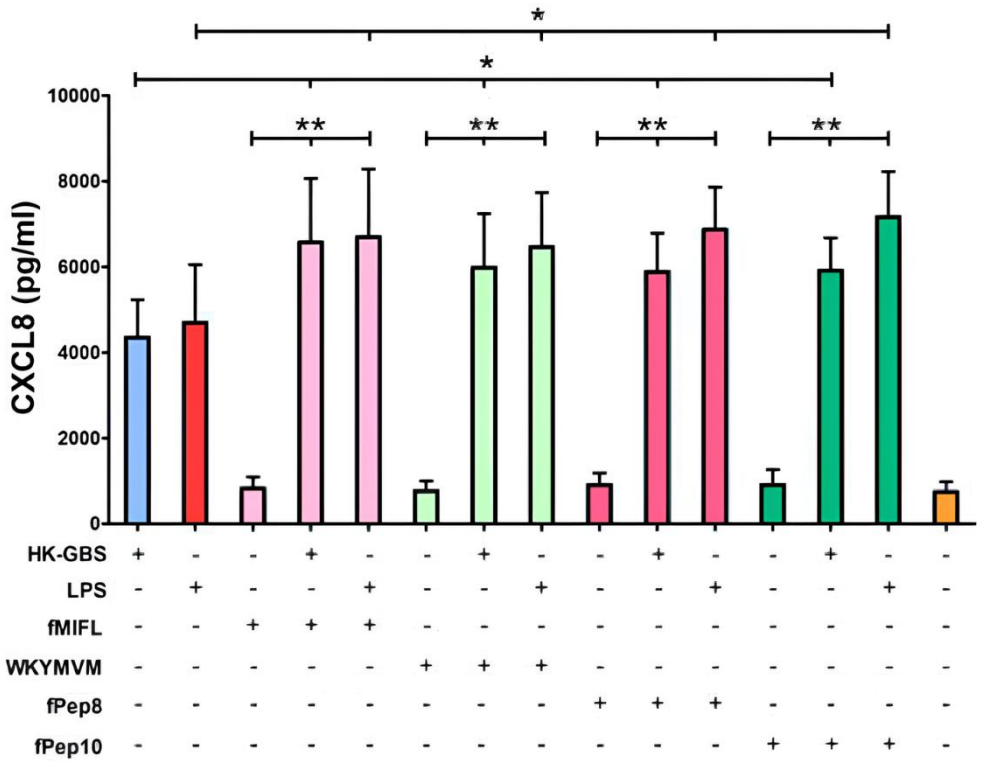
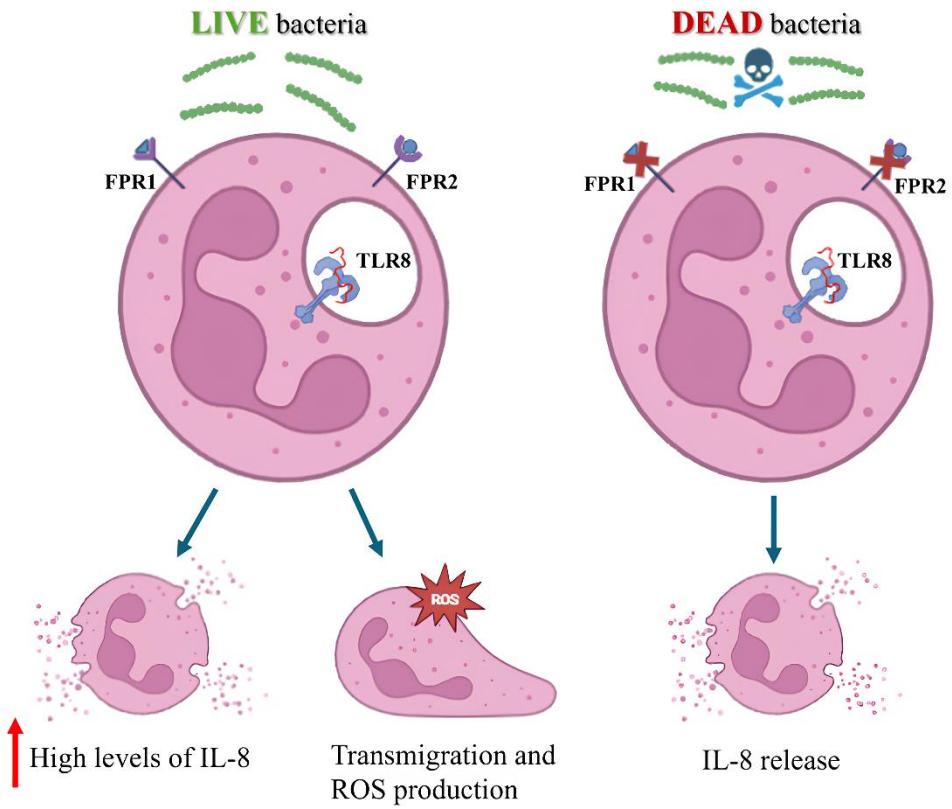


Figure 24: GBS signal peptides cooperate with TLR8 agonists in IL-8 production. IL-8 release in neutrophil culture supernatants following stimulation with combinations of heat-killed GBS (HK-GBS), formylated GBS-derived peptides, or TLR agonists. Neutrophils were pre-treated with FPR agonists (fMIFL, WKYMVM, fPep8, or fPep10) at a concentration of 1 µM for 1 hours prior to the addition of TLR8 agonists, including HK-GBS (10 µg/mL) or LPS (1 µg/mL). Data are presented as means ± standard deviations from five independent experiments, each performed in duplicate. *$p < 0.05$ and **$p < 0.01$, as determined by the Mann-Whitney test; ns, not significant.

Main findings:

The second objective of the project made it possible to define the responses and signaling pathways through which human neutrophils can discriminate between live and dead bacteria, through the release of CXCL8 (IL-8), the production of reactive species, and transmigration. This ability is not limited to GBS but also extends to other types of bacteria such as *S. aureus* and *K. pneumoniae*. It has been clarified that the main receptors responsible for conferring this ability to neutrophils are FPRs, specifically FPR1 and FPR2, by specific inhibitors and at specific concentrations. From an in-silico analysis, it has been observed that two of these synthetic compounds can induce such responses, in particular, fPep8, an agonist for FPR1, and fpep10, an agonist for FPR2. Additionally, it has been determined that the toll-like receptor involved in the recognition of live and dead bacteria is TLR8, which is involved in the recognition of bacterial nucleic acids and improving human neutrophils response by synergic effects with FPRs.



DISCUSSION

Inflammation is a protective response involving immune cells, blood vessels, and molecular mediators. The function of inflammation is to eliminate the initial cause of cell injury, remove pathogens, clear out damaged cells and tissues, and initiate tissue repair. Too little or excessive inflammation could lead to progressive tissue destruction by the harmful stimulus and compromise the survival of the organism. Innate immune recognition receptors detect microorganisms or damaged cells at key sites, including extracellular fluids and intracellular compartments. As consequence, innate immune cells release mediators that recruit inflammatory cells to sites of infection or tissue injury[172]. Macrophages and neutrophils are the first line of the organism's defense[4].

Macrophages are key players in regulating immune responses to viral infections via pathogen recognition and activation of inflammatory reactions which trigger both innate and adaptive immune responses [154,155]. Following virus internalization, macrophages may undergo cell death [131]. Under homeostatic conditions, cell death regulates tissue homeostasis, response to infections, and tissue repair following injury. However, when it spirals out of control, cell death can become a significant contributor to the propagation of inflammation. Numerous studies have implicated lytic forms of cell death in the development and progression of chronic inflammatory diseases [153]. In fact, lytic cell death is associated with the release of alarmins and DAMPs, such as S100 proteins, heat shock proteins and other mediators that enhance the recruitment of leukocytes, potentiate the inflammatory processes and trigger remodelling events [154,155]. A better knowledge of the mechanisms leading to programmed cell death in response to RNA viruses may provide tools for a better control of infections.

Herein, we investigated cell death mechanisms that are activated in human macrophages following Poly(I:C) stimulation. Poly(I:C) is a viral dsRNA mimic and it is generally used to study signaling pathways triggered by dsRNA upon its binding to intracellular receptors such as TLR3, RIG-I and MDA-5[121–123]. The use of Poly(I:C) makes it possible to mimic the signaling cascades enacted by cells as response to dsRNA, without these being affected by the other viral components.

Our data show that the transfection of Poly(I:C) led to apoptotic and necroptotic cell death, since this was inhibited by combination of z-VAD-fmk/GSK872 pre-treatment. We verified it by observing the presence of caspase-8 and -3 cleaved form and phospho-MLKL. Then, we wondered which up-stream factors were responsible of caspase-8 activation. As described by Buscetta *et al.,* TLR4 can activate caspase-8 by TRIF recruitment[132]; but it is also known that TLR3 is able to recruit TRIF involved in IFN-β and TNF-α gene expression induction[173,174]. However, it is still unclear whether TRIF can activate caspase-8 by TLR3 activation with dsRNA in human macrophages. We found that the pre-treatment with TRIF inhibitor reduced the cleavage of caspase-8 and -3 in cytosolic Poly(I:C)-stimulated hMDMs. Surprisingly, the RIPK3 inhibitor GSK872 induced a reduction in caspase-8 and

-3 cleavage. We hypothesized that this could be due to the inflammatory axis initiated by RIPK3 activation, which amplify cell death response. In particular, the literature shows that RIPK3 can induce, through the activation of IRF3 and Nf-kB, the expression and release of pro-inflammatory factors, such as IFN and TNF, respectively[175–177]. These factors, acting in a paracrine and autocrine manner, could enhance the activation of apoptotic caspases. Work is currently underway to investigate the role of RIPK3 as a mediator of inflammation and cell death. On the other hand, it is also known that active caspase-3 cleaves GSDME and GSDMD, by generating an active and inactive form, respectively[159,178]. In our experimental model, the dsRNA mimetic induced the cleavage of GSDME, which was inhibited by z-VAD-fmk, thereby indicating the activation of caspase 8/3/GSDME axis and causing pyroptosis secondary to apoptosis. On the other hand, cytosolic Poly(I:C) led to the inactivation of GSDMD by generating a fragment with molecular weight of 43 kDa which was abolished by z-VAD-fmk. This confirmed that dsRNA mimetic did not involve GSDMD activation. This selective engagement of GSDME over GSDMD is particularly relevant, suggesting that viral RNA sensing favors a pyroptotic phenotype linked to apoptosis rather than canonical inflammasome-driven pyroptosis. Further studies will be conducted to define how TRIF recruits caspase 8 and where GSDME can be localized in human macrophages stimulated with Poly(I:C).

The exploration of responses induced by dsRNA led to wonder us whether these signaling pathways changed compared to the context of viral infections. It is known that airways viruses such as Sars-Cov-2 or IAV leads to NLRP3 activation[179,180]. However, it is unclear whether NLRP3 activation is induced exclusively by dsRNA or whether components are necessary to act as priming signals. To explore this question, we used LPS as priming signal to compare with hMDMs unprimed. First, we have shown that the pre-treatment with NLRP3 inhibitor MCC950 did not affect the LDH release, by suggesting that the NLRP3 inflammasome was not involved in Poly(I:C)-induced cell death. Additionally, cytosolic Poly(I:C) did not induce transcriptional priming of the NLRP3 inflammasome nor pro-IL-1β in unprimed cells. Moreover, the IL-1β release and cleavage, caspase-1 activation and ASC speck formation were not induced by dsRNA mimetic in unprimed hMDMs, by indicating that inflammasome is not involved.

On the other hand, our data suggested that dsRNA mimetic probably acted as second signal in LPS-primed human macrophages, by inducing IL-1β release and cleavage, caspase 1 activation and ASC speck formation. However, Lipofectamine alone, used as negative control, induced the activation of inflammasome in LPS-primed cells, by generating artifacts. For this reason, as our preliminary data show, we are conducting new experiments using Fugene.

Overall, the data support a model in which cytosolic dsRNA induces intertwined apoptosis, necroptosis, and GSDME-mediated pyroptosis independently of NLRP3, driven by a TLR3–TRIF–caspase-8/3 axis. This model refines current understanding of viral RNA sensing and highlights the need to further investigate TRIF-mediated caspase-8 recruitment and the subcellular localization of GSDME during cell death.

The second main objective concerns the ability of human neutrophils to discriminate the live from dead bacteria. Neutrophils are the first innate immune cells mobilized to combat bacterial infections. *Streptococcus agalactiae*, or group B Streptococcus (GBS), is a common colonizer of the human gastrointestinal tract but can cause a wide range of severe infections (including sepsis, meningitis, endocarditis, soft tissue infections, and arthritis) particularly in vulnerable individuals. The incidence of GBS infections in adults, especially the elderly and those with underlying chronic conditions, has steadily increased in recent years[181–185]. In neonates, GBS remains a major cause of life-threatening diseases such as sepsis and meningoencephalitis, often resulting in long-term neurological complications[186]. Recognition of GBS by the innate immune system and the subsequent immune response are essential for host protection. Upon sensing GBS, innate immune pathways trigger a cytokine cascade that drives neutrophil recruitment, enhances phagocytosis, and promotes bacterial clearance[109,187,188]. Neutrophil recruitment to the site of infection is critical for effective anti-GBS defence. For example, experimental neutrophil depletion in animal models results in overwhelming septicaemia and death, whereas control animals control bacterial growth and clear the infection[187]. Consistently, clinical studies show that neonates with reduced neutrophil counts are more susceptible to GBS-induced sepsis[189,190]. Despite their central role, the molecular mechanisms by which neutrophils recognize and respond to GBS remain incompletely understood.

Upon encountering GBS, neutrophils produce a variety of cytokines and chemokines that shape subsequent immune responses and promote the recruitment of additional immune cells[187]. They can further amplify their own recruitment through the release of neutrophil-attracting chemokines, particularly Cxcl2[161,191]. Although individual neutrophils produce lower amounts of cytokines than mononuclear phagocytes, their high abundance makes them major contributors to the early cytokine milieu during infection[192]. In this study, we demonstrate that human neutrophils primarily sense GBS through Toll-like receptor 8 (TLR8), which is abundantly expressed in the endosomes of human myeloid cells, including neutrophils, as a functional cleavage product[193]. To assess the role of TLR8, we employed a selective chemical antagonist previously shown to completely inhibit cytokine production in monocytes stimulated with TLR8 agonists, but not with TLR2 or TLR4 agonists[170].

Upon challenge with GBS and other bacterial pathogens, including *S. pneumoniae* and *K. pneumoniae*, the TLR8 inhibitor markedly reduced the production of all tested pro-inflammatory and chemotactic cytokines, as well as reactive oxygen species (ROS). In contrast, the TLR2 inhibitor C29 had no detectable effect on cytokine production induced by any of the three bacterial species. These findings are consistent with our previous work showing that TLR2 is dispensable for mouse macrophage responses to whole GBS, although concentrated, lipoprotein-rich bacterial supernatants can elicit TLR2-dependent activation[194]. However, our data do not exclude the possibility that diffusible GBS lipoproteins may prime neutrophils via TLR2 for enhanced responses upon direct bacterial contact, and additional studies will be required to clarify this point. Conversely, studies in human monocytes and macrophages have shown that TLR2 ligands can suppress, rather than potentiate, TLR8-dependent cytokine production[193].

Given that TLR8 recognizes bacterial RNA, we assessed the cytokine-inducing potential of purified GBS RNA and found that it robustly activates human neutrophils in a TLR8-dependent manner. Overall, our findings indicate that TLR8-mediated sensing of bacterial RNA is a dominant mechanism driving neutrophil activation by GBS, consistent with previous studies using human monocytes and monocyte-derived macrophages[110] or whole blood[190]. However, the overall contribution of TLR8 to host defense against GBS and other encapsulated bacteria remains insufficiently defined and requires further investigation. Progress in this area is hindered by the lack of suitable small-animal models, as murine TLR8 differs substantially from the human receptor and does not respond to the same agonists [195]. Nevertheless, murine studies have demonstrated that TLR13, absent in humans but functionally analogous to TLR8 in sensing bacterial RNA, plays a significant role in host defense against GBS[195], *S. pneumoniae* [196] and *K. pneumoniae*[197]. Collectively, evidence from human cells and mouse models supports the emerging concept that bacterial RNA represents a key target for innate immune recognition by both mononuclear and polymorphonuclear phagocytes.

Our findings extend previous observations that neutrophils respond more robustly to live bacteria than to killed ones, particularly with respect to ROS production and chemokine release[162]. Using human neutrophils, we show here that full cell activation in response to live GBS requires the combined engagement of FPRs and TLR8. Of note, FPR1 and FPR2 appear to selectively recognize distinct formylated peptides derived from GBS. This has been demonstrated in murine models genetically lacking these receptors[162] and is further supported here using specific pharmacological inhibitors in human neutrophils. For example, we identified fpep8 (the N-terminal hexapeptide signal sequence of the CAMP factor, a well-characterized GBS cytotoxin[198]) as a selective agonist of both mouse and human FPR1. Conversely, fpep10 (a signal sequence found in the FbsA virulence factor[199])

preferentially activates FPR2 in both species. We propose that the requirement of multiple receptors (involving FPR1, FPR2 and TLR8) for high-level ROS and IL-8 production serves as a safeguard, ensuring that neutrophils are fully activated and the associated tissue-damaging effector functions are unleashed only when live bacteria pose an immediate threat.

CONCLUSIONS AND FUTURE PERSPECTIVES

Collectively, this study advances our understanding of how innate immune cells regulate inflammatory responses to viral and bacterial challenges. The investigation led to define a TLR3–TRIF–caspase-8/3–GSDME axis as a key driver of Poly(I:C)-induced lytic cell death in human macrophages, with the contemporary presence of necroptosis, operating independently of NLRP3 inflammasome activation. These findings refine the current model of dsRNA sensing by demonstrating that macrophages engage intertwined apoptotic, necroptotic, and pyroptotic pathways in response to cytosolic viral mimetics. Future studies will be essential to determine how TRIF recruits caspase-8 in this context, to define the subcellular localization of GSDME during Poly(I:C)-induced death, and to assess what factors are involved in necroptosis triggering.

In parallel, our work provides new insight into how neutrophils discriminate between live and dead bacteria. We show that effective activation of human neutrophils by GBS requires the coordinated engagement of TLR8, which senses bacterial RNA, and formyl peptide receptors that detect viability-associated formylated peptides. This synergize allows to enhance neutrophils responses to live and dead bacteria, applied not only to GBS but also to other pathogens such as *S. aureus* and *K. pneumoniae*. Future research should clarify how TLR8 and FPRs pathways integrate at the molecular level and investigate new strategies to enhance the recruitment and effector functions of polymorphonuclear leukocytes to improve the treatment of bacterial infections.

Collectively, these two lines of investigation reveal how innate immune cells calibrate their inflammatory programs by integrating both pathogen identity (viral dsRNA, bacterial RNA) and pathogen viability (formyl peptides), shaping cell-intrinsic death pathways and effector activation.

Overall, these findings reveal fundamental principles governing innate immune responses during viral and bacterial infections and open new avenues for therapeutic strategies aimed at modulating inflammation, either by limiting excessive tissue damage or by enhancing host defense against infections.

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