Non-canonical inflammasome activation in Natural Killer cells: should all the focus be on macrophages and neutrophils?

Mr. Alexander Hogg

Master of Science (by research)

University of York

Biology

31st October 2022

Abstract

The non-canonical inflammasome (NCI) is a crucial intracellular lipopolysaccharide (LPS) sensor and inflammatory activator in the host immune response to bacteria. This caspase-activating platform indirectly induces secretion of proinflammatory cytokines such as IL-1β and promotes a lytic form of cell death termed pyroptosis. The study of the NCI has mainly included the use of macrophages, neutrophils and epithelial cells which have all been reported to possess functional NCI activity. However, there are many lymphoid cell populations involved in the immune response to bacteria including invariant natural killer T cells (iNKTs), natural killer cells (NKs) and CD8+ T cells that so far have not been investigated for NCI activity. In this study, we utilised primary cells from mouse spleen and human blood to assess NCI activation in NK cells. In the context of intracellular bacterial infection NK cells can utilise their known cytotoxic and cytokine producing functions to resolve infection. Overactivation of this proinflammatory state in NK cells has been linked to increased sepsis disease severity. However, so far the NCI has not been implicated in these responses for NK cells. Our preliminary results presented in this thesis demonstrate that both mouse and human NK cells express inflammasome related genes at the transcriptional level but that only human NK cells are able to fully activate the NCI in response to intracellular LPS transfection through secretion of IL-1β. However, LPS transfection did not induce pyroptotic cell death in NK cells. Only with infection experiments using *Salmonella Typhimurium* could pyroptosis be induced. Further work is needed to unravel the cell specific mechanisms underpinning this phenotype. As such, our research indicates that lymphoid cell populations merit further investigation in the field of inflammasome biology.

List of contents

Acknowledgements

Dr Dave Boucher (supervisor). I would like to thank Dave for all his help, feedback and guidance throughout my MSc by Research. Not many supervisors would give up so much of their time to answer pages of questions and stay interested and willing to help. Your positive outlook on life in scientific research and how much you value the contribution of young researchers has been inspiring. The support you have given not only me but all the capable students that have passed through your lab is phenomenal. I truly could not have asked for a better supervisor.

Dr Jillian Barlow (supervisor). Special thanks to Jillian for all your guidance during my masters. I have been incredibly lucky to have not one but two superb supervisors helping me with my project. You have given up a crazy amount of time to help me and push me to better myself during the last year. I hope the work presented in this thesis lives up to that and does justice to the excellent standard of supervision I have had from you and Dave.

Dr Ben Savidge and Gemma Banister from the Boucher lab. Thank you to Gemma and Ben for helping me to get through the last 12 months. The lab environment has been one full of cooperation, fun and many laughs without which the days can drag. So, thank you for your help with the lab work but also for keeping this MSc by Research student sane.

Dr James Hewitson (TAP member). Thank you to James for your contribution and thorough scrutiny of my work during my thesis advisory panel (TAP) meetings. You helped immensely to focus my mind on the experimental details of this project which I hope has increased the robustness of this work.

Joanna Greenman. One of the most dedicated and hardworking PhD students I have met at the University of York. Thank you for finding the time to help me whenever I asked for it despite the busy nature of your PhD. From coding in R studio to flow cytometry guidance to live imaging help, your wealth of expertise has been crucial at educating me throughout the last year. You are a credit to James and the rest of the Hewitson lab.

Karen Hogg, Graeme Park, Karen Hodgkinson. Thank you to the imaging and cytometry team at the University of York technology facility for all their assistance with flow cytometry over the last year which made much of this work possible.

Dr David Kent and the Kent lab. Special thanks to the Kent lab at the University of York for all their assistance during my MSc by research. An excellent example of scientific collaboration in action!

Grace Boyd. Without the help of Grace, there would not be half as many data points on the figures above. Grace was instrumental in helping me collect blood from human donors and spleens from mice. Thank you for your crucial contribution.

James Fox. Thank you to James for setting up an anonymous blood donation program in the department and for collecting blood from donors for use in these experiments.

Dr Daniel Bode. Dan helped me immensely with coding at the beginning of my MSc by research. Thank you for all your help.

Sally James. Thanks to Sally and her team in the genomics lab at the University of York technology facility. qPCR was a focus of this work and your help with the instruments was crucial in being able to use this powerful technique.

Beatrice Rix Thank you Bea for your help with flow cytometry during my MSc by research.

Declaration

I declare that this thesis is a presentation of original work and I am the sole author. This work has not previously been presented for an award at this, or any other, University. All sources are acknowledged as references.

1.0 Introduction

Inflammatory diseases affect millions of people worldwide. A potential contributing factor in these conditions is dysregulated innate immune responses which can lead to abnormal cytokine secretion, sometimes leading to ‘cytokine storm’. In this process a medley of inflammatory signals exacerbate the disease state and contribute to autoinflammation and chronic disease (1). The key 'player' in this overactivated cell phenotype is the inflammasome which drives many inflammatory disease pathologies (2–4). Conventionally, the inflammasome is described as an inflammatory caspase-activating platform which allows for processing of inflammatory cytokines such as interleukin-1 beta (IL-1β) and activates an inflammatory, protective form of cell death called pyroptosis (5). Pyroptosis is a lytic form of cell death which is vital in the immune response to infection. However, overactivation of inflammasomes and uncontrolled pyroptosis directly causes increased disease severity, cytokine storm and autoinflammation in a whole host of disease conditions including rheumatoid arthritis, lupus, sepsis and COVID19 (**Figure 1**) (2,6–9). Although anti-IL-1β therapies are a recent development in treating aspects of chronic inflammation, (10) it is vitally important that more work is undertaken to understand the inflammasome pathway, the inflammatory cell death it triggers, and which cell types are especially affected.

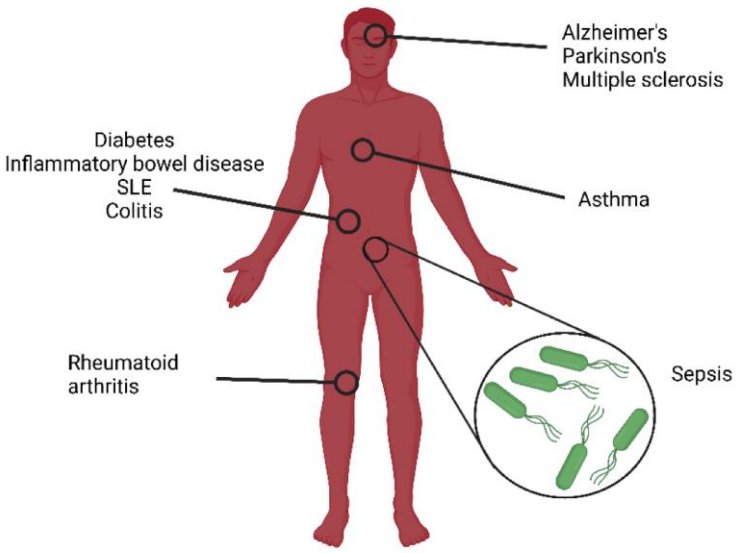


Figure 1. Diseases linked to the activation of inflammasomes.

1.1 Innate immune recognition of danger signals and infection

Cells have evolved many proteins, termed pattern recognition receptors (PRRs), to sense a wide range of cell homeostatic changes, pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs). They include the C-type lectin receptor (CLRs), NOD-like receptors (NLRs), Toll-like receptors (TLRs), RIG-I-like receptors (RLR), and the AIM2-like receptors (ALRs) (11–15). Additionally, guanylate-binding proteins (GBPs) have also been recently suggested to be a novel class of PRR (16,17). PRRs recognise a range of DAMPs and PAMPs with diverse and unique biochemical properties, ranging from lipids, to nucleic acids and proteins, with TLRs and NLRs receiving particular focus. TLRs are present at the cell surface and on endosomes and respond to a wide range of extracellular and intracellular signals (18). NLRs are found in the cytoplasm and orchestrate the response to extracellular and intracellular PAMPs and DAMPs. Crucially, activation of TLRs and cell surface cytokine receptors by extracellular cytokines, PAMPs, and DAMPs provide the priming signal required for the transcriptional upregulation of many proinflammatory proteins, such as inflammasome-related proteins (including inflammatory caspases, cytokines and NLRs) (19,20). Recognition of DAMPs and PAMPs by a subset of NLRs and TLRs can activate signalling immune platforms called inflammasomes. Each sensor protein is activated by specific PAMP/DAMP(s) and can initiate specific signal transduction pathways

Inflammasomes are subdivided into 2 different types; the canonical inflammasome, which activates the signalling protease caspase-1 and the non-canonical inflammasome (NCI), which activates the signalling protease caspase-4 and -5 in humans. Common sensor proteins that form canonical inflammasomes include NLRP1, NLRP3, AIM2, NLRC4 and Pyrin which each respond to different PAMP/DAMPs (5). Both priming at the cell surface and activation of sensors in the cytosol are important for full inflammasome assembly, activation and subsequent effector function. The NCI inflammasome activation is facilitated by GBPs and is specialised in responding to lipopolysaccharides (LPS), one of the most extensively studied PAMPs.

1.2 Lipopolysaccharide (LPS) recognition

Lipopolysaccharide is a key PAMP and has been studied extensively. LPS recognition generates immune responses in various organisms, from plants, invertebrates to higher vertebrates (21). LPS is a conserved component of gram-negative bacteria surface membranes and as such is a prominent inflammatory signal that is recognised by most cells in the body (22). LPS is very abundant (over 2 x 10^6 molecules/ bacteria) and is the main component of the bacterial outer membrane. LPS is composed of 3 chemically unique

structures. The O-antigen is the outer part and is a glycosylated polymer of repetitive polysaccharides units. This structure is highly-variable amongst gram-negative bacteria and is believed to mediate immune evasions under certain situations. The middle counterpart, the core domain, is composed of various oligosaccharide chains that link the third part (lipid A) through α-linked 3-deoxy-d-*manno*-oct-2-ulosonic acid (Kdo). Charges on the sugars present in the core domain are believed to be recognised by a few immune receptors (16). Finally, Lipid A anchors LPS in the outer bacterial membrane. This domain is highly hydrophobic and composed of 5-7 acyl chains, with variations between bacteria subspecies. Lipid A modification is a common strategy bacteria use to avoid immune recognition (21). It was originally thought that LPS recognition could only take place at the cell surface in a TLR4-dependent manner (**Figure 2**).

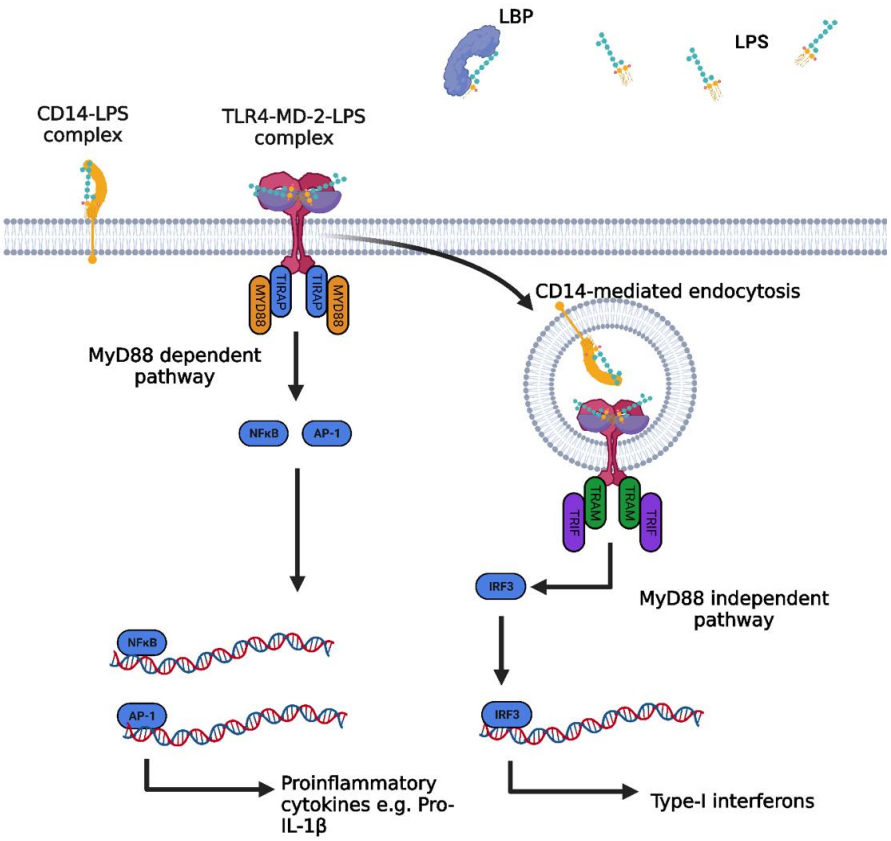


Figure 2. Extracellular LPS recognition via TLR4. LPS recognised by a receptor complex at the cell surface induces a signalling cascade resulting in the upregulation of proinflammatory cytokines. LPS, Lipopolysaccharide; LBP, LPS binding protein; CD, cluster of differentiation; TLR, Toll-like receptor; MyD, myeloid differentiation factor; TIRAP, TIR domain containing adaptor protein; NF, nuclear factor; AP, Activator protein; TRIF, TIR domain-containing adaptor-inducing interferon beta; TRAM, Translocation-associated membrane; IRF, Interferon regulatory factor; IL, interleukin.

In this process LPS is bound by LBP (lipopolysaccharide- binding protein) in the extracellular space and delivered to CD14 (cluster of differentiation 14) at the plasma membrane which trafficks LPS to TLR4. TLR4 exists in preformed dimers that bind the lipid A counterpart of LPS in complex with MD-2 (Myeloid differentiation factor 2). TLR4 detection in certain murine

backgrounds was found to be protective with TLR4-MD-2 complexes dimerising to allow intracellular signal transduction (23). This occurs by two distinct mechanisms. The first occurs at the plasma membrane and involves MyD88 directed signalling which induces expression of proinflammatory cytokines such as IL-6 (interleukin-6) and TNFα (tumour necrosis factor alpha) via NF-κB (24). The second occurs by CD14-mediated endocytosis of the LPS-TLR4-MD-2 complex and utilises TRIF signalling which induces expression of type-I interferons via IRF3 (**Figure 2**) (13,25). TRIF signalling also contributes to NF-κB signalling however this is delayed compared to MyD88. Therefore, LPS can directly promote upregulation of a plethora of proinflammatory cytokines.

In addition, secretion of newly produced type-I interferon by the cell (induced by IRF3) can act in an autocrine manner to further induce proinflammatory gene expression by interaction with its receptor (IFNAR1) at the plasma membrane (26,27). Therefore, LPS recognition at the plasma membrane can elicit a positive feedback loop to boost expression of protective inflammatory genes. As a result, pathogens such as *Mycobacterium tuberculosis* have developed mechanisms to prevent this type-I interferon autocrine signalling in order to increase bacterial proliferation within the host cell (28).

Despite this important role for TLR4 in LPS recognition, it is now known that LPS recognition can occur in a TLR4-independent fashion. For example, the lipid A moiety of LPS can be directly recognised in the cytoplasm by a newly described complex called the NCI (13,29,30). Intracellular bacteria proliferate within the host cell by distinct mechanisms (31,32). A well-characterised example of this is *Salmonella*. *Salmonella* invades epithelial cells within a membrane structure called the *Salmonella*-containing vacuole (SCV), utilises a membrane bound vacuole, and escapes into the host cytosol where it can hyper-proliferate. *Salmonella* uses a range of effectors that support cellular invasion and intracellular survival (33). Within the vacuole, *Salmonella* hijacks host autophagy chaperone components to supply itself with nutrients (34). Furthermore, to aid its growth in the cytosol *Salmonella* utilises the type III secretion system 1 to release SipA prior to escape from the vacuole. SipA enables *Salmonella* to evade host defence mechanisms and replicate in the cytosol upon escape from the vacuole (35). In contrast, *Shigella flexneri* survives and proliferates in the cytosol by quickly exiting its vacuole and evades the host immune response by shielding the lipid A moiety of LPS through a complex O-antigen modification (36) and various bacterial effectors targeting inflammasomes components (37,38).

Both of these examples illustrate the necessity of the host cell to recognise bacterial LPS within the cytosol. Extracellular bacteria, such as *E.coli*, generate membrane-derived

structures called outer-membrane vesicles (OMVs). These LPS-containing vesicles can be delivered inside cells and be detected by intracellular immune receptors. This intracellular recognition pathway is important in mediating immune responses to infection (39). Thus, the canonical inflammasome pathway (extracellular detection) and the non-canonical pathway (intracellular detection) are both relevant in the early response to LPS (40,41).

1.3 The canonical inflammasome

Intracellular sensor proteins such as NLRP3 (Nod-like receptor protein 3) contain conserved domains which allow oligomerisation and recruitment of the adaptor protein ASC (apoptosis-associated speck-like protein) and the inflammatory caspase-1 (4). Two crucial protein-protein interaction domains used to form an inflammasome are the pyrin domain (PYD) (found on most inflammasome sensor proteins) and the caspase-activation and recruitment domain (CARD). The canonical inflammasome forms when these domains interact by either sensor-adaptor-caspase complex or sensor-caspase complex. Upon direct or indirect DAMPs and PAMPs sensing, inflammasome-activating PRRs oligomerise and recruit ASC. ASC forms a large structure called the speck, which allows for the recruitment and activation of caspase-1. The NLRP3 inflammasome is an example of a sensor-adaptor-caspase complex (**Figure 3**) (42). The purpose of these multi-protein complexes is to provide a platform for inflammatory caspase activation by proximity-induced activation (43). Caspases are cysteine proteases that cleave proteins after aspartic acid residues. Inflammatory caspases exist as zymogen caspases (caspase-1/4/5/11) and consist of a CARD domain, a large subunit (p20) and a small subunit (p10) as shown in **Figure 3** (44,45).

Upon recruitment to the inflammasome complex via CARD-CARD interactions, caspase-1 proteins proteolytically cleave each other, releasing the CARD domain, to generate catalytically active caspases (p33/p10 form) (46) . These mature caspases can then cleave downstream targets such as the pore-forming protein Gasdermin-D (GSDMD) and cytokines such as pro-IL-1β and pro-IL-18 (5,47). The N terminal mature form of GSDMD oligomerise and form pores in the plasma membrane to induce pyroptosis. The pore also allows release of mature IL-1β and IL-18 which can signal to surrounding cells and induce a proinflammatory phenotype (47,48). Pyroptosis is characterised by cell swelling and subsequent plasma membrane rupture regulated by the protein Ninjurin-1 (Ninj1) which is necessary for the final membrane rupture stage of pyroptosis. Macrophages from Ninj1 knockout mice exhibit increased cell swelling and impaired membrane rupture. In addition, a lack of Ninj1 is associated with increased susceptibility to bacterial infection in mice highlighting the importance of pyroptosis in host defence (49).

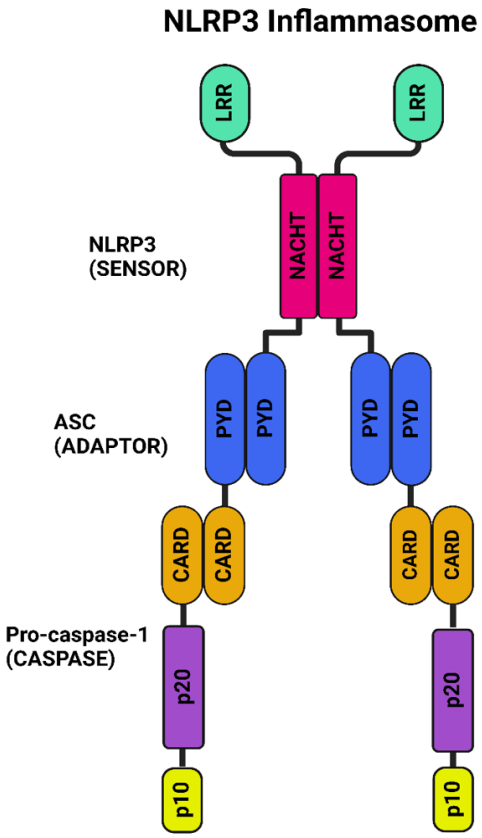


Figure 3. NLRP3 inflammasome assembly. Consists of NLRP3, ASC and pro-caspase-1. LRR, Leucine-rich repeat; PYD, Pyrin domain; CARD, Caspase activation and recruitment domain. (Created in Biorender).

1.4 The non-canonical inflammasome (NCI) pathway

The NCI specifically senses intracellular LPS either on the surface of intracellular bacteria with the assistance of interferon-inducible guanylate-binding proteins (GBPs) or by recognising free LPS in the cytosol (16,29,50). Free LPS can be delivered to the cell cytosol through release of LPS-containing OMVs by bacteria, which activate caspase-11 in the cytosol (39). Caspase-11 in mice and caspase-4 and 5 in human are the inflammatory caspases specifically activated by the NCI and are activated in the same way as caspase-1: by proximity-induced dimerisation that mediates auto-cleavage to generate catalytically active proteases (in this case the p32/p20 form by cleaving between the p20 and p10 subunits) (51).

Currently, the mechanism of assembly of the NCI is not fully understood but GBPs are thought to be important in early NCI formation and are suggested to be the intracellular LPS sensor along with caspase-4 and 5 (caspase-11 in mice). GBP genes are directly upregulated as a consequence of interferon gamma (IFN-γ) priming or signalling and thus follow the dogma associated with inflammasome activation where priming is thought to be required (50). Interferon-inducible GBPs have been shown to be crucial for host immunity to infection and

genetic deletion of GBP genes in mice has been shown to increase susceptibility to bacterial pathogens (50,52). In addition, GBPs have been reported to release bacteria hiding in pathogen-containing vacuoles into the cytosol and form a multi-protein platform on the bacterial surface consisting of a hierarchical network of GBP proteins (17). Furthermore, human GBP1 has been shown to directly bind LPS and thus acts as a sensor of intracellular bacterial infection. This binding of GBPs to LPS is thought to disrupt the bacterial surface coat and allow caspase-4 (or 11) recruitment and subsequent LPS binding and caspase activation (16). In infection, GBPs have been shown to lyse Salmonella-containing vacuoles to expose the bacteria to the cell cytoplasm (53). However, in contrast to canonical inflammasome activation, caspase-11 (as well as caspase 4 and 5 in human) has been shown to bind LPS, specifically the lipid A region, without the need for a sensor or adaptor protein (25,29).

This suggests a distinct mechanism must exist for NCI oligomerisation and activation. However, the effect of caspase-11 auto-cleavage is very similar to caspase-1. It has been reported that NCI activation is sufficient to induce pyroptosis in the absence of caspase-1 and that caspase-11 can directly cleave GSDMD to trigger pyroptosis (**Figure 4**) (54,55). However, the ability of caspase-4 and 5 (or 11) to directly cleave proinflammatory cytokines remains contentious and it is widely accepted that it instead induces IL-1β processing indirectly via the canonical inflammasome. The accepted mechanism is that caspase-mediated cleavage (4, 5, or 11) of GSDMD triggers potassium efflux that activates the NLRP3 inflammasome which in turn results in caspase-1 dependent cleavage of proinflammatory cytokines including IL-1β and IL-18 (**Figure 4**) (56).

Caspase-4 is also able to cleave IL-18 directly, and thus (in combination with caspase-1 cleavage of IL-18) stimulate IFN-γ production which is necessary to control intracellular bacterial infection. Caspase-1 knockout mice exhibit lower IL-18 secretion and subsequent reduction of IFN-γ levels in the blood which makes them more susceptible to intracellular bacterial infection (57,58). In the context of mucosal immunity, caspase-4 mediated IL-18 cleavage is important to control bacterial pathogens by enabling recruitment and activation of natural killer cells (NK cells) to the site of infection (59). IL-18 secretion promotes adaptive immune responses by boosting Th1 responses through activation of CD8+ T cells. IL-18 also acts on NK cells, increasing surface expression of the cell death ligand FasL and enabling the secretion of IFN-γ. Furthermore, bacteria have developed effectors that specifically target caspase-4 to enable survival in the gut epithelium. *Shigella flexneri* utilises the effector OspC3 to directly inhibit caspase-4 activity characterised by an inability to induce pyroptosis and thus allowing *Shigella* to proliferate within epithelial cells of the gut (60). This effector is believed to directly modify caspase-4 and -11 (38). *Shigella* has also been described to use an E3

ubiquitin ligase to target GSDMD and GBPs for degradation and thus prevent pyroptosis and bacterial release from the cell (37,61). Activation of caspase-11 and caspase-1 has also been shown to restrict growth of *Salmonella* within the cell independantly of pyroptosis (62).

Caspase-5 activation is less understood but has been shown to have a role during infections (63). Caspase-5 cleaves GSDMD and IL-1α (64).

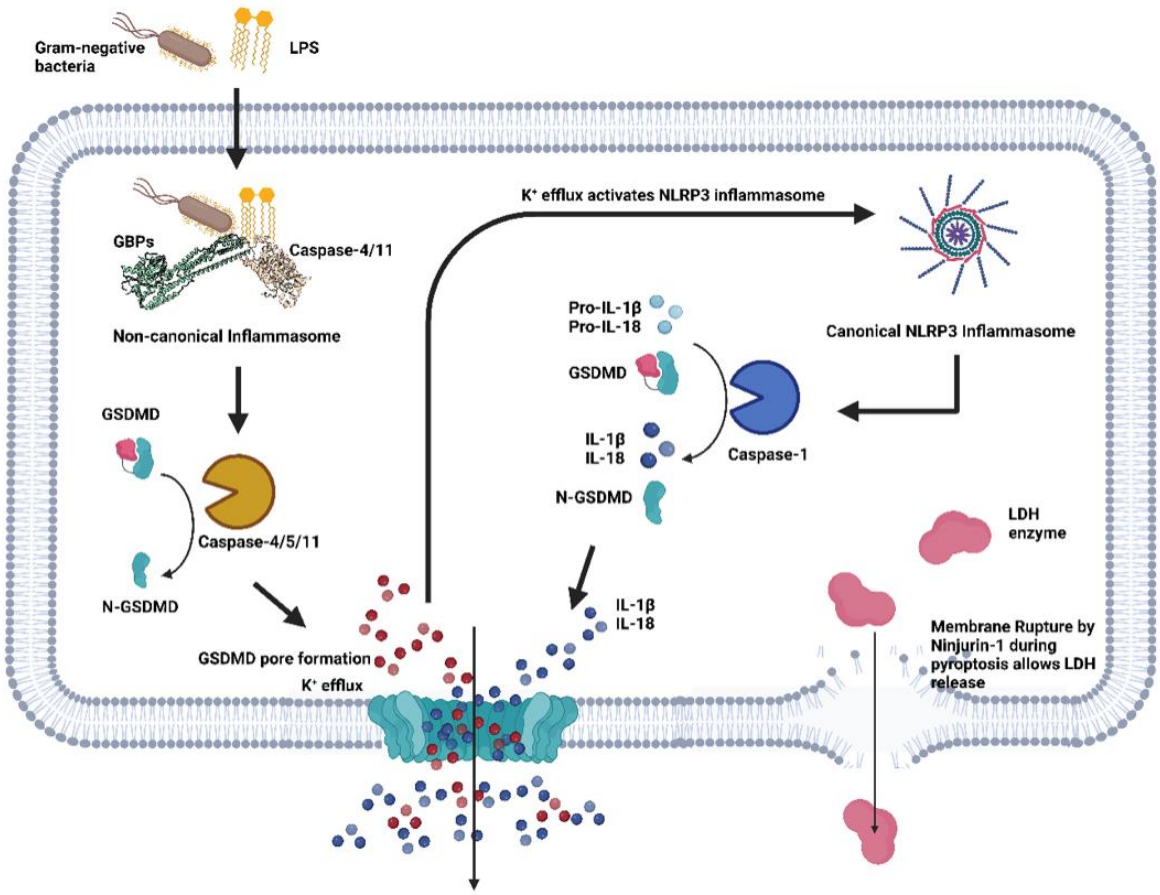


Figure 4. The non-canonical inflammasome pathway. TLR-independent recognition of LPS in an intracellular context leads to cleavage of GSDMD by caspase activity (caspase 4 and 5 in human and caspase 11 in mice). This allows pore formation, cytokine release, pyroptosis and subsequent activation of the canonical inflammasome. GBP, Guanylate binding protein; GSDMD, GasderminD; K, Potassium; NLRP3, NLR family pyrin domain containing-3; LDH, Lactodehydrogenase, IL, Interleukin.

The NCI is thought to have both protective and detrimental effects during bacterial infection. In most cases activating this inflammasome is vitally important in clearance of bacterial infection. However, caspase-11 activation can be damaging to the host in the absence of caspase-1 activity and cleavage of downstream proinflammatory cytokines (65). Furthermore, caspase-11 has been linked to increased disease severity in the context of sepsis and is thought to exhibit different cell-type specific roles (66). Other proteins are thought to contribute to this such as

high mobility group box 1 (HMGB1). HMGB1 has been reported to be required for caspase-11-dependent lethality in sepsis by delivering LPS into the cytosol for caspase-11 recognition and subsequent activation (67).

1.5 The non-canonical inflammasome pathway in macrophages and neutrophils

Inflammasomes are present in many specific cell types. However, the main research focus has surrounded myeloid cells such as macrophages and neutrophils. Initial papers discussing intracellular LPS recognition by caspase-1 and IL-1β secretion through GSDMD pores used macrophages in their experiments. Furthermore, GBPs have been shown to lyse SCVs and release bacteria into the cytoplasm in epithelial cells (30,48,53). Interest has grown into NCI functions in neutrophils in recent years due to their ability to release neutrophil extracellular traps (NETs) and induce a neutrophil-specific form of cell death called NETosis in response to intracellular LPS (68). NETs are DNA-based structures that capture bacteria to allow clearance by other immune cells. NETs are thought to have a positive role in bacterial clearance in the context of sepsis (69). However, studies have reported that overactivation of NETosis and reduced clearance of NETs contribute to autoimmunity, tissue damage and sepsis severity (70,71). Furthermore, overactivation of the NCI is directly linked to this phenotype and inhibition of GSDMD is reported to alleviate the disease severity associated with sepsis (68,72). This overactivation is thought to be worsened by type-I interferon dependent upregulation of caspase-11 and GSDMD (8).

1.6 The non-canonical inflammasome pathway in epithelial cells.

Over recent years, functions of the NCI have emerged as central to control gram-negative bacteria infections in epithelial cells. During infections, epithelial cells are amongst the first cell types to encounter bacteria and are often the point of entry into the body (73). Following epithelial cells infection, many gram-negative bacteria can access the cytosol where their LPS is detected by GBPs and elicit the formation of the NCI (16,17). In murine and humans, caspase-4/11 activation in these cells is important to decrease bacterial burden in a GSDMD-independent manner (62). In epithelium, caspase-4 activation leads to pyroptosis and to the maturation and secretion of IL-18. NCI is also promoting the extrusion and death of infected cells from the epithelium, promoting bacterial clearance (74). Caspase-4 activation has been reported in the gut, the skin and the lung epithelium and restrict pathogens proliferation (16,17,75,76). Research has mostly focussed on inflammasome activity in the gut epithelium. It has been reported that intestinal epithelial cells express most inflammasome components and that inflammasome activity in these cells is important for tissue homeostasis. However, the role of inflammasomes in pathogen exposure in the gut is not fully understood with reports

of delections of inflammatory caspases being both beneficial and detrimental depending on the study (77). The NAIP-NLRC4 canonical inflammasome has been linked to expulsion of epithelial cells of the gut during Salmonella infection as a necessary compromise to maintain epithelial cell barrier function (74).

During *Burkholderia thailandensis* infection of epithelial cells, the NCI restricts the formation of large multinucleated cells, limiting pathogen proliferation (78). NCI also reduces bacterial spreading by controlling cell-to-cell spreading of pathogens such as *Shigella* and *Burkholderia (61)*.

Epithelial cell expulsion and NETosis are good examples that inflammasome activation has different tissue and cell-type specific functions. As such it would be worthwhile to examine NCI activity in other cell types.

1.7 The innate lymphoid cell family

Although macrophages and neutrophils are a key component of innate immune responses during bacterial infection, both having the capacity to induce early effector responses, including cytokine release via the NCI, it is interesting to speculate as to the role of other less well characterised cells. Indeed, in the last decade it has been shown that rather than lymphoid lineage cells only forming an adaptive response (B cells, $CD8^+$ T cells, Th1, Th2, Th17 cells) that there is a complex innate lymphoid cell family that participates in the innate immune network (79). The founding member of these 'innate lymphoid cells' (ILCs) was actually identified in 1975 as the natural killer (NK) cell, as $CD3^-CD56^+$ in human and $NK1.1^+$ in mouse (80). Initially, NK cells were thought to exist as cytotoxic cells, expressing killer-cell immunoglobulin-like receptors (KIRs) to first detect and then eliminate pathological (infected or cancerous) cells. Their cytotoxicity was shown to derive from the release of anti-microbial components such as perforin, granzyme A and granzyme B. However NK cells also rapidly and robustly release large quantities of proinflammatory cytokines (81).

Since the publication of a number of articles in and around 2010 (82–84), it has become clear that NK cells exist in a developmental network with the potential to produce different types of ILC depending on the host insult . Thus, it is now thought that ILCs constitute both cytotoxic and non-cytotoxic cell types. They are classified into three sub-groups termed type 1, 2 and 3 innate lymphoid cells (ILC1s, ILC2s and ILC3s) which are thought to roughly mirror the function of their adaptive immune system counterparts T helper type 1 (Th1), T helper type 2 (Th2) and T helper 17 (Th17) cells respectively (85,86). This classification has been attributed to their similar cytokine profiles and functionality. Under this nomenclature, type 1 ILCs

constitute cytotoxic NK cells and cytokine-secreting ILC1s, with evidence currently lacking as to whether this represents a continuum of cell states or two distinct lineages (87). ILC1s are thought to secrete IFN-γ and TNFα like and be important in intracellular bacterial and viral infections, but also with a possible role in anti-tumour responses. ILC2s secrete IL-4, IL-5, IL-9 and IL-13 like Th2 cells and have been implicated in helminth infection, tissue repair and allergy (88–91). Finally, ILC3s are characterised by their secretion of IL-17A cytokine like Th17 cells and are involved in extracellular antibacterial immunity, chronic inflammation, and in autoimmune diseases such as multiple sclerosis (85,92).

The defining features of this family of ILCs, distinguishing them from T cells and other innate cell populations, are an inability to directly recognise both danger signals (PAMPs and DAMPs) and peptide antigen due to the lack of PRRs and TCR (T-cell receptor) on their cell surface. These cells also lack the expression of any other mature cell surface markers, and are therefore often defined as lineage negative (Lin^-) (93). It has been shown that ILC development derives from a shared $Id2^+$ (DNA-binding inhibitor 2) progenitor cell that is distinct from T cell development, with differentiation occurring both in the adult bone marrow and foetal and adult thymus (94,95). In addition, rather than constantly circulating via the blood and lymph like conventional T cells, they largely differentiate directly in tissues. Indeed, they have been identified in most tissues in both mice and humans, including skin, liver, lung, fat, intestine, and tonsil (96). It is thought that this tissue network allows an immediate response to epithelial-derived ‘inducer’ cytokines, IL-15 and IL-18 (ILC1s), IL-25 and IL-33 (ILC2s), and IL-23 (ILC3s) that then promote ILC proliferation and rapid secretion of large quantities of appropriate cytokine (92,97–100).

Although a growing number of mouse models allows us to more easily dissect the development, relatedness, and function of ILC1s versus NK cells, alongside the other ILC family members, the relationship between these cell types in humans is less well understood. Subsets of NK cells (human $CD56^{bright}$) that are non-cytotoxic and instead produce large amounts of IFN-γ like ILC1s have been identified, with recent and detailed flow cytometric analysis revealing three different populations of NK-related cells (conventional NK cells, $Nkp44^+CD56^+$, intraepithelial ILC1s, $Nkp44^-CD103^+$, and helper ILC1s, $Nkp44^-$, non-cytotoxic) (101,102). However, the ability of mixed populations of NK cells to become cytotoxic and produce granzyme and perforin (human $CD56^{bright}$ NK cells) has hampered their classification as ILC1s despite expression of cell surface cytokine receptors and a shared IFN-γ secretory phenotype (103). Thus, the dual functionality of NK cells (cytotoxic and proinflammatory cytokine producing) makes them an interesting cell type to study other mechanism of innate

responses in, particularly as they are so critical to the host response in viral and intracellular bacterial infection (104–106).

1.7.1 Natural killer (NK) cell development

As mentioned previously, it is still unknown as to the exact developmental relationship between conventional or 'cytotoxic' NK cells and 'helper' or cytokine-producing ILC1s. In mice, data suggests the initial site of ILC1 development to be the bone marrow, with immature cells being released for further maturation stages in sites such as the liver (87). A number of recent reports using multi-colour transcription factor reporter mice have identified a working paradigm by which ILC development, requiring GATA3 at a very early stage, derives from an haematopoietic $Id2^+$ ILC progenitor cell. It is suggested that pre-NKPs and refined NKP stages diverge towards immature NK cells from this 'ILCP', however it is unclear if this is the only developmental pathway since deficiencies in GATA3 still allowed for some mature NK cells (85,94,107) .

Initial commitment to the immature NK cell lineage has been linked to the expression of NK1.1, CD122 (IL-15 receptor), and KIRs (CD94/NKG2D and Ly49), with expression of CD11b, CD49b, and a downregulation of CD27 also being required at later stages (108–110). Furthermore, downstream commitment to either ILC1 or NK cell is thought to be dependent on two master transcription factors, Tbet and Eomes (Eomesdermin homolog), with T bet (T-box transcription factor) expression found in both cell types, and Eomes found only in NK cells (111). Although the relationship between these two proteins is complex, overexpression of Tbet in mice gave an increase in $Eomes^-$ NK/ILC1s, whilst ectopic overexpression of Eomes led to expansion of the $Eomes^+$ conventional NK cells (112). In addition, deletion of Eomes under the control of *Vav*-Cre led to a significant reduction in cytotoxic NK cells, whereas *Tbet*$^{-/-}$ mice appear to prevent ILC1 development in the bone marrow (113,114).

In humans it has been suggested by Mjosberg *et al* (2018) that three distinct lineages of mature ILC1s exist (conventional NK cells, intraepithelial ILC1s, and helper ILC1s) (102). However, lineage commitment of these subsets is unclear as *in vitro* experiments suggest human ILCs to be highly plastic. For example, data suggests that ILC2s and ILC3s may both have the ability to downregulate GATA3 and RORgammat, respectively and upregulate NK cell-related functions (115,116). Exposure of human NK cells to a cocktail of interleukins and type-I interferons furthers their differentiation via JAK/STAT signalling cascades (117). In humans $CD56^{bright}$ NK cells are suggested to further develop into mature $CD56^{dim}$ NK cells that express CD16 and exhibit a more cytotoxic phenotype characterised by granzyme and perforin production (101,117). Thus further work is needed on human NK and ILC1 populations.

1.7.2 NK cell function

NK cells have wide ranging functions from antiviral responses to their use as a potential antitumor immunotherapy (106,118,119). NK cells are experts of immune surveillance and patrol the body for signs of cell stress and infection. They express a variety of cell surface proteins that bind to markers of cell stress on neighbouring cells. One example is NKG2D which binds to a whole host of cell surface glycoproteins that indicate if a cell is undergoing stress or is infected with a pathogen. Upon binding to one of its ligands, NKG2D can elicit cytotoxic effects of NK cells. However, other costimulatory molecules are required or accessory cytokines such as IL-2 or IL-15 for full NK cell activation (120). IL-2, IL-15 and IL-18 are well documented to activate NK cells and encourage maintenance and proliferation (121,122). NK cell receptor-ligand interactions induce granzyme and perforin release which can trigger apoptosis of the target cell (123). Due to their impressive cytotoxic effects and IFN-γ production, interest has grown in developing them as a potential immunotherapy to target tumours (103,119). In addition, neutralising antibodies against HIV have been developed to allow NK cell mediated killing through antibody dependent cellular cytotoxicity (ADCC) (118). Evidently, NK cells are crucial for immune protection against infection and destruction of transformed or stressed cells. Research into their antitumor function is ongoing to assess their potential use as a cancer immunotherapy.

1.7.3 The role of lymphoid cells in bacterial responses

Currently, most of the research into the NCI is conducted using cell lines, macrophages, neutrophils and epithelial cells. However there are many other cell types that are important in the host response to bacterial infection that have not been shown to possess active NCI. NK cells have been shown to produce inflammatory cytokines in the context of bacterial infection, mainly through secretion of IFN-γ (124). IFN-γ is reported to be protective in bacterial infection and depletion of NK cells prior to induction of sepsis in mice resulted in higher bacterial burden and lower macrophage phagocytic activity (125,126). This phenotype could be observed in *Shigella* infection of mice lacking NK cells and other lymphoid cell populations including B and T cells with IFN-γ playing a crucial role again in protection (104). Interestingly, overactivation of NK cells and subsequent excessive IFN-γ production is known to drive sepsis, indicating the potential downside of NK cell activity during infection (127). Some lymphoid populations have killer functionality which can be utilised in bacterial infection. CD8+ T cells can kill cells infected with intracellular bacteria and even directly kill *Mycobacterium tuberculosis* through secretion of granulysin (128). Furthermore, innate T cells are thought to respond early on in

bacterial infection and facilitate clearance of bacteria from infected tissue (129). Removal of one type of innate T cell, invariant natural killer T cell (iNKT), has been shown to increase susceptibility to bacterial infection. The ability of iNKT cells to release IFN-γ or IL-4 depending on the type of bacterial infection exhibits their inherent diversity among subsets and their importance during early immune responses (130). Early studies identified the expansion of innate T cells, specifically gamma-delta T cells (γδ T cells) upon injection of bacteria in mice (131). Their ability to respond to intracellular bacterial infection has since been reported including their ability to respond to *Mycobacterium tuberculosis* infected cells by secretion of granzyme A (132).

In summary, there are several cell types that contribute to immune responses against invasive bacteria (133). Inflammasome activity in many of these cells is currently poorly characterised. As such it is important to establish whether inflammasome activation in these cells can contribute to bacterial clearance, or be disadvantageous and contribute to disease phenotypes including sepsis. NK cells are currently believed to express no functional inflammasome and to mediate inflammatory responses only downstream of inflammasome activation in myeloid and epithelial cells (134,135).

1.8 Project outline

We hypothesised that lymphoid cells could express functional NCI components in the context of cytosolic LPS delivery or during intracellular bacterial infection. To test this hypothesis, initial work focussed on analysing existing RNA-sequencing data to find promising candidate cell(s) expressing inflammasome components for further experiments. After an appropriate cell type was selected the decision was taken to examine NCI activation in both primary human and mouse cells. For physiological relevance, we conducted human work using fresh blood-isolated immune cells. Work with primary mouse cells brought with it the opportunity to examine the NCI pathway in primary tissue-resident cell populations (liver) which would not have been possible for human studies. In addition, due to the different NCI caspases present between mouse and human (caspase-11 in mice, caspase-4 & 5 in human) and differences in GBPs expression (7 GBPs in human, 11 in mice), we hypothesised the existence of species specific activity. Furthermore, humans express IL-37, an anti inflammatory IL-1 family cytokine that is cleaved by caspases which could provide a species specific target for the NCI in lymphoid cells (47).

2.0 Materials and methods

Details of the materials used are listed in **Supplementary tables 1, 2 and 3**.

2.1 Animals

All mice were bred in specific pathogen free conditions in the BSF facility, at the University of York. Mice were matched for age, sex, and background strain and all experiments were conducted on surplus tissue, with the approval of the local AWERB committee, and under license with the UK Home Office. C57BL/6J are referred to as 'wild type' (WT) mice in figures. *Fgd5*$^{ZSGreen/+}$ were from Jackson Laboratories and originally generated and described by Gazit R. *et al* (2014), and were backcrossed onto the C57BL/6J strain. WT mice were maintained in-house (136).

2.2 Mouse tissue and processing

Following dissection, spleen was compressed through 70 u filter membranes, which were washed with 1xPBS/2%FCS (foetal calf serum), to achieve a single cell suspension. Cells were centrifuged at 300g for 5 mins and the pellet resuspended in RBC lysis buffer (ammonium chloride, 5 mins, r.t.). Final resuspension, following centrifugation, was in 1xPBS/2%FCS. Cells were then used for cell purification either by FACS or by negative magnetic bead separation (EasySep, STEMCELLTechnologies), according to manufacturer's instructions) For bead separation single spleen samples were pooled to attain sufficient numbers of output cells and were from that point treated as one biological replicate (n=1). This method was used for all 14 hr treatments and 3 hr priming plus 5 hr treatment experiments unless otherwise stated in the figure legend.

For fluorescence-activated cell sorting, murine NK cells ($CD45^+lineage^-NK1.1^+$) and macrophages ($CD45^+CD3^-CD11b^{high}$) were sorted into 1xPBS/50%FCS by FACS. 7AAD (Invitrogen) was used as a live/dead stain. Compensation beads (Invitrogen) were used for single stain controls. Antibodies used were against the following molecules: 'lineage' (CD4, GK1.5, CD8a, 53-6.7, CD19, eBio1D3, CD11c, N418, Gr1, RB6-8C5, FceR1, MAR-1) conjugated to PECy7, CD45 (clone 30F11) conjugated to Alexa700, CD11b (clone M1/70) conjugated to BV510, CD3 (clone 145-2C11) conjugated to PE, and NK1.1 (clone PK136) conjugated to BV421. Following sorting, cells were either plated at the stated density or prepared for qPCR (quantitative polymerase chain reaction).

2.3 Human donors and tissue processing

Up to 36 ml blood was taken from healthy human donors anonymously, with the approval of the Biology Ethics Committee, University of York (reference DB202111). No details of age, sex or lifestyle were recorded except for details about current medication which was shared at the discretion of the donor. For initial experiments each donor was assigned a unique letter (e.g. A, B). For later experiments this was substituted for a character code with the format D000 and recorded by the blood collection team. To ensure no overlap in donors between the two naming systems and prevent donor duplication, they were analysed separately for data presentation. Blood was collected in a heparin coated tube containing 2mM EDTA to prevent coagulation.

Human NK cells and neutrophils were purified either directly from human blood using magnetic separation negative selection kits (EasySep, STEMCELL Technologies) or from PBMCs obtained by Ficoll separation and subsequent magnetic separation using negative selection kits (EasySep, STEMCELL Technologies, according to manufacturer's instructions using DPBS/2%FBS/2mM EDTA as a diluent). When necessary, PBMCs were differentiated to macrophages for downstream functional assays. Briefly, after Ficoll separation PBMCs were plated in HMDM media (RPMI1640 (Gibco)/10%FBS/1%Penicillin/Streptomycin and 1% glutamine with Macrophage-Colony stimulatory factor 1 (M-CSF1 (Proteintech); 50 ng/ml)) in a sterile dish (non-tissue culture coated) for 1 week. On day 6 of differentiation, cells were harvested with a 16G needle in cold sterile DPBS and replated at a concentration of 25,000 cells per well in tissue-culture coated 96-well plates in HMDM media.

Between 50,000 – 150,000 whole blood cells or PBMCs were taken prior to the separation step to act as a comparison. After separation neutrophils and NK cells from all donors were assessed for purity, based on the percentage of $CD45^+$ (clone X, conjugated to X) cells that were expressing neutrophil or NK cell markers (CD11b, clone X, conjugated to FITC or CD56, clone X, conjugated to FITC). For mice only NK cells NK1.1 was used to check purity. 7AAD was used as a live/dead control. Samples were run on BD Fortessa, CytoFLEX S or CytoFLEX 375 and analysed in FlowJo or CytExpert softwares.

Cells were eventually resuspended at 50,000-200,000 NK cells per well in a 96 well or 12 well plate with either Opti-MEM alone or X-VIVO15 plus IL-2 (100ng/ml). X-VIVO15 plus IL-2 was used in later experiments as it was found to increase cellular viability. Neutrophils were seeded in a 12-well plate at 1 x 10^6 cells/well in Opti-MEM (Gibco) +Aprotinin 1µg/ml (Sigma).

2.4 Human NK cell expansion and *Salmonella* infection

Following magnetic separation NK cells were plated for expansion in non-tissue culture treated 6-well plates at 1 x 10^6 cells per well. 2 hrs prior to seeding, plates were coated as per manufacturer's protocols (STEMCELLTechnologies, Immunocult). Cells were seeded in an appropriate volume of Immunocult base media and supplement and allowed to expand for 14 or 28 days. After expansion, cells were re-seeded in 96 well plates in 100µl of Immunocult media (50,000 cells per well). When used directly, NK cells were resuspended in X-VIVO15 medium (Lonza) supplemented with IL-2 (100 ng/mL).

Salmonella typhimurium serovar ST4174 was grown overnight in 3ml LB media at 37°C, 220 rpm in a shaking incubator. After overnight incubation, bacteria were diluted 1 in 40 with LB and incubated for 2.5 hrs at 37°C, 220 rpm shaking to get late log phase *Salmonella*. Bacteria were then centrifuged at 1800 g for 2 mins at room temperature (RT) and washed with DPBS. For stationary phase *Salmonella*, the sample was not diluted and used directly after the overnight growth culture. For optical density (OD) measurement samples were diluted 1 in 10 with sterile DPBS and OD measured at 600 nm. All experiments used *Salmonella* with a multiplicity of infection (MOI) of 50 by adding 10 - 40 µl of bacteria per well (depending on volume of cell culture media). After 1 hour of incubation with NK cells, gentamicin was added at 20 µg/ml to kill extracellular bacteria. The plate was then incubated for a further 4 hours at 37°C 5% CO_2 and cells were prepared for analysis in the LDH assay.

2.5 Cell priming and treatment

Following mouse or human cell preparation, priming was undertaken in 96 well plates for a minimum of 3hrs where culture media was supplemented with ultrapure LPS EK (Invivogen, 100 ng/ml) or 16hrs for IFN-γ (Abclonal Biotechnology, 10 ng/ml). Cells were incubated at 37°C 5% CO_2.

VX-765 caspase inhibitor was added (50 µM) for 30 mins prior to other treatments to allow effective inhibition of caspases to occur. For treatments cells were used in serum-low or serum free media (Opti-MEM or X-VIVO15) to reduce serum interference. For macrophages, cells were resuspended in Opti-MEM for 5hr treatments to reduce serum interference.

In most instances following priming, cells were either prepared for qPCR experiments directly or treated with lipofectamine LTX only (Invitrogen, 1 in 40 in Opti-MEM) or lipofectamine plus ultrapure LPS EK (10 ug/ml in Opti-MEM, vortexed for 30 seconds, lipofectamine added for 10

mins, r.t.). Lipofectamine only and lipofectamine plus LPS transfection mixes were added to cell plates at a 1:10 dilution by slowly pipetting across the surface of the well with centrifugation at 500g room temperature for 5 minutes. For untreated cells, Opti-MEM was added at an equal volume to other cell treatments. For extracellular LPS treatment, ultrapure LPS was diluted 1 in 40 with Opti-MEM, vortexed for 30 seconds and added to the cell plate at a 1 in 10 dilution. Cells were incubated at 37°C 5% CO_2 for 5-14 hrs as detailed in the figure legend.

2.6 RNA extraction, cDNA preparation, and quantitative PCR

Wells containing cells were gently resuspended and transferred to microcentrifuge tubes for RNA extraction (Arcturus Picopure RNA isolation kit, following manufacturer's instructions). If cells were still adhering to the plate, 100 µl of trypsin was added to each well, incubated at 37°C for 10 - 15 minutes, quenched with an equal volume of DPBS/2% FBS and cells subsequently removed. Samples were stored at -80°C before later being isolated (Arcturus Picopure RNA isolation kit), with the addition of a DNAse I treatment (Qiagen RNase-free DNase set). Following RNA isolation cDNA was immediately synthesised according to manufacturer's instructions (Invitrogen, superscript IV). For qPCR all samples were run in triplicate using Taqman primers against specified targets (**Supplementary Table 3**). *Gapdh* was used as the housekeeping gene for both mouse and human samples and values were normalised using the delta C_t (ΔCt) method. Data was expressed as fold change.

2.7 Lactate Dehydrogenase (LDH) assay

Lactate Dehydrogenase (LDH) was used to measure lytic death using the LDH cytotoxicity detection kit according to manufacturer instructions (Takara). For functional assays a few wells were selected for 100% cell lysis controls. To control wells, 4 µl of Triton (10%) was added to lyse cells and provide a reference control. The plate was incubated for 5 - 10 minutes at 37°C. 15 - 20 µl of supernatant was removed to a new plate for LDH assay. LDH substrate was prepared as per manufacturer's protocol (substrate plus catalyst). In each experiment control wells were included that contained media only. An equal volume of the newly made substrate was added to each well (15 - 20µl) and allowed to incubate at room temperature in the dark for 10 - 15 minutes or until 100% lysis control wells exhibited a prominent colour change. Subsequently an equal volume of stopping solution was added to each well (15 - 20µl 1M HCl). The plate was immediately read by spectrophotometer and absorbance measured at 490 nm and 650 nm. For analysis, the absorbance values at 650 nm were subtracted from the absorbance values at 490 nm to normalise background. In addition, absorbance values were

normalised to the media only wells. Values were expressed as a percentage of the 100% lysis control average (% LDH release to control).

2.8 IL-1β Enzyme-Linked Immunosorbent Assay (ELISA)

To measure IL-1β release, IL-1β ELISA detection kit (Invitrogen) was used. ELISA plates (high-affinity binding plate, Greiner) were treated with IL-1β capture antibody (in coating buffer, as per manufacturer's instructions, Invitrogen) at 4°C for a minimum of 16hrs prior to experiment. Antibody was discarded and the wells washed with 1xPBST (Phosphate buffered saline +0.05% Tween). Wells were then blocked for 1 hour at room temperature (ELISPOT diluent, Invitrogen). Blocking buffer was then removed and IL-1β standards (2000 pg/ml or 150 pg/ml starting concentration) added in duplicate, with a 2/3 serial dilution over 12 wells. 40 µl of supernatant was added to the ELISA plate with media alone as a control (note for macrophage and neutrophils supernatant was diluted to ensure measurements were kept in the range of the standard curve and 40 µl added). The ELISA plate was sealed and incubated at 4°C overnight. Samples were then discarded and plate washed with 1xPBST.

Detection antibody was added (in ELISA diluent, according to manufacturer's instructions) and incubated at room temperature for 1 hour. Following washing, the plate was incubated with avidin-HRP (according to manufacturer's instructions) for 30 minutes at room temperature. After a final wash 40 µl of TMB solution (HRP substrate) was added and allowed to develop at room temperature until 2/3 of the standards exhibited a colour change. 20 µl of stop solution was then added (1M sulfuric acid) and absorbance measured at 450 nm. For analysis sample absorbance values were normalised to media only wells and standard curve generated in pg/ml to which all samples were compared and then multiplied by dilution factor.

2.9 Sample preparation and western blot analysis

Protein was concentrated from cell supernatant samples by methanol-chloroform precipitation. Methanol (100%) was added in a 1:1 ratio followed by chloroform in a 1:3 ratio to the supernatant/methanol mix. Samples were vortexed and centrifuged at 15,000g for 10 mins, r.t. The upper phase was discarded, 1.6x volume of methanol added, vortexed, centrifuged (15,000g 10 mins, r.t.), supernatant discarded, and the pellet allowed to air dry for 30 mins. Subsequently, the pellet was resuspended in 25µl of Nupage 1X SDS lysis buffer (2% SDS, 66mM Tris pH 7.6).

Cells were lysed directly in the wells of the cell culture plate with 25µl per well of boiling Nupage 1X SDS Lysis buffer and extracted with thorough scraping. Some samples were prepared by pooling lysates and supernatants together. This was performed by lysing cells in

preheated supernatant Nupage mix. Cell supernatant and lysate were frozen at -80°C for later use.

Prior to SDS-PAGE analysis, samples were heated to 95°C for 5 mins, vortexed and 15 µl loaded on 12% or 15% polyacrylamide gels for electrophoresis without quantification. Samples were run at 150V for 60 - 90 minutes. Following electrophoresis, samples were transferred to nitrocellulose membranes by semi-dry transfer (TransBlot turbo 7mins, 25V, 2.5A). Membranes were blocked in 5% milk in TBST for 30mins on a shaker and stained with primary antibody in 5% milk in TBST plus 0.2% sodium azide overnight on a roller. Membranes were subsequently washed three times in TBST, shaking, and stained with appropriate HRP-conjugated secondary antibody for 2 hours, r.t., on an orbital shaker. Following further washing steps with TBST, membranes were treated with HRP substrate and developed (ChemiDoc). Loading control was assayed using mouse anti-Histone-3 antibody (Biolegend). Membranes were stripped with 30% hydrogen peroxide (37°C 30mins), washed, blocked (5% milk in TBST 5 mins on shaker), and stained with a new primary antibody as above.

2.10 RNA sequencing analysis

Raw RNA sequencing data was obtained from the GEO database. The data was filtered for genes relevant to the NCI and the mean expression values log transformed. Heat maps were generated in R Studio using the pheatmap() package. Clustering was automatically applied to the gene list.

2.11 Statistical analysis

All qPCR, LDH and ELISA statistical analysis and plots were generated using GraphPad Prism 9. Due to a low number of biological replicates non-parametric tests were conducted as data could not fit a normal distribution. For cell type comparisons unpaired Mann-Whitney tests were conducted and p-values recorded. For comparisons between treatments paired Wilcoxon signed rank tests were used and p-values recorded. Statistical analysis was displayed on plots by use of the following notation: test ns = not significant, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

3.0 Results

3.1 RNASequencing analysis reveals expression of inflammasome-related genes in lymphoid cells

It is clear from a wealth of literature that both canonical and non-canonical inflammasome pathways are operative and functionally relevant in macrophage lineages in both mouse and human. Recent work from our lab has focussed on the role of the non-canonical inflammasome in these cell populations and we now wanted to investigate the presence or absence of this pathway in unconventional lymphoid cells. Initial analyses therefore focussed on investigating which cell types could express components of the inflammasome pathway. Publicly available RNAsequencing (RNAseq) data from the gene expression omnibus were used (137–139). Data sets were chosen that involved LPS treatment or bacterial infection to relate to the NCI. The data sets were then filtered for inflammasome-related genes and heat maps were generated (**Figure 5**).

Figure 5A shows the effect of LPS priming (PAMP), compared to the oxidised lipid PGPC (DAMP, binds Caspase 11 directly, released from dying cells inducing IL-1beta secretion), and Alum (not a classical PAMP or DAMP), in bone marrow cultured murine dendritic cell populations (DC1s and DC2s). Here it is shown that for both DC1 and DC2 a number of inflammasome-related genes are upregulated, including GBPs, Interferon gamma receptor, GSDMD and multiple IRFs, in response to LPS and PGPC. Co-treatment with LPS and PGPC induced a similar inflammasome gene expression profile and, for the DC1 subset, also further exacerbated the increase in gene expression of some inflammasome related genes. Thus, the data suggest that cell types related to macrophages, with the ability to directly recognise PAMPs and DAMPs, can upregulate the inflammasome following exposure to LPS.

In a subsequent RNAsequencing dataset (137) samples were taken from before and after treatment of LPS intravenously in mice, where cells were sorted from the spleen at 3, 18, and 72 hrs post-treatment (**Figure 5B**). After 3 hours several inflammasome-related genes were upregulated as expected in macrophage populations, including *Casp4* (gene encoding murine *Caspase-11*), *Gbp2,* and *Gbp4* as well as some interferon regulatory factors (IRFs) such as *Irf1* and *Irf7.* This upregulation is reduced considerably, close to that seen at 0 hrs, at the 18 and 72 hr timepoints. LPS is a known inflammasome priming signal that has been shown to upregulate key inflammasome genes such as *Nlrp3* and *Il1b* via NF-κB as well as activating the NLRP3 inflammasome-independent of gene upregulation in macrophages (19). Interestingly, this pattern of inflammasome gene upregulation is also mirrored in the unconventional lymphoid populations (NK, iNKT, and γδT cell) displayed in the **Figure 5B**,

with upregulation being maximal also at the 3 hr timepoint. For example, *Casp4* and *Gbp2*, as well as other *Gbp* genes (*Gbp3, 4, 7, 8,* and *9*) are upregulated. This suggests the potential for an active non-canonical inflammasome pathway in these unconventional lymphoid cells, similar to the pathway activated in macrophage and DC populations. Additionally, it seems that LPS could be a suitable priming agent for further *in vitro* experiments to test inflammasome activation in lymphoid cells.

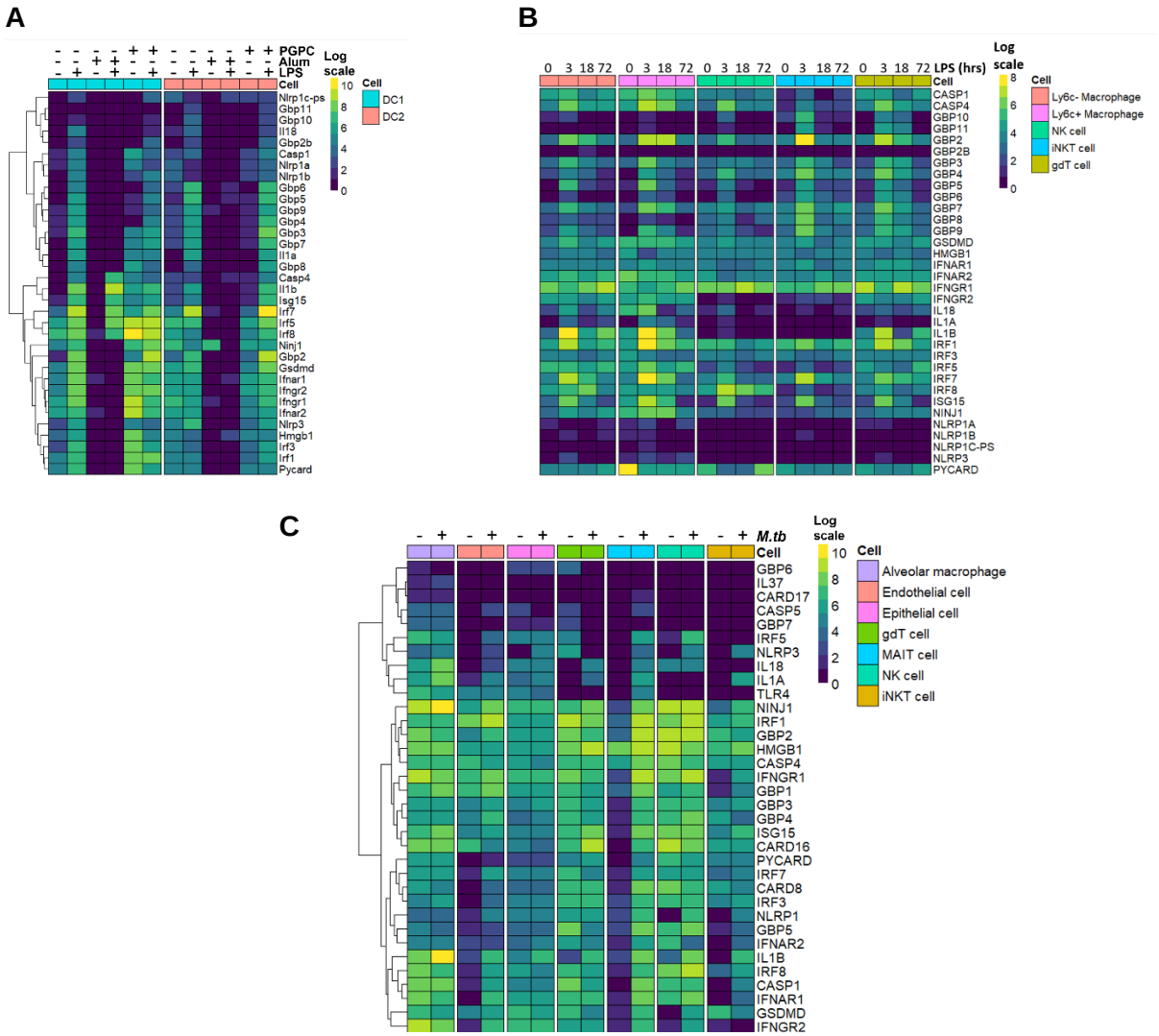


Figure 5. LPS treatment and intracellular bacterial infection upregulate inflammasome related gene expression in myeloid and lymphoid cell populations. **A** Data generated from GSE156159 (138). Dendritic cells differentiated from mouse bone marrow treated with LPS, PGPC and/or alum in vitro for high throughput RNA-sequencing. **B** Ex-vivo global transcriptomic RNA-seq analysis from GSE143943 (137). WT naive mice with intravenous injection (i.v.) of LPS. Cells sorted and lysate processed 0, 3, 18 and 72 hrs post i.v. **C** Data generated from GSE112483 (139). Lung tissue explants from human donors were infected with M. Tuberculosis by submerging in bacterial suspension for 30mins. Subsequently processed for cell sorting 20-24hrs post infection and RNA extraction performed for RNA-sequencing analysis.

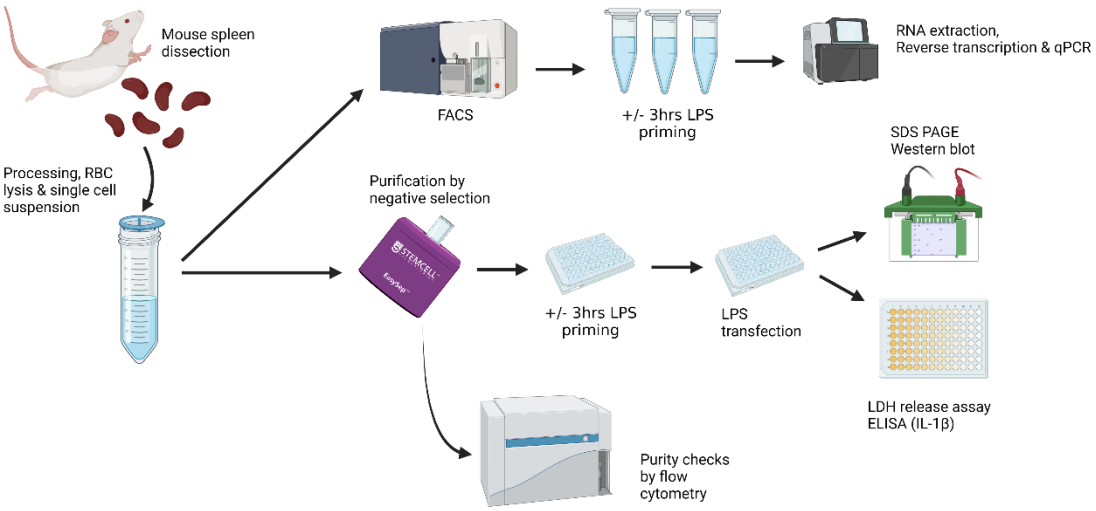
Additionally, it was also vital to establish whether lymphoid cell populations could upregulate inflammasome genes in humans, and in the event of an intracellular pathogen infection. Thus, a further RNASeq dataset was analysed (139) where human lung explant tissue was exposed to *Mycobacterium tuberculosis* infection. The heatmap in **Figure 5C** shows the expression of inflammasome-related genes before and after *M. tuberculosis* treatment in a number of different lung cell types, including macrophages, non-haematopoietic cells, iNKT cells and NK cells. Here again, macrophages show expression of non-canonical inflammasome genes, with upregulation of *NINJ1, IL1B,* and *GBP1* after infection. This pattern is broadly repeated in NK cells and γδT cells, with gene expression overall being lower for this gene set in human iNKT cells. This indicates a similar expression of the non-canonical pathway in at least some human lymphoid populations, as our data show in mouse populations. Here, NK cells are of particular interest as they are part of the innate response, alongside macrophages and DCs.

Therefore, it was decided to focus on NK cells for all further experiments based on the RNASeq data, using macrophages (and neutrophils in human samples) as a positive control for non-canonical inflammasome activation. NK cells upregulated inflammasome genes consistently across all three datasets and have been linked to bacterial infection. This includes studies describing their role in clearing bacteria via perforin release and expelling intracellular bacteria via lytic granules (140,141). Furthermore, molecules such as NKp46 have been indicated as potential receptors for bacterial antigen and thus activate NK cell cytotoxic functions (105). To pursue our interest in inflammasome activation in this cell type, gene expression analyses were undertaken *in vitro* using purified NK cells from mouse and human tissue (spleen and peripheral blood respectively) by qPCR to assess whether conventional methods of inflammasome priming were effective. To assess activation of the non-canonical inflammasome in NK cells, functional assays were performed *in vitro* using LPS transfection as the activator which has been widely used previously in other cell types (54,68).

3.2 Mouse NK cells do not express a functional non-canonical inflammasome

To validate some of the findings relating to NK cells in the RNASeq analysis, mouse splenic NK cells were purified using two different methods as depicted in **Figure 6A**. WT Mouse spleens were dissected and immune cell populations purified by FACS or use of EasySep purification kits (STEMCELL Technologies) for qPCR or functional assays respectively. The decision to use EasySep kits for functional assays was taken to maximise cell numbers for the downstream experiments. Due to the unknown effectiveness of the kits at purifying these immune cell populations flow cytometry was performed on samples pre and post purification to ensure high purity was maintained (**Figure 6B**).

A



B

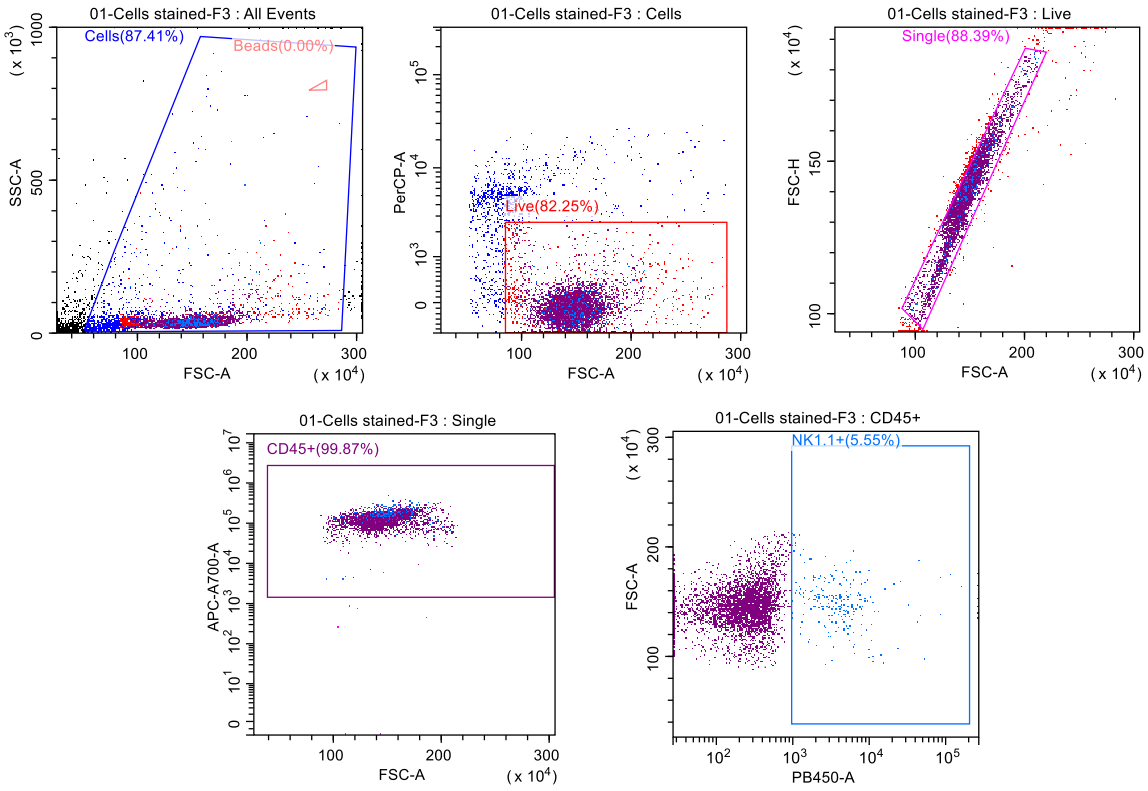


Figure 6. Experimental setup and flow cytometry gating strategy for mouse NK cell purity checks. A Workflow for mouse experiments **B** Example flow cytometry strategy for setting gates to assess NK cell purity post negative selection. APC-A700 =CD45, PB450 = NK1.1, PerCP = 7AAD (live/dead stain).

Results of the purity tests are displayed in **Figure 7** and ranged from 60-84% for NK cells. Percentages were based on the CD45+ gated cell population. Higher purity was expected from the kits, so this presented a challenge for further experiments. FACS purified macrophages and NK cells were used for RNA extraction and cDNA preparation. Splenic NK cells were sorted as $CD45^{+}lineage^{-}NK1.1^{+}$. As a control population where the non-canonical inflammasome is known to be active, we also sorted splenic macrophages as $CD45^{+}CD11b^{high}$. These samples were then subjected to qPCR analysis for known genes related to the NCI.

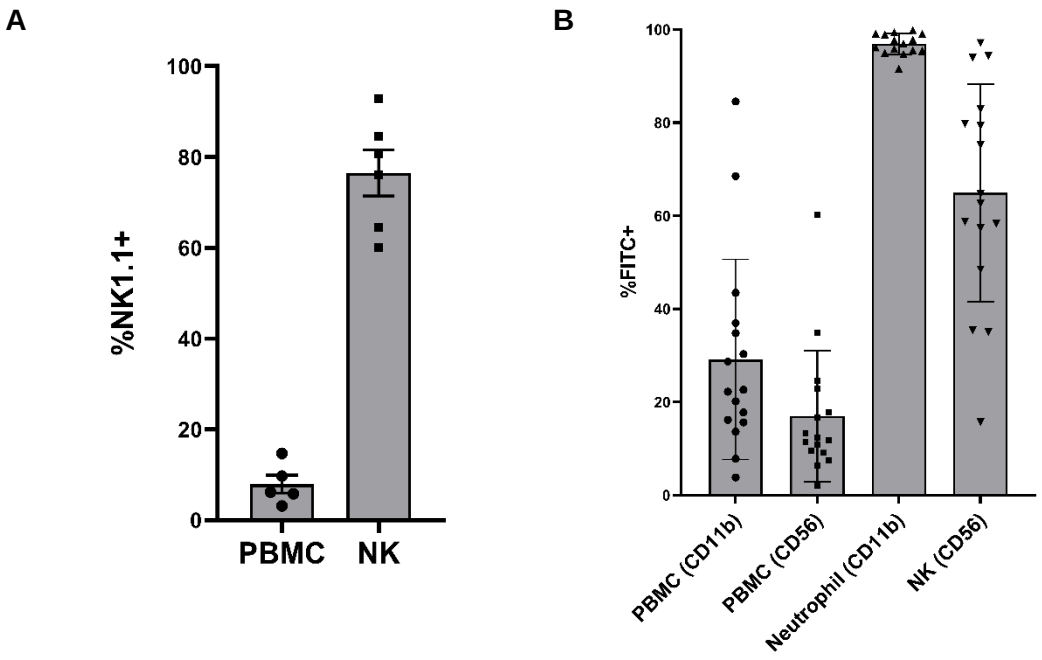


Figure 7. Flow cytometry purity checks show high variability in purity for EasySep purified human NK cells. A Purity of EasySep purified mouse splenic NK cells. Purity assessed based on NK1.1 (BV421) expression as a % of CD45+ cells n=5-6. PBMC fraction used as a pre-purification comparison. **B** Purity of EasySep purified human Neutrophils and NK cells from peripheral blood. Purity based on CD11b (FITC) or CD56 (FITC) expression as a % of CD45+ cells for Neutrophils and NK cells respectively. PBMC fraction used as a pre-purification comparison n=15-16.

qPCR experiments revealed that, in the absence of conventional inflammasome priming methods, NK cells ($NK1.1^{+}$) exhibited similar basal levels of *Casp1* and *Casp4* (gene encoding murine Caspase-11) compared to macrophages ($CD11b^{high}$) (**Figure 8A**). This was encouraging as caspase expression would be crucial for functional assays in these cells. Interestingly, NK cells showed higher basal levels of several GBP genes including *Gbp3, Gbp4* and *Gbp5* (**Figure 8B-C**). GBPs are a known intracellular LPS sensor and have been shown to be important in recruiting caspases to the bacterial cell surface for activation (17). Presence

of both GBPs and caspases in NK cells indicated a that NK cells may be able to form caspase-activating platforms in response to LPS. The surprisingly high level of GBP expression in NK cells compared to macrophages suggested a potential heightened ability to sense LPS in the cytosol and thus was a key finding in this experiment. Furthermore, other inflammasome-related genes were expressed with no significant differences to macrophages including *Gsdmd, Nlrp3* and *Ninj1* (**Figure 8D-E**). NLRP3 recruits the adaptor ASC to form the canonical inflammasome downstream of NCI activation (**Figure 3 and 4**) and therefore would be required for IL-1β processing and secretion. In addition, the presence of GSDMD and Ninj1 was interesting as both are required for pyroptotic cell death downstream of NCI activation. However, *Il1b* expression exhibited significantly lower expression compared to macrophages suggesting priming might be needed in this case (**Figure 8F**). Thus, splenic mouse NK cells seem to express many components of the NCI in an unprimed state. However, we decided to investigate gene expression post priming to examine whether inflammasome related genes could be further induced.

To determine which kind of priming would be effective, expression of cell surface receptors was also examined by qPCR with both *Tlr4* and *Ifngr1* manifesting similar or increased expression respectively compared to macrophages (**Figure 9A**). Therefore, the use of LPS or IFN-***γ*** as priming agents seemed appropriate. However, levels of *Cd14* were lower compared to macrophages in unprimed NK cells so not all LPS receptors were expressed to similar levels (**Figure 9B**). As priming NK cells to express inflammasome related genes has not been reported before, we decided to use LPS as it is known to upregulate inflammasome-related genes in other cell types. To assess the purity of the cells, NK cell markers were also included in the analysis including *Gzma* and *Prf1* which both demonstrated significantly increased expression compared to macrophages (**Figure 9C-D**). This was expected as the cells were FACS purified based on expression of NK1.1 which is a well-known marker of murine NK cells.

Other cytokine receptors were also included in the gene expression analysis including type-I interferon receptor and IL-18 receptor with the latter exhibiting high basal expression in murine NK cells (**Supplementary Figure 1**). Again, as NK cells are known to require accessory cytokines such as IL-18 during immune responses, this finding was to be expected. The proinflammatory cytokine TNFα was also included in qPCR experiments but showed low basal expression compared to macrophages (**Supplementary figure 1**). This increased the need to examine the effect of priming on expression of these genes to ensure that these NK cells could still elicit proinflammatory gene expression when exposed to TLR agonists.

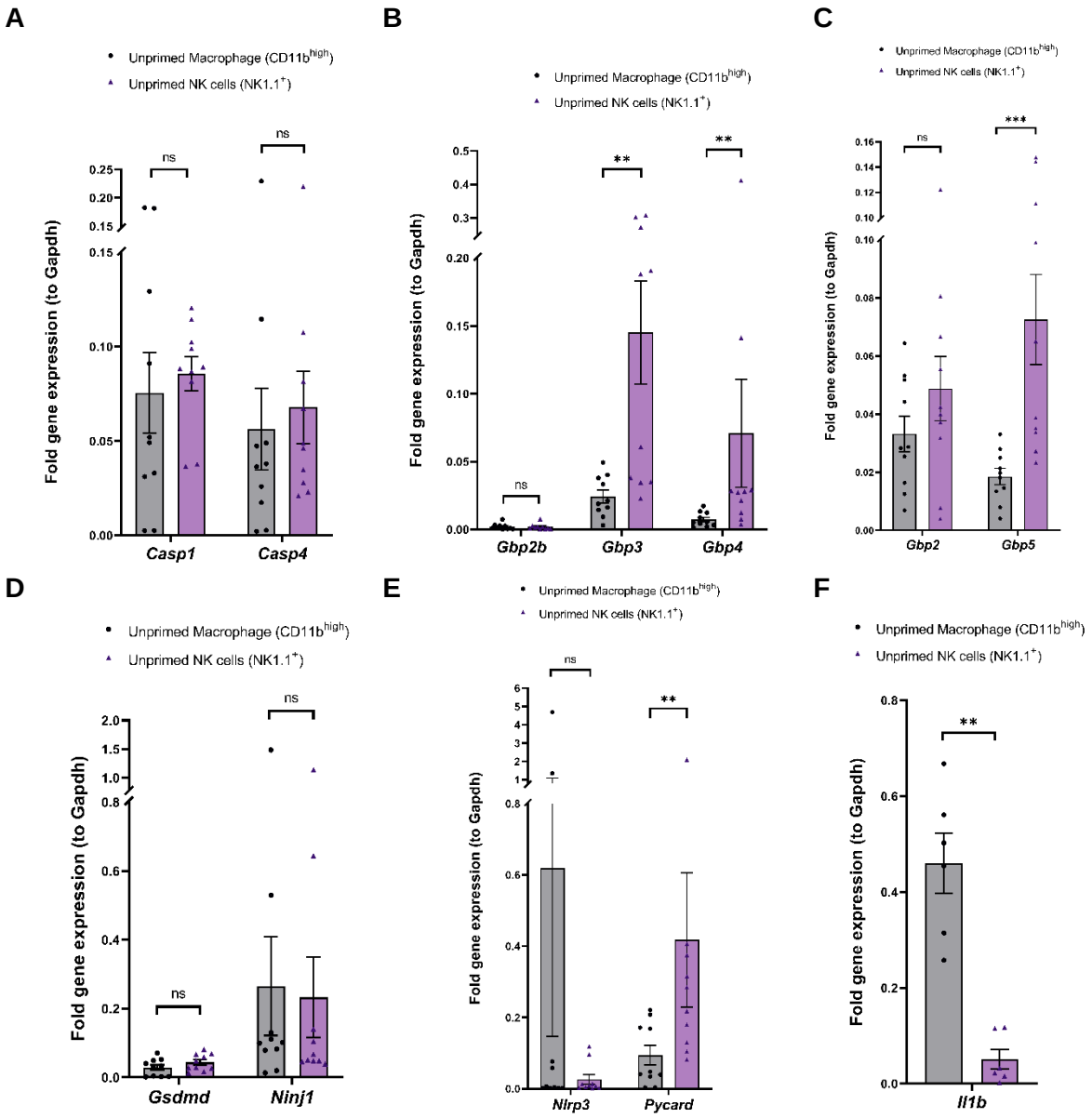


Figure 8. Unprimed murine NK cells express inflammasome related genes. A-E qPCR data of relative gene expression to *Gapdh* n=10. **F** qPCR data of *Il1b* relative gene expression to *Gapdh* n=5-6. Analysis by Mann Whitney test ns = not significant, * = p<0.05, ** = p<0.01, *** = p<0.001

It was decided to examine the effect of LPS priming on inflammasome related gene expression levels in murine NK cells. LPS seemed a sensible candidate as the cells expressed basal *Tlr4.* Furthermore, LPS is a known inflammasome priming agent and is a likely PAMP to be associated with intracellular bacterial infection in the context of NCI activation.

Macrophages and NK cells were primed for 3hrs with LPS prior to RNA extraction and reverse transcription. Analysis revealed that the primed had no effect on inflammasome related gene expression in NK cells with *Casp1, Casp4, Gbp2b, Gbp2, Gbp3, Gbp4, Gbp5, Pycard, Ninj1, Gsdmd* and *Nlrp3* exhibiting no significant change in expression with priming (**Figure 10D-I**). In addition, LPS priming had no significant effect on LPS receptor or NK cell marker expression with *Tlr4, Cd14, Gzma, Prf1* and *Tnf* levels remaining unchanged (**Figure 11**). This suggested that use of LPS as a priming agent was not effective in NK cells and that a different priming agent or altered timepoint for priming may be necessary. Macrophages were also included as a positive control in these experiments as they are known to upregulate inflammasome related genes in response to LPS. Surprisingly, macrophages also showed no change with LPS priming except for *Gbp2, Gbp3, Gbp4* and *Gbp5* (**Figure 10A-C** and **Supplementary Figure 2**). As such, repeated experiments with a longer LPS priming step or higher LPS concentration may be appropriate.

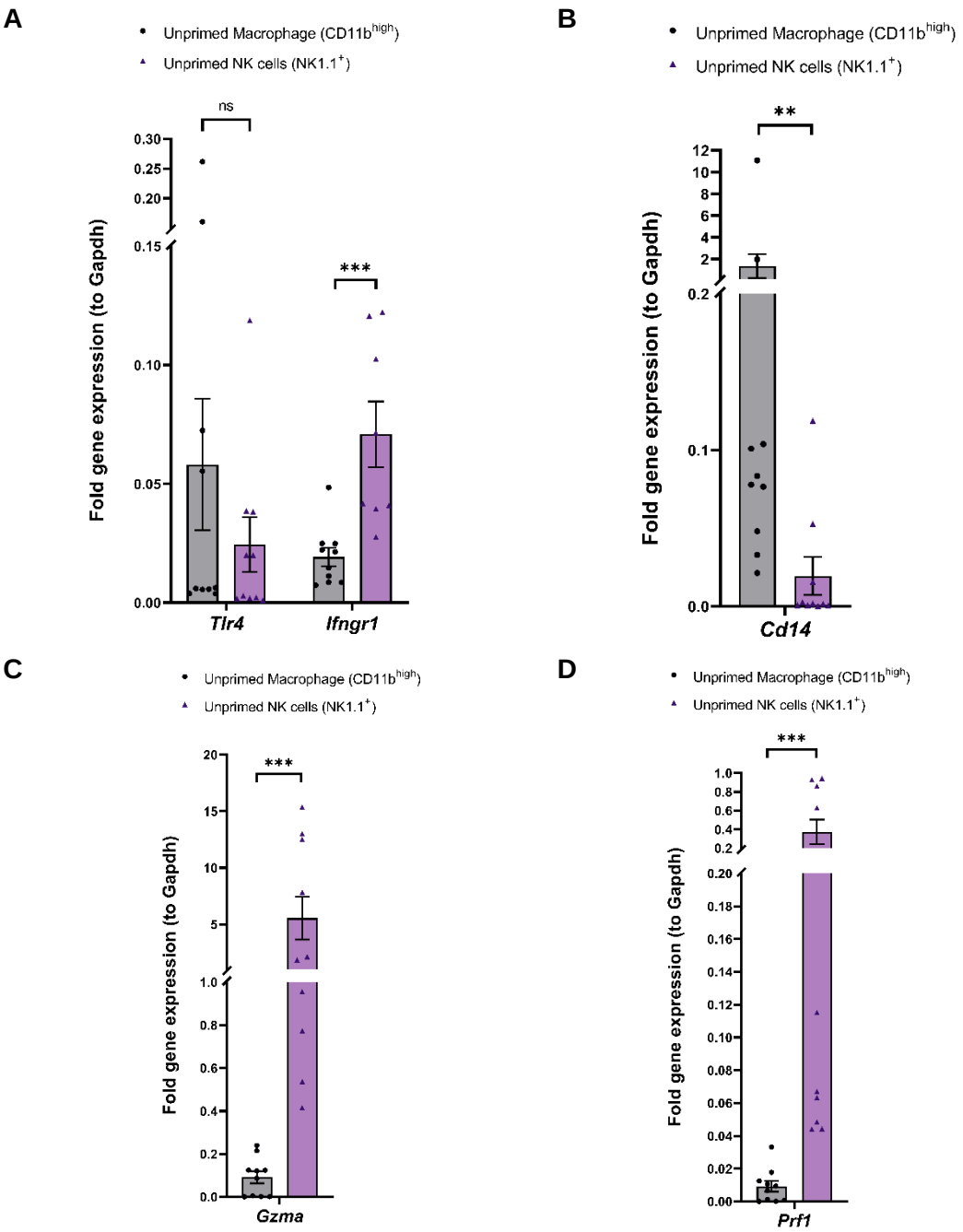


Figure 9. Murine NK cells express LPS and IFN-γ surface receptors. A-D qPCR data of relative gene expression to *Gapdh* n=8-10. Analysis by Mann Whitney test ns = not significant, * = p<0.05, ** = p<0.01, *** = p<0.001

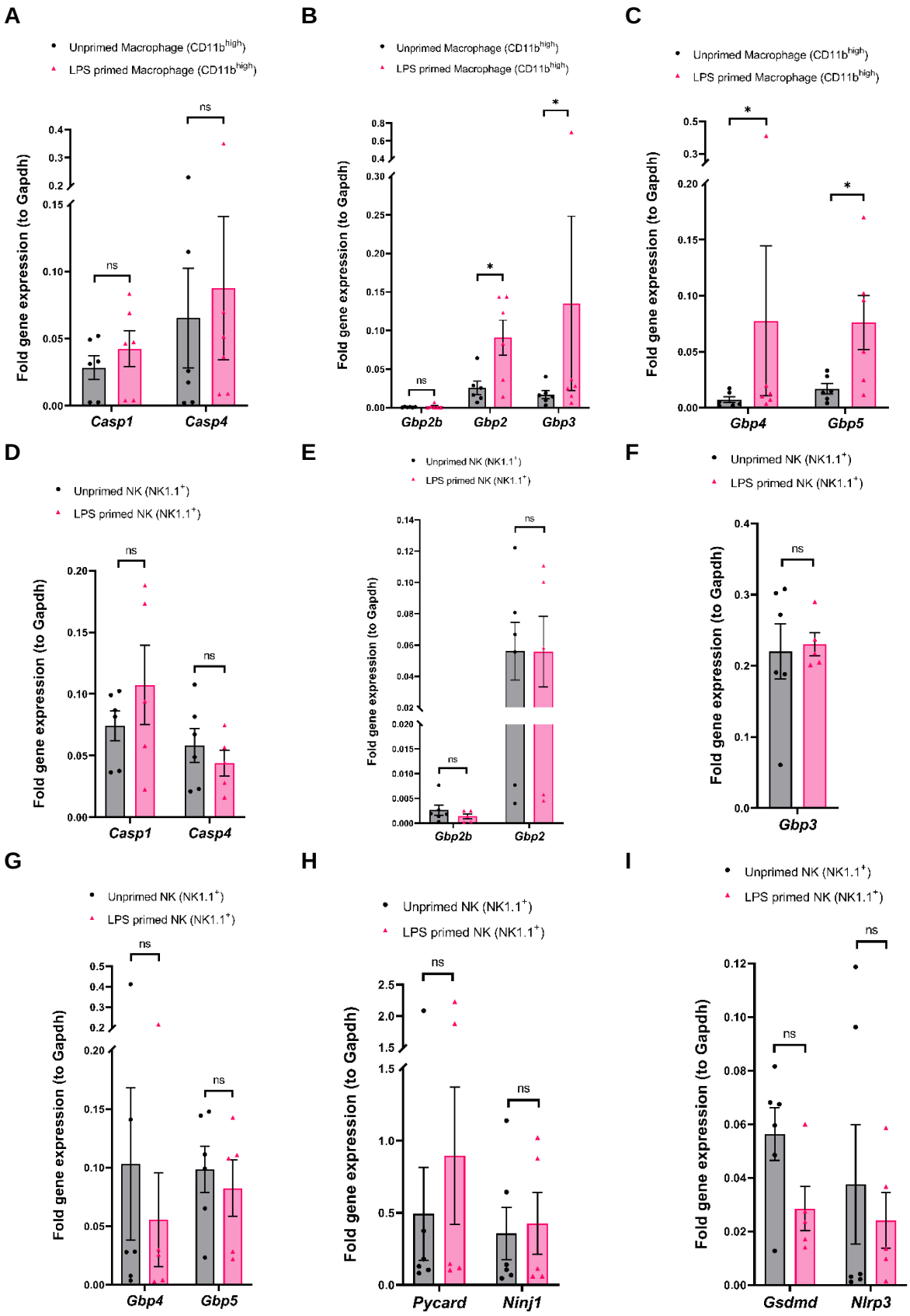


Figure 10: LPS priming has no effect on inflammasome related gene expression in murine NK cells. A-C Macrophage qPCR data of relative gene expression to *Gapdh* n=6. **D-I** NK qPCR data of relative gene expression to *Gapdh* n=5-6. Analysis by Wilcoxon signed rank test ns = not significant, * = p<0.05, ** = p<0.01, *** = p<0.001

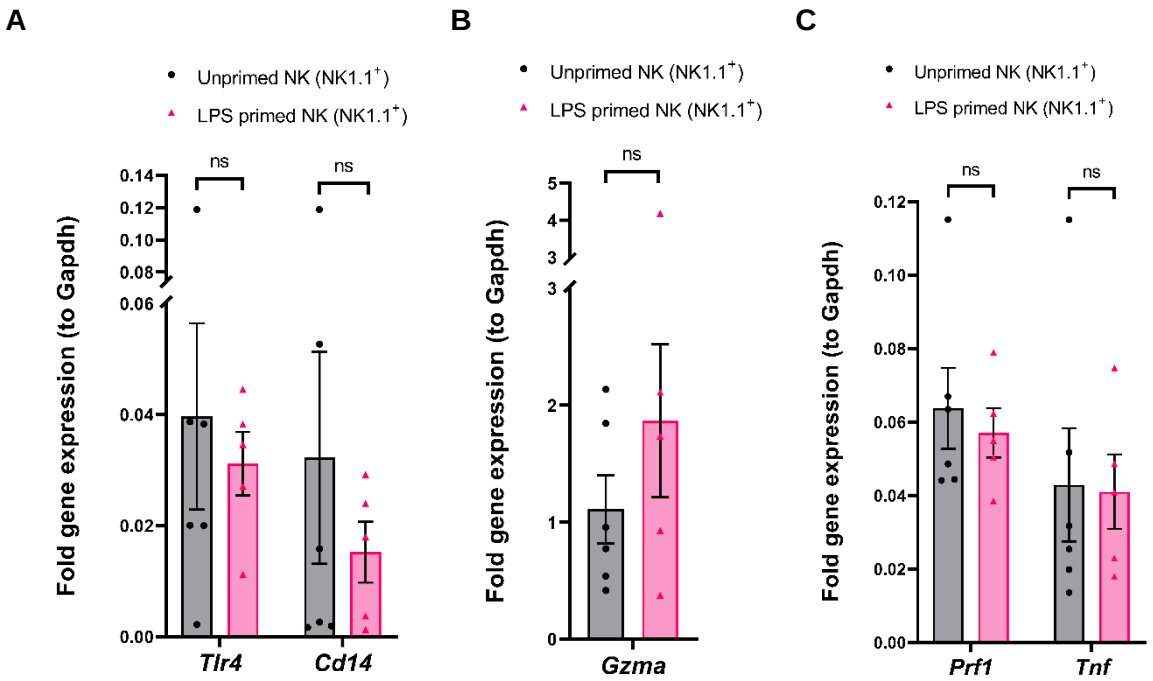


Figure 11. LPS priming has no effect on murine NK cell marker gene expression. A-C qPCR data of relative gene expression to *Gapdh* n=5-6. Analysis by Wilcoxon signed rank test. ns = not significant, * = p<0.05, ** = p<0.01, *** = p<0.001

Alongside gene expression analyses, functional assays were also performed to assess NCI activity in purified mouse splenic NK cells. Initially, cells were left unprimed and then subjected to 14 hours of LPS transfection with supernatant taken for LDH assay and IL-1**β** ELISA (**Figure 12A - F**). Cell lysate was also taken for western blot analysis (**Figure 12G**). LDH assays showed no significant difference in cell death upon LPS transfection in NK cells (**Figure 12B**). This was surprising given the findings by qPCR that NK cells expressed genes for the pore forming protein GSDMD and membrane rupture regulator Ninj1. Similarly, there was no increase in IL-1**β** secretion (**Figure 12E**). Both findings suggested that the NCI could not be induced in murine NK cells. Macrophages did exhibit an increase in cell death upon 14hr LPS transfection (**Figure 12A**) but no increase in IL-1**β** secretion (**Figure 12D**). Macrophages have been shown to release IL-1**β** secretion. As there was a high basal level of death after 14hrs in untreated cells it was decided to switch to an earlier time point (5hrs) and seed NK cells in different media designed to support NK cell growth (X-VIVO15). The rationale was that these changes would reduce the basal death observed and maintain NK cell viability. Cells were primed for 3hrs with LPS and then subjected to 5hrs of LPS transfection. However, a similar phenotype was observed with no change in cell death between treatments for NK cells and negligible levels of IL-1**β** secretion (**Figure 12C and F**). The 5 hr transfection time

point did alleviate the high basal death seen previously but no changes were observed upon LPS transfection.

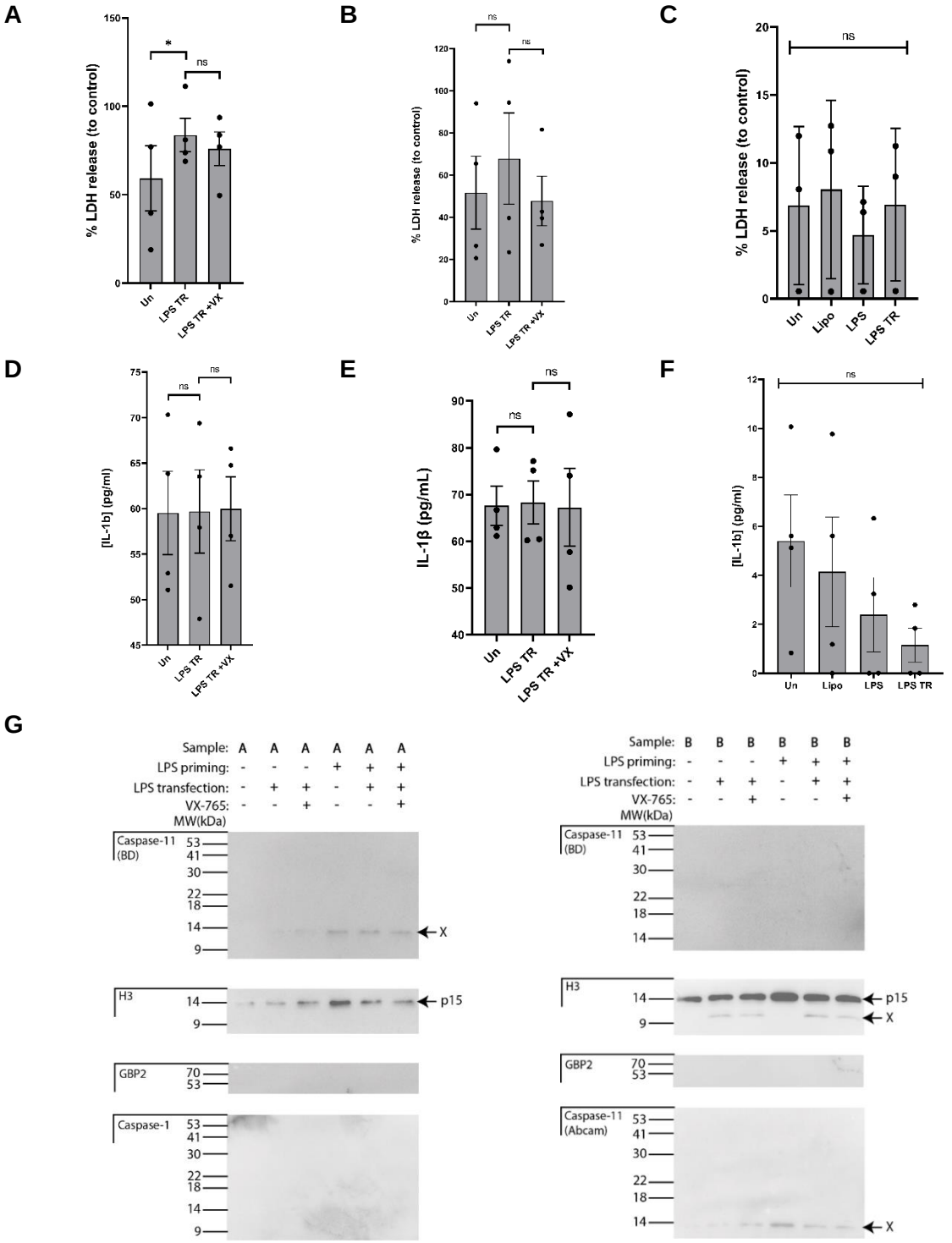


Figure 12. Non-canonical inflammasome is not functional in murine NK cells due to suspected inactive Caspase-11 isoform. Analysis by Wilcoxon signed rank test, ns = not significant, * = p<0.05, ** = p<0.01, *** = p<0.001 **A** LDH assay from FACS purified WT mouse macrophage 14hr treatments n=4. **B** LDH assay from FACS purified WT mouse NK cells 14hr treatments n=4. **C** LDH assay from EasySep purified WT mouse NK cells 3hrs LPS priming and

5hrs treatments n=3. **D** IL-1β ELISA from FACS purified WT mouse macrophage 14hr treatments n=4. **E** IL-1β ELISA from FACS purified WT mouse NK cells 14hr treatments n=4. **F** IL-1β ELISA from EasySep purified WT mouse NK cells 3hrs LPS priming and 5hrs treatments n=3. **G** Western blot with cell lysates from EasySep purified Fgd5+ mouse NK cells +/- 3hrs LPS priming. Treatments were run for 5hrs prior to cell lysate extraction. Stained with antibodies against Caspase-11, GBP2 and Caspase-1 (H3 as a loading control). X = Unexpected band. LPS TR = LPS transfection. VX = VX-765 caspase inhibitor. Lipo = Lipofectamine only control.

We hypothesised that a different priming agent may be required to elicit inflammasome activation in NK cells. Type-I interferon and IL-12 released by other cell types including dendritic cells have been shown to be important for NK cell activation and cytotoxicity. As such, mice deficient in type-I interferon receptors have impaired functionality (142,143). Furthermore, type-I interferon is a known inflammasome priming agent in mice that upregulates *Casp11* and *GSDMD* (8). Murine NK cells were primed with IFN-**β** for 3hrs prior to LPS transfection (5hrs) instead of LPS priming. However, the same phenotype was observed with no change in LDH release or IL-1**β** secretion after LPS transfection (**Supplementary Figure 3**). Collectively, the data suggested that the NCI was not present in an active state despite appropriate gene expression observed by qPCR.

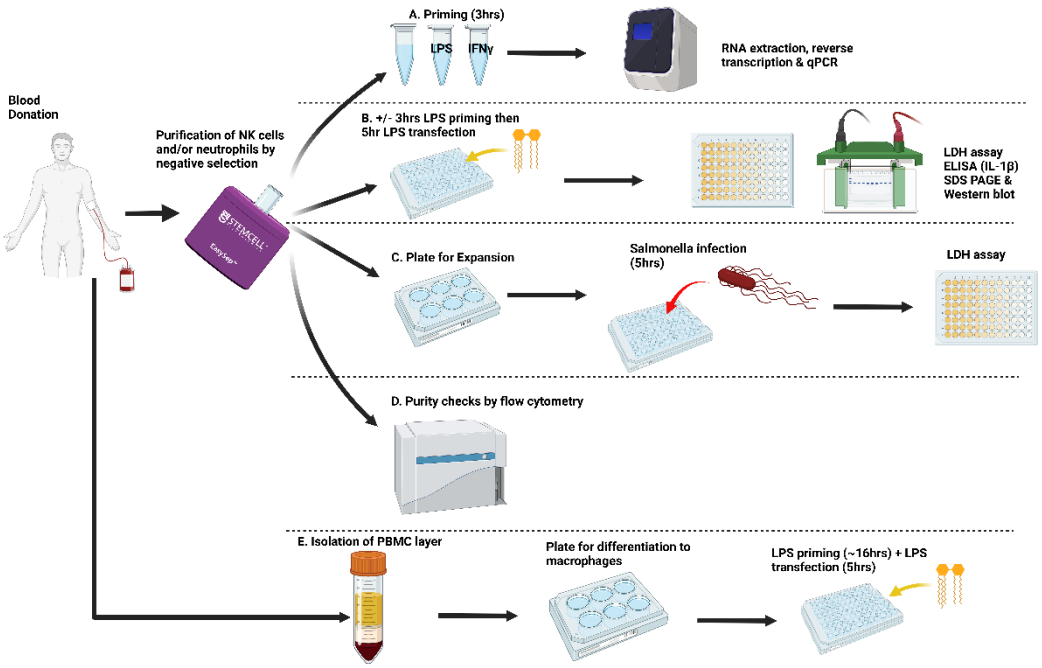
To further understand this, western blots were undertaken to see if components of the pathway could be activated such as Caspase-11, which requires self-cleavage to a p32/p10 fragment to become catalytically active. As seen in **Figure 12G**, the full length caspase-11 could not be detected (43kDa). However, a protein was observed with a low molecular weight. This was observed twice with two different antibodies against Caspase-11. Interestingly, the protein was more apparent after LPS priming. However, GBP proteins could not be detected suggesting the platform for Caspase-11 activation on cytosolic LPS cannot form.

3.3 Human NK cells shows activation of the non-canonical inflammasome, but with varying responses to LPS transfection and *Salmonella* infection

Alongside experiments into mouse splenic NK cells, peripheral blood was taken from healthy human donors. A similar experimental setup was utilised to the mouse experiments to see if any species differences could be observed. However, all qPCR experiments used samples purified using the EasySep column purification method (**Figure 13A**). Again, purity checks were performed by flow cytometry (**Figure 13B**). Surprisingly, there was far more variability in the purity of the human NK cell collections compared to mice with purity ranging from 35-97% (%CD56+) for humans. However, neutrophil purity remained consistently high in human samples, ranging from 94-99%(%CD11b+) (**Figure 7**). This led us to believe that a different marker may be needed to assess human NK cell purity by flow cytometry.

qPCR experiments were undertaken regardless, revealing that human NK cells express similar levels of inflammasome related genes to neutrophils (known to have active NCI) including *CASP1, CASP4, GBP1, GBP2* and *GSDMD* (**Figure 14**). However, NK cells had reduced expression of *NLRP3, PYCARD* and *CASP5* compared to neutrophils. Crucially, NK cells showed expression of *GZMA* (NK cell marker) and a lack of *TLR4* expression indicating that they cannot be primed or respond to extracellular LPS. Therefore, we hypothesised that LPS recognition could only take place intracellularly.

A



B

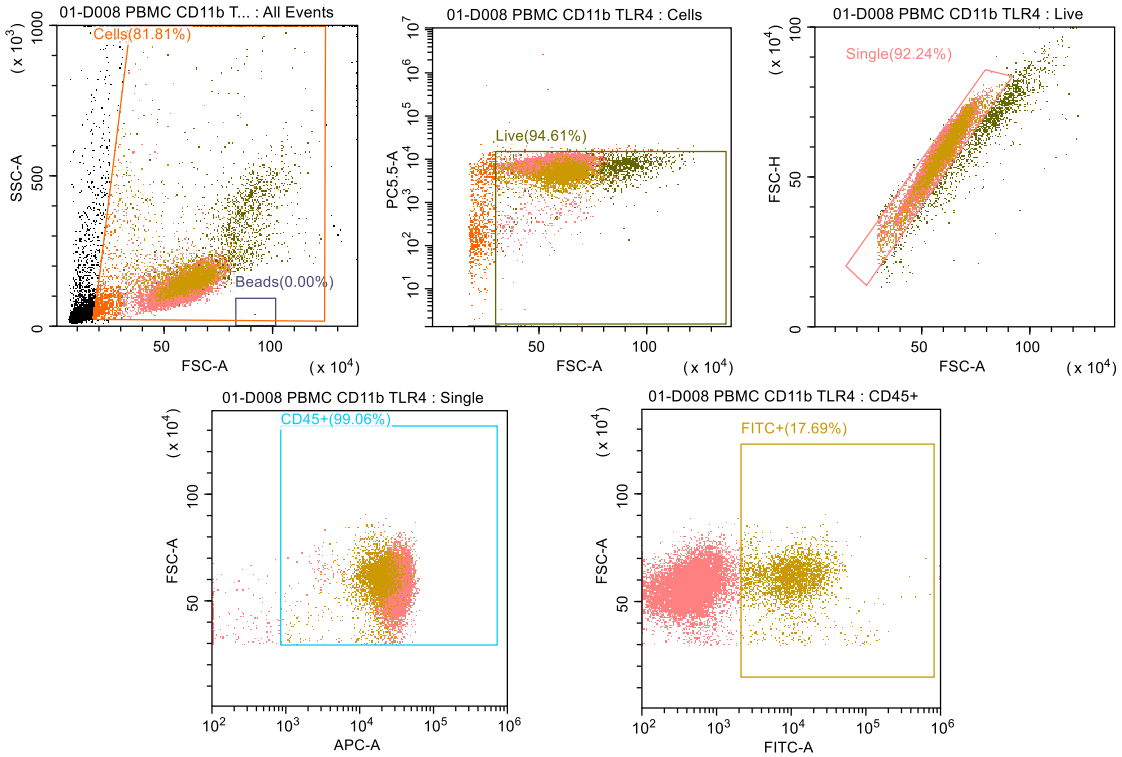


Figure 13. Human experimental setup and flow cytometry gating strategy for human NK cells and Neutrophils. A Human experiment workflow. **B** Example flow gating strategy for purity checks post negative selection. CD11b FITC and CD56 FITC used as markers of Neutrophils and NK cells respectively.

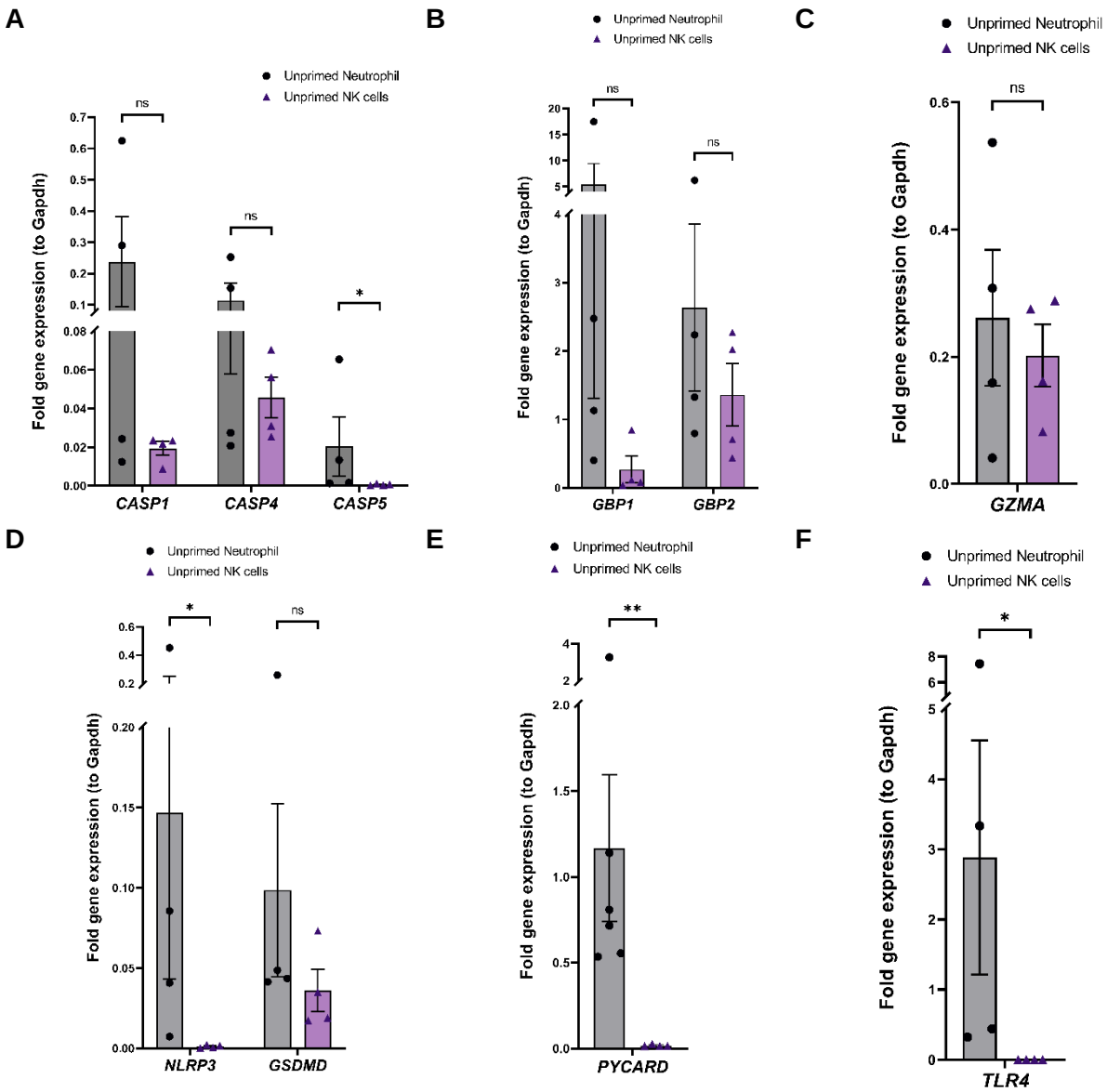


Figure 14. Human NK cells express inflammasome related genes. A-F qPCR data of relative gene expression to *GAPDH* n=4-6. Analysis by Mann Whitney test ns = not significant, * = p<0.05, ** = p<0.01, *** = p<0.001

For priming experiments, it was decided to include LPS priming as a negative control (for NK cells) and use IFN-**γ** instead as the main priming agent. IFN-**γ** has been used in other studies to prime the NCI and was demonstrated to be important in host defence against *Salmonella* infection (16). However, no changes in Caspase or GBP gene expression were observed (*CASP1, CASP4, GBP1, GBP2)* in human neutrophils or NK cells upon IFN-**γ** priming (**Figure 15**). However, this could be due to a low sample number (n=4) or an insufficient length of priming (3hrs). Similarly, other inflammasome related genes (*NLRP3, GSDMD, CASP5, PYCARD)* as well as *TLR4* and *GZMA* were unaffected by IFN-**γ** priming in both cell types (**Supplementary Figure 4 and 5**).

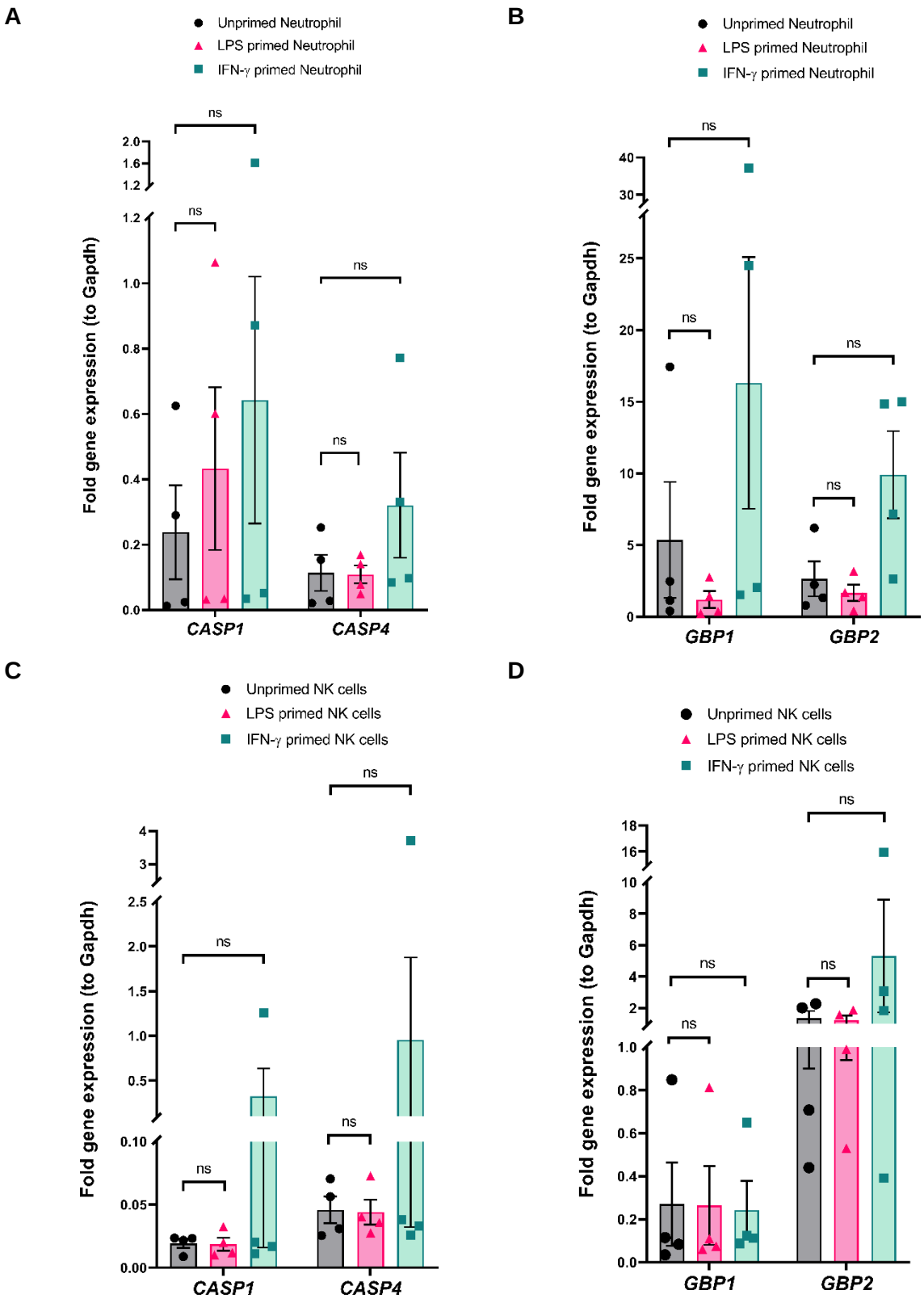


Figure 15. LPS and IFN-γ priming do not increase Caspase or GBP expression in human NK cells. A-B qPCR data of relative gene expression to *GAPDH* in human neutrophils n=4. **C-D** qPCR data of relative gene expression to *GAPDH* in human NK cells n=4. Analysis by Wilcoxon signed rank test. ns = not significant, * = p<0.05, ** = p<0.01, *** = p<0.001

Supernatant was taken from human peripheral blood NK cells transfected with LPS for LDH assay and IL-1**β** ELISA to assess NCI activity (3hrs LPS priming followed by 5hrs LPS transfection). No change in LDH levels could be detected upon LPS transfection indicating that pyroptosis is not induced in human NK cells (**Figure 16A**). However, secretion of IL-1**β** was significantly increased with LPS transfection in NK cells suggesting that pro-IL-1**β** had been cleaved by active caspases (**Figure 16B**). It was decided to include macrophages as a control cell type for these experiments which were differentiated from PBMCs. Macrophages exhibited an observational increase in LDH release and IL-1**β** secretion as expected, however more replicates are needed for this observation to be statistically significant (**Supplementary Figure 6**). The increase in IL-1**β** secretion in NK cells in response to LPS transfection were verified by western blot.

Western blots with NK cell lysates were able to detect full length Caspase-1 and Caspase-4 in human NK cells as well as GBP1 and GSDMD (**Figure 16C and D**) which could not be observed in murine NK cells. In addition, GSDMD cleaved fragments could also be observed with multiple human donors. However, cleaved GSDMD was present in wells without LPS transfection therefore the effect cannot be attributed to NCI activation. Crucially, no TLR4 protein could be detected by western blot confirming the previous findings by qPCR that human NK cells lack the extracellular LPS receptor.

For some donors Caspase-4 cleavage could also be observed in cell supernatant (**Supplementary Figure 7**) however this finding could not be repeated. Donor variability had a significant impact throughout the studies. Interestingly, Caspase-5 could not be detected by western blot even by repeating with another different primary antibody.

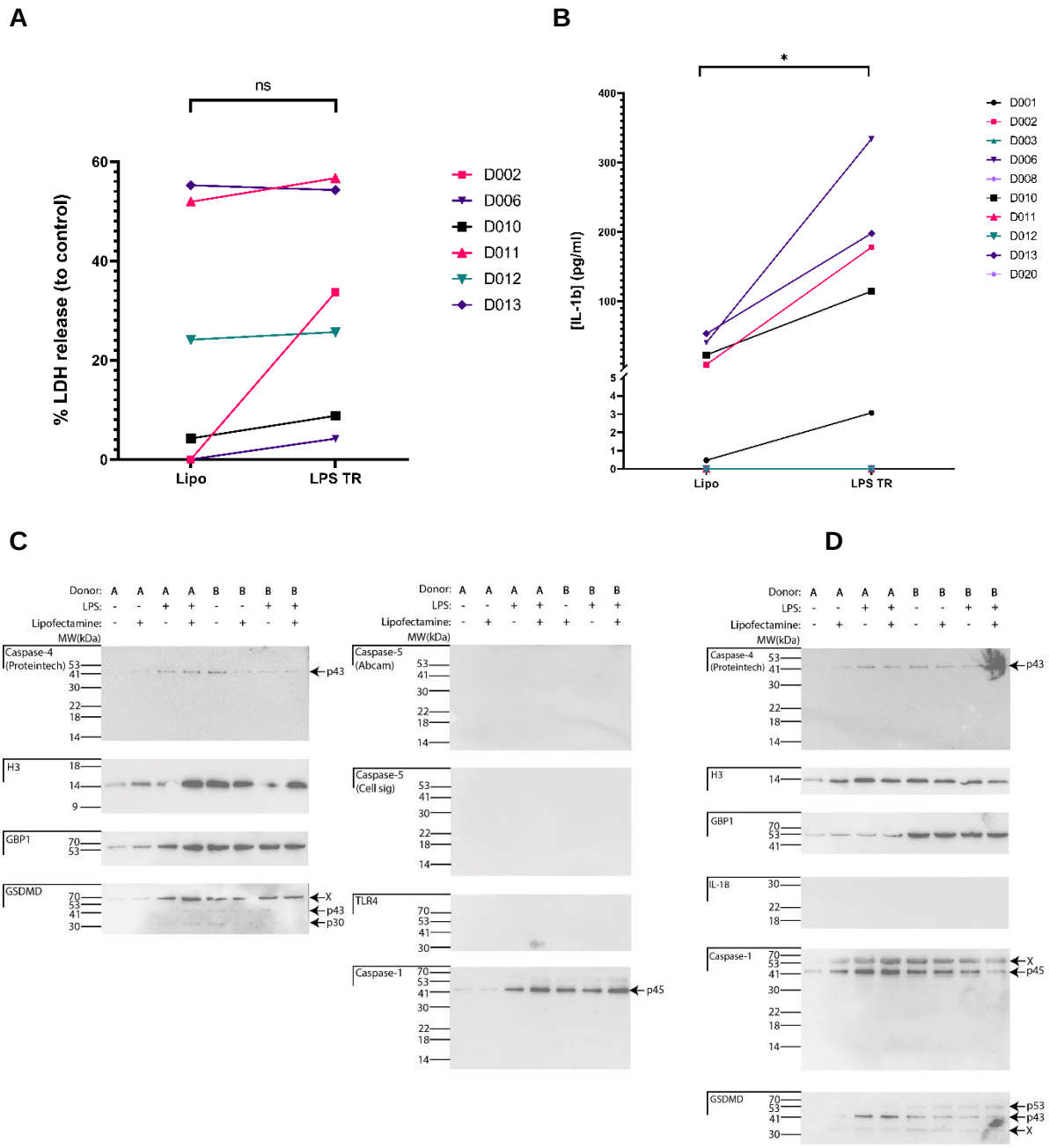


Figure 16. Inflammasome pathway partially active in human NK cells with LPS transfection. **A** LDH assay with Triton as 100% positive control. Human NK cells with 3hrs LPS priming followed by 5hrs of treatment (n=6). **B** IL-1β ELISA. Human NK cells with 3hrs LPS priming followed by 5hrs of treatment (n=10). **C** Western blot with human NK cell lysate from 2 donors. Cells were unprimed followed by 5hrs treatment. LPS transfection was performed for wells with Lipofectamine and LPS (++). **D** Same as **C** but with 2 different human donors. Statistical analysis for LDH and ELISA by Wilcoxon signed rank test ns = not significant, * = p<0.05, ** = p<0.01, *** = p<0.001

To relate the research more towards bacterial infection, experiments were conducted using *Salmonella* instead of LPS transfection. An increase in LDH release was observed in *Salmonella* infection of NK cells compared to untreated cells (**Figure 17**). However, more biological replicates are required to increase the statistical power for this finding.

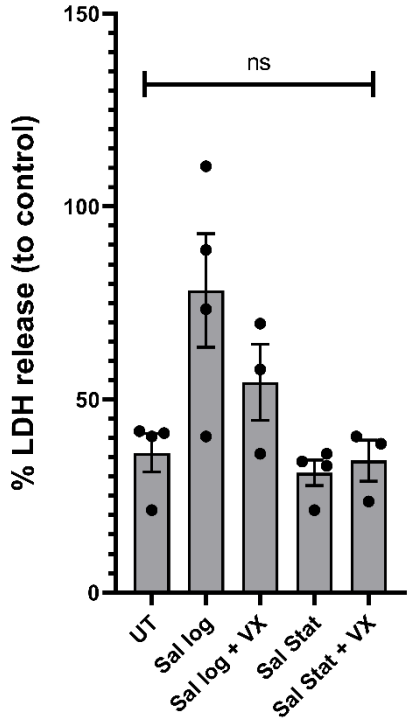


Figure 17. Human NK cells die by pyroptosis in response to log-phase Salmonella infection. Unprimed expanded human NK cells with 5hr Salmonella infection (n=4). Analysis by Wilcoxon signed rank test ns = not significant, * = p<0.05, ** = p<0.01, *** = p<0.001

4. Discussion

The above research provides an insight into NCI function in NK cells. Although still preliminary, the results suggest unexpected functions for the NCI in these cells. Gene expression of inflammasome related genes was observed in both mouse and human NK cells (**Figure 8 and 14**). However, functional activity was restricted to human NK cells characterised by IL-1β production (**Figure 16**). The identification of a possible caspase-11 isoform provided an explanation for the lack of response seen in mouse NK cells (**Figure 12**) and identified a novel level of regulation. Interestingly, we fail to detect pyroptosis, suggesting that human NK cells may avoid it like neutrophils and dendritic cells which utilise ESCRT machinery for membrane repair. Thus, the results provide evidence for a potential species-specific induction of the NCI in NK cells.

The work brought with it many challenges. As cells were isolated from fresh blood, the efficiency of the purification used was critical to allow the removal of other cell types expressing inflammasomes (e.g. neutrophils, monocytes). Central to this was the necessity to examine the purity of the cells used for the research. CD56 FITC was chosen as the human NK cell marker for the flow cytometry purity checks. It is widely reported that increasing CD56 expression is a marker of human NK cell maturation from IFN-γ producing to cytotoxic function (144–146). As such, post-purification high CD56 expression was expected. However, during the flow cytometry purity checks there was only a subtle shift observed post purification compared to the use of NK1.1 BV421 for murine NK cells or CD11b FITC for neutrophils (**Supplementary Figure 8**). Considering that good shifts were observed for the murine NK cells, it is unlikely that the human purification kit would be more ineffective at purifying NK cells. As such, another marker of NK cells may be appropriate for purity checks such as NKp80 or NKp46 with the former reported to identify $CD56^{neg}$ NK cell populations (147–149). CD56 is described as being ‘bright’ or ‘dim’ for different NK cell subsets. In addition, Vivier and colleagues suggested that 90% of human blood NK cells are $CD56^{dim}$ which may explain the low shift observed in **Supplementary Figure 8** and subsequent low numbers for purity post purification (**Figure 7**) (101). Further optimisation of the CD56 antibody may also be required to assess different concentrations of staining prior to analysis. Or indeed switching to another fluorophore for CD56 may be appropriate.

During human flow cytometry purity checks, antibodies were used against TLR4 (data not shown), which seemed to confirm our previous findings by qPCR (**Figure 14** and **Supplementary Figure 5**) and western blot (**Figure 16C**) that human NK cells express negligible amounts of TLR4. Other studies suggest that NK cells express very low levels of

TLR4 but that upon stimulation with accessory cytokines mouse and human NK cells can respond to TLR4 agonist FimH characterised by increased cytotoxicity and cytokine production (124,150). As such the use of accessory cytokines for the above experiments would be useful to properly assess TLR4 expression in purified NK cells.

Throughout the experiments, donor to donor variability was a common feature. The need to gather data from many human donors is paramount in order to observe any significant trends. Variability could be impacted by the age, genetic variability and lifestyle of the donor and affect the numbers of purified immune cells from blood which impacted the scope of the experiments. To this end, NK cells were expanded using Immunocult media (STEMCELL technologies) to try and increase NK cell numbers for functional assays. Although this protocol allows for cellular amplification, we did not compare with unexpanded cells to confirm that features of interest were not impacted or changed during the expansion. Although allowing us to use more cells, this approach still had limitations and did not allow us to genetically investigate the function of NCI genes. Attempts to perform siRNA in these primary cells were unsuccessful (data not shown). In the future it would be useful to use iPSC-derived NK cells or an NK cell-like cell line to increase the cell numbers and give greater scope to the experiments. It has been reported that NK cells derived from iPSCs exhibit functional cytotoxicity and IFN-γ production so provide a promising avenue for future work (151). CRISPR-Cas9 technology could be used to investigate caspase-4 roles in iPSC-derived NK cells. Furthermore, this would help to reduce donor to donor variability seen throughout experiments. **Supplementary Figures 4&5 and Figure 15** displayed high variation between donors for IFN-γ primed neutrophils and NK cells. A couple of donors consistently exhibited high gene expression across all genes which was inconsistent with other donors. Therefore, a greater sample size is required for these qPCR experiments to distinguish any anomalous data.

Comparisons have been made in the experiments between mouse splenic NK cells and human peripheral blood NK cells but the composition of NK cells in the spleen can differ significantly from blood in terms of the type of NK cells present (152). Single cell RNA sequencing studies have shown that NK-cell specific genes differ between mouse and human and that NK cell subsets differ between spleen and blood, with more active NK cells resident in the spleen rather than the blood for both mice and human (152). Therefore it would be interesting to investigate direct tissue comparisons between species for our experimental setup. However, access to human tissues for such investigations is restricted.

A key component of NCI activation in both species is the use of extracellular priming. The use of LPS and IFN-γ as priming agents failed to induce significant changes of inflammasome related gene expression in the qPCR experiments (**Figure 10, 11, 15 and Supplementary Figure 5**). In contrast to the mouse data, human qPCR data suggested a lack of TLR4 expression in NK cells which would explain the lack of responsiveness to LPS priming (**Figure 14**). As such it may be necessary to switch to another known inflammasome priming agent such as Pam3CSK4 or TNFα in an attempt to boost gene expression. Pam3CSK4 is a TLR2 agonist that has been shown to upregulate proinflammatory cytokine production including IL-1β and results in better antimicrobial responses in neutrophils against antibiotic resistance bacteria (153). A study by Yokose et al demonstrated that TNFα priming along with a secondary stimulus (monosodium urate) resulted in caspase-1 activation and IL-1β secretion in neutrophils (154). However, the lack of response seen by priming the above qPCR experiments may just be due to the length of priming. Priming for more than 3hrs with TLR agonists, IFN-β, IFN-γ or TNFα may be necessary to induce gene expression via NFκB and IRFs. Therefore simply changing the time point may be sufficient in this case. Furthermore, NK cell activation is known to rely on accessory cytokines such as IL-2, IL-15 and IL-18 secreted from neighbouring cells (such as dendritic cells), therefore it may be necessary to treat cells with a cocktail of these cytokines in concert with traditional inflammasome priming agents to induce a response at the transcription level (124,155).

IFN-β priming was also used as a priming agent in experiments with murine NK cells instead of LPS to try and induce inflammasome components, IL-1β secretion and increaseLDH release upon LPS transfection. However, priming with IFN-β did not induce this phenotype (**Supplementary Figure 3**). Autocrine IFN-β signalling downstream of TLR4 activation is reported to upregulate proinflammatory gene expression (26). However, the effect of IFN-β is ambiguous and studies have widely reported its immunomodulatory and anti-inflammatory effects including an inhibitory effect on IL-1β production and canonical inflammasome activation (156). Therefore it may not be effective at inducing inflammasome-related gene expression in murine NK cells.

Another central component of the NCI pathway is pyroptotic cell death. LDH assay data in **Figure 16A** shows a lack of lytic cell death in human NK cells upon LPS transfection in contrast to macrophages (**Supplementary Figure 6**) which are known to exhibit NCI activation in response to LPS transfection (30). Considering IL-1β secretion is observed (**Figure 16B**), pyroptosis would be expected as well. However, it is well documented that certain cell types express repair machinery that can keep cells viable even after GSDMD pore-formation.

Studies have shown that ESCRT-III repair machinery is activated upon calcium influx from the GSDMD pore and acts to maintain the viability of the plasma membrane and avoid pyroptosis (157). If a similar mechanism exists in NK cells then pyroptosis may not be detectable, and NK cell numbers would be maintained during infection which could be beneficial to the host by maintaining cytotoxic and IFN-γ producing functions. Certain cell types, such as neutrophils and dendritic cells, have been shown to resist cell death whilst still secreting IL-1β in response to defined inflammasome activators (158,159). The molecular basis for the latter is unknown but similar phenomena may happen in NK cells.

Live imaging approaches were undertaken (data not shown) as a substitute for LDH assay to assess cell death in human NK cells. This technique requires more optimisation as well as a way to quantify the level of cell death observed. However, interesting changes in morphology could be observed for LPS transfected NK cells suggesting that transfection resulted in changes to plasma membrane functionality. In future experiments it would be worthwhile to use imaging approaches to validate that *Salmonella* is able to directly infect the NK cells such as confocal microscopy with GFP-tagged *Salmonella Typhimurium* serovar SL13414. Furthermore, due to the lack of LDH release observed in the above experiments with LPS transfection (**Figure 12 and 16**) it would be interesting to use an imaging approach to observe cell death and GSDMD pore formation. A previous study utilised a GFP-tagged GSDMD that conserved its pore forming and pyroptotic functions (160). This would provide a good insight into whether the NCI is active in the NK cells and able to catalyse cleavage of GSDMD particularly in an experimental setup with and without the NLRP3 inhibitor MCC950.

Aside from pyroptosis, IL-1β secretion is a core marker of inflammasome activation. **Figure 16** suggests that human NK cells can secrete IL-1β in response to LPS transfection. There was variability in quality of the accompanying IL-1β standard curves (**Supplementary Figure 9**) which may affect the results observed. This could be due to repeated freeze-thaw cycles of aliquoted IL-1β standard or variability in pipetting quality during serial dilution. The optical density (OD) at 450nm was low even at high concentration of IL-1β standard which could be due to the quality of the plate reader available to measure absorbance accurately. Other secreted factors should also be considered alongside IL-1β when looking at NK cell activation.

To further investigate the effects of LPS transfection on IL-1β secretion and LDH release in NK cells, siRNA against caspase-4 could be utilised to add evidence that the NCI is driving the increase in LDH and IL-1β. Inhibitors of the canonical pathway could also be used to assess whether caspase-4 activity alone is sufficient to drive pyroptosis. Commonly used inhibitors include the NLRP3 inhibitor MCC950 which specifically targets the NACHT domain of NLRP3,

thus inhibiting subsequent caspase-1 activation (161). Therefore, MCC950 could be used in the NK cell experimental setup to help distinguish which inflammasome is responsible for LDH and IL-1β signal. LDH and IL-1β could be observed from direct action of caspase-4 or by caspase-4 mediated activation of NLRP3 via potassium efflux (**Figure 4**). The qPCR data suggests that *Nlrp3* expression is low in mouse and human (**Figure 8 and 14**) so the effects seen may already be completely NCI driven. However, with the addition of accessory cytokines such as IL-2, IL-12, IL-15 and IL-18 NK cell expression levels of canonical inflammasome components could increase. This is worth investigating further as NK cells have been reported to require a combination of these accessory cytokines in order to respond to TLR agonists and induce IFN-γ secretion (124).

It would be interesting to assess whether the NK cells purified for the experiments above express markers of cytotoxicity. NK cells are generally thought of as either cytokine producing (high IFN-γ) which are the $CD56^{bright}$ subset or cytotoxic (high granzyme and perforin) which are the $CD56^{dim}$ subset (90% of human blood) (101). Planned future experiments include the use of retained supernatant from the above experiments to test for IFN-γ and granzyme/perforin production from NK cells to observe any changes upon LPS transfection, such as hyperactivation observed by increased granzyme secretion. The experiment would involve the use of a bead array in a multiplex format to assess production of various secreted factors including NK cell markers and markers of inflammasome activation such as: IFN-γ, granzyme, perforin, IL-1α, IL-1β, IL-18 and IL-37. Due to variability in IL-1β ELISA readouts between donors for the human experiments, a sensitive bead array system would be more appropriate to assess IL-1β levels for these human samples.

Mouse western blot data suggested the presence of an inactive caspase-11 isoform (**Figure 12**). Caspase-11 has been reported to only be active in murine macrophages if they are primed with stimuli such as LPS, IFN-**γ** or IFN-**β** (162). There is also a known caspase-11 isoform with a molecular weight of 10.7kDa. We saw more protein upon LPS priming and at the known molecular weight of the isoform with two separate antibodies. It was assumed that we had indeed detected an inactive Caspase-11 isoform. This provided justification that the non canonical pathway could not be active in mouse splenic NK cells. This would explain the lack of cell death and IL-1β secretion observed for murine NK cells and suggests species differences between mice and humans for inflammasome activation (**Figure 12 & 16**). It would be important to confirm the identity of this isoform using another technique such as mass-spectrometry.

Another experimental change could be to use LPS EB instead of LPS EK for transfection. LPS EB is more representative of true bacterial LPS and could elicit a more representative phenotype. Furthermore, to get closer to the biological situation during infection, outer membrane vesicles (OMVs) have been shown to activate caspase-11 in the cytosol and could therefore be used in experiments with NK cells to assess NCI activation (39). For some experiments, Salmonella *Typhimurium* was used instead of LPS transfection which exhibited an increased trend in LDH release for human NK cells 5hrs post infection with log phase Salmonella (**Figure 17**). However, this increase could be attributed to another inflammasome such as the NLRC4 inflammasome which is activated in response to bacterial flagellin and utilises caspase-1 for pyroptosis induction (163,164). Interestingly, qPCR data in **Figure 14** and **Supplementary Figure 5** indicate a lack of *PYCARD* (gene encoding ASC) in human NK cells. There is debate around whether ASC is required for full NLRC4 activation, with suggestions that it is needed for effective cytokine processing downstream of NLRC4. However due to the presence of a CARD domain in NLRC4, it is reported to bind caspase-1 directly through CARD-CARD interactions and thus bypass ASC (42,158). As such it is unclear whether the LDH response observed could be attributed to flagellin recognition by NLRC4.

IRF3 is required for IFN-β expression downstream of TLR4 signalling and has implications in endotoxic shock. Mice deficient in IRF3 have been shown to have better survival outcomes after high dose injection of LPS. In the same study IFN-β levels were reduced in IFR3 deficient mice (165). LPS and IFN-β priming of inflammasome gene expression could be linked to this phenotype. It would be interesting to include IRF3 and other IRFs in qPCR analysis of NK cells to examine if these key inducers of IFN-β are present. Furthermore, RNA-sequencing data in **Figure 5** suggests LPS treatment in mouse spleen or Mycobacterium *tuberculosis* infection of human lung explants induces *IRF1* gene expression, which has been shown to induce IFN-β autocrine signalling as well (27). Therefore it would be interesting to validate the phenotype observed with RNA-sequencing by qPCR in the above experimental setup.

Due to variances in human NK cell purity (**Figure 7**), it would be useful to examine gene expression of NK cell markers by qPCR from purified human NK cell samples. One possible candidate is *KLRK1* (encodes NKG2D) which is a known activating receptor on the surface of NK cells crucial for clearance of infected host cells (120). In addition, PAD4 could be included as a neutrophil marker for the same experiment. PAD4 is central in Neutrophil NETosis as it citrullinates histones required for NET formation (166,167).

Additionally, expulsion of bacteria via NK cell degranulation presents a novel mechanism in the host defence to intracellular bacterial infection and provides evidence that NK cells can be directly infected by bacteria (140). Therefore, activation of the NCI in NK cells would be possible as bacteria would be exposed to GBPs and caspase-11/4 in the cytosol.

The role of NK cells in sepsis remains inconclusive with studies offering contrasting data. Studies have reported that an increased number of NK cells results in an overactive inflammatory phenotype which contributes to sepsis disease progression, particularly through a substantial increase in IFN-γ levels. In contrast, depletion of NK cells in sepsis has been shown to prevent clearance of bacteria and as such further disease progression (127). NK cells are thought to contribute to LPS induced shock through release of IFN-γ which can exacerbate the proinflammatory phenotype. This is thought to be dependent on exposure of NK cells to accessory cytokines such as IL-12 and IL-15 (121,168). Furthermore, studies have shown that depletion of NK cells in mice prolongs survival in pneumococcal pneumonia and this is dependent on interaction with iNKT cells. Inhibition of iNKT activation in this study resulted in elevated levels of NK cells in the spleen and subsequent increased levels of IFN-γ (169). However a study by Kim et al suggested that in the context of secondary bacterial infection post sepsis, IFN-γ acts in an immunosuppressive capacity characterised by increased survival of bacteria in the host. Interestingly, in this situation iNKTs were reported to drive this immunosuppressive phenotype through mTOR activation in NK cells resulting in higher susceptibility to bacterial infection. Deletion of the iNKT population or inhibition of mTOR with rapamycin rescued the immune response and prevented the establishment of bacterial infection (137). These studies highlight a complex interplay between iNKTs and NK cells in the context of sepsis and post-sepsis recovery of the early immune response.

Reports estimate the number of sepsis related deaths to potentially be as high as 5.3 million annually (170). As such there is an unmet clinical need for therapeutics to target this disease. Inflammasome activation is a key driver of sepsis disease progression with the NCI shown to drive this through its ability to sense intracellular LPS (171). With NK cell dependent release of IFN-γ thought to contribute towards sepsis, their lack of pyroptotic cell death as observed in **Figure 16** may contribute to their accumulation and overactive phenotype associated with sepsis severity. Indeed, caspase-4 directed cleavage of IL-1β as seen in **Figure 16** would add to this proinflammatory state observed in sepsis patients (172). To better link this study to human disease, use of tissue from sepsis patients would be beneficial and provide insight into NCI activation in humans. Studies have already implicated Caspase-11 activation in sepsis disease progression in mouse models (8).

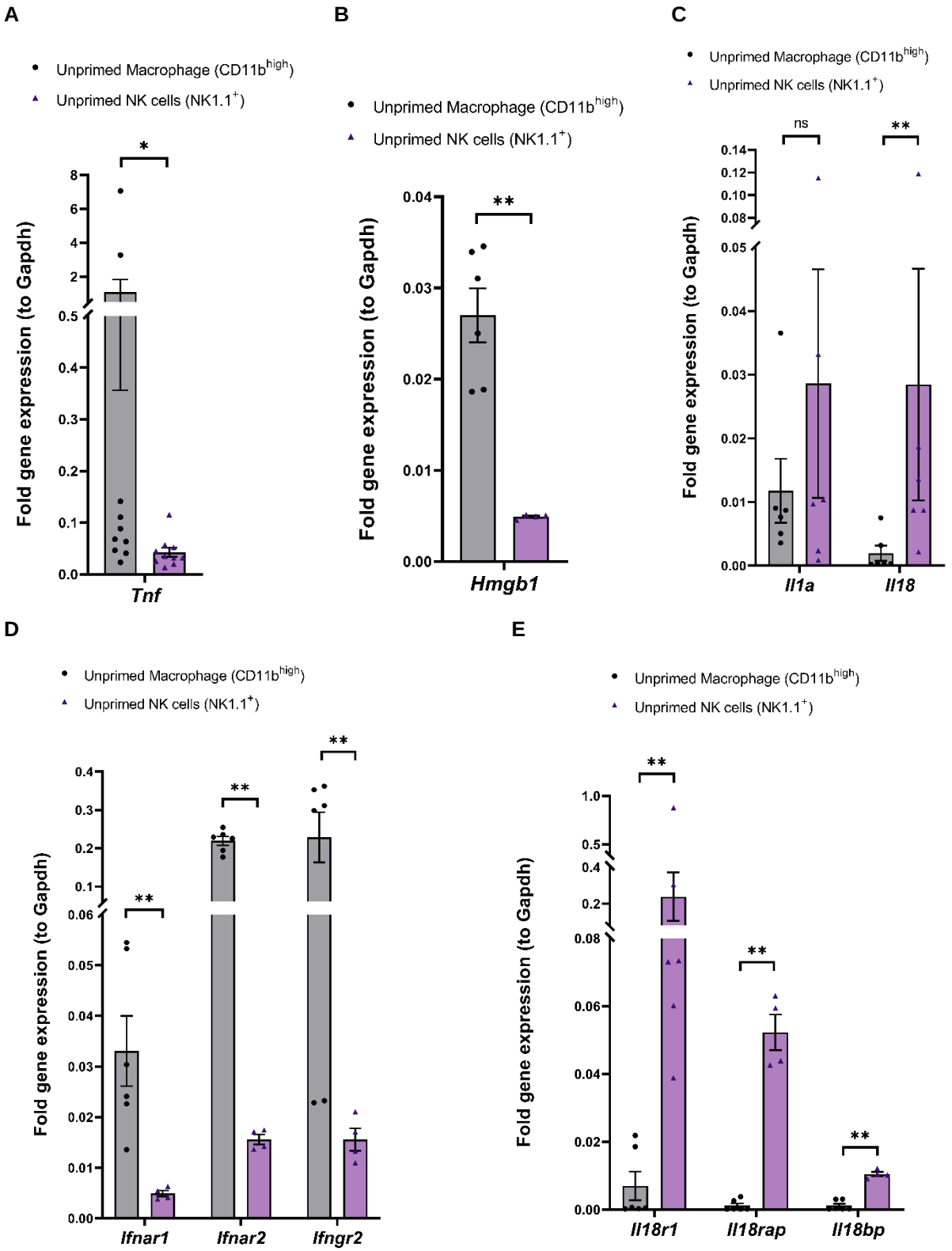
Recently, lymphoid cells have been found to possess functional inflammasome in response to Salmonella *Typhimurium*. Specifically, mouse group 3 innate lymphoid cells (ILC3s) were found to exhibit cell death upon S.*Typhimurium* infection. The researchers showed in a ILC3-like cell line (MNK-3) that cell death occurred in a GSDMD and Caspase-1 dependent manner upon S.*Typhimurium* infection (173). This study demonstrates that lymphoid cells can express functional inflammasome, however they describe a lack of caspase-11 expression at the transcriptional level during Salmonella infection and suggest it is not activated. However, no studies have focussed on the pathway in humans. **Figure 12** demonstrates a lack of NCI activation in mice, but **Figure 16** shows that NK cells in humans possess components of the inflammasome pathway at the protein level and that they can secrete IL-1β in response to LPS transfection. As such, activation of the NCI may be species specific for NK cells. In addition, mice used for laboratory experiments are not regularly exposed to pathogens, unlike humans. Humans may therefore have a greater need for the NCI and are able to respond differently to mice. Furthermore, ILC3s are a different lymphoid population so direct comparison to NK cells cannot be made in this case.

qPCR data in **Figure 14** suggests a lack of *NLRP3* and *PYCARD* (gene encoding ASC) in human NK cells which would remove the possibility of IL-1β processing by the NLRP3 inflammasome. As such, any IL-1β secretion observed would have to be due to the action of another inflammasome. This opens up the possibility that caspase-4 could cleave IL-1β directly without the need for activation of caspase-1 through the NLRP3 inflammasome as shown in **Figure 4**. Western blot analysis would be needed here to assess whether NLRP3 is expressed at the protein level. Furthermore, induction of NLRP3 was not observed with LPS or IFN-γ priming in NK cells but another priming agent could be effective for NLRP3 induction. Also the use of IL-15, IL-12 and IL-18 cytokines as described previously may help to activate NK cells and drive expression of NLRP3 (155). Previous studies showed that gram-negative bacteria could upregulate caspase-11 through TRIF dependent induction of type-I interferon which in turn acts in an autocrine manner to upregulate caspase-11. Only then could the NLRP3 inflammasome be activated. The study theorised that caspase-11 could directly activate caspase-1 through formation of a heterodimer and thus bypass NLRP3 and ASC (however they provided no evidence for this) (174). Therefore, during bacterial infection NK cells may be able to behave in this way and bypass NLRP3-dependent caspase-1 activation. The use of an NLRP3 inhibitor such as MCC950 would be useful to examine this hypothesis in NK cells.

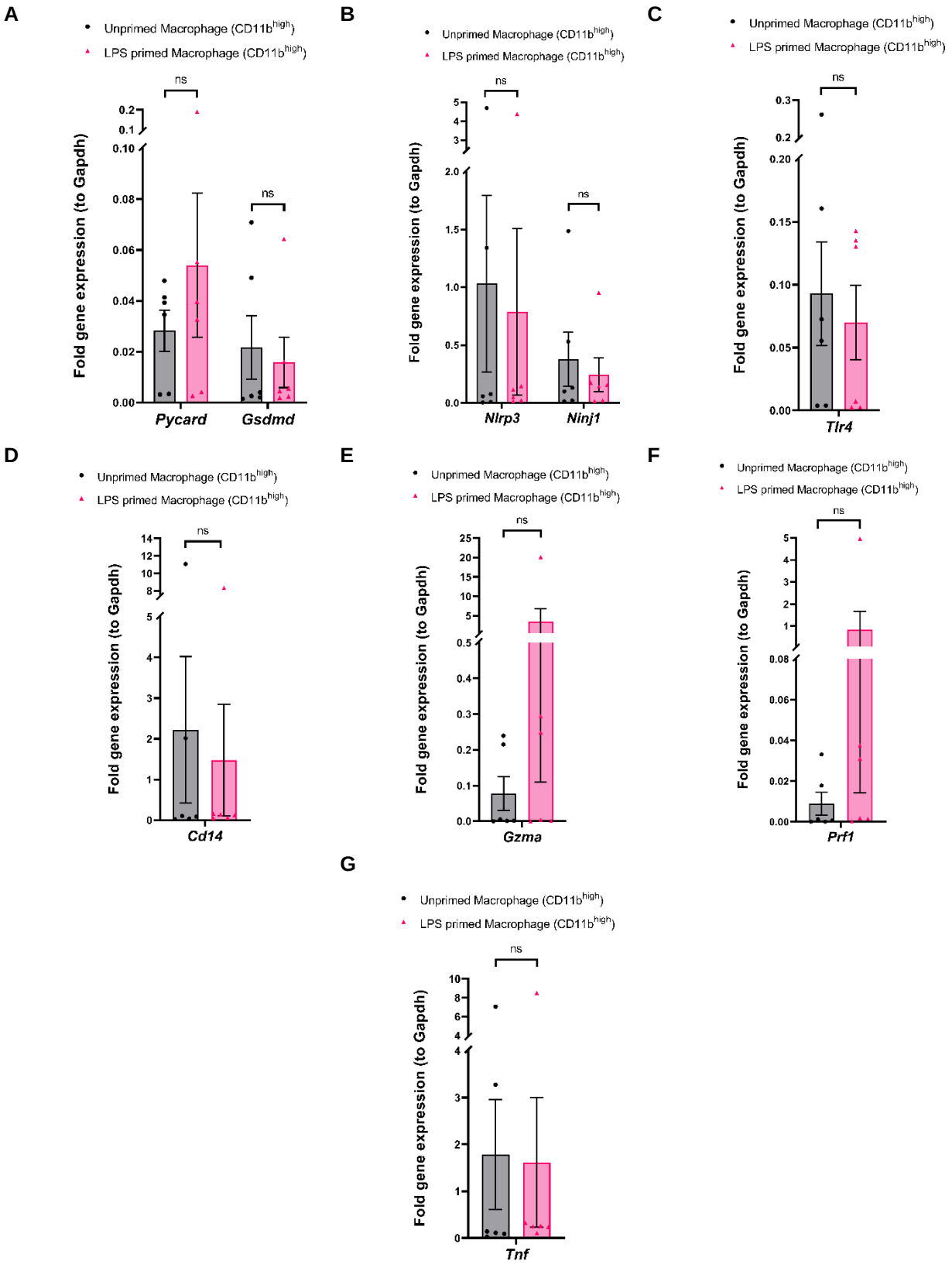
NCI activation has been solely attributed to select types of cell including macrophages and neutrophils. The research presented in this thesis exhibits the potential contribution of NK cells

in NCI-mediated IL-1β production. In conjunction with recent studies into NK cell function in sepsis and canonical inflammasome activation in ILC3s (127,173), this work provides an alternative view on the role of lymphoid cells in infection. Further work is needed to uncover the exact mechanisms responsible and explain the lack of pyroptosis in NK cells. However, through the use of multiple techniques there is scope to uncover the functionality of the NCI in NK cells. For example; microscopy to examine cell death and intracellular infection, bead array to provide an insight into the secretome of NK cells transfected with LPS, as well as the use of siRNA for caspase-4 and caspase-1 knockdowns. In addition, the use of inhibitors for caspases or NLRP3 (VX-765 or MCC950 respectively) could add further mechanistic insight. The role of NK cells and the NCI in sepsis has been uncovered and as such need to be assessed as a whole to fully understand the pathways contributing to this disease state. In conclusion, lymphoid cell populations should be given more focus in inflammasome research. After all, the immune response to infection encompasses a range of cell types and therefore lymphoid cells should be included in any further study.

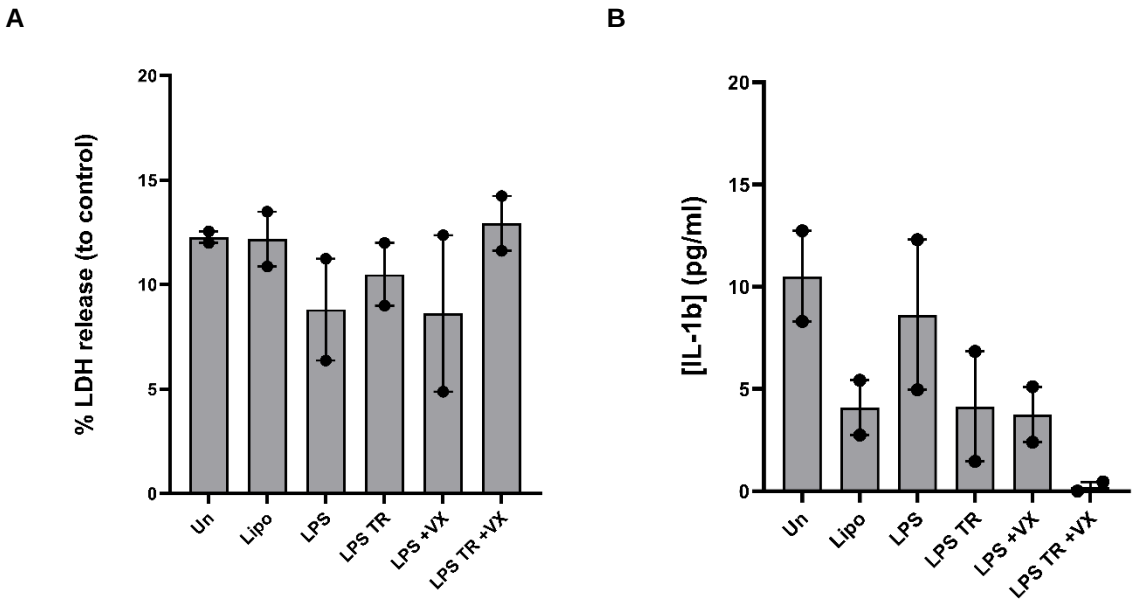
5.0 Appendices



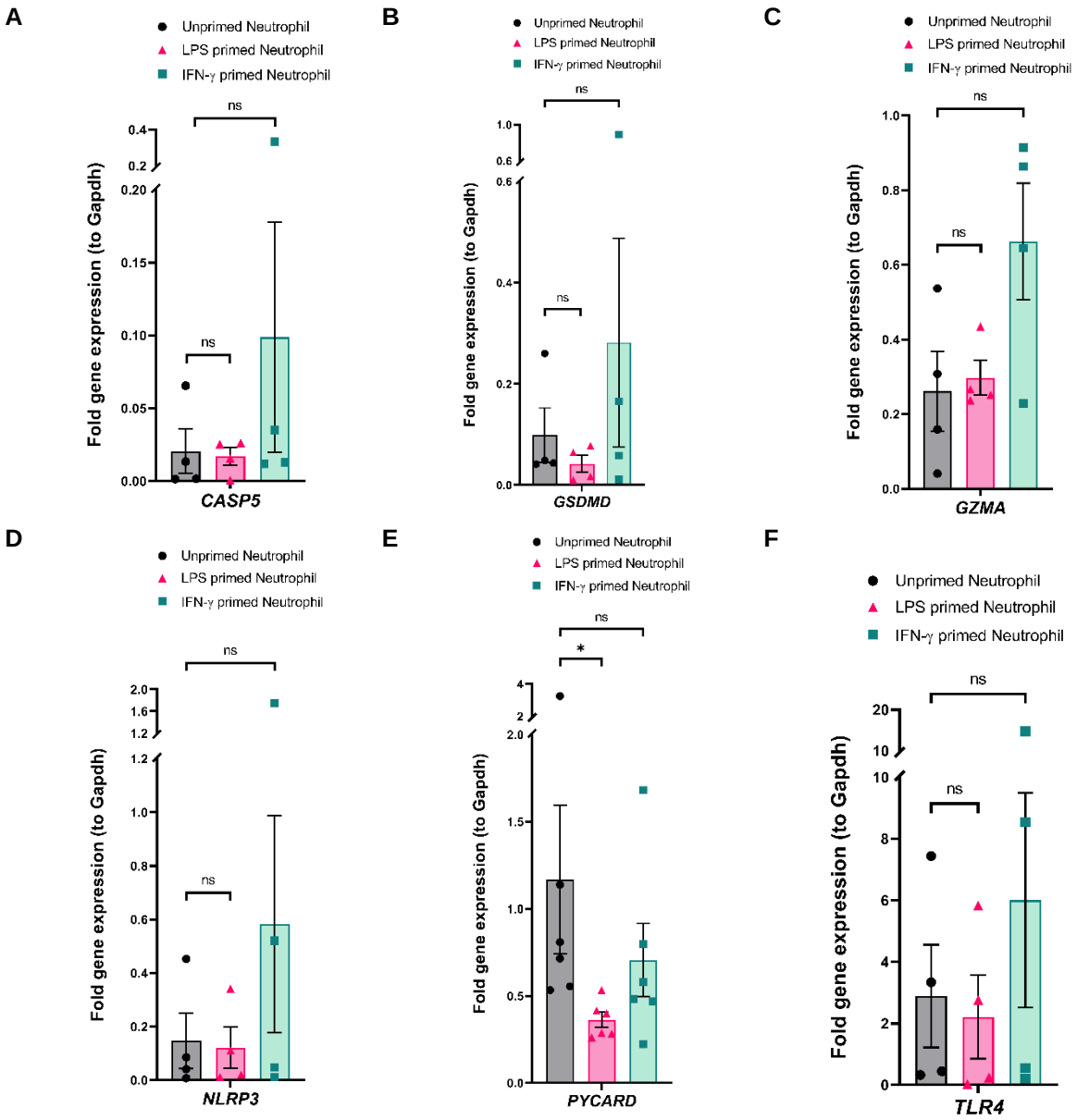
Supplementary figure 1. Murine NK cells express high levels of IL-18 related genes. A-E qPCR data of relative gene expression to *Gapdh* n=4-10. Analysis by Mann Whitney test ns = not significant, * = p<0.05, ** = p<0.01, *** = p<0.001



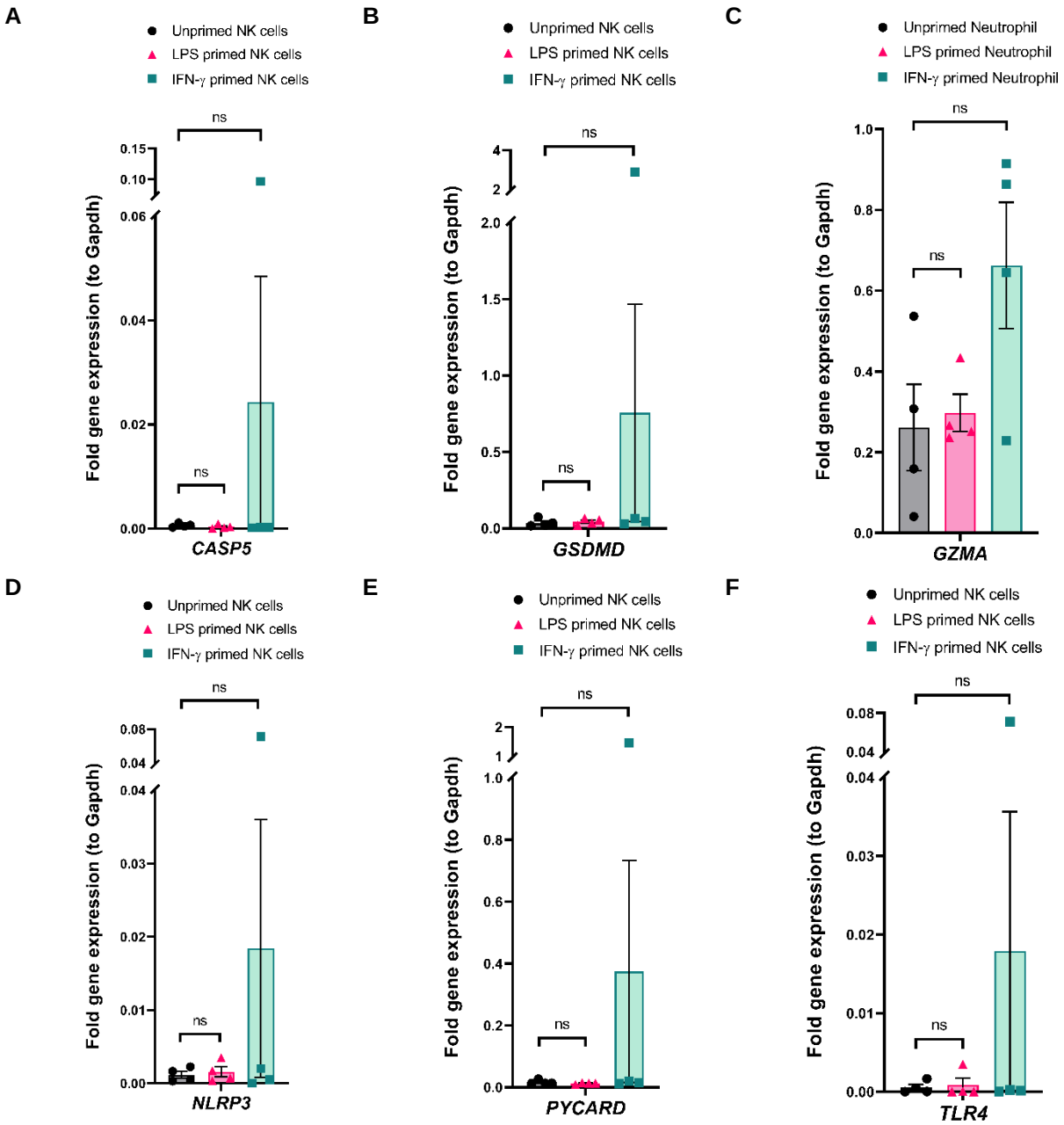
Supplementary figure 2. LPS priming does not impact gene expression in murine macrophages. **A-G** qPCR data of relative gene expression to *Gapdh* n=6. Analysis by Wilcoxon signed rank test. ns = not significant, * = p<0.05, ** = p<0.01, *** = p<0.001



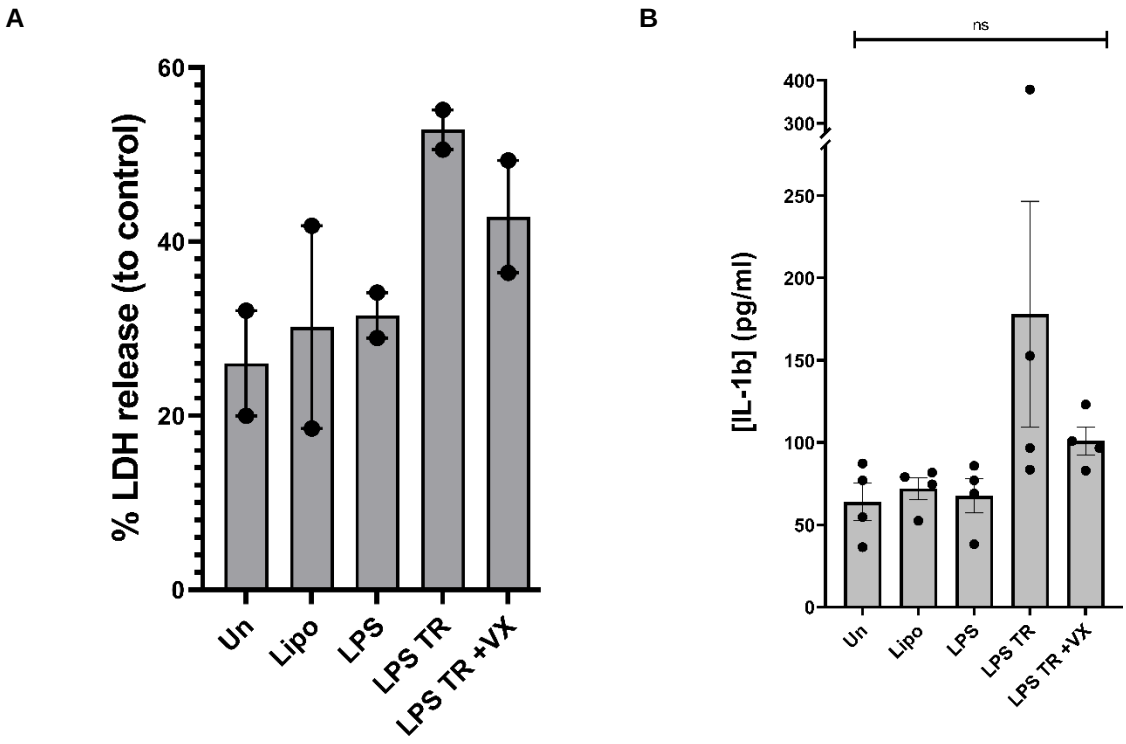
Supplementary figure 3. IFN-β primed murine NK cells do not respond to LPS transfection. Samples were primed for 3hrs with IFN-**β** followed by 5hrs of LPS transfection. **A** LDH assay with WT mouse splenic NK cells n=2. **B** IL-1**β** ELISA with WT mouse splenic NK cells n=2. Un = Untreated, Lipo = Lipofectamine only, LPS= Extracellular LPS treatment, LPS TR = LPS transfection, VX= VX-765 Caspase inhibitor.



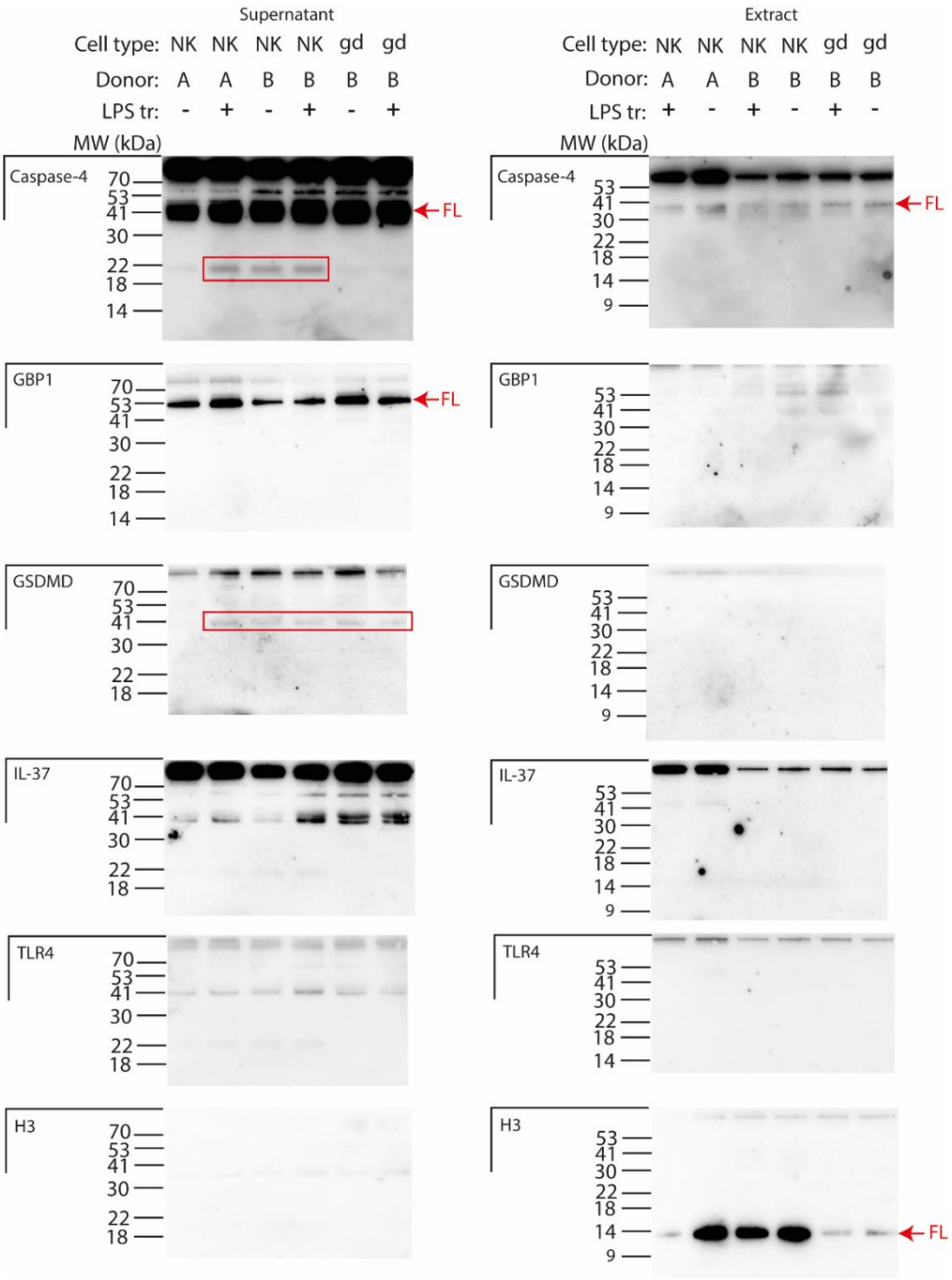
Supplementary figure 4: Human neutrophils show no change in gene expression in response to LPS or IFN-γ priming. A-D qPCR data of relative gene expression to *GAPDH* n=4-6. Analysis by Wilcoxon signed rank test. ns = not significant, * = p<0.05, ** = p<0.01, *** = p<0.001



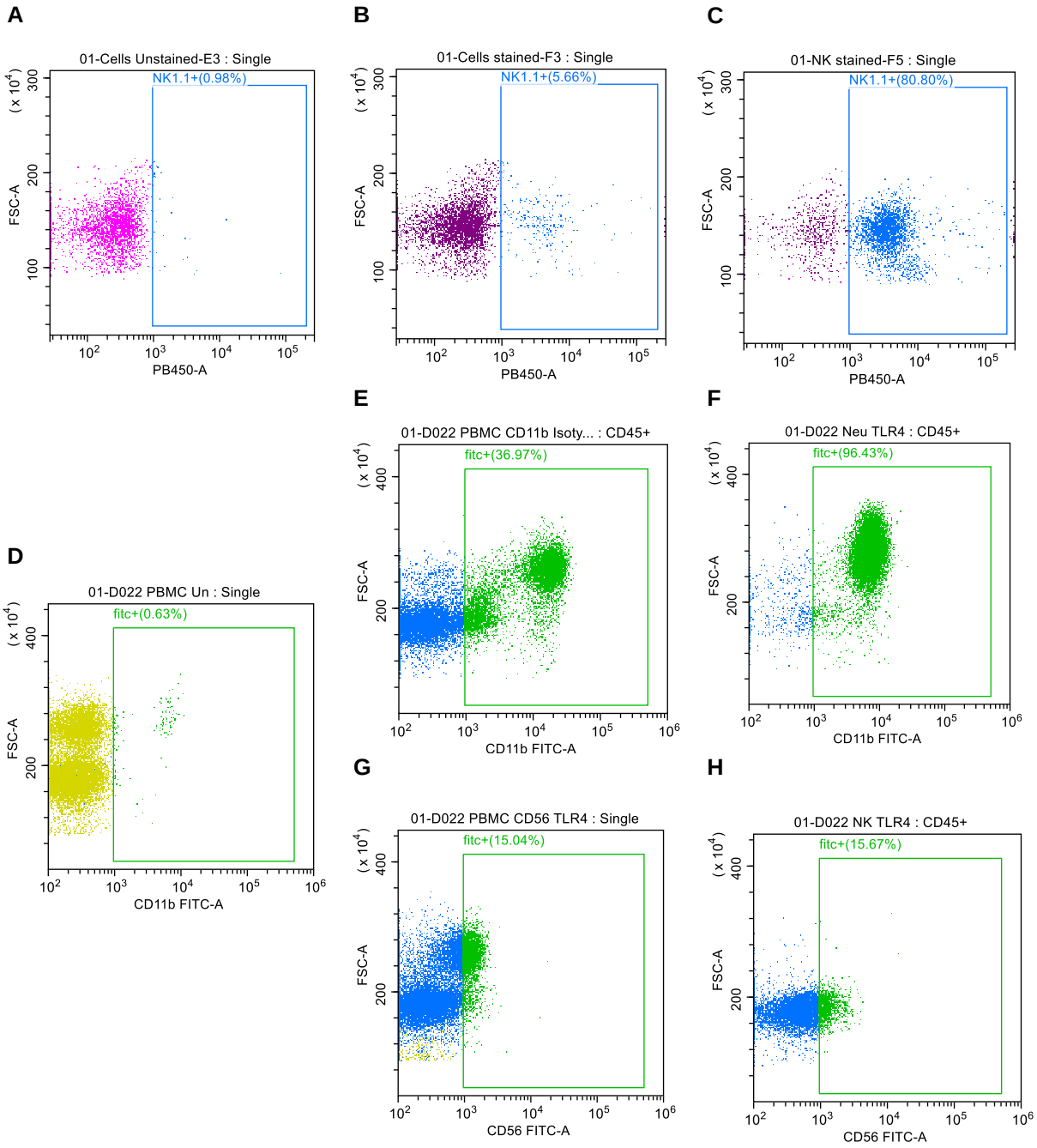
Supplementary figure 5. Human NK cells show no change in gene expression in response to LPS or IFN-γ priming. A-D qPCR data of relative gene expression to *GAPDH* n=4-6. Analysis by Wilcoxon signed rank test. ns = not significant, * = p<0.05, ** = p<0.01, *** = p<0.001



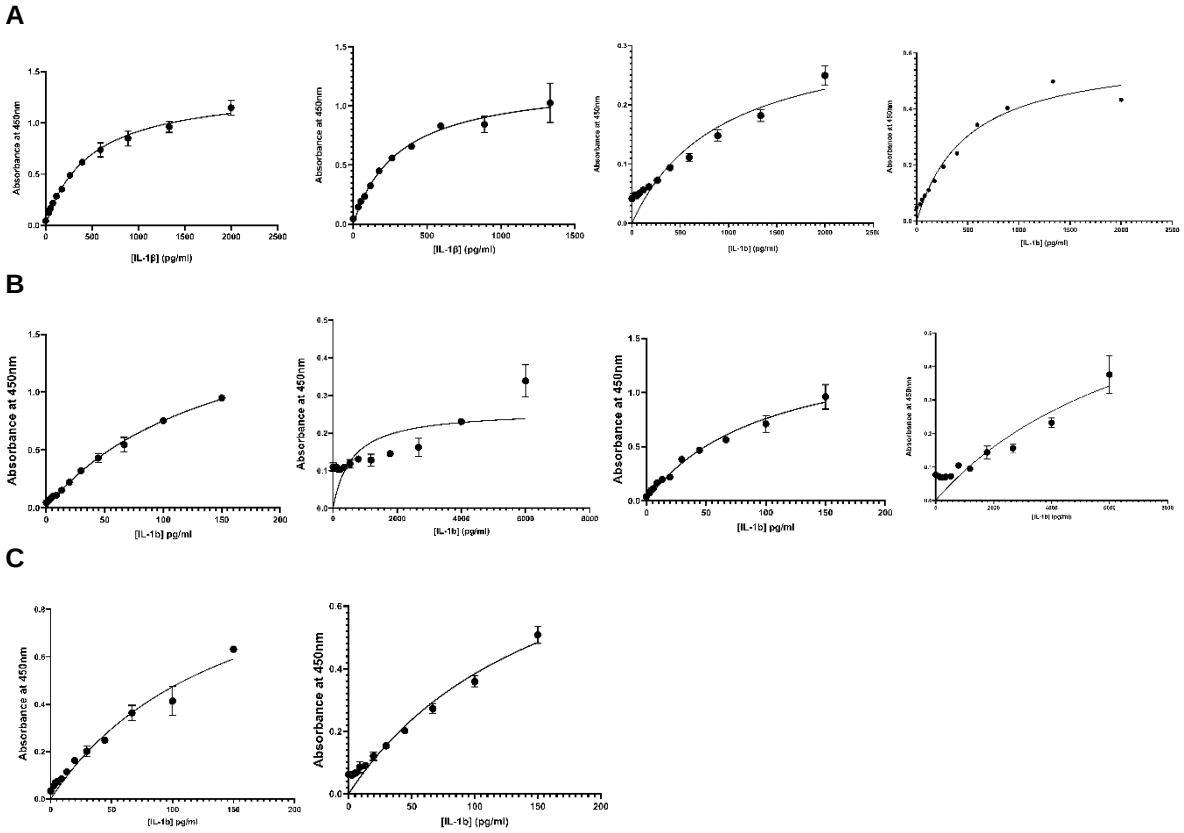
Supplementary figure 6. Human macrophages exhibit trends toward increased IL-1β secretion and LDH release in response to LPS transfection. Human macrophages differentiated from peripheral blood PBMCs n=2 **A** LDH assay. **B** IL-1β ELISA. Analysis by Wilcoxon signed rank test ns = not significant, * = p<0.05, ** = p<0.01, *** = p<0.001. Un = Untreated, Lipo = Lipofectamine only, LPS= Extracellular LPS treatment, LPS TR = LPS transfection, VX= VX-765 Caspase inhibitor.



Supplementary figure 7. Caspase-4 cleaved upon LPS transfection in human NK cells. Western blot with human NK cells (NK) or gamma-delta T cells (gd). Cell extract (lysate) and supernatant were run separately. Cells were unprimed followed by 14hrs treatment. FL= Full length. Cleaved proteins indicated by red boxes.



Supplementary figure 8. Weak shifts observed for human CD56 in flow cytometry purity checks. A Mouse unstained control gating for NK1.1+ cells. **B** Mouse PBMCs with flow antibody staining, gating for NK1.1+ cells**. C** Mouse NK cells with flow antibody staining, gating for NK1.1+ cells. **D** Human unstained control gating for CD11b or CD56+ cells. **E** Human PBMCs with flow antibody staining, gating for CD11b+ cells. **F** Human Neutrophils with flow antibody staining, gating for CD11b+ cells. **G** Human PBMCs with flow antibody staining, gating for CD56+ cells. **H** Human NK cells with flow antibody staining, gating for CD56+ cells.



Supplementary figure 9. Inconsistency for ELISA standard curves. A Mouse NK cell and Macrophage IL-1β ELISA standard curves. **B** Human NK cell IL-1β ELISA standard curves. **C** Human macrophage IL-1β ELISA standard curves.

Supplementary Table 1: Table of materials

Description	Manufacturer	Catalogue no	Use
Triton X-100	Sigma-Aldrich	9002-93-1	Detergent for LDH assay 100% lysis control
LDH Dye solution (INT/Na-lactate)	Takada	MK401-2	LDH substrate for LDH assay
LDH catalyst	Takada	MK401-1	LDH catalyst for LDH assay
DPBS (without calcium & magnesium)	Corning	21-031-CV	ELISA diluent, flow cytometry, tissue processing etc
DPBS	Sigma Life Sciences	D8537	ELISA diluent, flow cytometry, tissue processing etc
IL-1b mouse ELISA kit	Invitrogen	88-7013-88	IL-1β ELISA
IL-1b human ELISA kit	Invitrogen	88-7261-88	IL-1β ELISA

Skim milk powder	Sigma-Aldrich	-	To make 5% milk in TBST for western blot blocking buffer
Nupage LDS Sample buffer 4X	Novex (Life Technologies)	NP008	Lysis buffer for western blot
Nitrocellulose membranes (0.2µm)	Bio-RAD	-	Membranes for western blot
Trans-Blot Turbo 5X Transfer buffer	Bio-RAD	10026938	Transfer buffer for western blot semi-dry transfer
Broad range prestained protein marker	Proteintech	PL00002	Protein ladder for SDS PAGE
Prime-step prestained broad range protein ladder	Biolegend	773302	Protein ladder for SDS PAGE
Immobilon Forte western HRP substrate	Millipore	WBLUF0500	Western HRP substrate
Immobilon Crescendo western HRP substrate	Millipore	WBLUR0500	Western HRP substrate
Hydrogen peroxide 35%	Merck	-	Stripping western blot membranes
Lipofectamine LTV plus reagent	Invitrogen	15338-100	LPS transfection reagent
Gentamicin	Sigma-Aldrich	-	Antibiotic used for *Salmonella* infection experiments
LPS-EK Ultrapure	InvivoGen	tlrl-peklps	For LPS priming, Extracellular LPS treatments and LPS transfections
VX-765	Selleckchem	-	Caspase inhibitor used in cell treatments
Opti-MEM (1X reduced serum medium +L-glutamine, +HEPES -Phenol red)	Gibco	11058-021	Media for cell culture and treatments
Ficoll – PaquePLUS	cytiva	17144002	Blood separation for EasySep and PBMC layer collection
7AAD	Invitrogen	A1310	Live/dead stain for flow cytometry
7-AAD Viability Staining Solution	Biolegend	420404	Live/dead stain for flow cytometry
X-VIVO15	Lonza	BF02-060F	NK cell culture medium
STEMCELL EasySep Human NK Cell Isolation Kit	STEMCELL Technologies	17955	Purification of immune cells

STEMCELL EasySep Direct Human NK Cell Isolation Kit	STEMCELL Technologies	19665	Purification of immune cells
STEMCELL EasySep Direct Human Neutrophil Isolation Kit	STEMCELL Technologies	19666	Purification of immune cells
STEMCELL EasySep Mouse NK Cell Isolation Kit	STEMCELL Technologies	19855	Purification of immune cells
STEMCELL ImmunoCult NK Cell Expansion kit	STEMCELL Technologies	100-0711	NK cell expansion
STEMCELL SepMATE tubes	STEMCELL Technologies	85450	Blood separation
EasySep Magnet	STEMCELL Technologies	18000	Purification of immune cells
Interleukin-2 Human	Sigma Life Sciences	H7041-IOVG	Supplement for NK cell culture
Recombinant Human IL-2	Biolegend	791904	Supplement for NK cell culture
High-affinity binding plate	Greiner	2015477-1	96-well plate for ELISA
IFN-γ	AbClonal Biotechnology	-	Priming of immune cells
HumanKine M-CSF (Recombinant Human)	Proteintech	Hz-1192	Differentiation of PBMCs to macrophages
RPMI 1640	Gibco	31870-025	Macrophage culture medium
UltraCompeBeads Compensation Beads	Invitrogen	01-2222-42	Single stain controls for flow cytometry
Arcturus PicoPure RNA Isolation Kit	Applied Biosystems	12204-01	RNA extraction and isolation
Superscript IV First strand	Invitrogen	18091050	Reverse transcription of RNA to cDNA
RNase-free DNase Set	Qiagen	79254	Column DNase treatment during RNA isolation
Ultrapure 0.5M EDTA pH 8.0	Invitrogen	15575-038	Added to culture media
HEPES Solution	Sigma	7365-45-9	Added to culture media
Taqman Universal PCR Master Mix	Applied Biosystems	4304437	qPCR master mix
MicroAMP Optical 96-well Reaction Plate with barcode	Applied Biosystems	4306737	qPCR plates
IFN-b Mouse, His	PROSPEC protein specialists	cyt-651-a	Priming of immune cells

Supplementary Table 2: Table of antibodies

Antibody	Clone	Manufacturer	Catalogue no	Dilution
Goat anti-mouse IgG Fc Secondary Antibody HRP	-	Invitrogen	31437	1:3333
Goat anti-rabbit IgG Secondary Antibody HRP	-	Invitrogen	31460	1:3333
Caspase-4 mouse monoclonal antibody	-	Proteintech	67398-1-Ig	1:2000
Rat anti-mouse Caspase-11 ab	17D9	BD Pharmingen	564971	1:750
Anti-Caspase-11 antibody [EPR 22717-11]	-	Abcam	ab246496	1:1000
Caspase-5 (D3G4W) Rabbit mAb	-	Cell signalling Technologies	46680	1:1000
Caspase 1/p20/p10 polyclonal antibody	-	Proteintech	22915-1-AP	1:1000
Mouse anti-H3 ab	-	Biolegend	-	1:2000
GSDMD antibody -100ug	-	Cusabio	CSB-PA009956LA01HU	1:2000
GBP2 Rabbit Polyclonal antibody ab	-	Proteintech	11854-1-AP	1:1000
FITC anti-human CD11b	ICRF44	Biolegend	301329	1:200
FITC anti-human CD56	MEM-188	Biolegend	304603	1:200
Alexa 700 anti-mouse CD45	30-F11	Biolegend	103128	1:200
APC anti-human CD45	HI30	Biolegend	304011	1:200
BV421 anti-mouse NK1.1	PK136	Biolegend	108732	1:200
GBP1 Rabbit polyclonal antibody	-	Proteintech	15303-1-AP	1:2000

TLR4 Mouse monoclonal antibody	-	Proteintech	66350-1-Ig	1:1000
IL-37 Mouse monoclonal antibody	-	Proteintech	6029C-1-Ig	1:500
IL-18 Rabbit polyclonal antibody	-	Proteintech	10663-1-AP	1:2000
Anti-Caspase-5 antibody [EP876Y] - BSA and Azide free	-	Abcam	Ab247301	1:1000

Supplementary Table 3: Table of Taqman primers (ThermoFisher)

Gene name	**Target**	**Taqman assay number**	**Catalogue number**
Casp1	Mouse	Mm00438023_m1	4331182
Casp4	Mouse	Mm00432304_m1	4331182
Gbp2b	Mouse	Mm00657086_m1	4331182
Gbp2	Mouse	Mm00494575_m1	4331182
Gbp3	Mouse	Mm00497606_m1	4331182
Gbp4	Mouse	Mm00657752_m1	4331182
Gbp5	Mouse	Mm00463735_m1	4331182
Il1a	Mouse	Mm00439620_m1	4331182
Il1b	Mouse	Mm00434228_m1	4331182
Il18	Mouse	Mm00434225_m1	4331182
Ifnar1	Mouse	Mm00439544_m1	4331182
Ifnar2	Mouse	Mm00494916_m1	4331182
Ifngr1	Mouse	Mm00599890_m1	4331182
Ifngr2	Mouse	Mm00492626_m1	4331182
Gsdmd	Mouse	Mm00509958_m1	4331182
Ninj1	Mouse	Mm00479014_m1	4331182
Nlrp3	Mouse	Mm00840904_m1	4331182
Pycard	Mouse	Mm00445747_g1	4331182
Hmgb1	Mouse	Mm00849805_gH	4331182
Tlr4	Mouse	Mm00445273_m1	4331182
Tnf	Mouse	Mm00443258_m1	4331182

Gzma	Mouse	Mm01304452_m1	4331182
Prf1	Mouse	Mm00812512_m1	4331182
Il18r1	Mouse	Mm00515178_m1	4331182
Il18rap	Mouse	Mm00516053_m1	4331182
Il18bp	Mouse	Mm00456733_m1	4331182
Cd14	Mouse	Mm00438094_g1	4331182
Gapdh	Mouse	Mm99999915_g1	4331182
CASP1	Human	Hs00354836_m1	4331182
CASP4	Human	Hs01031951_m1	4331182
CASP5	Human	Hs07290189_m1	4331182
GBP1	Human	Hs00266717_m1	4331182
GBP2	Human	Hs00894837_m1	4331182
GBP3	Human	Hs00544385_m1	4331182
GBP4	Human	Hs00364728_m1	4331182
GBP5	Human	Hs00369472_m1	4331182
IL1B	Human	Hs01555410_m1	4331182
IL18	Human	Hs01038788_m1	4331182
IFNGR1	Human	Hs00166223_m1	4331182
IFNGR2	Human	Hs00194264_m1	4331182
GSDMD	Human	Hs00226875_m1	4331182
NINJ1	Human	Hs00982607_m1	4331182
NLRP3	Human	Hs00918082_m1	4331182
PYCARD	Human	Hs00203118_m1	4331182
TLR4	Human	Hs00152939_m1	4331182
TNF	Human	Hs00174128_m1	4331182
GZMA	Human	Hs00989184_m1	4331182
PRF1	Human	Hs00169473_m1	4331182
CD14	Human	Hs00169122_g1	4331182
IL37	Human	Hs00367201_m1	4331182
GAPDH	Human	Hs02758991_g1	4331182

6.0 Abbreviations

Abbreviation	Meaning
γδT	Gamma-delta T cell
7AAD	7-aminoactinomycin D
ADCC	Antibody-dependent cellular cytotoxicity
AIM2	Absent in melanoma 2
ALRs	AIM2-like receptors
ASC	Apoptosis-associated speck-like protein containing a CARD
CARD	Caspase activation and recruitment domain
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CLP	Common lymphoid progenitor
CLRs	C-type lectin receptors
DAMPs	Danger-associated molecular patterns
DC	Dendritic cell
DPBS	Dulbecco's phosphate buffered saline
Eomes	Eomesdermin homolog
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ESCRT	Endosomal sorting complex required for transport
FACS	Fluorescence-activated cell sorting
FBS	Foetal bovine serum
FCS	Foetal calf serum
GATA3	GATA binding protein 3
GBP	Guanylate binding protein
GFP	Green fluorescent protein
GSDMD	Gasdermin-D
HCl	Hydrochloric acid

HMDM	Human monocyte derived macrophages
HMGB1	High mobility group box 1
HRP	Horseradish peroxidase
Id2	DNA-binding protein inhibitor 2
IFN-γ	Interferon gamma
IFNAR1	Interferon alpha and beta receptor subunit 1
IL	Interleukin
ILCs	Innate lymphoid cells
iPSC	Induced pluripotent stem cell
iNKT	Invariant natural killer T cell
IRF	Interferon regulatory factor
JAK	Janus family tyrosine protein kinase
KIRs	Killer-cell immunoglobulin-like receptors
LB	Luria-Bertani
LBP	Lipopolysaccharide binding protein
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
Ly6C	Lymphocyte antigen 6C
M-CSF	Macrophage colony stimulating factor
MD2	Myeloid differentiation factor 2
MOI	Multiplicity of infection
mTOR	Mammalian target of rapamycin
MyD88	Myeloid differentiation primary response protein
NAIP	Neuronal apoptosis inhibitory protein
NCI	Non-canonical inflammasome
NETs	Neutrophil extracellular traps
NFκB	Nuclear factor kapa-light-chain-enhancer of activated B cells
Ninj1	Ninjurin-1
NK	Natural killer cells
NKG2D	NKG2-D type II integral membrane protein

NKP	NK lineage progenitor
Nkp44	Natural cytotoxicity triggering receptor 2
NLRC4	NLR family CARD domain containing protein 4
NLRP3	NLR family PYD domain containing protein 3
NLRs	Nod-like receptors
OD	Optical density
OMVs	Outer membrane vesicles
PAD4	Peptidylarginine deiminase 4
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline tween
PGPC	1-palmitoyl-2-glutaryl-sn-glycero-3-phosphocholine
PRRs	Pattern recognition receptors
PYD	Pyrin domain
qPCR	Quantitative polymerase chain reaction
RBC	Red blood cell
RLRs	RIG-I-like receptors
RNA	Ribonucleic acid
RNAseq	RNA sequencing
SCV	Salmonella-containing vacuole
SDS	Sodium dodecyl sulphate
siRNA	Small interfering ribonucleic acid
SLE	Systemic lupus erythematosus
STAT	Signal transducer and activator of transcription
Tbet	T-box transcription factor
TBST	Tris buffered saline tween
TCR	T-cell receptor
Th	T-helper cell
TLRs	Toll-like receptors

TNFα	Tumour necrosis factor alpha
TRIF	TIR-domain-containing adaptor-inducing interferon β
WT	Wild type

7.0 References

1. Karki R, Kanneganti T-D. The 'cytokine storm': molecular mechanisms and therapeutic prospects. Trends Immunol. 2021 Aug;42(8):681–705.

2. Lin L, Xu L, Lv W, Han L, Xiang Y, Fu L, et al. An NLRP3 inflammasome-triggered cytokine storm contributes to Streptococcal toxic shock-like syndrome (STSLS). PLoS Pathog. 2019 Jun;15(6):e1007795.

3. Guo H, Callaway JB, Ting JP-Y. Inflammasomes: mechanism of action, role in disease, and therapeutics. Nat Med. 2015 Jul;21(7):677–87.

4. Schroder K, Tschopp J. The inflammasomes. Cell. 2010 Mar 19;140(6):821–32.

5. Broz P, Dixit VM. Inflammasomes: mechanism of assembly, regulation and signalling. Nat Rev Immunol. 2016 Jul;16(7):407–20.

6. Li Z, Guo J, Bi L. Role of the NLRP3 inflammasome in autoimmune diseases. Biomed Pharmacother. 2020 Oct;130:110542.

7. Uresti-Rivera EE, García-Hernández MH. AIM2-inflammasome role in systemic lupus erythematous and rheumatoid arthritis. Autoimmunity. 2022 Nov;55(7):443–54.

8. Wang Y, Zhang X, Guo Y, Li X, Guo G, Niu Z, et al. Type 1 interferon aggravates lipopolysaccharide-induced sepsis through upregulating Caspase-11 and Gasdermin D. J Physiol Biochem. 2021 Feb;77(1):85–92.

9. Rodrigues TS, de Sá KSG, Ishimoto AY, Becerra A, Oliveira S, Almeida L, et al. Inflammasomes are activated in response to SARS-CoV-2 infection and are associated with COVID-19 severity in patients. J Exp Med [Internet]. 2021 Mar 1;218(3). Available from: http://dx.doi.org/10.1084/jem.20201707

10. Abbate A, Toldo S, Marchetti C, Kron J, Van Tassell BW, Dinarello CA. Interleukin-1 and the Inflammasome as Therapeutic Targets in Cardiovascular Disease. Circ Res. 2020 Apr 24;126(9):1260–80.

11. Geijtenbeek TBH, Gringhuis SI. Signalling through C-type lectin receptors: shaping immune responses. Nat Rev Immunol. 2009 Jul;9(7):465–79.

12. Motta V, Soares F, Sun T, Philpott DJ. NOD-like receptors: versatile cytosolic sentinels. Physiol Rev. 2015 Jan;95(1):149–78.

13. Kagan JC. Lipopolysaccharide Detection across the Kingdoms of Life. Trends Immunol. 2017 Oct;38(10):696–704.

14. Rehwinkel J, Gack MU. RIG-I-like receptors: their regulation and roles in RNA sensing. Nat Rev Immunol. 2020 Sep;20(9):537–51.

15. Wang B, Tian Y, Yin Q. AIM2 Inflammasome Assembly and Signaling. Adv Exp Med Biol.

2019;1172:143–55.

16. Santos JC, Boucher D, Schneider LK, Demarco B, Dilucca M, Shkarina K, et al. Human GBP1 binds LPS to initiate assembly of a caspase-4 activating platform on cytosolic bacteria. Nat Commun. 2020 Jun 24;11(1):3276.

17. Wandel MP, Kim B-H, Park E-S, Boyle KB, Nayak K, Lagrange B, et al. Guanylate-binding proteins convert cytosolic bacteria into caspase-4 signaling platforms. Nat Immunol. 2020 Aug;21(8):880–91.

18. Fitzgerald KA, Kagan JC. Toll-like Receptors and the Control of Immunity. Cell. 2020 Mar 19;180(6):1044–66.

19. Schroder K, Sagulenko V, Zamoshnikova A, Richards AA, Cridland JA, Irvine KM, et al. Acute lipopolysaccharide priming boosts inflammasome activation independently of inflammasome sensor induction. Immunobiology. 2012 Dec;217(12):1325–9.

20. Christgen S, Place DE, Kanneganti T-D. Toward targeting inflammasomes: insights into their regulation and activation. Cell Res. 2020 Apr;30(4):315–27.

21. Steimle A, Autenrieth IB, Frick J-S. Structure and function: Lipid A modifications in commensals and pathogens. Int J Med Microbiol. 2016 Aug;306(5):290–301.

22. Di Lorenzo F, Duda KA, Lanzetta R, Silipo A, De Castro C, Molinaro A. A Journey from Structure to Function of Bacterial Lipopolysaccharides. Chem Rev [Internet]. 2021 Jul 21; Available from: http://dx.doi.org/10.1021/acs.chemrev.0c01321

23. Park BS, Song DH, Kim HM, Choi B-S, Lee H, Lee J-O. The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. Nature. 2009 Apr 30;458(7242):1191–5.

24. Andreakos E, Sacre SM, Smith C, Lundberg A, Kiriakidis S, Stonehouse T, et al. Distinct pathways of LPS-induced NF-kappa B activation and cytokine production in human myeloid and nonmyeloid cells defined by selective utilization of MyD88 and Mal/TIRAP. Blood. 2004 Mar 15;103(6):2229–37.

25. Zamyatina A, Heine H. Lipopolysaccharide Recognition in the Crossroads of TLR4 and Caspase-4/11 Mediated Inflammatory Pathways. Front Immunol. 2020 Nov 27;11:585146.

26. Hassanzadeh-Kiabi N, Yáñez A, Dang I, Martins GA, Underhill DM, Goodridge HS. Autocrine Type I IFN Signaling in Dendritic Cells Stimulated with Fungal β-Glucans or Lipopolysaccharide Promotes CD8 T Cell Activation. J Immunol. 2017 Jan 1;198(1):375–82.

27. Venkatesh D, Ernandez T, Rosetti F, Batal I, Cullere X, Luscinskas FW, et al. Endothelial TNF receptor 2 induces IRF1 transcription factor-dependent interferon-β autocrine signaling to promote monocyte recruitment. Immunity. 2013 May 23;38(5):1025–37.

28. Banks DA, Ahlbrand SE, Hughitt VK, Shah S, Mayer-Barber KD, Vogel SN, et al. Mycobacterium tuberculosis Inhibits Autocrine Type I IFN Signaling to Increase Intracellular Survival. J Immunol. 2019 Apr 15;202(8):2348–59.

29. Shi J, Zhao Y, Wang Y, Gao W, Ding J, Li P, et al. Inflammatory caspases are innate immune receptors for intracellular LPS. Nature. 2014 Oct 9;514(7521):187–92.

30. Kayagaki N, Wong MT, Stowe IB, Ramani SR, Gonzalez LC, Akashi-Takamura S, et al. Noncanonical inflammasome activation by intracellular LPS independent of TLR4. Science. 2013 Sep 13;341(6151):1246–9.

31. McClure EE, Chávez ASO, Shaw DK, Carlyon JA, Ganta RR, Noh SM, et al. Engineering of obligate intracellular bacteria: progress, challenges and paradigms. Nat Rev Microbiol. 2017 Sep;15(9):544–58.

32. Weiss G, Schaible UE. Macrophage defense mechanisms against intracellular bacteria. Immunol Rev. 2015 Mar;264(1):182–203.

33. Lockwood TD. Distinction between major chloroquine-inhibitable and adrenergic-responsive pathways of protein degradation and their relation to tissue ATP content in the Langendorff isolated perfused rat heart. Biochem J. 1988 Apr 15;251(2):341–6.

34. Singh V, Finke-Isami J, Hopper-Chidlaw AC, Schwerk P, Thompson A, Tedin K. Salmonella Co-opts Host Cell Chaperone-mediated Autophagy for Intracellular Growth. J Biol Chem. 2017 Feb 3;292(5):1847–64.

35. Chong A, Starr T, Finn CE, Steele-Mortimer O. A role for the Salmonella Type III Secretion System 1 in bacterial adaptation to the cytosol of epithelial cells. Mol Microbiol. 2019 Oct;112(4):1270–83.

36. Günther SD, Fritsch M, Seeger JM, Schiffmann LM, Snipas SJ, Coutelle M, et al. Cytosolic Gram-negative bacteria prevent apoptosis by inhibition of effector caspases through lipopolysaccharide. Nat Microbiol. 2020 Feb;5(2):354–67.

37. Luchetti G, Roncaioli JL, Chavez RA, Schubert AF, Kofoed EM, Reja R, et al. Shigella ubiquitin ligase IpaH7.8 targets gasdermin D for degradation to prevent pyroptosis and enable infection. Cell Host Microbe. 2021 Oct 13;29(10):1521–30.e10.

38. Li Z, Liu W, Fu J, Cheng S, Xu Y, Wang Z, et al. Shigella evades pyroptosis by arginine ADP-riboxanation of caspase-11. Nature. 2021 Nov;599(7884):290–5.

39. Vanaja SK, Russo AJ, Behl B, Banerjee I, Yankova M, Deshmukh SD, et al. Bacterial Outer Membrane Vesicles Mediate Cytosolic Localization of LPS and Caspase-11 Activation. Cell. 2016 May 19;165(5):1106–19.

40. Bierschenk D, Boucher D, Schroder K. Salmonella-induced inflammasome activation in humans. Mol Immunol. 2017 Jun;86:38–43.

41. Clare B. Inflammasome activation by Salmonella. Curr Opin Microbiol. 2021 Dec;64:27–32.

42. Broz P, von Moltke J, Jones JW, Vance RE, Monack DM. Differential requirement for Caspase-1 autoproteolysis in pathogen-induced cell death and cytokine processing. Cell Host Microbe. 2010 Dec 16;8(6):471–83.

43. Lamkanfi M, Dixit VM. Mechanisms and functions of inflammasomes. Cell. 2014 May 22;157(5):1013–22.

44. Bateman G, Hill B, Knight R, Boucher D. Great balls of fire: activation and signalling of inflammatory caspases. Biochem Soc Trans. 2021 Jun 30;49(3):1311–24.

45. Ross C, Chan AH, von Pein JB, Maddugoda MP, Boucher D, Schroder K. Inflammatory Caspases: Toward a Unified Model for Caspase Activation by Inflammasomes. Annu Rev Immunol. 2022 Apr 26;40:249–69.

46. Boucher D, Monteleone M, Coll RC, Chen KW, Ross CM, Teo JL, et al. Caspase-1 self-cleavage is an intrinsic mechanism to terminate inflammasome activity. J Exp Med. 2018 Mar 5;215(3):827–40.

47. Chan AH, Schroder K. Inflammasome signaling and regulation of interleukin-1 family cytokines. J Exp Med [Internet]. 2020 Jan 6;217(1). Available from: http://dx.doi.org/10.1084/jem.20190314

48. Evavold CL, Ruan J, Tan Y, Xia S, Wu H, Kagan JC. The Pore-Forming Protein Gasdermin D Regulates Interleukin-1 Secretion from Living Macrophages. Immunity. 2018 Jan 16;48(1):35–44.e6.

49. Kayagaki N, Kornfeld OS, Lee BL, Stowe IB, O'Rourke K, Li Q, et al. NINJ1 mediates plasma membrane rupture during lytic cell death. Nature. 2021 Mar;591(7848):131–6.

50. Kim B-H, Shenoy AR, Kumar P, Das R, Tiwari S, MacMicking JD. A family of IFN-γ-inducible 65-kD GTPases protects against bacterial infection. Science. 2011 May 6;332(6030):717–21.

51. Ross C, Chan AH, Von Pein J, Boucher D, Schroder K. Dimerization and auto-processing induce caspase-11 protease activation within the non-canonical inflammasome. Life Sci Alliance. 2018 Dec;1(6):e201800237.

52. Marinho FV, Fahel JS, de Araujo ACVSC, Diniz LTS, Gomes MTR, Resende DP, et al. Guanylate binding proteins contained in the murine chromosome 3 are important to control mycobacterial infection. J Leukoc Biol. 2020 Oct;108(4):1279–91.

53. Meunier E, Dick MS, Dreier RF, Schürmann N, Kenzelmann Broz D, Warming S, et al. Caspase-11 activation requires lysis of pathogen-containing vacuoles by IFN-induced GTPases. Nature. 2014 May 15;509(7500):366–70.

54. Kayagaki N, Stowe IB, Lee BL, O'Rourke K, Anderson K, Warming S, et al. Caspase-11 cleaves gasdermin D for non-canonical inflammasome signalling. Nature. 2015 Oct 29;526(7575):666–71.

55. Kayagaki N, Warming S, Lamkanfi M, Vande Walle L, Louie S, Dong J, et al. Non-canonical inflammasome activation targets caspase-11. Nature. 2011 Oct 16;479(7371):117–21.

56. Schmid-Burgk JL, Gaidt MM, Schmidt T, Ebert TS, Bartok E, Hornung V. Caspase-4 mediates non-canonical activation of the NLRP3 inflammasome in human myeloid cells. Eur J Immunol. 2015 Oct;45(10):2911–7.

57. Gaggero A, De Ambrosis A, Mezzanzanica D, Piazza T, Rubartelli A, Figini M, et al. A novel isoform of pro-interleukin-18 expressed in ovarian tumors is resistant to caspase-1 and -4 processing. Oncogene. 2004 Sep 30;23(45):7552–60.

58. Pedra JHF, Sutterwala FS, Sukumaran B, Ogura Y, Qian F, Montgomery RR, et al. ASC/PYCARD and caspase-1 regulate the IL-18/IFN-gamma axis during Anaplasma phagocytophilum infection. J Immunol. 2007 Oct 1;179(7):4783–91.

59. Sellin ME, Müller AA, Hardt W-D. Consequences of Epithelial Inflammasome Activation by Bacterial Pathogens. J Mol Biol. 2018 Jan 19;430(2):193–206.

60. Kobayashi T, Ogawa M, Sanada T, Mimuro H, Kim M, Ashida H, et al. The Shigella OspC3 effector inhibits caspase-4, antagonizes inflammatory cell death, and promotes epithelial infection. Cell Host Microbe. 2013 May 15;13(5):570–83.

61. Wandel MP, Pathe C, Werner EI, Ellison CJ, Boyle KB, von der Malsburg A, et al. GBPs Inhibit Motility of Shigella flexneri but Are Targeted for Degradation by the Bacterial Ubiquitin Ligase IpaH9.8. Cell Host Microbe. 2017 Oct 11;22(4):507–18.e5.

62. Thurston TLM, Matthews SA, Jennings E, Alix E, Shao F, Shenoy AR, et al. Growth inhibition of cytosolic Salmonella by caspase-1 and caspase-11 precedes host cell death. Nat Commun. 2016 Nov 3;7:13292.

63. Baker PJ, Boucher D, Bierschenk D, Tebartz C, Whitney PG, D'Silva DB, et al. NLRP3 inflammasome activation downstream of cytoplasmic LPS recognition by both caspase-4 and caspase-5. Eur J Immunol. 2015 Oct;45(10):2918–26.

64. Wiggins KA, Parry AJ, Cassidy LD, Humphry M, Webster SJ, Goodall JC, et al. IL-1α cleavage by inflammatory caspases of the noncanonical inflammasome controls the senescence-associated secretory phenotype. Aging Cell. 2019 Jun;18(3):e12946.

65. Broz P, Ruby T, Belhocine K, Bouley DM, Kayagaki N, Dixit VM, et al. Caspase-11 increases susceptibility to Salmonella infection in the absence of caspase-1. Nature. 2012 Oct 11;490(7419):288–91.

66. Kumari P, Russo AJ, Wright SS, Muthupalani S, Rathinam VA. Hierarchical cell-type-specific

functions of caspase-11 in LPS shock and antibacterial host defense. Cell Rep. 2021 Apr 20;35(3):109012.

67. Deng M, Tang Y, Li W, Wang X, Zhang R, Zhang X, et al. The Endotoxin Delivery Protein HMGB1 Mediates Caspase-11-Dependent Lethality in Sepsis. Immunity. 2018 Oct 16;49(4):740–53.e7.

68. Chen KW, Monteleone M, Boucher D, Sollberger G, Ramnath D, Condon ND, et al. Noncanonical inflammasome signaling elicits gasdermin D-dependent neutrophil extracellular traps. Sci Immunol [Internet]. 2018 Aug 24;3(26). Available from: http://dx.doi.org/10.1126/sciimmunol.aar6676

69. McDonald B, Urrutia R, Yipp BG, Jenne CN, Kubes P. Intravascular neutrophil extracellular traps capture bacteria from the bloodstream during sepsis. Cell Host Microbe. 2012 Sep 13;12(3):324–33.

70. Kaplan MJ, Radic M. Neutrophil extracellular traps: double-edged swords of innate immunity. J Immunol. 2012 Sep 15;189(6):2689–95.

71. Chen Z, Zhang H, Qu M, Nan K, Cao H, Cata JP, et al. Review: The Emerging Role of Neutrophil Extracellular Traps in Sepsis and Sepsis-Associated Thrombosis. Front Cell Infect Microbiol. 2021 Mar 17;11:653228.

72. Silva CM, Wanderley CWS, Veras FP, Sonego F, Nascimento DC, Gonçalves AV, et al. Gasdermin D inhibition prevents multiple organ dysfunction during sepsis by blocking NET formation. Blood [Internet]. 2021 Aug 18; Available from: http://dx.doi.org/10.1182/blood.2021011525

73. Dougan G, Baker S. Salmonella enterica serovar Typhi and the pathogenesis of typhoid fever. Annu Rev Microbiol. 2014;68:317–36.

74. Rauch I, Deets KA, Ji DX, von Moltke J, Tenthorey JL, Lee AY, et al. NAIP-NLRC4 Inflammasomes Coordinate Intestinal Epithelial Cell Expulsion with Eicosanoid and IL-18 Release via Activation of Caspase-1 and -8. Immunity. 2017 Apr 18;46(4):649–59.

75. Sollberger G, Strittmatter GE, Kistowska M, French LE, Beer H-D. Caspase-4 is required for activation of inflammasomes. J Immunol. 2012 Feb 15;188(4):1992–2000.

76. Srisaowakarn C, Pudla M, Ponpuak M, Utaisincharoen P. Caspase-4 Mediates Restriction of Burkholderia pseudomallei in Human Alveolar Epithelial Cells. Infect Immun [Internet]. 2020 Feb 20;88(3). Available from: http://dx.doi.org/10.1128/IAI.00868-19

77. Winsor N, Krustev C, Bruce J, Philpott DJ, Girardin SE. Canonical and noncanonical inflammasomes in intestinal epithelial cells. Cell Microbiol. 2019 Nov;21(11):e13079.

78. Dilucca M, Ramos S, Shkarina K, Santos JC, Broz P. Guanylate-Binding Protein-Dependent Noncanonical Inflammasome Activation Prevents Burkholderia thailandensis-Induced Multinucleated Giant Cell Formation. MBio. 2021 Aug 31;12(4):e0205421.

79. Spits H, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, et al. Innate lymphoid cells--a proposal for uniform nomenclature. Nat Rev Immunol. 2013 Feb;13(2):145–9.

80. Kiessling R, Klein E, Pross H, Wigzell H. 'Natural' killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell. Eur J Immunol. 1975 Feb;5(2):117–21.

81. Herberman RB, Nunn ME, Holden HT, Lavrin DH. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. II. Characterization of effector cells. Int J Cancer. 1975 Aug 15;16(2):230–9.

82. Moro K, Yamada T, Tanabe M, Takeuchi T, Ikawa T, Kawamoto H, et al. Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells. Nature. 2010 Jan 28;463(7280):540–4.

83. Neill DR, Wong SH, Bellosi A, Flynn RJ, Daly M, Langford TKA, et al. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. Nature. 2010 Apr 29;464(7293):1367–70.

84. Price AE, Liang H-E, Sullivan BM, Reinhardt RL, Eisley CJ, Erle DJ, et al. Systemically dispersed innate IL-13-expressing cells in type 2 immunity. Proc Natl Acad Sci U S A. 2010 Jun 22;107(25):11489–94.

85. Artis D, Spits H. The biology of innate lymphoid cells. Nature. 2015 Jan 15;517(7534):293–301.

86. Colonna M. Innate Lymphoid Cells: Diversity, Plasticity, and Unique Functions in Immunity. Immunity. 2018 Jun 19;48(6):1104–17.

87. Daussy C, Faure F, Mayol K, Viel S, Gasteiger G, Charrier E, et al. T-bet and Eomes instruct the development of two distinct natural killer cell lineages in the liver and in the bone marrow. J Exp Med. 2014 Mar 10;211(3):563–77.

88. Barlow JL, Bellosi A, Hardman CS, Drynan LF, Wong SH, Cruickshank JP, et al. Innate IL-13-producing nuocytes arise during allergic lung inflammation and contribute to airways hyperreactivity. J Allergy Clin Immunol. 2012 Jan;129(1):191–8.e1–4.

89. Klein Wolterink RGJ, Kleinjan A, van Nimwegen M, Bergen I, de Bruijn M, Levani Y, et al. Pulmonary innate lymphoid cells are major producers of IL-5 and IL-13 in murine models of allergic asthma. Eur J Immunol. 2012 May;42(5):1106–16.

90. Kim HY, Chang Y-J, Subramanian S, Lee H-H, Albacker LA, Matangkasombut P, et al. Innate lymphoid cells responding to IL-33 mediate airway hyperreactivity independently of adaptive immunity. J Allergy Clin Immunol. 2012 Jan;129(1):216–27.e1–6.

91. Halim TYF, Krauss RH, Sun AC, Takei F. Lung natural helper cells are a critical source of Th2 cell-type cytokines in protease allergen-induced airway inflammation. Immunity. 2012 Mar 23;36(3):451–63.

92. Buonocore S, Ahern PP, Uhlig HH, Ivanov II, Littman DR, Maloy KJ, et al. Innate lymphoid cells drive interleukin-23-dependent innate intestinal pathology. Nature. 2010 Apr 29;464(7293):1371–5.

93. Vivier E, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, et al. Innate Lymphoid Cells: 10 Years On. Cell. 2018 Aug 23;174(5):1054–66.

94. Walker JA, Clark PA, Crisp A, Barlow JL, Szeto A, Ferreira ACF, et al. Polychromic Reporter Mice Reveal Unappreciated Innate Lymphoid Cell Progenitor Heterogeneity and Elusive ILC3 Progenitors in Bone Marrow. Immunity. 2019 Jul 16;51(1):104–18.e7.

95. Ferreira ACF, Szeto ACH, Heycock MWD, Clark PA, Walker JA, Crisp A, et al. RORα is a critical checkpoint for T cell and ILC2 commitment in the embryonic thymus. Nat Immunol. 2021 Feb;22(2):166–78.

96. Gasteiger G, Fan X, Dikiy S, Lee SY, Rudensky AY. Tissue residency of innate lymphoid cells in lymphoid and nonlymphoid organs. Science. 2015 Nov 20;350(6263):981–5.

97. Happel KI, Dubin PJ, Zheng M, Ghilardi N, Lockhart C, Quinton LJ, et al. Divergent roles of IL-23 and IL-12 in host defense against Klebsiella pneumoniae. J Exp Med. 2005 Sep 19;202(6):761–9.

98. Fort MM, Cheung J, Yen D, Li J, Zurawski SM, Lo S, et al. IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies in vivo. Immunity. 2001 Dec;15(6):985–95.

99. Hurst SD, Muchamuel T, Gorman DM, Gilbert JM, Clifford T, Kwan S, et al. New IL-17 family members promote Th1 or Th2 responses in the lung: in vivo function of the novel cytokine IL-25. J Immunol. 2002 Jul 1;169(1):443–53.

100. Schmitz J, Owyang A, Oldham E, Song Y, Murphy E, McClanahan TK, et al. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T

helper type 2-associated cytokines. Immunity. 2005 Nov;23(5):479–90.

101. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. Nat Immunol. 2008 May;9(5):503–10.

102. Mjösberg J, Spits H. Human innate lymphoid cells. J Allergy Clin Immunol. 2016 Nov;138(5):1265–76.

103. Chiossone L, Dumas P-Y, Vienne M, Vivier E. Natural killer cells and other innate lymphoid cells in cancer. Nat Rev Immunol. 2018 Nov;18(11):671–88.

104. Le-Barillec K, Magalhaes JG, Corcuff E, Thuizat A, Sansonetti PJ, Phalipon A, et al. Roles for T and NK cells in the innate immune response to Shigella flexneri. J Immunol. 2005 Aug 1;175(3):1735–40.

105. Mody CH, Ogbomo H, Xiang RF, Kyei SK, Feehan D, Islam A, et al. Microbial killing by NK cells. J Leukoc Biol. 2019 Jun;105(6):1285–96.

106. Björkström NK, Strunz B, Ljunggren H-G. Natural killer cells in antiviral immunity. Nat Rev Immunol. 2022 Feb;22(2):112–23.

107. Xu W, Cherrier DE, Chea S, Vosshenrich C, Serafini N, Petit M, et al. An Id2RFP-Reporter Mouse Redefines Innate Lymphoid Cell Precursor Potentials. Immunity. 2019 Apr 16;50(4):1054–68.e3.

108. Kim S, Iizuka K, Kang H-SP, Dokun A, French AR, Greco S, et al. In vivo developmental stages in murine natural killer cell maturation. Nat Immunol. 2002 Jun;3(6):523–8.

109. Chiossone L, Chaix J, Fuseri N, Roth C, Vivier E, Walzer T. Maturation of mouse NK cells is a 4-stage developmental program. Blood. 2009 May 28;113(22):5488–96.

110. Hayakawa Y, Smyth MJ. CD27 dissects mature NK cells into two subsets with distinct responsiveness and migratory capacity. J Immunol. 2006 Feb 1;176(3):1517–24.

111. Gordon SM, Chaix J, Rupp LJ, Wu J, Madera S, Sun JC, et al. The transcription factors T-bet and Eomes control key checkpoints of natural killer cell maturation. Immunity. 2012 Jan 27;36(1):55–67.

112. Townsend MJ, Weinmann AS, Matsuda JL, Salomon R, Farnham PJ, Biron CA, et al. T-bet regulates the terminal maturation and homeostasis of NK and Valpha14i NKT cells. Immunity. 2004 Apr;20(4):477–94.

113. Jenne CN, Enders A, Rivera R, Watson SR, Bankovich AJ, Pereira JP, et al. T-bet-dependent S1P5 expression in NK cells promotes egress from lymph nodes and bone marrow. J Exp Med. 2009 Oct 26;206(11):2469–81.

114. Robbins SH, Tessmer MS, Van Kaer L, Brossay L. Direct effects of T-bet and MHC class I expression, but not STAT1, on peripheral NK cell maturation. Eur J Immunol. 2005 Mar;35(3):757–65.

115. Ohne Y, Silver JS, Thompson-Snipes L, Collet MA, Blanck JP, Cantarel BL, et al. IL-1 is a critical regulator of group 2 innate lymphoid cell function and plasticity. Nat Immunol. 2016 Jun;17(6):646–55.

116. Klose CSN, Kiss EA, Schwierzeck V, Ebert K, Hoyler T, d'Hargues Y, et al. A T-bet gradient controls the fate and function of CCR6-RORγt+ innate lymphoid cells. Nature. 2013 Feb 14;494(7436):261–5.

117. Gotthardt D, Trifinopoulos J, Sexl V, Putz EM. JAK/STAT Cytokine Signaling at the Crossroad of NK Cell Development and Maturation. Front Immunol. 2019 Nov 12;10:2590.

118. Bernard NF, Kiani Z, Tremblay-McLean A, Kant SA, Leeks CE, Dupuy FP. Natural Killer (NK) Cell Education Differentially Influences HIV Antibody-Dependent NK Cell Activation and Antibody-Dependent Cellular Cytotoxicity. Front Immunol. 2017 Aug 24;8:1033.

119. Myers JA, Miller JS. Exploring the NK cell platform for cancer immunotherapy. Nat Rev Clin Oncol. 2021 Feb;18(2):85–100.

120. Lanier LL. NKG2D Receptor and Its Ligands in Host Defense. Cancer Immunol Res. 2015 Jun;3(6):575–82.

121. Guo Y, Luan L, Patil NK, Wang J, Bohannon JK, Rabacal W, et al. IL-15 Enables Septic Shock by Maintaining NK Cell Integrity and Function. J Immunol. 2017 Feb 1;198(3):1320–33.

122. Abel AM, Yang C, Thakar MS, Malarkannan S. Natural Killer Cells: Development, Maturation, and Clinical Utilization. Front Immunol. 2018 Aug 13;9:1869.

123. Grudzien M, Rapak A. Effect of Natural Compounds on NK Cell Activation. J Immunol Res. 2018 Dec 25;2018:4868417.

124. Adib-Conquy M, Scott-Algara D, Cavaillon J-M, Souza-Fonseca-Guimaraes F. TLR-mediated activation of NK cells and their role in bacterial/viral immune responses in mammals. Immunol Cell Biol. 2014 Mar;92(3):256–62.

125. Scott MJ, Hoth JJ, Gardner SA, Peyton JC, Cheadle WG. Natural killer cell activation primes macrophages to clear bacterial infection. Am Surg. 2003 Aug;69(8):679–86; discussion 686–7.

126. Lai H-C, Chang C-J, Lin C-S, Wu T-R, Hsu Y-J, Wu T-S, et al. NK Cell-Derived IFN-γ Protects against Nontuberculous Mycobacterial Lung Infection. J Immunol. 2018 Sep 1;201(5):1478–90.

127. Guo Y, Patil NK, Luan L, Bohannon JK, Sherwood ER. The biology of natural killer cells during sepsis. Immunology. 2018 Feb;153(2):190–202.

128. Lin PL, Flynn JL. CD8 T cells and Mycobacterium tuberculosis infection. Semin Immunopathol. 2015 May;37(3):239–49.

129. Gao Y, Williams AP. Role of Innate T Cells in Anti-Bacterial Immunity. Front Immunol. 2015 Jun 11;6:302.

130. Joyee AG, Qiu H, Wang S, Fan Y, Bilenki L, Yang X. Distinct NKT cell subsets are induced by different Chlamydia species leading to differential adaptive immunity and host resistance to the infections. J Immunol. 2007 Jan 15;178(2):1048–58.

131. Skeen MJ, Ziegler HK. Induction of murine peritoneal gamma/delta T cells and their role in resistance to bacterial infection. J Exp Med. 1993 Sep 1;178(3):971–84.

132. Rasi V, Wood DC, Eickhoff CS, Xia M, Pozzi N, Edwards RL, et al. Granzyme A Produced by γ9δ2 T Cells Activates ER Stress Responses and ATP Production, and Protects Against Intracellular Mycobacterial Replication Independent of Enzymatic Activity. Front Immunol. 2021 Aug 3;12:712678.

133. Medzhitov R. Recognition of microorganisms and activation of the immune response. Nature. 2007 Oct 18;449(7164):819–26.

134. Deets KA, Vance RE. Inflammasomes and adaptive immune responses. Nat Immunol. 2021 Apr;22(4):412–22.

135. Deets KA, Nichols Doyle R, Rauch I, Vance RE. Inflammasome activation leads to cDC1-independent cross-priming of CD8 T cells by epithelial cell-derived antigen. Elife [Internet]. 2021 Dec 23;10. Available from: http://dx.doi.org/10.7554/eLife.72082

136. Gazit R, Mandal PK, Ebina W, Ben-Zvi A, Nombela-Arrieta C, Silberstein LE, et al. Fgd5

identifies hematopoietic stem cells in the murine bone marrow. J Exp Med. 2014 Jun 30;211(7):1315–31.

137. Kim EY, Ner-Gaon H, Varon J, Cullen AM, Guo J, Choi J, et al. Post-sepsis immunosuppression depends on NKT cell regulation of mTOR/IFN-γ in NK cells. J Clin Invest. 2020 Jun 1;130(6):3238–52.

138. Zhivaki D, Borriello F, Chow OA, Doran B, Fleming I, Theisen DJ, et al. Inflammasomes within Hyperactive Murine Dendritic Cells Stimulate Long-Lived T Cell-Mediated Anti-tumor Immunity. Cell Rep. 2020 Nov 17;33(7):108381.

139. Maertzdorf J, Tönnies M, Lozza L, Schommer-Leitner S, Mollenkopf H, Bauer TT, et al. Mycobacterium tuberculosis Invasion of the Human Lung: First Contact. Front Immunol. 2018 Jun 12;9:1346.

140. Matthiesen S, Zaeck L, Franzke K, Jahnke R, Fricke C, Mauermeir M, et al. Coxiella burnetii-Infected NK Cells Release Infectious Bacteria by Degranulation. Infect Immun [Internet]. 2020 Oct 19;88(11). Available from: http://dx.doi.org/10.1128/IAI.00172-20

141. Theresine M, Patil ND, Zimmer J. Airway Natural Killer Cells and Bacteria in Health and Disease. Front Immunol. 2020 Sep 25;11:585048.

142. Swann JB, Hayakawa Y, Zerafa N, Sheehan KCF, Scott B, Schreiber RD, et al. Type I IFN contributes to NK cell homeostasis, activation, and antitumor function. J Immunol. 2007 Jun 15;178(12):7540–9.

143. Dalod M, Salazar-Mather TP, Malmgaard L, Lewis C, Asselin-Paturel C, Brière F, et al. Interferon alpha/beta and interleukin 12 responses to viral infections: pathways regulating dendritic cell cytokine expression in vivo. J Exp Med. 2002 Feb 18;195(4):517–28.

144. Gunesch JT, Dixon AL, Ebrahim TA, Berrien-Elliott MM, Tatineni S, Kumar T, et al. CD56 regulates human NK cell cytotoxicity through Pyk2. Elife [Internet]. 2020 Jun 8;9. Available from: http://dx.doi.org/10.7554/eLife.57346

145. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. Trends Immunol. 2001 Nov;22(11):633–40.

146. Crinier A, Narni-Mancinelli E, Ugolini S, Vivier E. SnapShot: Natural Killer Cells. Cell. 2020 Mar 19;180(6):1280–1280.e1.

147. Orrantia A, Terrén I, Izquierdo-Lafuente A, Alonso-Cabrera JA, Sandá V, Vitallé J, et al. A NKp80-Based Identification Strategy Reveals that CD56neg NK Cells Are Not Completely Dysfunctional in Health and Disease. iScience. 2020 Jul 24;23(7):101298.

148. Vitale M, Falco M, Castriconi R, Parolini S, Zambello R, Semenzato G, et al. Identification of NKp80, a novel triggering molecule expressed by human NK cells. Eur J Immunol. 2001 Jan;31(1):233–42.

149. Mandelboim O, Porgador A. NKp46. Int J Biochem Cell Biol. 2001 Dec;33(12):1147–50.

150. Mian MF, Lauzon NM, Andrews DW, Lichty BD, Ashkar AA. FimH can directly activate human and murine natural killer cells via TLR4. Mol Ther. 2010 Jul;18(7):1379–88.

151. Cichocki F, Bjordahl R, Gaidarova S, Mahmood S, Abujarour R, Wang H, et al. iPSC-derived NK cells maintain high cytotoxicity and enhance in vivo tumor control in concert with T cells and anti-PD-1 therapy. Sci Transl Med [Internet]. 2020 Nov 4;12(568). Available from: http://dx.doi.org/10.1126/scitranslmed.aaz5618

152. Crinier A, Milpied P, Escalière B, Piperoglou C, Galluso J, Balsamo A, et al. High-Dimensional Single-Cell Analysis Identifies Organ-Specific Signatures and Conserved NK Cell Subsets in Humans and Mice. Immunity. 2018 Nov 20;49(5):971–86.e5.

153. Chen Y, Lu S, Zhang Y, Yu J, Deng L, Chen H, et al. TLR2 agonist Pam3CSK4 enhances the antibacterial functions of GM-CSF induced neutrophils to methicillin-resistant Staphylococcus aureus. Microb Pathog. 2019 May;130:204–12.

154. Yokose K, Sato S, Asano T, Yashiro M, Kobayashi H, Watanabe H, et al. TNF-α potentiates uric acid-induced interleukin-1β (IL-1β) secretion in human neutrophils. Mod Rheumatol. 2018 May;28(3):513–7.

155. Newman KC, Riley EM. Whatever turns you on: accessory-cell-dependent activation of NK cells by pathogens. Nat Rev Immunol. 2007 Apr;7(4):279–91.

156. Guarda G, Braun M, Staehli F, Tardivel A, Mattmann C, Förster I, et al. Type I interferon inhibits interleukin-1 production and inflammasome activation. Immunity. 2011 Feb 25;34(2):213–23.

157. Rühl S, Shkarina K, Demarco B, Heilig R, Santos JC, Broz P. ESCRT-dependent membrane repair negatively regulates pyroptosis downstream of GSDMD activation. Science. 2018 Nov 23;362(6417):956–60.

158. Chen KW, Groß CJ, Sotomayor FV, Stacey KJ, Tschopp J, Sweet MJ, et al. The neutrophil NLRC4 inflammasome selectively promotes IL-1β maturation without pyroptosis during acute Salmonella challenge. Cell Rep. 2014 Jul 24;8(2):570–82.

159. Zanoni I, Tan Y, Di Gioia M, Broggi A, Ruan J, Shi J, et al. An endogenous caspase-11 ligand elicits interleukin-1 release from living dendritic cells. Science. 2016 Jun 3;352(6290):1232–6.

160. Rathkey JK, Benson BL, Chirieleison SM, Yang J, Xiao TS, Dubyak GR, et al. Live-cell visualization of gasdermin D-driven pyroptotic cell death. J Biol Chem. 2017 Sep 1;292(35):14649–58.

161. Coll RC, Hill JR, Day CJ, Zamoshnikova A, Boucher D, Massey NL, et al. MCC950 directly targets the NLRP3 ATP-hydrolysis motif for inflammasome inhibition. Nat Chem Biol. 2019 Jun;15(6):556–9.

162. Hagar JA, Powell DA, Aachoui Y, Ernst RK, Miao EA. Cytoplasmic LPS activates caspase-11: implications in TLR4-independent endotoxic shock. Science. 2013 Sep 13;341(6151):1250–3.

163. Zhao Y, Yang J, Shi J, Gong Y-N, Lu Q, Xu H, et al. The NLRC4 inflammasome receptors for bacterial flagellin and type III secretion apparatus. Nature. 2011 Sep 14;477(7366):596–600.

164. Kortmann J, Brubaker SW, Monack DM. Cutting Edge: Inflammasome Activation in Primary Human Macrophages Is Dependent on Flagellin. J Immunol. 2015 Aug 1;195(3):815–9.

165. Sakaguchi S, Negishi H, Asagiri M, Nakajima C, Mizutani T, Takaoka A, et al. Essential role of IRF-3 in lipopolysaccharide-induced interferon-beta gene expression and endotoxin shock. Biochem Biophys Res Commun. 2003 Jul 11;306(4):860–6.

166. Münzer P, Negro R, Fukui S, di Meglio L, Aymonnier K, Chu L, et al. NLRP3 Inflammasome Assembly in Neutrophils Is Supported by PAD4 and Promotes NETosis Under Sterile Conditions. Front Immunol. 2021 May 28;12:683803.

167. Thiam HR, Wong SL, Qiu R, Kittisopikul M, Vahabikashi A, Goldman AE, et al. NETosis proceeds by cytoskeleton and endomembrane disassembly and PAD4-mediated chromatin decondensation and nuclear envelope rupture. Proc Natl Acad Sci U S A. 2020 Mar 31;117(13):7326–37.

168. Heinzel FP, Rerko RM, Ling P, Hakimi J, Schoenhaut DS. Interleukin 12 is produced in vivo during endotoxemia and stimulates synthesis of gamma interferon. Infect Immun. 1994 Oct;62(10):4244–9.

169. Christaki E, Diza E, Giamarellos-Bourboulis EJ, Papadopoulou N, Pistiki A, Droggiti D-I, et al. NK and NKT Cell Depletion Alters the Outcome of Experimental Pneumococcal Pneumonia:

Relationship with Regulation of Interferon-γ Production. J Immunol Res. 2015 May 31;2015:532717.

170. Fleischmann C, Scherag A, Adhikari NKJ, Hartog CS, Tsaganos T, Schlattmann P, et al. Assessment of Global Incidence and Mortality of Hospital-treated Sepsis. Current Estimates and Limitations. Am J Respir Crit Care Med. 2016 Feb 1;193(3):259–72.

171. Downs KP, Nguyen H, Dorfleutner A, Stehlik C. An overview of the non-canonical inflammasome. Mol Aspects Med. 2020 Dec;76:100924.

172. Cecconi M, Evans L, Levy M, Rhodes A. Sepsis and septic shock. Lancet. 2018 Jul 7;392(10141):75–87.

173. Xiong L, Wang S, Dean JW, Oliff KN, Jobin C, Curtiss R 3rd, et al. Group 3 innate lymphoid cell pyroptosis represents a host defence mechanism against Salmonella infection. Nat Microbiol. 2022 Jul;7(7):1087–99.

174. Rathinam VAK, Vanaja SK, Waggoner L, Sokolovska A, Becker C, Stuart LM, et al. TRIF licenses caspase-11-dependent NLRP3 inflammasome activation by gram-negative bacteria. Cell. 2012 Aug 3;150(3):606–19.