The role of innate immune system receptors in smoking related disease.

A thesis submitted to the Imperial College London for the degree of Doctor of Philosophy.

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DECLARATION

I, Katarzyna Parzych, hereby declare that the work presented in this thesis is my own. Information derived from other sources and work done in collaboration has been appropriately cited.

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This thesis is dedicated to my parents, Anna and Tadeusz

for their love, endless support

and encouragement.

ABSTRACT

Cigarette smoke induces inflammation, in part, by activation of Toll like receptor (TLR) 2 and TLR4. TLRs are present on most cells and recognize pathogen associated molecular patterns (PAMPs) and non-pathogenic molecules, known as danger associated molecular patterns (DAMPs). Activation of TLRs results in the propagation of a signalling pathway leading to the de novo synthesis of pro-inflammatory mediators that include CXCL8 and IL-1β. TLRs initiate inflammation and are involved in tissue repair processes in a number of diseases such as chronic obstructive pulmonary disease (COPD) and cancer. Therefore, it is the aim of this thesis to explore the contribution of TLRs in inflammation and cellular proliferation, as these processes are integral to the pathology in inflammatory lung disease and cancer.

Activation of IL-1β is a two-step process. The first step synthesises pro-IL-1β and can be activated by TLR ligands. The second induces inflammasome complex assembly and results in the cleavage of pro-IL-1β to mature IL-1β by caspase-1. IL-1β is implicated in the inflammation caused by bacteria during exacerbations in lung disease. Interestingly, we found a difference in the activation and release of IL-1β from monocytes activated with the TLR2 ligand, Pam3CSK4 and the TLR4 ligand, LPS. The difference was seen at the level of inflammasome assembly, where, unlike TLR4, TLR2-induced IL-1β release was reliant on the hemichannel protein, pannexin-1.

We have previously found that cigarette smoke causes sensitisation of the blood of smokers to bacterial and viral PAMPs. Cigarette smoke is the major contributing factor in COPD and it is known that bacterial and viral induced exacerbations play a major role in the deterioration of lung function in these patients. COPD patients are also more prone to lung infections than their healthy age-matched equivalents. We therefore hypothesised that blood from COPD patients is more sensitive to challenge with bacterial and viral

PAMPs than blood from age-matched controls. COPD patient's blood had an increased sensitivity to challenge with TLR2/1 and TLR3 ligands with respect to IL-1β release. Regarding CXCL8 release in this system, a greater response to stimulation with TLR2/1, TLR3, TLR4 agonists and IL-1β was observed in blood from COPD patients. There was a negative correlation between CXCL8 levels and COPD GOLD staging in response to TLR4 agonism.

TLR2 has been implicated in the proliferation of cancer cells. There is an emerging interest in the link between chronic inflammation and cancer. It is well documented that COPD patients have an increased risk of developing lung cancer, which has poor prognosis. Our aim was to investigate whether TLRs are a novel drug target for inhibiting the proliferation of lung cancer cells. TLR2, 3, 4 and 7 agonists had no effect on lung cancer cell proliferation; however, activation of TLR8 induced apoptosis in these cells.

In conclusion, my thesis shows that TLRs are important receptors involved in the initiation of inflammation and inhibition of cellular proliferation. We show, for the first time, that TLR4-induced CXCL8 release in COPD patients may be a good biomarker of disease severity, and that TLR8 offers a good prospect as an adjuvant in the treatment of lung cancer.

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Others:

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LIST OF ABBREVATIONS

ACAT1	acetyl-Coenzyme A acetyltransferase
Akt	serine/threonine-specific protein kinase
AM	alveolar macrophage
ANOVA	analysis of variance
AP-1	activating protein-1
APC	antigen presenting cell
apoE	apolipoprotein E
ASK	activator of S phase kinase
ASM	airway smooth muscle
ATF	activating transcription factor
ATP	adenosine-5'-triphosphate
BCG	Bacillus Calmette–Guérin
BCL	B-cell lymphoma 6
BclxL	B-cell lymphoma-extra large
BLF1	Burkholderia pseudomallei toxin
BMDM	bone-marrow-derived macrophage
BP	binding protein
BSA	bovine serum albumin
BTSM	bovine tracheal smooth muscle
CAF	carcinoma-associated fibroblasts
CARD	caspase recruitment domain
CASP	caspase
COPD	chronic obstructive pulmonary disease
CCL	chemokine (C-C motif) ligand
CD	cluster of differentiation
cDC	classical dendritic cell
cGMP	cyclic guanosine monophosphate
CHX	cycloheximide
cIAP	cellular inhibitor of apoptosis
COX	cyclooxygenase
CRP	C-reactive protein

CS	cigarette smoke
CSE	cigarette smoke extract
CTD-PAH	PAH in association with connective tissue disease
CTR	control
CXCL	chemokine (C-X-C motif) ligand
CYLD	cylindromas
DAMP	danger associated molecular pattern
DAPI	4',6-diamidino-2-phenylindole
DB	dissociation buffer
DC	dendritic cell
DMEM	dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DR	death receptor
dsRNA	double-stranded RNA
DUBA	deubiquitinase
ECACC	European collection of cell cultures
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular-signal-regulated kinase
FACS	fluorescence-activated cell sorting
FADD	Fas-Associated protein with death domain
FCS	foetal calf serum
FEV1	forced expiratory volume
FITC	fluorescein isothiocyanate
FLICE	FADD-like interleukin-1β converting enzyme
FLIP	FLICE-like inhibitory protein
FSL-1	
FVC	forced vital capacity
G	gap
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GOLD	global initiative for obstructive lung disease

HepG2	liver hepatocellular cells
HMGB	high mobility group
HMOX	heme oxygenase
HPV	human papillomavirus
HSP	heat shock protein
HTSMC	human tracheal smooth muscle cells
IBD	inflammatory bowel disease
ICAM	intercellular adhesion molecule 1
IFN	Interferon
IFNAR	interferon-α/β receptor
Ig	immunoglobulin
IKK	IκB kinase
IL	interleukin
IL-1R	interleukin-1 receptor
iNOS	inducible nitric oxide synthases
CXCL10	IFN-inducible protein 10
iPAH	idiopathic pulmonary arterial hypertension
IRAK	IL-1R-associated kinase
IRF	interferon –regulatory factor
ISGF	interferon-stimulated gene factor 3
IκB	inhibitor of κB
JNK	c-Jun N-terminal kinase
LAM	lipoarabinomannan
LBP	LPS-binding protein
LDL	low-density lipoprotein
LLC	Lewis lung carcinoma
LPS	lipopolysaccharides
LRR	leucine-rich repeat
LTA	lipoteichoic acid
M	mitosis
MAL	MyD88 adaptor-like protein
MALP-2	macrophage-activating lipopeptide
MAPK	mitogen-activated protein kinase

MC	medium-age control
MCP-1	monocyte chemoattractant protein-1
MHC	major histocompatibility complex
MIP-1	macrophage inflammatory protein 1
MMPs	matrix metalloproteinase
mRNA	messenger RNA
MSU	monosodium urate
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
MyD88	myeloid differentiation primary response protein 88
NACHT	
NADP	nicotinamide adenine dinucleotide phosphate
NALP	NACHT domain, leucine-rich-repeat and PYD-containing protein
NCoR	nuclear co-repressor
NF-κB	nuclear factor κB
ND	not detected
NK	natural killer
NLR	NOD-like receptors
NLRC4	NLR family CARD domain-containing protein 4
NLRP3	NOD-like receptor family, pyrin domain containing 3
NO	nitric oxide
NOD	nucleotide-binding and oligomerisation domain
NOS	nitric oxide synthase
NS	not significant
NYHA	New York Heart Association
OC	older control
ODN	oligodeoxynucleotides
OSCC	oral squamous cell carcinoma
P2X7R	purinoreceptor 7
PI3K	phosphoinositide 3-kinase
PAH	pulmonary arterial hypertension
PAMP	pathogen-associated molecular pattern
Panx-1	pannexin-1
PAT	pulmonary artery thrombosis

PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pDC	plasmacytoid dendritic cells
PFA	paraformaldehyde
PG	prostaglandin
PGN	peptidoglycan
PI	Propidium iodide
PIN1	peptidyl-prolyl cis/trans isomerase
PNM	polymorphonuclear
Poly I:C	polyinosine-polycytidylic acid
PPAR	peroxisome proliferator-activated receptor
PRR	pattern recognition receptor
PYD	pyrin domain
QOL	quality of life
qPCR	quantitative polymerase chain reaction
RA	rheumatoid arthritis
RAC1	Ras-related C3 botulinum toxin substrate
RANTES	regulated on activation, normal T cell expressed and secreted
REDOX	reduction-oxidation
RIP	receptor-interacting protein
ROS	reactive oxygen species
RNA	ribonucleic acid
RP105	radioprotective protein 105
RPMI	Roswell Park Memorial Institute medium
RT	reverse transcriptase
RVSP	right ventricle systolic pressure
S	synthesis
SARM	sterile α and HEAT–Armadillo motifs
S.E.M.	standard error of the mean
siRNA	small interfering RNA
SMC	smooth muscle cells
SNP	single-nucleotide polymorphism

SOCS-1	suppressor of cytokine signalling 1
Src	non-receptor tyrosine kinase
ssRNA	single-stranded RNA
STAT-1	signal transducer and activator of transcription
TAB	TAK1-binding protein
TAK	TGFβ-activated kinase
TAM	tumor-associated macrophage
Tc	T cytotoxic cell
TGF	transforming growth factor
TIR	Toll/IL-1R
TLR	Toll-like receptor
Th	T helper cell
TIRAP	TIR-domain-containing adaptor protein
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TOLLIP	Toll interacting protein
TRAF	tumor necrosis factor-associated factor
TRAIL	TNF-related apoptosis-inducing ligand
TRAM	TRIF-related adaptor molecule
TReg	T-regulatory cell
TRIAD3A	Triad domain-containing protein 3
TRIF	TIR-domain-containing adaptor protein inducing IFN-β
TRIM30α	tripartite-motif protein
TYK	tyrosine kinase
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
VSMC	vascular smooth muscle cell
xIAP	X-linked inhibitor of apoptosis
YC	young control

Chapter 1

Introduction

1.1. Inflammation: a historical perspective. (Reviewed by Spector and Willougby, 1968)

Inflammation is a localised response to a tissue injury or infection. The processes involved in inflammatory response are necessary for the elimination of pathogens or other inflammatory factors, clearing the damage tissue components and initiating the repair process. During the process of inflammation, activated immune cells release factors leading to the well-know clinical symptoms of inflammation [1]. The term *edema* was introduced by Hippocrates in the 5th century BC. The main four signs of inflammation: redness, heat, swelling and pain, were first described by Celsus, a Roman writer who lived between 30BC and 45 AD. Eighteenth-century applications of the microscope lead to descriptions of blood flow changes in inflamed tissue and the proposal by Gaubius that inflammation can promote the “disposition to coagulation.” The term angiogenesis was first time introduced by John Hunter in 1794 to describe the development of growing vessels in healing wounds, followed by description of leukocyte rolling attributed to Wagner in 1839. The contemporary understanding of this process we owe to 19th-century milestone discoveries by Rudolph Virchow, Julius Cohnheim and Elie Metchnikoff, who gave rise to cell-based definitions of inflammation [2, 3]. In the 20th century, the development of new technological tools allowed the rapid expansion of knowledge of the cells and mediators of inflammatory processes, as well as the molecular mechanisms of the microvascular responses to inflammation. Development of molecular biology and immunology, gene-targeted knock-out mice, discovery of cytokines, chemokines, and their receptors, as well as leukocyte and endothelial cell adhesion molecules, have proven to be immensely useful in the research of molecular mechanisms of inflammation *in vivo*.

1.2. The molecular basis of inflammation.

In the acute phase, inflammation is characterized by increased blood flow and vascular permeability, accumulation of fluid, leukocytes, and inflammatory mediators such as cytokines and chemokines. The development of specific humoral and cellular immune responses to pathogens present at the site of tissue injury is seen in the chronic phase of inflammatory response. During both processes, a variety of soluble factors are released and involved in leukocyte recruitment through expression of cellular adhesion molecules and chemoattractants. Many of these soluble mediators regulate the activation of fibroblasts, endothelial cells, tissue macrophages, and mast cells. These newly recruited inflammatory cells such as monocytes, lymphocytes, neutrophils, and eosinophils and also some of mediators released by these cells, result in the systemic responses to the inflammatory process [4].

1.2.1. Acute inflammation.

Mast cells, tissue-resident macrophages, DCs, stromal cells, white blood cells and endothelial cells are responsible for the vascular and cellular changes necessary for the initiation of inflammation. After the injury or infection they are recruited to the inflammatory lesion and they release factors such as cytokines, chemokines, histamine and prostaglandins [5]. Histamine released from mast cells acts on target tissues to induce vasodilation, which elevates the regional blood flow to the affected area, and increases vascular permeability. It allows an extravasation of neutrophils, and leakage of plasma to the affected tissue. This results in an accumulation of fluid and plasma proteins into the inflamed tissue through large venular junction gaps. Cytokines released during the inflammatory process can also induce acute phase proteins such as C-reactive protein (CRP) and coagulation factor which in turn activate production of prostaglandins. PGE_2 is a major proinflammatory prostaglandin which promotes so-called sickness behaviour:

fever, anorexia, fatigue and sleepiness [6]. Some prostaglandins affect the aggregation of platelets, which is a part of the clotting process, preventing the spread of infectious agents. Activated immune cells release more chemical substances recruiting dendritic cells, macrophages and neutrophils to remove foreign debris by phagocytosis, antigen processing and secretion of cytokines which play a role in slower lymphocyte-mediated adaptive immune response [7].

The adaptive immune system does not form the major part of this thesis, it is described only briefly. The adaptive immune system is comprised of T and B lymphocytes which act as a host surveillance mechanism against invading pathogens [8]. The innate immune responses can activate adaptive immunity by increasing the flow of lymph containing antigens and antigen presenting cells (APC). The peptide fragments are then carried to the surface of the presenting cell on special molecules called major histocompatibility complex (MHC) proteins, which present the fragments to T cells [9]. B lymphocytes mediate antibody responses and are activated to secrete immunoglobulins. These antibodies can circulate in bloodstream and recognize foreign antigen that stimulated their production. T cells are responsible for the cell-mediated immune response. They react directly against a foreign antigen that is presented to them on the surface of a host cell, eliminating the infected cells before they had a chance to replicate. T cells can also produce signals to activate macrophages to destroy the microbes that have been phagocytosed [9]. There are two main classes of T cells—cytotoxic T cells (Tc) and helper T cells (Th). Effector Tc cells directly kill cells that are infected with a virus or some other intracellular pathogen. Effector Th cells, by contrast, help stimulate the responses of other cells—mainly macrophages, B cells, and Tc cells. Components on the surfaces of the microbes can also induce changes in phagocytic cells to synergize with lymphocytes whose receptors bind to specific microbial agents. Dendritic cell upon activation matures into a highly effective antigen-presenting cell, and undergoes changes that enable it to

activate pathogen-specific lymphocytes. Macrophages and DCs can also activate T lymphocytes and initiate adaptive immune response [10].

The cross-talk between innate and adaptive immunity can be bidirectional. Atherosclerosis is a disease where major cytokines are produced by both, innate and adaptive immunity acting upon one another. Interferon (IFN) γ produced by effector Th1 cells activates macrophages and interleukin (IL)-4 and IL-5 produced by Th2 cells may stimulate macrophage and DC subsets [11]. On the other hand, IL-12, IL-18, and type I IFNs promote the differentiation of Th1 cells, and IL-12 can stimulate the production of IFNγ by natural killer (NK) cells [12, 13]. CXCL10, which is produced by T cells, dendritic cells, and macrophages, inhibits IL-12 production and Th1-mediated inflammation [14].

1.2.2. Chronic inflammation.

Acute inflammation is rapid in onset and lasts as long as a few days. Chronic inflammation is characterized by persistent inflammation and lasts from a few days to a few years. It can cause a considerable damage to the tissues and interferes with the function of various organs [15]. Pro-inflammatory signalling pathways have the capacity to induce the parallel expression of anti-inflammatory mediators, such as CXCL10. A deficiency in any of negative regulators may result in exaggerated inflammatory response to insult or injury that culminates in severe inflammation and damage to the host. Genetic studies in mice have shown that even the absence of one negative regulator is sufficient to result in serious inflammatory disorders [16]. Thus, aberrations in such negative regulatory pathways can contribute to the development of chronic inflammatory diseases. The phagocytosis of apoptotic cells plays an important role in the negative regulation of macrophage activation; apoptotic leucocytes may well fit into the category of endogenous anti-inflammatory mediators; therefore, the mechanisms of apoptosis and the clearance of apoptotic cells may be critical in the development of chronic inflammation [16].

Proliferation of endothelial cells resulting in the formation of new capillaries (a process known as angiogenesis) leads to restoration of the vascular supply, and promotes the proliferation of fibroblasts which restores the connective tissue matrix. These important processes involved in the chronic inflammatory cycle are orchestrated by mediators secreted by resident cells, neutrophils, macrophages, T and B lymphocytes, plasma cells and platelets [17-20]. The spectrum of inflammatory conditions can shift from acute inflammatory reactions in response to wounds and infections to chronic inflammatory disease such as (COPD), atherosclerosis, asthma, type 2 diabetes and cancer [6]. In all these patients, systemic inflammation is considered a key factor in the pathogenesis of the multicomponent disease manifestations. Inflammation is not just isolated to the affected organ, but also results in an elevation of inflammatory mediators in the circulation, which is responsible for systemic effects observed in most inflammatory diseases [21].

1.3. Toll-like receptors.

Toll-like receptors (TLRs) are type I transmembrane receptors that recognize structurally conserved pathogen associated molecular patterns (PAMPs). They participate in the first line of defence against invading pathogens and play a significant role in inflammation, immune cell regulation, survival and proliferation [22]. TLRs are human homologues of the *Drosophila* protein Toll. This protein controls the dorsal-ventral patterning in the developing fly embryo and is critical for the fly's recognition of Gram-positive bacteria and fungi, and the production of anti-microbial peptides [23]. TLRs posses extracellular leucin-rich repeat (LRR) domain which consist of varying numbers of repeats, each 24–29 amino acids in length, containing the motif *XX*L*X*L*XX* and other conserved leucines [24]. This domain is involved in the recognition of a variety of pathogens and is followed by a single transmembrane region. The carboxyterminal intracellular tail contains a conserved region of ~200 amino acids called the Toll/interleukin-1 receptor (TIR) homology domain which is

also found in the IL-1 receptor (Fig. 1). TIR domain binds one or more of four adaptor proteins and mediates signal transduction [23, 24]. In 2001 a new adaptor was described; sterile α and HEAT–Armadillo motifs (SARM) [25] which it is a negative regulator of nuclear factor κB (NF-κB) and interferon regulatory factor (IRF) activation [26].

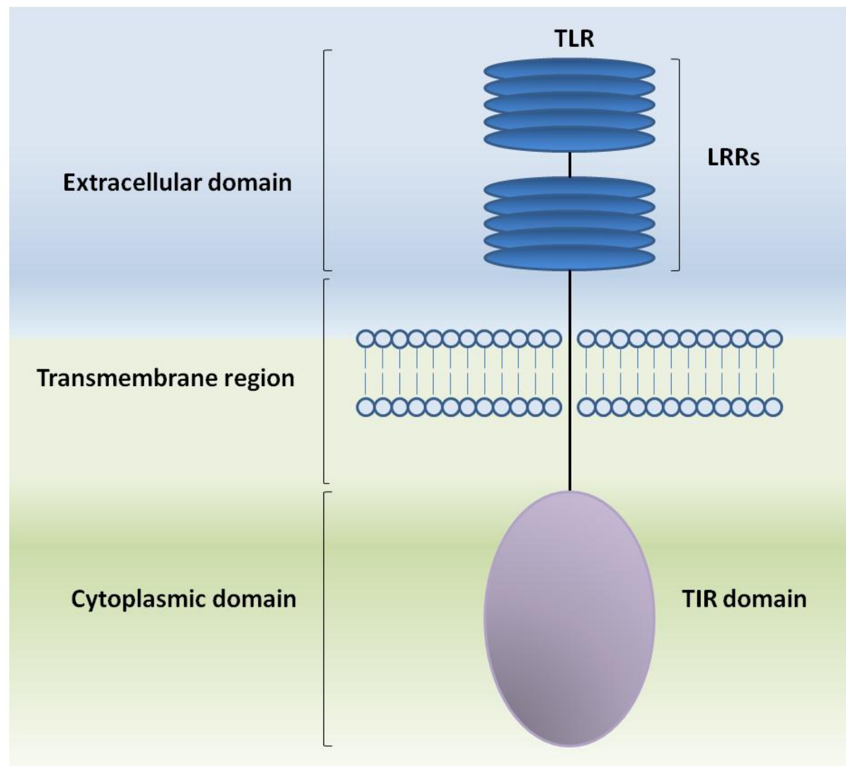


Figure 1. Structure of Toll-like receptors.

Apart from pathogenic ligands, TLR can also recognize non-pathogenic molecules; known as danger associated molecular patterns (DAMPs) which can contribute to sterile inflammation. Many studies showed that these endogenous molecules or alarmins released from necrotic cells, activate leukocytes and play a critical role in inflammation [27]. The first suggestion of DAMP recognition came from studies by Erridge et al. where heat shock proteins (HSPs) were recognized by TLRs [28]. After that, a number of other endogenous ligands including the extra domain A of fibronectin and hyaluronic acid were

also shown to activate these receptors [29, 30]. Recognition of DAMPs by TLRs may also contribute to the autoimmune responses. The high mobility group box protein 1 (HMGB1) which normally resides in the cell nucleus can activate TLR2 and induce symptoms of lupus-like disease when released from apoptotic cells [31, 32].

There are 10 Toll-like receptors discovered in humans and they can be found in different subcellular compartments depending on the nature of the molecule they recognize. Endogenous viral and bacterial ligands for human TLRs are shown in Table 1. TLR1, TLR2, TLR4, TLR5, TLR6, TLR10 recognize bacterial, fungal and protozoan cell wall components and are localized to the cell membrane. Activation of these receptors leads to NF-κB activation and an innate immune response. TLRs that detect viral or microbial nucleic acids (TLR3, TLR7, TLR8, TLR9) are localized to intracellular membranes and are thought to encounter their ligands in phagosomes or endosomes. TLRs are expressed on monocytes/macrophages, DCs, NK cells and lymphocytes.

	Ligand(s)
TLR1/2	triacyl lipopeptides, soluble factors, OspA
TLR2	lipoproteins/lipopetides, peptodoglycan, lipoteichoic acid, glycolipids, zymosan, porins, atypical LPS, oxidants and many more
TLR3	dsRNA, Poly I:C
TLR4	LPS, HSP60, HSP70, taxol, envelope proteins, fibrinogen, heparin sulfate fragments
TLR5	flagellin
TLR2/6	diacyl lipopeptides
TLR7	ssRNA, imidazoquinoline, loxoribine (a guanosine analogue) bropirimine
TLR8	small synthetic compounds, ssRNA
TLR9	unmethylated CpG DNA
TLR10	triacyl lipopeptides and a wide variety of other microbial-derived agonists shared by TLR1, but not TLR6

Table 1. Toll-like receptors ligands. Adapted from Gay and Gangloff, 2007 [33].

Respiratory epithelium is the first line of defence against invasion of microorganisms, therefore it is not surprising that TLRs are also expressed on epithelial cells and play crucial role in innate immunity of the lung. Respiratory epithelium is continuously exposed to microbial challenges and environmental stress as a result of breathing, and constitutively express TLRs in alveolar and bronchial epithelial cells [34].

1.3.1. TLR1/2/6/10.

TLR2 is involved in recognition of broad range of products including peptidoglycan from Gram-positive bacteria, lipoteichoic acid (LTA) bacterial lipoproteins, zymosan, mycobacterial cell-wall components and yeast cell walls. [35]. TLR2 ligand recognition

involves the co-operation with other TLR family members, TLR1 and TLR6, but it is unknown whether dimerization of TLR2 with other TLRs occurs constitutively or if it is induced in response to ligand stimulation. TLR1 is highly homologous to TLR6 and both are able to heterodimer with TLR2. TLR2/1 heterodimer recognizes of triacyl peptides and lipoprotein from mycobacteria as macrophages from TLR1 deficient mice showed impaired production of inflammatory cytokines in response to these ligands. TLR1 is also responsible for recognizing the differences between lipid moieties of lipoproteins [36]. TLR2 co-operates with TLR6 in recognition of macrophage-activating lipopeptide-2 (MALP-2) and other diacetylated lipoproteins. TLR2-deficient macrophages are unresponsive to bacterial and mycoplasmal lipoproteins, whereas TLR6-deficient cells are unresponsive to MALP-2, but respond normally to bacterial lipoproteins [37]. A study by Hoebe et al. using forward-genetic mutagenesis demonstrated that TLR2/6 heterodimers also require CD36 to sense diacylated lipoproteins, whereas TLR2/1 heterodimers do not [38]. TLR1/2 heterodimers induce also a different immune response against pathogens as compared to TLR2/6 heterodimers. When TLR1/2 heterodimers were absent from cells, there was a reduction of early cytokine release. In contrast, TLR2/6 heterodimeric receptors played a role in modulating the balance between a Th1/Th2 immune response [39].

Recent studies showed that generation and analysis of chimeric receptors containing the extracellular recognition domain of TLR10 and the intracellular signalling domain of TLR1, senses triacylated lipopeptides and a wide variety of other microbial-derived agonists shared by TLR1, but not TLR6. TLR10 requires TLR2 for innate immune recognition, and these receptors colocalize in the phagosome and physically interact in an agonist-dependent fashion [40]. One major hindrance to TLR10 studies is that it lacks a rodent homologue [41]. This has hampered the assignation of a natural or synthetic ligand to TLR10 because the majority of TLR ligands have been defined using mutant or genetically

deficient mouse models. Thus, it has been speculated that TLR10 could potentially act as a TLR2 coreceptor [42, 43]. Polymorphisms in TLR10 have conferred increased susceptibility to a number of chronic inflammatory diseases, which include prostate and urothelial cancer (rs4129009) [44], asthma and atopy (1031G->A and 2322A->G) [45]

1.3.2. TLR3.

TLR3 recognizes double-stranded RNA (dsRNA) and has been studied in the context of immediate innate responses to virus infections [46]. It is a potent inducer of type I IFNs and it is expresses in conventional DCs (cDCs) and in a epithelial cells found in the airways, uterine and intestine. It can also been found in the brain, specifically in astrocytes and glioblastoma cells [47]. TLR3 uses the TIR domain containing adaptor inducing interferon-beta (TRIF) adaptor protein to activate IRF3, NF-κB and activating protein 1 (AP-1) transcription factors. Activation of these molecules leads to the release of hundreds of cellular genes including IFN genes [48]. TLR3 lacks the proline residue that is conserved among other TLRs [36]. This is particularly important for the immune responses to viruses that do not directly infect dendritic cells, as TLR3 was demonstrated to promote crosspriming of CD8 T cells in response to dsRNA [49].

1.3.3. TLR4.

TLR4 recognizes lipopolysaccharides (LPS) from Gram-negative bacteria, LTA and heat-sensitive cell-associated factor derived from *Mycobacterium Tuberculosis* and many non-pathogenic molecules such as HSP60 [35], fibrinogen and surfactant protein A (SP-A). It was the first functional TLR to be identified. There are two mouse stains, C3H/HeJ and C57BL10/ScCr that were known to be hyperresponsive to LPS. It was shown by two independent groups that a mutation in TLR4 gene was responsible for LPS resistance [36]. Recognition of LPS also requires another co-receptor, CD14, which is preferentially expressed in monocytes, macrophages and neutrophils, and which helps maneuver the

LPS-LPS-binding protein complex to TLR4 [50]. MD-2 is another molecule that is associated with the extracellular portion of TLR4 and enhances LPS responsiveness [51]. MD-2 associated TLR4 homodimers do not bind LPS directly, LPS must be first bound by soluble LPS binding protein (LBP) in a complex that then recruits CD14. The exact mechanism of this binding is not clear but it is suggested that LBP might transfer LPS to CD14 thereby facilitating the activation of TLR4 [52]. TLR4 possesses two options for signal transduction, either to signal via MyD88 or through TRIF. MyD88-dependent and MyD88-independent pathways utilize distinct adapter proteins but both pathways involve the activation and nuclear translocation of NF-κB which leads to the expression of numerous proinflammatory cytokines [53]. TLR4 is expressed in monocytes/macrophages, DCs, mast cells, B lymphocytes and also epithelial and endothelial cells [54].

1.3.4. TLR5.

Flagellin is a primary component of flagella, a structure that extends from the outer membrane of Gram-negative bacteria and is recognized by TLR5. Flagellin-induced immune responses are mediated by Myd88, which results in the activation of NF-κB [36]. It is the only TLR that is conserved in vertebrataes from fish to mammals and it is expressed in epithelial cells, monocytes and dendritic cells [55]. Gut epithelial cells are hyporesponsive to LPS, but respond well to flagellin, which induces a robust proinflammatory response. Flagellin has been suggested to be a major target of innate and adaptive immunity in Crohn’s disease and, in certain ethnicities, heterozygous carriage of a dominant negative allele for TLR5 is associated with a protection against this condition [56].

1.3.5. TLR7/8.

TLR7 and TLR8 show a high degree of homology to each other and recognize single-stranded RNA (ssRNA). Similarly to TLR3 and TLR9 they are expressed within endosomal membranes where they will encounter enveloped viruses [47]. Both TLR7 and TLR8 have been shown to be stimulated by sequences containing GU- or poly-U. Activation of these receptors results in NF-κB activation and induction of cytokines, chemokines, adhesion molecules and apoptosis-related proteins. Synthetic imidazoquinolines which are recognized by TLR7 are potent antiviral and antitumor agents and have the ability to induce type I IFNs such as IFN-α [36]. Moreover, TLR7 and TLR8 activation can induce IFNs in a NF-κB independent fashion [57]. TLR7 and TLR8 are expressed in DCs and epithelial cells. The strongest TLR8 mRNA expression was found in monocytes, monocytes derived DCs, macrophages and Langerans cells. TLR7 is strongly expresses in plasmacytoid DCs (pDCs) and human B cells. T cells also express TLR7 and TLR8 [58, 59].

1.3.6. TLR9.

Viral DNA is recognized by TLR9. The immune stimulatory activity of bacterial DNA is attributed to the unmethyleated CpG motifs which are not present in mammalian genome. Some studies have reported that CpG recognition occurs in the endosome and activation of signalling pathways such as c-Jun N-terminal kinase (JNK) and NF-κB is delayed compared with LPS-induced activation of macrophages [60, 61]. Upon TLR9 stimulation mediated by distinct classes of CpG oligonucleotides (ODNs), pDCs are activated to produce IFN-α/β and various chemokines. B lymphocytes are also induced to proliferate and secrete IgM and IL-6 [62]. There is variation in the results of studies investigating the subcellular location of TLR9. In dendritic cells and macrophages, TLR9 is located in the endoplasmic reticulum of resting cells but upon stimulation with CpG DNA is endocytosed

and moves to the lysosomal compartment and subsequently binds directly to TLR9. Surface expression of TLR9 has also been found to occur in human embryonic kidney 293 cells (HEK293) cells transfected with TLR9-containing vectors in gastric and intestinal epithelial cells, and in some peripheral blood mononuclear cells (PBMCs). Recently, a cytosolic innate immune response to DNA has been identified, which triggers a potent type I IFN response. It remains unknown whether expression or localization of TLR9 changes in response to native bacterial DNA [63].

1.4. TLR signalling.

Pathogen recognition by TLRs causes rapid activation of innate immunity by inducing the production of proinflammatory cytokines and the upregulation of costimulatory molecules [64]. Once TLRs are activated they recruit adaptor molecules that facilitate a signalling cascade, which results in the initiation of the innate immune response by the cell. There are five known adaptor proteins, four of which positively regulate signalling: MyD88, MyD88-adaptor-like (Mal), TRIF and TRAM, and sterile-alpha and Armadillo motif containing protein (SARM) that acts as a negative regulator of TLR activation [65]. TLR signalling pathways consist of a Myd88-dependent pathway that is common for all TLRs except TLR3, and an alternative pathway, Myd88-independent that is activated only by TLR4 and TLR3 [66].

1.4.1. Myd88-dependent signalling pathway.

Once the adaptor proteins are associated with the TLR complex, the IL-1 receptor-associated kinase (IRAK) family is recruited through interaction of the death domains of both molecules. IRAK1 and IRAK4 are activated by phosphorylation, and then actively associate with the ubiquitin E ligase TNF receptor associated factor 6 (TRAF6). TRAF6 becomes auto-ubiquitinated and forms a complex with TGFβ-activated kinase (TAK1)-binding protein (TAB2)/TAB3/TAK1, which in turn results in the activation of the MAP

kinase (MAP3K) and TAK-1. TAK-1 phosphorylates the kinases upstream of p38, MAPKs and JNK. In addition, TAK-1 can also activate the inhibitor of nuclear factor kappa-B (IKK) complex, which consists of IKKα and IKKβ and scaffolding protein IKKγ. Phosphorylation of the IκBα inhibitory unit leads to its degradation and the release of NF-κB that mediate the de novo synthesis of a number of proinflammatory cytokines. Moreover, TLR signalling pathway can activate other transcription factors such as AP-1 also leading to the release of proinflammatory cytokines (Fig. 2). TLR7/8/9 stimulation activate IRF5 and IRF7, which are transcription factors playing a role in antiviral defence, cell growth and immune regulation, and are transducers of virus-mediated TLR signalling [67, 68].

1.4.2. Myd88-independent pathway.

This pathway was first described when the study by Kawai el al. showed that Myd88 knockout mice responded to LPS stimulation and activated NF-κB and JNK with delayed kinetics [69]. TRIF interacts with receptor-interacting protein 1 (RIP1), TRAF6 and TRAF3 [67]. TRAF6 activation leads to the same signalling pathway as Myd88 activation, but TRAF3 associates with TRIF and two non-canonical IKKs, TBK1 and IKKε. These kinases preferentially phosphorylate the transcription factors IRF3 and IRF7. IRF3 translocates to the nucleus as a homodimer and induces the expression of IFNβ, which in turns activates the receptor for IFNα/β and the JAK/STAT pathway leading to the phosphorylation of IRF7. IRF7 is activated by TBK1/IKKε and induces the expression of IFNα/β, forming a positive feedback loop for the release of large amounts of type I IFNs [70].

1.4.3. Negative regulators of TLR signalling.

Excessive activation of TLR pathways can contribute to the pathogenesis of many human diseases, thus their regulation needs to be tightly controlled. There are several negative regulators of TLR pathways that have been described. The best-known example is the ubiquitin-modifying enzyme, Triad domain-containing protein 3 (triad3A), which can

degrade TLR3/4/5/9 using ubiquitin/proteasome machinery. Another example is suppressor of cytokine signalling-1 (SOCS-1), which interacts with the adaptor protein Mal, a key mediator of TLR4 and TLR2 signalling. SOCS-1 upregulation results in Mal degradation, thereby inhibiting Mal-mediated phosphorylation of the p65 subunit of NF-κB and thus the expression of NF-κB–dependent genes [71, 72]. Prolyl isomerase (Pin)1 suppresses TLR3 mediated and IRF3-dependent transcriptional activation. Tripartite-motif protein (TRIM30α) is another protein which stimulates degradation of TAB2 and TAB3 [73, 74]. Recent research provides increasing numbers of examples proving the importance of de-ubiquitinating enzymes in regulation of TLR signalling pathways. De-ubiquitinating enzyme A (DUBA) interacts with TRAF3 and inhibits the its self-ubiquitination. Cylindromatosis protein (CYLD) negatively regulated TLR-mediated immune and inflammatory responses by inhibiting activation of TRAF6 and TRAF7 [75, 76]. SARM was also shown to be a negative regulator of TRIF-dependent signalling [26]. Another example is IRF-4 which interacts with MyD88 and competes with IRF-5, but not with IRF-7, for MyD88 interaction [77]. Radioprotective 105 (RP105) and its helper molecule, MD-1, have a physical association with TLR4/MD2, and this association inhibits LPS–TLR4/MD2 complex formation [78]. TLR pathways can also be negatively regulated by IRAK-M, which associates with IRAK-1 and inhibits its signalling [79]. Recent research also demonstrated activating transcription factor-3 (ATF3) as a negative regulator not only in the TLR4-stimulated inflammatory response, but also in TLR2/6 heterodimer, TLR3, TLR5, TLR7, and TLR9 pathways [80]. Another example is Tollip which associates directly with TLR2 and TLR4 and inhibits TLR-mediated cell activation [81] [82].

TLRs have also been shown to regulate cell death. Activation of PI3K-Akt signalling pathway promotes cell survival and TLR-mediated increased expression of anti-apoptotic proteins Bcl-2-related protein A (BCL2A1), inhibitor of apoptosis 1 (cIAP1), cIAP2, X-linked inhibitor of apoptosis protein (XIAP) and other Bcl-2 family members [83].

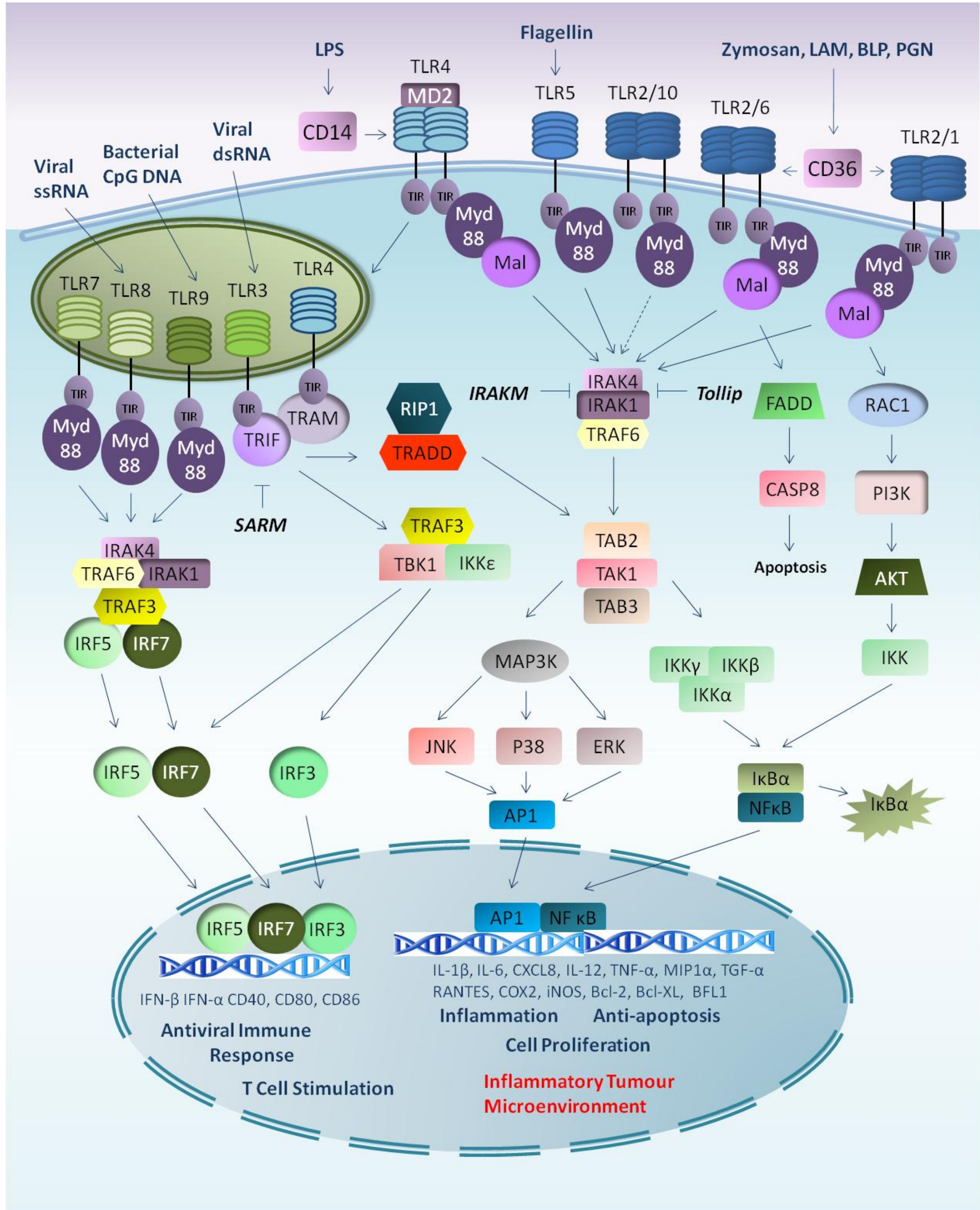


Figure 2. TLR signalling pathway. Adapted from O'Neill and Bowie, 2007 [65].

1.5. The role of TLRs in inflammasome activation.

It was described previously that recognition of PAMPs and DAMPs by the TLRs triggers a series of events that lead to the expression of many immune and inflammatory genes (Fig. 3). Activation of TLRs by various ligands leads to NF-κB activation which is needed to induce the expression of cytokines such as CXCL8 and IL-1β [84]. The release of IL-1β has to be tightly controlled, as excess levels of this cytokine not only causes fever, anorexia, and other constitutional symptoms but also triggers tissue damage and remodelling. IL-1β itself can drive the activity of cryopyrin, thereby inducing its own secretion leading to autoinflammatory diseases such as Muckle–Wells syndrome. It requires processing by the enzyme, caspase-1, which cleaves the inactive precursor, pro-IL-1β, to its mature form. Activation of caspase-1 takes place via a protein complex called the inflammasome [85]. Several protein platforms/inflammasomes have been described for the activation of caspase-1, each of them include members of the NOD-like receptor (NLR) family of proteins [86]. The NLR family is characterized by the presence of a central nucleotide-binding and oligomerization (NACHT) domain, which is involved in the regulation of homo-oligomerization or hetero-oligomerization and inflammasome assembly. It is flanked by C-terminal LRRs and N-terminal caspase recruitment (CARD) or pyrin (PYD) domains. LRRs function in ligand sensing and autoregulation, whereas CARD and PYD domains mediate homotypic protein-protein interactions for downstream signalling [87]. The most studied inflammasome, NACHT, LRR and PYD domains-containing protein 3 (NLRP3) can be activated by a variety of both, exogenous and endogenous signals. Activation of inflammasome requires two signals. A “priming” signal that results in transcription of pro-IL-1β, and a second signal that promotes inflammasome assembly, activation of caspase-1, followed by the maturation and secretion of IL-1β. The first signal is under NF-κB activation and can be achieved by TLRs stimulation, whereas numerous molecules can provide second signal including various pore-forming toxins,

amyloid-β, reactive oxygen species (ROS), adenosine triphosphate (ATP), K^+ efflux, as well as silica and uric acid crystals [88-90], however, the underlying mechanism of activation is poorly understood. The first danger-associated signal described was ATP that activates P2X7 ion channel receptors [91]. P2X7 receptors belong to a family of ion channel receptors activated by extracellular ATP [85]. ATP has a high intracellular concentration and is kept low in the extracellular space by the activity of ATPases. It is released from dying cells and activated platelets, and it is usually present locally in stressed tissues. Binding of extracellular ATP to the P2X purinoceptor 7 (P2X7R) leads to IL-1β maturation via the effects of purinergic stimulation of the hemichannel pannexin-1 (panx1), which forms large pores in the plasma membrane and acidifies endosomal compartments [1]. The hemichannel protein pannx1 was suggested to form large, nonselective pores after ATP stimulation of P2X7R, thereby allowing DAMPs and PAMPs access to cytosolic NLRP3 [92]. Inflammasomes have been extensively studied in recent years but more research is needed to investigate the role of various inflammasomes in human inflammatory diseases and infections.

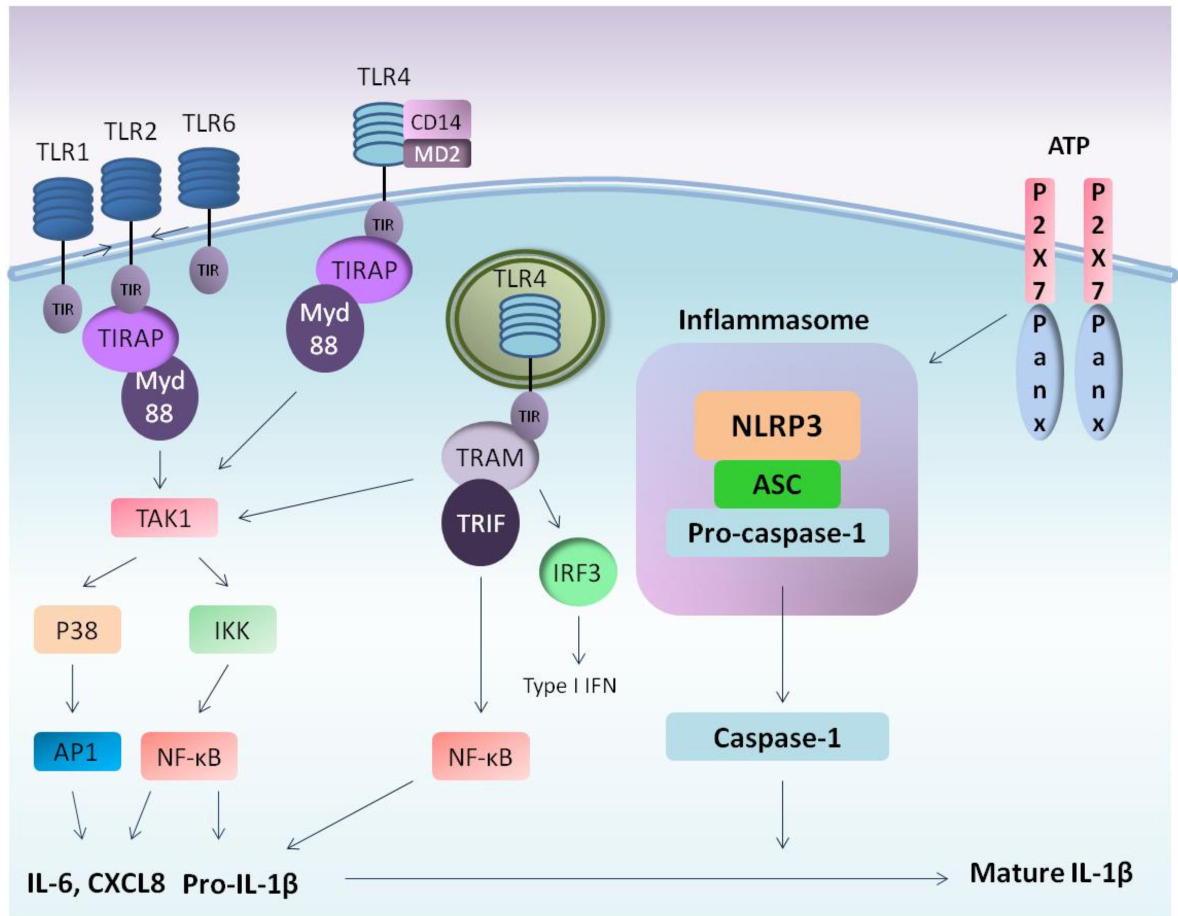


Figure 3. Activation of the inflammasome.

1.6. Smoking and inflammation.

Cigarette smoking is the major risk factor in many inflammatory diseases such as cardiovascular disease, COPD and a number of malignancies including lung adenocarcinoma. Cigarette smoke (CS) contains more than 4500 components in its gaseous and particulate phases including direct carcinogens (methylcholanthrene, benzo-α-pyrenes and acrolein); toxins (carbon monoxide, ammonia, acetone, nicotine and hydroquinone) and oxidants (superoxide and nitrogen oxides). It is chemically a highly reactive condensate that contains billions of oxidative moieties. Furthermore, CS affects the immune system by chemically modifying signalling pathways and the extracellular matrix through acetylation, nitrosylation, carbonylation and oxidation which can have an effect on cell survival, activation and differentiation. Cigarette smoke has been shown to

have both stimulatory and inhibitory effects on immune cell activation. These findings have made the interpretation of the literature related to animal models difficult to translate to the human situation [93]. In the innate immune system cigarette smoke increases the number of alveolar macrophages (AM) which upon activation release lysosomal enzymes and elastases. These enzymes contribute to the damage of connective tissue and parenchyma of the lung. In addition, AM from smokers produce more oxygen radicals and have higher myeloperoxidase activity and a reduced ability to phagocytose and kill bacteria [94, 95]. It was also shown that NK cells activity against cultured cancer cells was reduced in smokers and animal models [96, 97]. Moreover, cigarette smoking in humans can cause leukocytosis and greatly reduce the function of these cells [97]. Some studies also demonstrated that long-term smoking reduces serum levels of immunoglobulins, confirmed in animal models, as well as increasing susceptible to influenza and sarcoma viruses [97, 98]. T cells from smokers and animals that were exposed to CS have a decreased ability to proliferate in response to T cell mitogens, which indicates a deficient cell-mediated immune response [98, 99].

1.7. The role of TLRs in sensing oxidants.

Oxidants which can be found in CS play a part in damage caused by inflammation but it is not fully understood how exactly oxidants are sensed and propagate the inflammatory reaction. A specific function for TLR2 as a 'receptor' for oxidants was first suggested by Frantz et al. using cardiac myocytes or fibroblasts stimulated with H_2O_2, where it was shown that activation of NF-κB by H_2O_2 was only seen in TLR2-expressing cells and was prevented after blocking of TLR2 with antibody against this receptor [100]. After this study, many other papers showed that inflammatory responses after cigarette smoke exposure were mediated by TLRs. A study by Karimi et al. showed that human monocytes and monocyte-derived macrophages produced high amounts of CXCL8 after cigarette smoke stimulation. CXCL8 production was dependent on TLR4 activation where LPS was not

present. Moreover, neutralization of TLR4 but not TLR2 inhibited CS medium-induced CXCL8 secretion by human macrophages [101]. Another study demonstrated that CS increased the expression of TLR4 and NF-κB activation from the bronchial epithelial cell line (16-HBE) [102]. A number of studies from our group, using animal models, showed that oxidant-induced inflammation associated with cigarette smoke extract or ozone was absent in TLR2 or TLR4 knockout animals, suggesting that both TLR2 or TLR4 could act as sensors for oxidants [103-106]. In 2009, study from our group showed that oxidants evoked the release of CXCL8 from monocytes/macrophages which was abrogated by pretreatment with N-acetylcysteine or binding antibodies to TLR2. Oxidative activation of TLRs was associated with a rapid phosphorylation of IRAK1. Oxidants added to HEK293 cells transfected with TLR2, TLR1/2, or TLR2/6 but not TLR4/MD2-CD14 or control HEK nulls also resulted in the release of CXCL8. These findings clearly substantiate the hypothesis that TLRs are the sensors of early oxidant-induced inflammation. The involvement of TLR4 in oxidant-induced inflammation *in vivo*, was shown at later time points (≥6h), and it may be due to a secondary inflammatory insult mediated by bacterial leakage from the gut or the lungs [64]. TLR9 has also been implicated in TLR-mediated inflammatory responses after CS exposure. It was demonstrated using neutrophils and HEK293 cells, that CS induced CXCL8 release via TLR9. It also increased expression of TLR9 and this upregulation was suppressed by N-acetylcysteine [107]. These results were confirmed by Nadigel et al. where CD8+ T cells exposed to CS had increased TLR4 and TLR9 levels and increased cytokine production [108]. Cigarette smoke also induces the release of ATP and activates P2X7 receptor signal transduction pathways of human neutrophils [109]. A study by Mortaz et al. demonstrated that the inflammasome activator monosodium urate crystals (MSU) induced the release of CXCL8 and IL-1β from HBE-14o and that the caspase-1 inhibitor, Z-VADDCB, suppressed the CS-induced release of CXCL8. In addition, CS, CpG ODN, LPS and monosodium urate crystals (MSU) all increased the expression of caspase-1 and IL-1β suggesting that CS releases CXCL8

from HBE-14o cells via TLR4, TLR9 and inflammasome activation [110]. Similar results were obtained by Eltom et al. where mice were exposed to CS twice a day to induce COPD-like inflammation and the role of the P2X7 receptor was investigated. CS-induced neutrophilia in a pre-clinical model was temporally associated with increased caspase-1 activity and the release of IL-1β/IL-18 in the lungs. A selective P2X7 receptor antagonist and P2X7 knockout mice, attenuated caspase-1 activation, IL-1β release and airway neutrophilia in response to CS. This pathway was not restricted to early stages of disease development by showing increased caspase-1 activation in lungs from a more chronic exposure to CS and from patients with COPD. These results demonstrated the critical role of the P2X7/caspase-1 and inflammasome in CS-induced inflammation [111] [112]. Cigarette smoke has also been shown to induce COX-2 in lung epithelial cells in vitro in a TLR4-dependent manner [113].

1.8. The role of TLRs in smoking related disease.

The role of TLRs has been implicated in many diseases induced by cigarette smoking. This may be through the involvement of TLRs in innate and adaptive immunity. Here, I describe two diseases, which are relevant to my thesis, where TLRs may play a role in disease pathogenesis and may therefore provide an attractive therapeutic target for drug development.

1.8.1. Chronic obstructive pulmonary disease (COPD).

It is well known that cigarette smoking is a major risk for developing COPD. COPD is a chronic inflammatory disease which includes chronic obstructive bronchitis with fibrosis and obstruction of small airways, emphysema with enlargement of airspaces, destruction of lung parenchyma, loss of lung elasticity, and closure of small airways. It is characterized by the presence of CD8+ cytotoxic T cells and Th1 cells [114]. Upon stimulation of epithelial cells and macrophages by noxious particles, these cells release

high levels of pro-inflammatory cytokines such as CXCL8, IL-6, TNF-α and monocyte chemoattractant protein-1 (CCL2) that recruit neutrophilic granulocytes and macrophages [115]. Activated neutrophils release inflammatory mediators such as serine proteases contributing to tissue destruction [116].

Exacerbations of COPD are episodes of worsening of symptoms, accompanied by a reduction in FEV1, which impose a substantial burden on health-care systems worldwide; they are a major cause of morbidity, mortality, and reduced health status. They are now the most common cause of medical hospital admission in the UK, at a cost to the National Health System of over £253 million a year. COPD exacerbations are heterogeneous events that are now thought to be caused by complex interactions between the host, respiratory viruses, airway bacteria, and environmental pollution, leading to an increase in the inflammatory burden [117]. The first step in COPD management is the reduction of risk factors. Smoking cessation is currently the only effective therapy for a decline of COPD progression. Inhaled long acting bronchodilators in combination with glucocorticosteroids form the basis of symptomatic treatment in COPD but a lot of patients show insensitivity to these drugs [116]. Pulmonary hypertension (PH) is a common complication of COPD. The increase in pulmonary artery pressures is often mild to moderate. However, 5–10% of patients with advanced COPD may suffer from severe pulmonary hypertension and present with a progressively downhill clinical status because of right heart failure added to ventilatory handicap [118]. Since TLRs play a role in the defence against pathogens and other danger molecules, they might be important in pathogenesis of COPD as infections in airways worsen the disease process. This is normally associated with a clinical worsening of FEV1. TLR2 downregulation was found in alveolar macrophages from COPD patients and smokers [119]. However, another study showed increased expression of TLR2 in peripheral blood monocytes from COPD patients, suggesting a difference between alveolar and systemic effects in terms of TLR

expression [119, 120]. TLR4 gene expression was reduced in nasal epithelium of COPD patients and smokers [121]. However, in airway epithelium TLR4 expression was increased in subjects with mild-moderate COPD compared to normal controls, while with increasing severity of disease and fall in FEV1, TLR4 expression was reduced [122]. A recent study by Wang et al. demonstrated that TLR5 was downregulated at mRNA, protein and functional levels in airway epithelium in healthy smokers and in smokers with COPD [123]. COPD is also characterized by progressive and irreversible airway obstruction [124]. Small airway remodelling includes adventitial fibrosis and mucus hyperplasia and may involve increased airway smooth muscle cells mass [125]. It is also possible that the destruction of the epithelial cells and the release of growth factors cause thickening of the reticular basement membrane and proliferation of smooth muscle cells [126]. In addition to initiating inflammatory responses, TLRs have been shown to regulate cell proliferation and survival in a variety of biological settings. Most of the evidence for TLRs and proliferation in non-cancerous cells has been shown in vascular smooth muscle cells (VSMC) and in cardiovascular models. TLRs can regulate the compensatory proliferative responses and suppression of apoptosis [64]. The mechanisms responsible for the control of these outcomes are poorly defined. In 2007, Schultz and collaborators revealed that IL-1R signalling promotes basal and TLR agonist-stimulated CCL2 release, as well as TLR-induced proliferation of VSMCs. They demonstrated that IL-1α is a key mediator of the phenotypic responses of human VSMC to TLR agonist and that it also contributes to growth factor-induced proliferation in human vascular disorders [127]. Another study revealed that early aortic lipid accumulation and expression of proinflammatory mediators markedly dependend on TLR4, and to a lesser extent TLR2, at lesion prone sites in apolipoprotein E (ApoE)–deficient mice. Furthermore, VSMCs incubated in cholesterol-enriched media in vitro expressed CCL2 and the cholesterol-esterifying enzyme acyl-coenzyme A: cholesterol acyltransferase (ACAT1). VSMCs were also shown to accumulate intracellular cholesteryl ester in a TLR4-dependent fashion,

which suggests that TLR4 contributes to proinflammatory events and foam cell formation in early atherogenesis. A study by Pera et al. demonstrated that CSE and LPS, both of which can act via TLRs, induced a profound increase in DNA synthesis in bovine tracheal smooth muscle cells (BTSM) [125]. Since TLR4 is responsible for sensing LPS and CS they may contribute to airway remodelling in COPD through direct effects on airway smooth muscle (ASM) cells causing a proliferative phenotype that may be involved in increased ASM mass in this disease. There is a lot of evidence that TLRs provide a fascinating and rapidly expanding area of research; it is likely that therapies that target these receptors and their signalling could be of use in COPD [112].

1.8.2. Lung cancer.

Chronic infection and inflammation are widely known to be risk factors for tumorigenesis. Infiltrating host leukocytes, such as neutrophils, tumor-associated macrophages (TAMs), dendritic cells (DC), mast cells and lymphocytes are known to be present at tumor sites and in the surrounding stroma. The damage caused by invading pathogens induces destructive cycles that are initiated within tissues and result in excessive tissue remodeling, loss of function and in some cases DNA mutations. In this way chronic infection and inflammation are widely known to be risk factors for tumorigenesis [128]. Activated TLRs on cancer cells may promote cancer progression, anti-apoptotic activity and resistance to host immune response. Ligation of TLRs increases production of proinflammatory mediators like TNFα, IL-1α, IL-6, intercellular adhesion molecule 1 (ICAM-1) and vascular endothelial growth factor (VEGF). Their presence releases various factors that have multiple effects on tumor, immune and normal cells. An activated immune system can induce cytokine release that stimulates cancer associates fibroblasts (CAFs) and impairs the function of APCs, effector T cells and TAA-specific immunity [129]. Summary of these effects maintain tumor environment and promote further progression. Recently, the expression or up-regulation of TLRs has been detected in

many tumor cell lines or tumors, especially epithelial derived cancers. Several studies have shown a link between TLR activation and tumor growth. For example, Kim and collaborators demonstrated that the proteoglycan versican, which is upregulated in many human tumors, was produced by Lewis Lung Carcinoma (LLC) and strongly enhanced LLC metastatic growth through activation of TLR2/6 and the secretion of TNF-α [130]. TLR2, TLR3, TLR6, and TLR9 were consistently expressed in the hepatocellular carcinoma cell line, Hep G2. mRNA for TLR4 was also detected in these cells. Breast cancer cell responsiveness to LPS suggests that functional TLR4 can be expressed in breast cancer cells [131-133].

Lung cancer is one of the leading cause of death worldwide and only 15% of patients survive longer than 5 years from the time of diagnosis [134]. It is a non-immunogenic cancer, resistant to the surveillance of immune system, which should recognize and eliminate transformed cells [135]. A study by He et al. demonstrated that TLR4 was expressed on human lung cancer cell lines. TLR4 ligation promoted production of immunosuppressive cytokines such as transforming growth factor beta (TGF-β), VEGF and CXCL8. In addition, TLR4 ligation induced resistance of human lung cancer cells to TNF-α or TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis. p38 activation was necessary for increased VEGF and CXCL8 secretion, and NF-κB activation contributed to apoptosis resistance of human lung cancer cells induced by LPS [136]. Droemann and colleagues showed that TLR9 is expressed in the cytoplasm of tumor cells in the majority of lung cancer specimens as well as in all tested tumor cell lines. Non-malignant lung tissues showed only sporadically weak expression. Stimulation of HeLa and A549 cells with CpG ODN induced secretion of CCL2 and reduction of spontaneous and TNF-α-induced apoptosis [137]. The induction of Th1-like and cytotoxic immunity by TLR signalling should lead to tumor regression or arrest. However, results obtained from

basic research and clinical trials have suggested that there is no antitumor activity seen when TLRs are activated [125].

1.9. Therapeutic potential of TLRs.

TLRs play a key role in the detection of microbes and in sterile inflammation. Excessive TLR responses may have important consequences during infection, leading to exaggerated responses such as in chronic disease, as well as more acute settings such as severe sepsis. This knowledge has led to a great deal of research effort directed towards developing TLR agonists and antagonists as therapeutic targets for the prevention and treatment of diseases. Inappropriate TLR responses may have important consequences during infection, leading to exaggerated responses such as in severe sepsis [138]. To reduce signal transduction through TLRs in sepsis, different strategies are being developed. Firstly, soluble TLRs that can bind and neutralize the microbial ligands; secondly, antibodies neutralizing TLRs blocking the binding of the ligands; thirdly, small molecular mass inhibitors blocking signalling pathways activated by TLRs [139].

TLRs are also very attractive targets for the treatment of chronic inflammatory diseases that are caused by dysregulation of the immune system. TLR agonist or adjuvant therapy can be very successful, especially during episodes of exacerbations. Activation of TLRs can boost the protective inflammatory response that destroys pathogens and protects the host [116]. Certain TLR activators have also the potential to reverse Th2-type responses to allergens and thus, restore the balance of the immune system [140]. However, these two approaches may be harmful for the host and might amplify unwanted tissue destructive inflammation. TLR inhibitors are currently being developed for the treatment of inflammation. Antagonist therapies can downregulate inflammatory responses by blocking the interaction between TLRs and their ligands or by blocking the downstream signalling cascades. However, so far no studies have addressed a potential role for TLR antagonists

in the treatment of asthma or COPD specifically [116]. Better understanding of the pathways and mechanisms leading to inflammatory responses and chronic inflammation may lead to better therapeutic approaches for COPD. Further studies of the inflammatory signalling networks and the cross talk between them are important to develop a greater understanding of the pathogenesis of COPD. It is still debatable whether COPD occurs as a result of excessive inflammatory drive or a lack of inhibitory feedback loops. However, it is clear that many of these pathways are abnormally activated in COPD and that interference with these signalling pathways could shed light on disease processes and provide novel therapeutic approaches [141].

There is also a growing evidence to indicate therapeutic interest in TLR7/8 agonists for cancer treatment. In the 19th century William Coley observed that repeated injection with bacterial toxins from Staophylococcus pneumoniae and Serratia marcescens produced an efficient antitumor effect. It was discovered later that LPS from the Coley's toxins stimulated TLR4 and resulted in anti-tumor activity [83]. Nowadays, clinical trials investigating novel anticancer therapies are based on TLR ligand delivery. A successful example is imiquimod. This TLR7 agonist is used extensively to treat actinic keratosis and basal cell carcinoma, and it is being studied as an adjuvant therapy for melanoma. Imiquimod induces IFNα, IFNγ and IL-12, which activate APC function and TAA-specific immunity, thereby correcting the aberrant conditions of the tumor microenvironment [142]. Another example is Bacillus Calmette-Guérin (BCG) which was originally developed as a tuberculosis vaccine, but was later discovered to be effective against superficial bladder tumors. BCG targets TLR2 and TLR4 and enhances immune responses [143]. Stimulation of TLR9 activates human pDCs and B cells, and induces potent innate immune responses in preclinical tumor models and in patients. As such, the TLR9 agonist, CpG ODN, has shown promising results as vaccine adjuvant and in the treatment of cancers, infections, asthma and allergy [144]. Vaccine adjuvants are perhaps the most extensively explored

applications for TLR agonists. The rational design of specific TLR agonists with reduced toxicity but increased potency, as compared to adjuvant candidates from only a decade ago, offers the opportunity to meet the stringent safety criteria required for prophylactic vaccines [145]. TLR stimulation can also have a negative role in tumorigenesis. It can increase proliferation, decrease rates of apoptosis and enhance the growth of adoptively transferred cells. TLRs are believed to be directed against tumor cells, but they also have been found to be involved in tumor development. It is crucial to develop further studies to establish the exact role of TLRs in human disease.

TLRs offer a promising new therapeutic strategy for the treatment of human inflammatory diseases. However, the potential for adverse effects when targeting such fundamental pathways of the host defence mechanism needs to be carefully assessed, especially for not usually life-threatening diseases [139].

Hypothesis and aims of this thesis.

The overall hypothesis of this thesis is that TLR signalling is central to inflammation and cellular proliferation in lung pathologies, and that understanding how specific functions of TLRs mediate responses at the cellular level and in tissue from patients will be of value in the search for new biomarkers and drug therapies.

The specific aims are as follows:

1) To investigate the signalling mechanisms involved in inflammasome activation and IL-1β release from human monocytes after activation with TLR2 and TLR4 ligands.

2) To assess the sensitivity of the blood of COPD patients to produce CXCL8 and IL-1β after challenge with bacterial and viral TLR ligands.

3) To study the role of TLRs in proliferation and death of human lung cancer cells.

Chapter 2

Methods

2.1. Cell culture.

Type II pneumocyte-delivered human lung cancer cell line A549 (ECACC) was cultured in T175cm^2 culture flasks (Nunc) and incubated at 37°C containing 5% CO_2. A culture medium of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% filtered heat-inactivated Foetal Calf Serum (FCS), 2mM glutamine and100U/ml penicillin/streptomycin was used to sustain the cells. Cells were observed to be confluent after approximately 3 days. For T175cm^2 flasks cells were seeded at 2mln cells/flask, for 6 well plates cells were seeded at 3x10^{5}cells/well, for 96 well plates cells were seeded and 1x10^4 or 2.4x10^4 cells/well.

Human acute monocytic leukemia cell line THP-1 (ECACC) and freshly isolated PBMCs were cultured in T175cm^2 culture flasks and incubated at 37°C containing 5% CO_2. A culture medium of RPMI 1640 supplemented with 10% filtered heat-inactivated Foetal Calf Serum (FCS), 2mM glutamine and 100U/ml penicillin/streptomycin was used to sustain the cells. Cells were observed to be confluent after approximately 3 days. For T175mm^2 flask cells were seeded at 2mln cells/flask and for 96 well plates cells were seeded at 1x10^5 cells/well.

2.1.1. Cell passage.

For adherent cells, media was removed from the cells with an aspiration pipette and washed once with PBS at room temperature. 10 mls of trypsin solution made up with PBS was added to detach cells from the flask surface (5ml for T75cm^2, 10ml for T175cm^2). After 5min incubation at 37°C, equal volume of media was added to neutralize the trypsin. For non-adherent cells, cell suspension was simply transferred from the flask to a centrifuge tube. Both, adherent and non-adherent cell lines, were centrifuged at 190xg for

5min at room temperature. The cell supernatant was removed and 1ml of media was added. Cells were counted and either used for experiments or frozen into aliquots for long-term storage.

2.1.2. Cell counting.

10µl of cell suspension was added to 90µl Turk's solution (0.01% crystal violet, 3% acetic acid in H_2O). 10µl of the suspension was added onto haemocytometer (Neubauer) and observed under a bright-field microscope. The total number of cells was counted in 25 0.004mm^3 squares. 25 squares with combined volume of 25x(0.004)=0.1mm^3. There are 1000 cubic mm in one cubic cm, so the number of cells was multiply by 1000 and the dilution factor (10x). The concentration of cells in the original suspension (cell/ml) is represented by:

Cell/ml = number of cells in 25 squares /0.1 x 1000 x 10

2.1.3. Cell freezing/storage/thawing.

Cells were resuspended to the concentration 1mln cells/ml. 10% of DMSO was added to the suspension and placed in the cryotubes. The cryotubes were then placed in a storage device containing isopropanol and placed in the -80ºC freezer. This ensured freezing at no more that 1ºC per min. After 24h cryotubes were transferred to the liquid nitrogen container for long-term storage. To thaw cells, cryotube was placed in the 37 ºC water bath until cells had just started to thaw. Cells were carefully resuspended and added to the flask with complete media.

2.2 Pharmacology of TLR2 and TLR4 agonists on IL-1β and CXCL8 release by human monocytes and peripheral blood mononuclear cells.

2.2.1. Isolation and culture of human peripheral blood mononuclear cells.

Blood samples were collected from healthy volunteers (ethics number: 08/H0708/69). Informed consent was obtained from all subjects. Prior to blood collection 3ml of Histopaque 1077 was added to the 15ml centrifuge tubes. Peripheral blood samples were taken from healthy volunteers. For each donor 20 ml of blood was collected into 50ml centrifuge tube containing sodium citrate (ratio of sodium citrate:blood was 1:9). Citrated blood was diluted with RPMI media which had been warmed to 37ºC. 6ml of diluted blood was layered on top of 3ml Histopaque (prepared earlier) and centrifuged at 400xg (with a very low brake) for 30min at room temperature. Layer of PBMCs, which at this point was between the Histopaque and the serum, was carefully removed from each tube using Pasteur pipette. PBMCs were transferred into 15ml centrifuged tubes (up to 7ml per tube) and made up to 14ml with warm RPMI media. Tubes were centrifuges at 200xg for 15min at room temperature to remove any remaining Histopaque. After spinning, supernatants were discarded from each tube. Cell pellets were pooled into 50ml falcon tubes with 20ml of fresh media and centrifuged again at the same conditions for a final wash. After final wash, supernatant was removed and cell pellet was resuspended in 1ml of media and counted using haemocytometer. PBMC were seeded in 96 well plates at $1x10^5$ cells per well.

2.2.2. Measurement of cytokine release (ELISA).

Levels of cytokines (IL-1β, CXCL8 and CXCL10) in the cell culture supernatants and plasma were measured by enzyme-linked immunosorbent assay (ELISA) using commercially available kits (R&D Systems, UK) following the manufacturer's instruction. Briefly, 96 well plates were coated overnight with capture antibody specific for the cytokine

being measured. Plates were washed 3 times with PBS/Tween (Sigma) and blocking buffer was added to block any remaining spaces on the well. After an hour, plates were washed three times with PBS/Tween and incubated with samples (diluted it reagent specified in protocol if necessary) and standard solution of the cytokine being measured. After 2h plates were washed 3 times in PBS/Tween and incubated with biotinylated detection antibody specific for the cytokine being measured. After 2h plates were washed 3 times in PBS/Tween and incubated with a streptavidin/horseradish peroxidise conjugate solution. After 20 min plates were washed 3 times with PBS/Tween and then incubated with substrate solution, a mix of H_2O and tetramethylbenzidine (BD Bioscience, UK). Reaction was stopped with addition of 2N H_2SO_4. Optical density of each well was obtained by reading the plate at 450nm with wavelength correction at 570nm using microplate reader. Levels of cytokine in samples were calculated by interpolation from the standard curve, which is generated from optical density reading for the wells containing standards.

The sensitivities of the assays used:

- IL-1β - 3.91-250 pg/ml
- CXCL8 – 31.2-2000 pg/ml
- CXCL10 – 31.2-2000 pg/ml

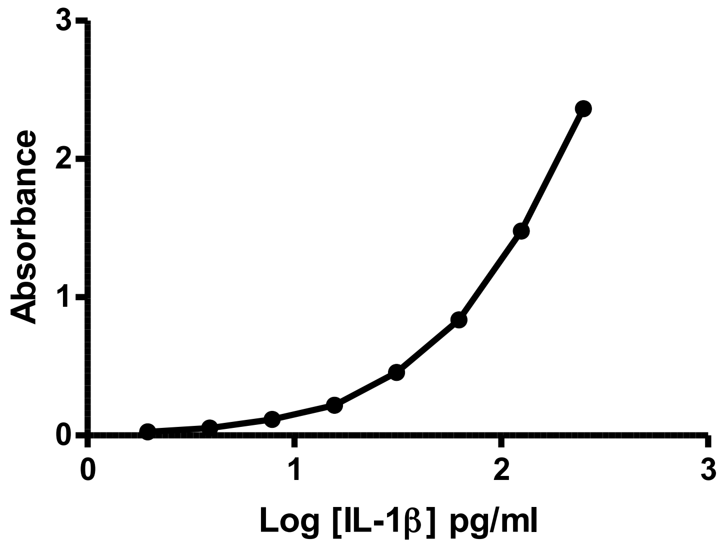


Figure 4. Example of the results of human IL-1β ELISA standard curve. Absorbance read at 450nm with wavelength correction at 570nm.

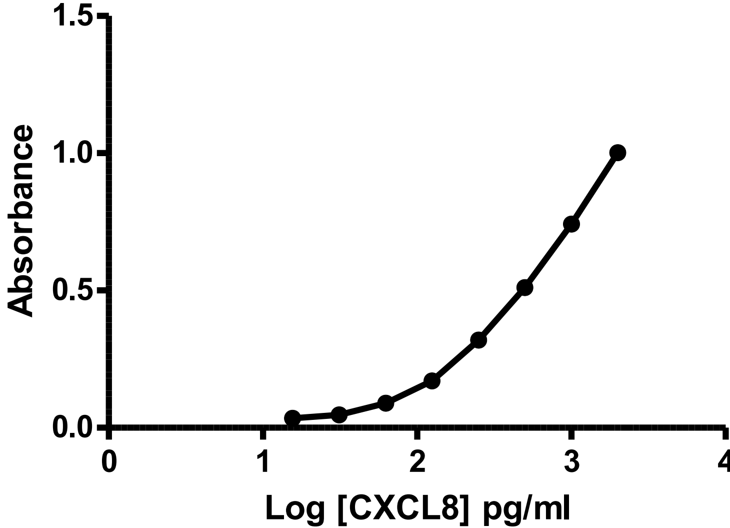


Figure 5. Example of the results of human CXCL8 ELISA standard curve. Absorbance read at 450nm with wavelength correction at 570 nm.

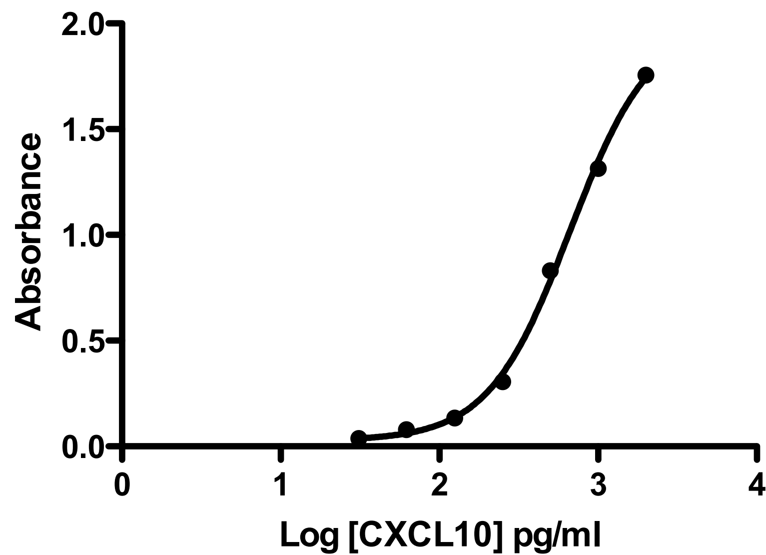


Figure 6. Example of the results of human CXCL10 ELISA standard curve. Absorbance read at 450nm with wavelength correction at 570 nm.

2.2.3. Cell treatment.

THP-1 cells

THP-1 cells were seeded at $1x10^5$ cells/well in 96 well plates in complete media and treated with the following inhibitors:

- Steroid- Prednisone (0.1-10 µg/ml)
- IKK inhibitor- SC-514 (0.1-10 µg/ml)
- TAK1 inhibitor- 5Z-7 (0.1-10 µg/ml)
- p38 inhibitor- BIRB (0.001-10 µg/ml)
- Caspase inhibitor- Z-VAD-FMK (0.001-10 µg/ml)
- Panx1 inhibitor- Carbenoxolone (0.001-10 µg/ml)
- P2X7R inhibitor- AZ (0.001-10 µg/ml)
- Media with 0.01% DMSO (control)

After 30min LPS (0.1µg/ml) and Pam_3CSK4 (0.1µg/ml) were added for 24h. Cells were centrifuged at 400xg for 5min at room temperature. Supernatants were collected and stored at -20ºC for cytokines measurement by ELISA (as described in section 2.2.2). Cells were kept for AlamarBlue cell viability assay (section 2.4.2).

Experiments that included an ATP pulse were performed as follows*:* THP-1 monocytes were plated and treated with PAMPs for 24 hours as described above. After 24 hours, cells were centrifuged (190xg, 5min) and supernatants collected. Media was then replaced with media alone or media containing 5mM ATP for 30min. Cells were then centrifuged (190xg, 5min) and supernatants collected for ELISA.

PBMCs

PBMCs were seeded at $1x10^5$ in 96 well plates in 100µl of media and treated with the following inhibitors:

- p38 inhibitor- BIRB (0.001-10 µg/ml)
- Caspase inhibitor- Z-VAD-FMK (0.001-10 µg/ml)
- Pannexin-1 inhibitor- Carbenoxolone (0.001-10µ g/ml)
- P2X7R inhibitor- AZ (0.001-10µg/ml)
- Media with 0.01% DMSO (control)

After 30min LPS (1µg/ml) and Pam_3CSK4 (1µg/ml) were added for 24h. Cells were centrifuged at 400xg for 5min at room temperature. Supernatants were collected and stored at -20ºC for cytokines measurement by ELISA (as described in section 2.2.2). Cells were kept for AlamarBlue cell viability assay (section 2.4.2).

2.2.4. Cytokine release after activation of Myd88 and TRIF signalling pathways.

Freshly isolated PBMCs were seeded at density $1x10^5$ cells/well. On the next day cells were treated with:

- TLR2/1 agonist- Pam_3CSK4 (0.01-1 μg/ml)
- TLR2/6 agonist- FSL-1 (0.01-1 μg/ml)
- TLR3 agonist- Poly I:C (0.1-10 μg/ml)
- TLR4 agonist- LPS (0.001-1 μg/ml)
- Media (control)

After 24h IL-1β and CXCL8 levels were measured by ELISA as described in section 2.2.2.

2.2.5. Cytokine release after inhibition of Myd88 and TRIF adaptor proteins.

2.2.6. Preparation of cigarette smoke extract (CSE).

CSE was produced as described previously [146]. Briefly, CSE was prepared by combusting four full-strength Marlboro cigarettes (filters removed) using a 60ml syringe apparatus. Cigarette smoke was passed through 100ml of RPMI media, supplemented with 10% foetal calf serum, Penicillin Streptomycin (100U/ml), and L-Glutamine (200 mM). Each full draw of the syringe took approximately 10s to complete, yielding approximately 5-6 draws in total. CSE was then filtered using a 0.25-μ to sterilise and remove large particulates. At this point CSE is considered to be at 100% and was used immediately, unless otherwise stated.

2.2.7. Cell treatment.

Freshly isolated PBMCs were seeded at density $1x10^5$ cells/well. On the next day cells were treated with:

- TLR2/1 agonist- Pam_3CSK4 (1 µg/ml)
- TLR3 agonist- Poly I:C (10 µg/ml)
- TLR4 agonist- LPS (1 µg/ml)
- CSE (10%)
- Media (control)

After 6h, 10µM or 50µM of inhibitors was added to the cells.

IL-1β, CXCL8 and CXCL10 levels were measured by ELISA as described in section 2.2.2.

2.2.8. Transfection of THP-1 cells using siRNA.

THP-1 cells were maintained as described in section 2.1 and harvested by centrifufation (200*x*g, 21°C, 5min). Media was removed and cells were resuspended in solution V (Lonza cat no VCA–1003). For each nucleofection, siRNA was added (20nM-100nM) per sample and transferred to a new electroporation cuvette (Lonza). Cuvette was inserted to Amaxa Nucleofector transfection system and electroporated using program V-001. Immediately after the pulse, pre-warmed media was added to the cuvette and aspirated to a new eppendorf tube using the supplied pipettes. After 10min incubation at 37°C, cells were transferred onto the 96 well plate. After 48h, cells were treated with:

- TLR2/1 agonist Pam_3CSK4 (1 µg/ml)
- TLR4 agonist- LPS (1 µg/ml)
- Media (control)

Supernatant was collected after 24h and subjected to ELISA as described in section 2.2.2.

siRNA sequence

AllStars Negative Control siRNA (Qiagen)

ON-TARGET plus SMARTpool, Human Panx1 (Dharmacon)

- UAAGUGAGGUCAAGUCAUA
- CGGCAGAGCUCCAAGGUAU
- CAUAUUUGCUCAGACUUGA
- CACUGUGGCUGCAUAAGUU

ON-TARGET plus SMARTpool, Human P2X7 (Dharmacon)

- GGAUAGCAGAGGUGAAAGA
- GCUUUGCUCUGGUGAGUGA
- GGAUCCAGAGCAUGAAUUA
- GCGGUUGUGUCCCGAGUAU

2.2.9. RNA extraction (Macherey-Nagel).

RNA was extracted by Macherey-Nagel kit according to the manufacturer instruction. Briefly, cells were lysed by adding 350μl of RA1 buffer (containing 10μl β-mercaptoethanol per ml of RA1) and pipetting up and down 5 times. Viscosity of the lysate was reduced by filtration through Nucleospin Filter and centrifuged at 11,000xg for 1min. 350μl of 70% Ethanol (Sigma) was added to the homogenized lysate and transferred to a Nucleospin RNA II Column. After centrifugation, membrane was desalted by adding 350μl of MDB buffer and spun at the same conditions. 10μl of DNase was added to 90μl Reaction Buffer for DNase in a clean Eppendorf tube and added directly to the membrane. Column was incubated at room temperature for 15min. 200μl Buffer RA2 was added directly to the column and spun for 30s at 11,000xg. To wash the column, 600μl of buffer RA3 was added and after centrifugation additional 250μl of the same buffer was added. After

spinning at the same conditions RNA was eluted with 60µl of DNase/RNase free water. Concentration of the RNA was measured at 260/280nm using a UV spectrophotometer (Nanodrop ND-1000, Thermo).

2.2.10. RT-PCR.

RNA (stored at -80°C) was thawed at room temperature. 250ng of RNA was used to make up the first strand reaction as outlined below.

RNA	200ng
Oligo (dT)$_{12\text{-}18}$ (Invitrogen)	1µl
10mM dNTP Mix (Fisher Scientific)	1µl
DNase / RNAse Free Water (Qiagen)	Make up to 20µl

After 5min incubation at 65°C, tube was placed on ice for 1min. Then following reagents were added:

Reagent	Volume(µl)
Reverse Transcriptase (Fermenetas)	1
RT Buffer (Fermentas)	4

Tubes were placed in Techne Cycler and incubated at 25°C for 10min, 42°C for 60min and 70°C for 10min.

Master mix for the PCR reaction was made up using following instruction:

Reagent	Volume per well (µl)
SyBr Green Mastermix (Sabiosciences)	12.5
Primer Mix (Sigma)	1
cDNA from first strand reaction	1
Dnase / RNAse Free Water (Qiagen)	10.5

25µl per well was loaded and run on the ABI7500 qPCR machine with the following cycle:

Cycles	Duration	Temperature
1	10 minutes	95°C
40	15 Seconds	95°C
40	1 minute	63°C

Primers:

GAPDH

Forward 5' GAAGGTGAAGGTCGGAGTC

Reverse 5' GAAGATGGTGATGGGATTTC

P2X7

Forward 5' TCCTACGTTTGCTTTGCTCTGG

Reverse 5' ATTCCTTTGCTCTGCGGGTC

Panx1

Forward 5' TCACGTGCATTGCGGTGGGG

Reverse 5' GCGAAACGCCAAGAACAGCGG

2.3 COPD and PAH study – cytokine release measurement from whole blood.

2.3.1. Whole blood culture – Ethics statement.

Patients with COPD from Greater London area, who were attending respiratory clinics at the Royal Brompton Hospital, were invited to participate. The study was approved by the Royal Brompton, Harefield and National Heart and Lung Institute Research Ethics Committee (ethics number 07/Q0404/17; the study was adopted by UK Clinical Research

Network; http://www.crncc.nihr.ac.uk/). Signed informed consent was obtained from all subjects. The diagnosis of COPD was made when the FEV1/FVC ratio was less than 70%. Patients were diagnosed according to the GOLD guidelines (http://www.goldcopd.org/gold-around-the-world.html). COPD severity was classified as mild (stage I – FEV1 ≥ 80%), moderate (stage II – FEV1 50-79%) and severe (stage III – FEV1 30-49%) or very severe (stage IV – FEV1 <30%). Smoking history and pack years smoked were also recorded.

Patients with idiopathic pulmonary hypertension (PAH) were invited to participate. The study was approved by the Royal Brompton Hospital Cardiovascular Biomedical Research Unit Tissue Bank Ethics Committee, reference 09/H0504/104. Signed informed consent was obtained from all patients. Disease severity was based on NHYA score (from I-IV); where higher RVSP (right ventricular systolic pressure from echo); lower PAT (pulmonary arterial acceleration time) and lower 6MW (6-minute walk) value all indicated worse PH.

2.3.2. Study Design.

We calculated the mean age of COPD patients and chose the volunteers for the control group to give us as similar an age range as possible. We also divided our control subjects into three groups:

- Young controls (<30yrs)
- Medium-age controls (30-50yrs)
- Older controls (>50yrs)

to investigate whether any changes in cytokines release were caused by disease or were associated with aging. 12ml of whole blood from volunteers was collected into 2.7ml vacutainers (BD Bioscience) containing sodium citrate (3.2%). 190µl of blood was aliquoted onto 96 well plate and treated with 10µl of the following agonists:

- TLR2/1 agonist- Pam_3CSK4 (0.001-1 µg/ml)
- TLR2/6 agonist- FSL-1 (0.001-1 µg/ml)
- TLR3 agonist- Poly I:C (0.01-10 µg/ml)
- TLR4 agonist- LPS (0.001-1 µg/ml)
- NOD1 agonist- C12-iE-DAP (0.001-1 µg/ml)
- IL-1β (0.001-1 ng/ml)
- Media (control)

Cells were incubated at 37°C containing 5% CO_2 for 24h. Plates were centrifuged at 1000xg at room temperature for 5min with very low breaking. Serum was carefully transferred to the dummy plate and frozen at -20ºC for future analysis of IL-1β and CXCL8 release by ELISA (as described in section 2.2.2).

2.4 The Role of TLRs in lung cancer cells proliferation and survival.

2.4.1. Cytokine release measurement from A549 cells.

A549 cells were seeded at $1x10^4$ cells/well in 96 well plates in media (with 10% FCS and 0% FCS) and treated with:

- TLR2/1 agonist- Pam_3CSK4 (0.1-1 µg/ml)
- TLR2/6 agonist FSL-1 (0.1-1 µg/ml)
- TLR3 agonist- Poly I:C (0.1-10 µg/ml)
- TLR4 agonist- LPS (0.01-1 µg/ml)
- TLR7 agonist- Imiquimod (0.1-10 µg/ml)
- TLR8 agonist- ssRNA40/Lyovec (0.1-10 µg/ml)
- Media (control)

After 24h, 48h, 78 and 144h supernatants were collected and frozen at -20ºC. ELISA was performed on the following day (section 2.2.2).

2.4.2. AlamarBlue assay.

A549 cells were seeded in complete media at density 1×10^4 cells/per in 96 well plates and left overnight to become adherent. The next day, cell supernatant was aspirated and new culture medium containing stated concentrations of FCS (0-10%) was added. Cells were treated with the following agonists:

- TLR2/1 agonist- Pam_3CSK4 (0.01-1 µg/ml)
- TLR2/6 agonist- FSL-1 (0.01-1 µg/ml)
- TLR3 agonist- Poly I:C (0.1-10 µg/ml)
- TLR4 agonist- LPS (0.01-1 µg/ml)
- TLR7 agonist- Imiquimod (0.1-10 µg/ml)
- TLR8 agonist- ssRNA40/Lyovec (0.1-10 µg/ml)
- Media (control)

After 24h AlamarBlue reagent (Invitrogen) was added to each well ($1/10^{th}$ of the total volume in the well). Plate was incubated in the dark at 37ºC for 4h and cell viability was measured by reduction assay. This assay incorporates a specially selected oxidation-reduction (REDOX) indicator that both fluoresces and undergoes colorimetric change in response to cellular metabolic reduction**.** The active ingredient of AlamarBlue (resazurin) is a nontoxic, cell permeable compound that is blue and nonfluorescent. Upon entering cells, resazurin is reduced to resorufin, which produces very bright red fluorescence. Hence, cell viability can be monitored as reduction is accompanied by a measurable shift in color (blue to pink).

2.4.3. Cell Cycle BioApplication (Cellomics).

A549 cells were seeded in complete media at density 1×10^4 cells/per in 96 well plates and left overnight to become adherent. The next day, cell supernatant was aspirated and new

culture medium containing stated concentrations of FCS (0-10%) was added. Cells were treated with the following agonists:

- TLR2/1 agonist- Pam_3CSK4 (0.01-1 µg/ml)
- TLR2/6 agonist- FSL-1 (0.01-1 µg/ml)
- Media (control)

After, 24h, 48h and 72h cells were washed once in PBS and fixed immediately in 4% para-formaldehyde (PFA) for 10min at room temperature. PFA was removed and plates were then washed again in PBS and left for 5min. This step was repeated 3 times. Cell nuclei were stained with DAPI. Plates were then stored in PBS at 4°C prior to imaging using a Cellomics VTi HTS Arrayscanner (Thermo Fisher, Pittsburgh, US). This BioApplication is designed to classify cells and define cell cycle stage by measuring the nuclear DNA content using the total intensity of a DNA binding dye (DAPI) for each individual cell. Correspondingly, the cells are classified into one of five classes (Fig. 7): less than 2N (apoptotic/damaged), 2N (G1), 2N-4N (S), 4N (G2/M), and >4N (polyploid or necrotic). Cellomics arrayscanner is an automated fluorescence microscope that provides comprehensive data on the spatial and temporal distribution of fluorescence intensities in 96-well plates. In these experiments the system scanned 49 fields per well. After adjusting the parameters for our cell type so that the camera was able to pick up individual cells and their nuclei distinctly, DNA content was measured using fluorescent intensity recorded on a camera. This fluorescence was characteristically bimodal and corresponded to the cell cycle position for individual cells. To corroborate findings of this system, individual capture frames were looked at by eye and compared with the automated readouts.

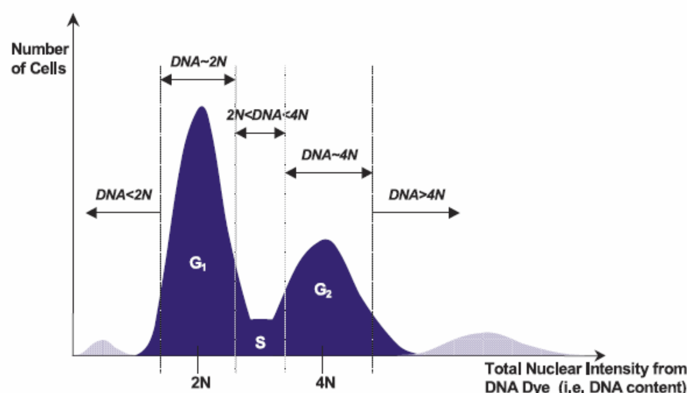


Figure 7. Schematic of the total fluorescence intensity distribution from the DNA binding dye and the corresponding populations of cells in the different cell cycle phases.

2.4.4. CyQuant Assay.

A549 cells were seeded in complete media at density $1x10^4$ cells/per in 96 well plates and left overnight to become adherent. On the following day media from half of the plate was removed and replaced with media without FCS. Cells were treated with the following agonists:

- TLR2/1 agonist- Pam_3CSK4 (0.01-1 µg/ml)
- TLR2/6 agonist- FSL-1 (0.01-1 µg/ml)
- TLR4 agonist- LPS (0.01-1 µg/ml)
- TLR8 agonist- ssRNA40/Lyovec (0.1-10 µg/ml)
- Media (control)

After, 24h, 48h and 72h CyQuant cell proliferation assay (Invitrogen, San Diego, USA) was performed following the instruction provided by the manufacturer. A solution of CyQuant GR dye, which exhibits strong fluorescence enhancement when bound to cellular nucleic acids, was made in cell lysis buffer prior to each experiment. Cells were lysed using a freeze-thaw cycle, frozen at -80°C and thawed at room temperature. 200µl of Cyquant GR dye/lysis buffer was added to each well. The samples were incubated in darkness for 2 to 5min. The fluorescence of each sample was at 485nm excitation and

530nm emission filters. For each experiment, a DNA standard curve was prepared from bacteriophage λ DNA that was provided with the assay. The DNA content of samples was calculated using the standard curve generated each time.

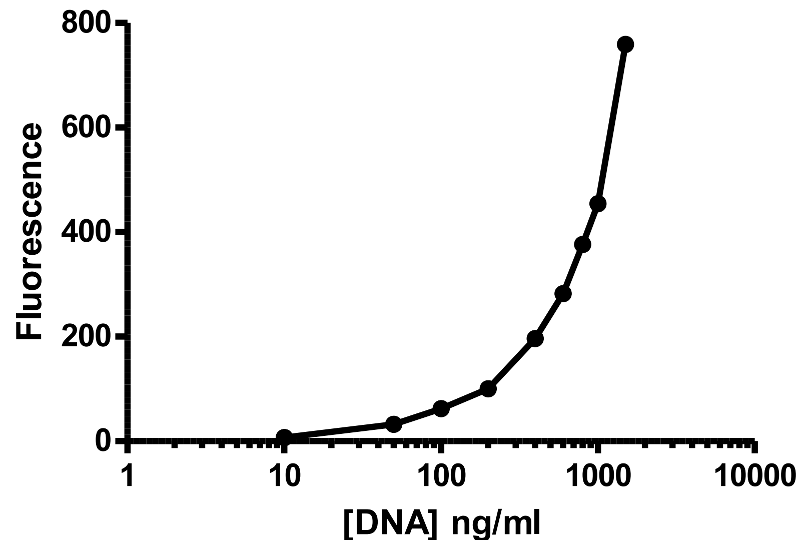


Figure 8. Example of standard curve used for CyQuant proliferation assay. Fluorescence was read at 485nm excitation and 530nm emission filters.

2.4.5. Cell viability assay.

A549 cells were seeded in complete media at density $1x10^4$ cells/per in 96 well plates and left overnight to become adherent. The next day, cell supernatant was aspirated and new culture medium containing stated concentrations of FCS (0-10%) was added. Cells were treated with the following agonists:

- TLR2/1 agonist- Pam_3CSK4 (0.01-1 µg/ml)
- TLR2/6 agonist- FSL-1 (0.01-1 µg/ml)
- TLR4 agonist- LPS (0.01-1 µg/ml)
- Media (control)

After 4h cells were treated with apoptotic agents:

- Cycloheximide (3µg/ml)
- TNF-α (3ng/ml)
- H_2O_2 (0.6 mM)
- TNF-α+CHX (3ng/ml+3µg/ml)
- Media (control)

Alamar Blue assay was performed 24h later as described in the section 2.4.2.

2.4.6. Flow Cytometry.

Annexin V and propidium iodide (PI) can be used to quantitatively determine the percentage of cells that undergo apoptosis within a population. During the apoptosis, the membrane phospholips phosphatidylserine (PS) is translocated to the outer leafleat exposing PS to external environment. Annexin V is a phospholipid-binding protein that has high affinity to PS and PI can bind nucleic acid therefore, these two markers can be used to distinguish viable cells from apoptotic/dead ones. Cells that stain positive for both annexin V and PI are apoptotic or dead and cells that stain negative for both annexin V and PI are alive and not undergoing measurable apoptosis. For FACS analysis A549 cells were harvested after treatment and washed twice with media containing 10% FCS. After final wash with cold PBS and centrifuging at 200xg for 5min at room temperature, cells were resuspended in the binding buffer and 100µl of cells at density 10^5 cells/tube were transferred to a 5ml FACS tube. Cells were filtered through a mesh cloth to discriminate doublets and 5µl of annexin V and PI were added to the each tube. After 15min incubation in the dark at room temperature, 400µl of binding buffer was added to each tube and cells were analysed on FACS Canto II machine. The emission/excitation wavelengths were 530/488 nm for annexin V FITC and 650nm/488nm for PI, according to the manufacturer's specifications of wavelength combinations. Data were analysed by FACS Diva software. Cells were classified as: annexin V and PI negative (viable, with no measurable

apoptosis), annexin V positive and PI negative (early apoptosis, membrane integrity is present), annexin V and PI positive (late apoptosis and death).

2.4.7. Apoptotic agents concentration curves.

A549 cells were seeded in complete media at density $3x10^5$ cells/well in 6 well plates and left overnight to become adherent. The next day, cells were treated with the following apoptotic agents:

- TRAIL (50-1000 ng/ml)
- Cycloheximide (1-100 µg/ml)
- TNF-α (15ng/ml) + Cycloheximide (1-100 µg/ml)
- Cycloheximide (1µg/ml) + TNF- α (1-20 ng/ml)
- Media (control)

After 24h cells were harvested and stained with annexin V and PI and subjected to FACS analysis.

2.4.8. Stimulation of TLRs and apoptosis

A549 cells were seeded in complete media at density $3x10^5$ cells/per in 6 well plates and left overnight to become adherent. The next day, cells were treated with the following agonists:

- TLR2/1 agonist- Pam_3CSK4 (0.01-1 µg/ml)
- TLR2/6 agonist- FSL-1 (0.01-1 µg/ml)
- TLR4 agonist- LPS (0.01-1 µg/ml)
- TLR3 agonist- Poly I:C (0.1-10 µg/ml)
- TLR7 agonist- Imiquimod (0.1-10 µg/ml)
- TLR8 agonist ssRNA40\Lyovec (0.1-10 µg/ml)

After 4h the following apoptotic agents were added:

- TRAIL (1000ng/ml)
- TNF-α (15ng/ml) + Cycloheximide (1μg/ml)
- Media (control)

After 24h cells were harvested and stained with annexin V and PI and subjected to FACS analysis.

2.5. Statistical analysis.

All statistical tests were performed using GraphPad Prism 4 software. Results are expressed as the mean ± standard error (S.E.M). We used one-way ANOVA to compare the means of several different groups with one independent variable, and the two-way ANOVA to compare the means of two or more different groups with more than one independent variable. Dunnett's post-test was used when all groups were compared against one control group and Bonferroni's post-test was used when multiple comparisons were made between selected columns. We also used column analysis with one-sample t-test for normalized data. Differences at a probability value (P) of ≤ 0.05 were considered as significant.

2.6. Reagents.

All cell culture plastics, general disposables and enzyme linked immunosorbent assay (ELISA) plates were obtained from Fisher Scientific. Unless stated otherwise, all cell culture reagents were supplied by Invitrogen. General laboratory reagents were purchase from Sigma-Aldrich. Agonists for TLRs were obtained from Sigma and Invivogen unless stated otherwise.

Chapter 3

Pharmacology of TLR2 and TLR4 agonists on IL-1β and CXCL8 release by human monocytes and peripheral blood mononuclear cells.

Rationale

IL-1β and CXCL8 are pro-inflammatory cytokines that play a critical role in innate and adaptive immunity. CXCL8 is a major chemoattractant for the recruitment of neutrophilic granulocytes and is secreted by endothelial cells, fibroblasts and local immune cells [147]. IL-β is a pro-inflammatory cytokine and its secretion is tightly controlled [148]. The release of IL-1β requires two well-defined signals: a “priming signal” to induce pro-IL-1β synthesis, and a second signal to trigger assembly of the inflammasome. The induction of pro-IL-1β is under regulation of NF-κB and it occurs upon activation of TLRs, whilst inflammasome assembly is associated with a number of highly varying stimuli, including ATP [1]. The P2X7 receptor is an ion channel gated by high concentrations of extracellular ATP, and it has been demonstrated to be responsible for ATP-mediated processing and the release of IL-1β from LPS primed cells [149]. Panx1 exists as a transmembrane channel allowing the passage of ions and small molecules. It is upregulated by LPS stimulation and it co-immunoprecipitates with P2X7R. Inhibition of panx1 with siRNA blocked caspase-1 cleavage as well as IL-1β processing and release from a repertoire of LPS-primed mouse and human macrophages in response to P2X7R activation [150]. Recently, a role for TLRs in inflammasome activation and IL-1β release has been identified. For example, stimulation of TLR4 on monocytes or macrophages by LPS results in the induction of pro-IL-1β synthesis. The second step of activation of the inflammasome and subsequent release of bioactive IL-1β is governed by other factors as previously mentioned. Less is known regarding the effect of TLR2 ligands on the release of IL-1β. Thus in the present study we hypothesised that there are differences in TLR2 and TLR4-induced

inflammasome activation and subsequent release mature IL-1β release. Since TLRs and the inflammasome are now highly implicated in respiratory disease, such as COPD [111], we performed a study where we have directly characterized the signalling pathways in TLR4 versus TLR2 induced release of IL-1β. In the present study we stimulated THP-1 cells and PBMCs with TLR ligands and measured the levels of CXCL8 and IL-1β. We also inhibited components of TLR and inflammasome signalling pathways and knocked-down panx1 and P2X7R genes using siRNA to investigate whether there are any differences between TLR2 and TLR4-induced release of CXCL8 and IL-1β. TIR domains of TLRs are composed of five β-strands alternating with five α-helices. A functionally important proline-glycine combination that is highly conserved among TIR domains is located in the loop connecting the second β-strand with the second helix, the "BB loop". In our study we used Myd88 and TRIF BB loop inhibitory peptides which block MyD88 signalling by inhibiting its homodimerization through binding, and which block TRIF signalling by interfering with TLR-TRIF interaction respectively.

Methods

For the experiments in this chapter, THP-1 cells and PBMCs were cultured in 96 well plates as described in chapter 2.1. Cells were treated in sterile conditions with:

- TLR agonists (LPS, Pam_3CSK4, FSL-1, Poly I:C) with or without ATP pulse.
- TLR signalling and inflammasome inhibitors 30min prior to TLR2 and TLR4 activation with Pam_3CSK4, FSL-1 and LPS.
- siRNA to knock-down *P2X7R* and *panx1* 48h prior to TLR activation with LPS and Pam_3CSK4.
- Myd88, TRIF and Myd88/TRIF inhibitory peptides 6h prior treatment with LPS, Pam_3CSK4, FSL-1 and CSE.

Cells were incubated for 24h after which media was removed and stored at -20°C for future analysis. IL-1β, CXCL8 and CXCL10 levels were measured by ELISA as described in chapter 2.2.2. 100µl of fresh media and 10µl of AlamarBlue solution was added to the cells to measure cell viability/respiration (see chapter 2.4.2. for further details of methods)

For the knock-down experiments, THP-1 cells were cultured in 96 well plates. P2X7R, pannexin-1 or control siRNA were added to the media and cells were transfected using the Amaxa Nucleofector system (Lonza), according to the manufacturer's protocol. Transfection efficiency was analysed by PCR. IL-β and CXCL8 levels were measured by ELISA according to the protocol furnished by the manufacturer. More details of the methods are described in materials and methods chapter and in figure legends in the results section.

Results

3.1. Effect of TLR stimulation on IL-1β and CXCL8 release from THP-1 cells.

THP-1 monocytes upon stimulation with TLR2/1 agonist Pam_3CSK4, TLR2/6 agonist FSL-1 and TLR4 agonist LPS produced measurable quantities of IL-1β after either 3h or 24h (Fig. 9A,B). In contrast, TLR3 agonist Poly I:C failed to result in the release of significant levels of IL-1β from THP-1 cells. These agonists also caused a concentration-dependent increase in CXCL8 levels after 24h (Fig. 10A), it is worth noting that non of the TLR ligands tested release CXCL8 at 3h (data not shown). This is not surprising as in previous studies CXCL8 mRNA was not significantly elevated above baseline until 8h [146].

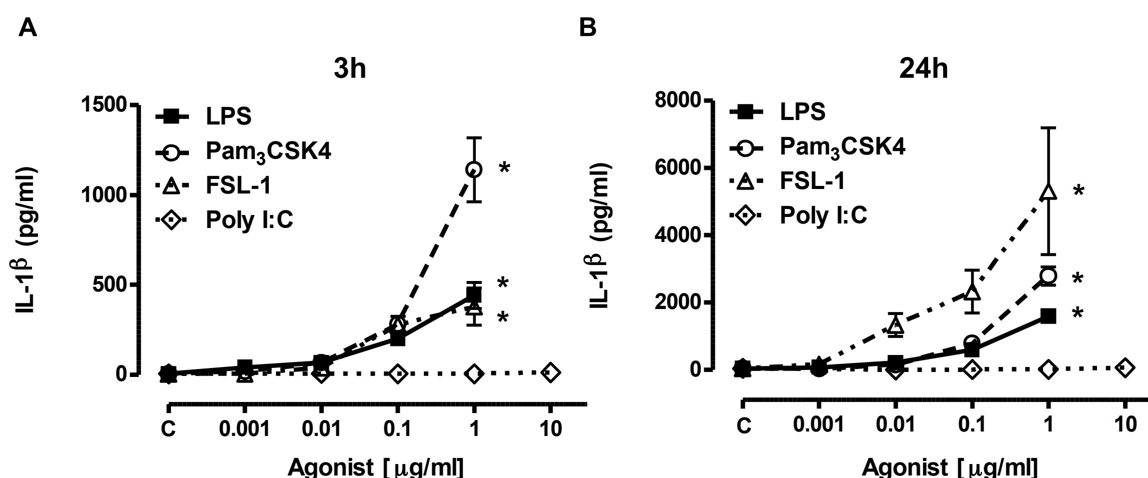


Figure 9. Mature IL-1β release from THP-1 cells in response to LPS, Pam_3CSK4 and FSL-1. THP-1 monocytes were seeded in a 96 well plates at 1 x 10^5 cells per well in RPMI with 10% FCS and allowed to equilibrate for 12h. After this time period cells were treated with media, Pam_3CSK4, FSL-1 and Poly I:C for 3h (A) and 24h (B). IL-1β was measured in the supernatants by ELISA. Data represented are the mean ± SEM of a total of n=9 replicates from 3 experimental days. *denotes $p \leq 0.05$ as assessed using a one-way ANOVA followed by a Dunnett's multiple comparison post-hoc test.

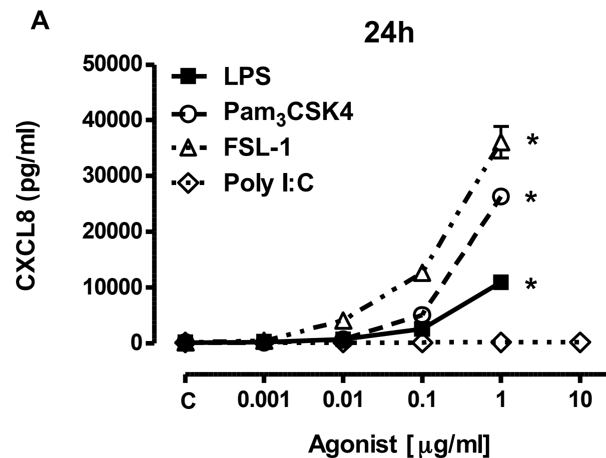


Figure 10. CXCL8 release from THP-1 cells in response to LPS, Pam3CSK4 and FSL-1. THP-1 monocytes were seeded in a 96 well plates at 1 x 10^5 cells per well in RPMI with 10% FCS and allowed to equilibrate for 12h. After this time period cells were treated with media, Pam3CSK4, FSL-1 and Poly I:C for 24h. CXCL8 release was measured in the supernatants by ELISA. Data represented are the mean ± SEM of n=9 replicates from 3 experimental days. *denotes $p \leq 0.05$ as assessed using a one-way ANOVA followed by a Dunnett's multiple comparison post-hoc test.

3.2. Role of ATP in IL-1β release from human monocytes stimulated with TLR2 and TLR4 ligands.

It has recently been documented that monocytes, unlike macrophages, are capable of releasing IL-1β in response to TLR activation alone, as they release sufficient endogenous ATP to stimulate NLRP3 inflammasome assembly [151]. ATP, via P2X7 receptors, opens a pannexin pore, which leads to assembly and activation of the inflammasome. For this reason ATP was added in selected protocols. Cells were stimulated with PAMPs followed by a 30min pulse with exogenous ATP. When THP-1 monocytes were pre-stimulated for 3h and 24h with Pam3CSK4, FSL-1 or LPS, followed by 30 min ATP pulse (1mM or 5mM), we observed a significant increase in IL-1β release compared with media pulsed cells (Fig. 11).

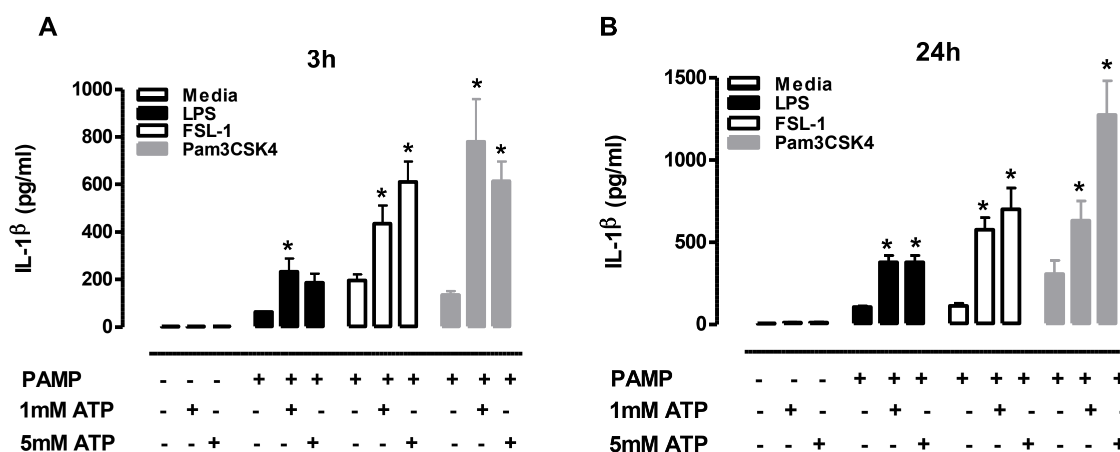


Figure 11. The effect of ATP pulse on the release of IL-1β in human monocytes after PAMP stimulation. THP-1 monocytes were seeded in a 96 well plates at 1 x 10^5 cells per well in RPMI with 10% FCS and allowed to equilibrate for 12h. After this time period cells were treated with media, LPS (1 µg/ml), FSL-1 (1 µg/ml) and Pam_3CSK4 (1 µg/ml) for 24h after which media was removed and cells were pulsed for 30 min with either ATP or fresh media. IL-1β release was measured by ELISA after 3h (A) and 24h (B). Data represented are the mean ± SEM of a total of n=9 replicates from 3 experimental days. *denotes p≤ 0.05 as assessed using a one-way ANOVA followed by a Bonferroni's multiple comparison post-hoc test.

3.3. Effect of signalling inhibitors on TLR2 and TLR4-induced IL-1β release by THP-1 monocytes.

To investigate whether there is any difference between TLR2 and TLR4-induced IL-1β release, we treated THP-1 cells with increasing concentrations of TLR signalling inhibitors, 30min prior to activation with LPS, Pam3CSK4 and FSL-1. All of the inhibitors that we used (Prednisone, p38, IKK and TAK-1 inhibitors) significantly reduced cytokine release with no difference observed between TLR4 and TLR2 signalling pathway (Fig. 12). The only difference that we noted was the potency and efficacy of the drugs to reduce IL-1β release. To determine whether inhibition of cytokine release was caused by the toxicity of the drug and/or cell death, we performed an AlamarBlue assay to measure cell respiration/viability. Our results showed that using the top concentration of TAK-1 inhibitor 5Z-7, caused a significant reduction in THP-1 respiration/viability and it might have been the cause of IL-1β reduction (Tab. 2).

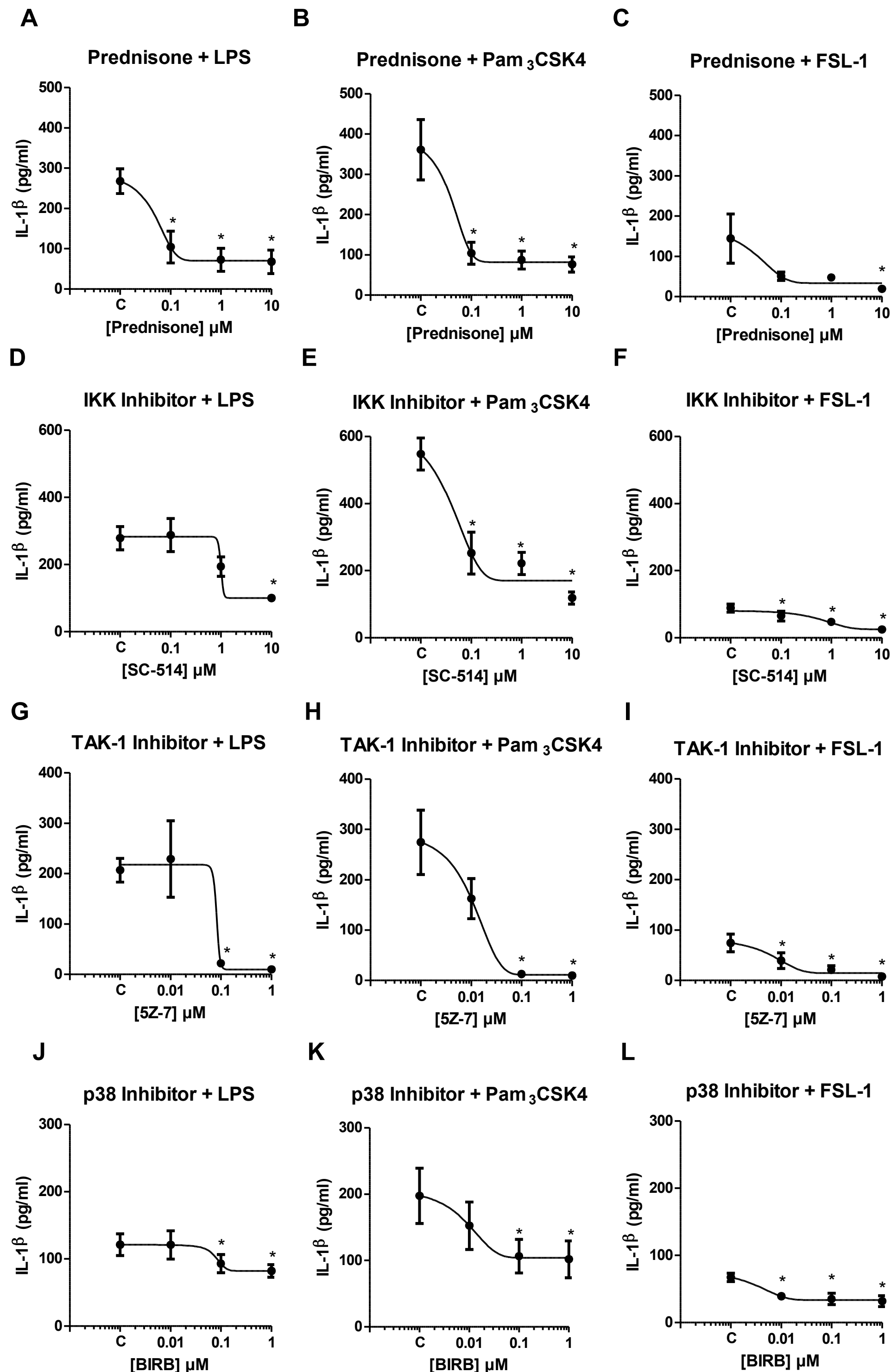


Figure 12. Effect of signalling inhibitors on TLR2 and TLR4 induced IL-1β release from THP-1 cells. THP-1 cells were seeded in a 96 well plates at 1 x 10^5 cells per well in RPMI with 10% FCS and allowed to equilibrate for 12h. After this time period cells were treated with increasing concentration of Prednisone (A-C), SC-514 (D-F), 5Z-7 (G-I) and BIRB (J-L). After 0.5h, LPS (0.1μg/ml), Pam_3CSK4 (0.1μg/ml) and FSL-1 (0.1μg/ml) were added. IL-1β release was measured by ELISA after 24h. Data represented are the mean ± SEM of n=9 replicates measured over 3 separate experimental days. *denotes $p \leq 0.05$ as assessed using a one-way ANOVA followed by a Dunnett's multiple comparison post-hoc test.

3.4. Effect of inflammasome inhibitors on TLR2 and TLR4-induced IL-1β release by THP-1 monocytes.

TLR ligands induce activation of NF-κB and trigger the gene expression and synthesis of pro-IL-1β. Conversion of pro-IL-1β to its active form requires activation of caspase-1 and a second stimulus to induce the formation of the inflammasome. Inflammasome assembly is associated with a number of highly varying stimuli which include ATP. To determine whether there are any differences between TLR2 and TLR4-induced IL-1β release, we used three inhibitors of inflammasome activation. Caspase-1 inhibition reduced IL-1β release after TLR2 and TLR4 stimulation with no differences seen between the two pathways (Fig. 13A,B, C). P2X7 inhibition (Fig. 13D,E,F) caused a decrease in cytokine secretion in TLR2 stimulated cells. After LPS stimulation there was a reduction in IL-1β only when the highest concentration of the inhibitor was used. Interestingly, pannexin-1 inhibition (Fig.13G,H,I) reduced IL-1β production only after TLR2 stimulation showing that TLR4 pathway does not require pannexin-1 in the absence of exogenous ATP (Fig. 13G). Cell viability data showed that Z-VAD-FMK induced very low level of cell death (Tab. 2).

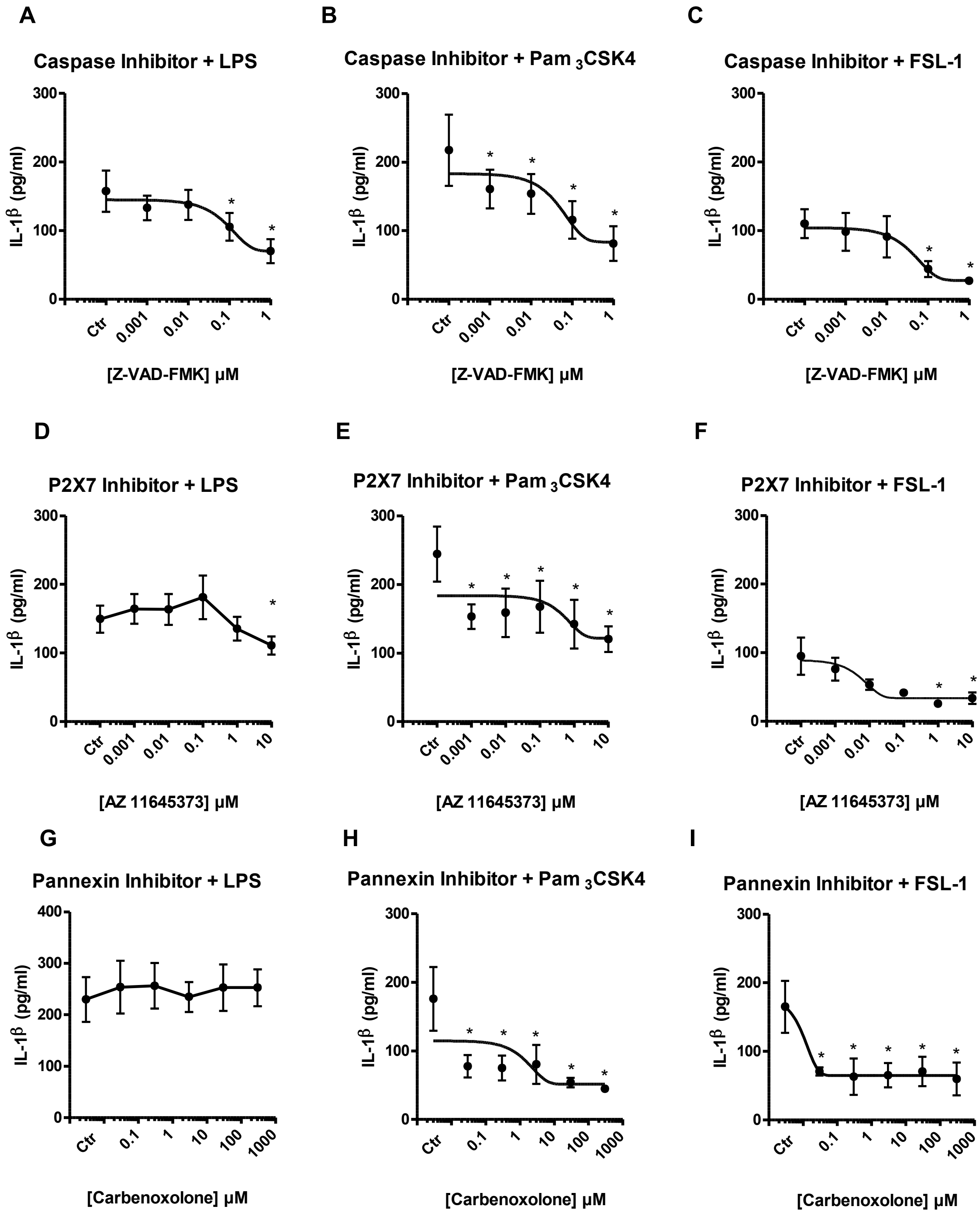


Figure 13. Effect of inflammasome inhibitors on TLR2 and TLR4 induced IL-1β release from THP-1 cells. THP-1 cells were seeded in a 96 well plates at 1 x 10^5 cells per well in RPMI with 10% FCS and allowed to equilibrate for 12h. After this time period cells were treated with increasing concentration of Z-VAD-FMK (A-C), AZ 11645373 (D-F) and Carbenoxolone (G-I). After 0.5h, LPS (0.1μg/ml), Pam_3CSK4 (0.1μg/ml) and FSL-1 (0.1μg/ml) were added. IL-1β release was measured by ELISA after 24h. Data represented are the mean ± SEM of n=9 replicates measured over 3 separate experimental days. *denotes p≤ 0.05 as assessed using a one-way ANOVA followed by a Dunnett's multiple comparison post-hoc test.

3.5. Effect of signalling inhibitors on TLR2 and TLR4-induced CXCL8 release by THP-1 monocytes.

We performed the same experiments as previously, but we measured CXCL8 release instead of IL-1β. All inhibitors that we used caused significant reduction of CXCL8 production by THP-1 monocytes (Fig. 14A–L). Although there were some differences in the potency and efficacy of the drugs, we did not observe any difference in activation between the TLR2 and TLR4 pathways. The top concentration of TAK-1 inhibitor again caused a significant reduction in cell viability/respiration (Tab. 2).

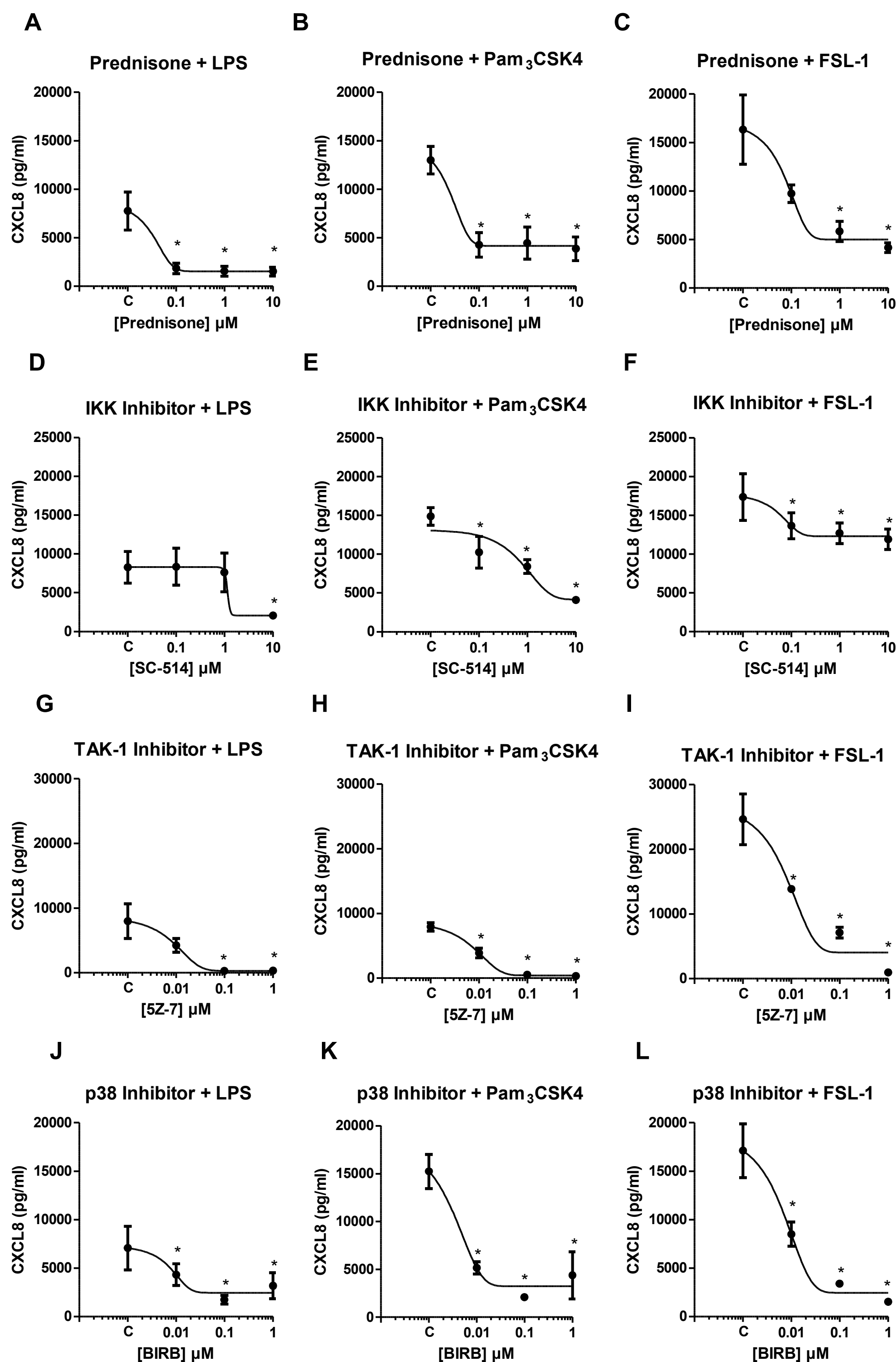


Figure 14. Effect of signalling inhibitors on TLR2 and TLR4 induced CXCL8 release from THP-1 cells. THP-1 cells were seeded in a 96 well plates at 1 x 10^5 cells per well in RPMI with 10% FCS and allowed to equilibrate for 12h. After this time period cells were treated with increasing concentration of Prednisone (A-C), SC-514 (D-F), 5Z-7 (G-I) and BIRB (J-L). After 0.5h, LPS (0.1μg/ml), Pam_3CSK4 (0.1μg/ml) and FSL-1 (0.1μg/ml) were added. CXCL8 release was measured by ELISA after 24h. Data represented are the mean ± SEM of n=9 replicates measured over 3 separate experimental days. *denotes p≤ 0.05 as assessed using a one-way ANOVA followed by a Dunnett's multiple comparison post-hoc test.

3.6. Effect of inflammasome inhibitors on TLR2 and TLR4-induced CXCL8 release by THP-1 monocytes.

CXCL8 release was slightly reduced after treating THP-1 monocytes with caspase-1 and P2X7 inhibitors but we did not observed a concentration dependent inhibition (Fig. 15B,F). CXCL8 release was slightly increased after pre-incubation with the P2X7 receptor inhibitor in LPS-primed monocytes and it showed as statistical significant but we think that this may be due to off target effects of AZ11645373 (Fig. 15A,D,H). Our results indicated that inflammasome signalling is not involved in CXCL8 production.

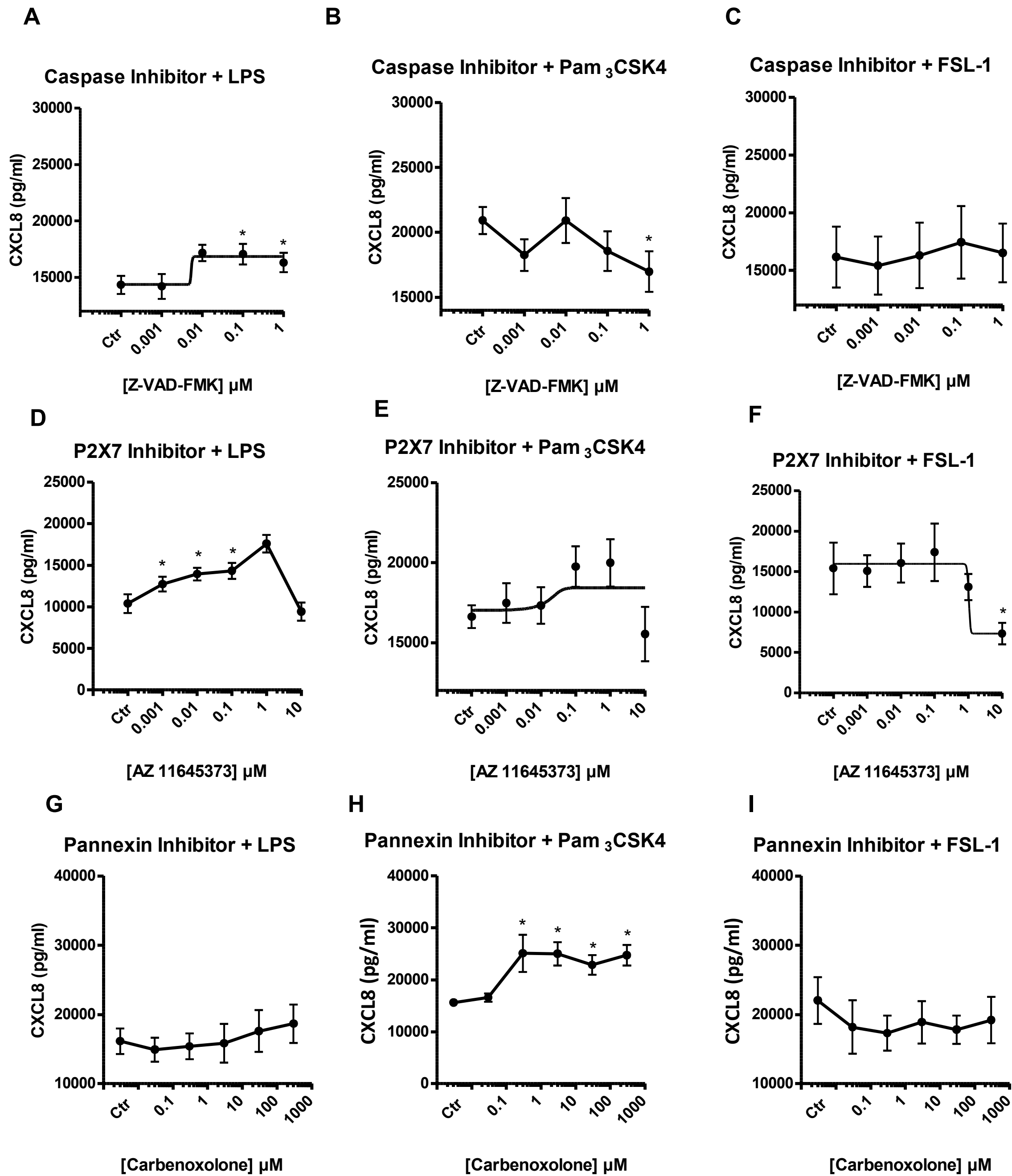
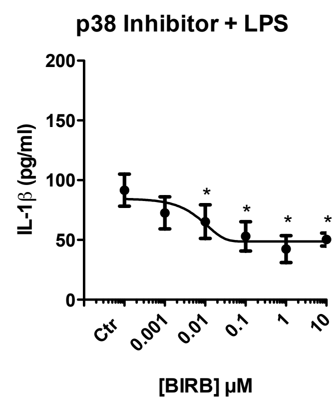


Figure 15. Effect of inflammasome inhibitors on TLR2 and TLR4 induced CXCL8 release from THP-1 cells. THP-1 cells were seeded in a 96 well plates at 1 x 10^5 cells per well in RPMI with 10% FCS and allowed to equilibrate for 12h. After this time period cells were treated with increasing concentration of Z-VAD-FMK (A-C), AZ 11645373 (D-F) and Carbenoxolone (G-I). After 0.5h, LPS (0.1µg/ml), Pam_3CSK4 (0.1µg/ml) and FSL-1 (0.1µg/ml) were added. CXCL8 release was measured by ELISA after 24h. Data represented are the mean ± SEM of n=9 replicates measured over 3 separate experimental days. *denotes p≤ 0.05 as assessed using a one-way ANOVA followed by a Dunnett's multiple comparison post-hoc test.

3.7. Effect of signalling inhibitors on TLR2 and TLR4-induced IL-1β release by PBMCs.

To give clinical relevance and to assess our model, experiments in THP-1 cells were repeated in freshly isolated PBMCs. We chose inhibitors where differences were found between TLR2 and TLR4-mediated cytokines release. We stimulated cells only with LPS and Pam_3CSK4, but not FSL-1, as there was no difference between TLR2/1 and TLR2/6-mediated responses in THP-1 cells. PBMCs were less sensitive to TLR stimulation than THP-1 cells and the response to LPS was much greater than to Pam_3CSK4. We observed a reduction in IL-1β release after inhibition of p38 (Fig. 16A,B), caspase-1 (Fig. 16E,F) and P2X7 (Fig. 16G,H) with no difference between TLR2 and TLR4. Similarly to THP-1 cells there was significant inhibition after pre-incubation with inhibitors of pannexin-1 (Fig. 16I,J), where we observed a reduction in TLR2 but not TLR4-induced IL-1β release. The same effect was seen after inhibition of IKK (Fig. 16C,D) which is probably associated with late NF-κB activation after LPS stimulation and recruitment of TRAM protein. We also observed a very small decrease in cell viability induced by the highest concentration of Z-VAD-FMK and AZ. However, unlike the effects on CXCL8, this effect was not concentration dependent and therefore unlikely to account for the inhibitory effects of these compounds on cytokine release (Tab. 3).

A



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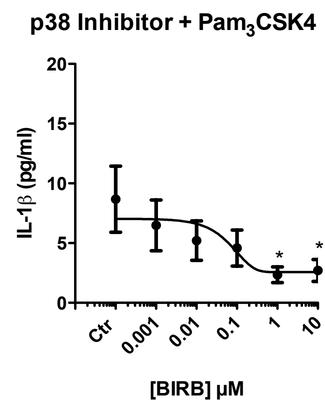

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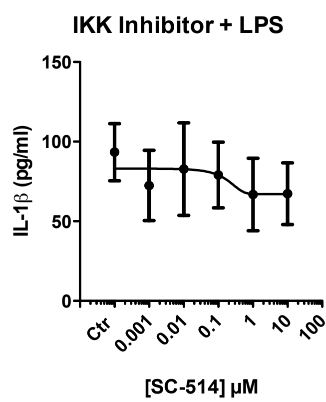

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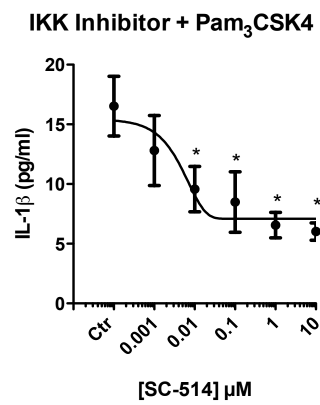
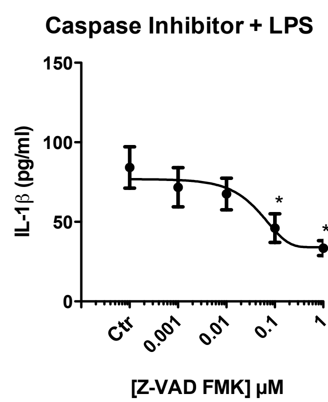


E



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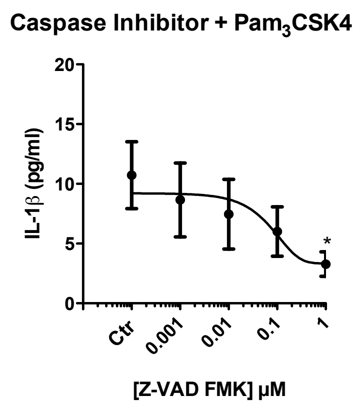


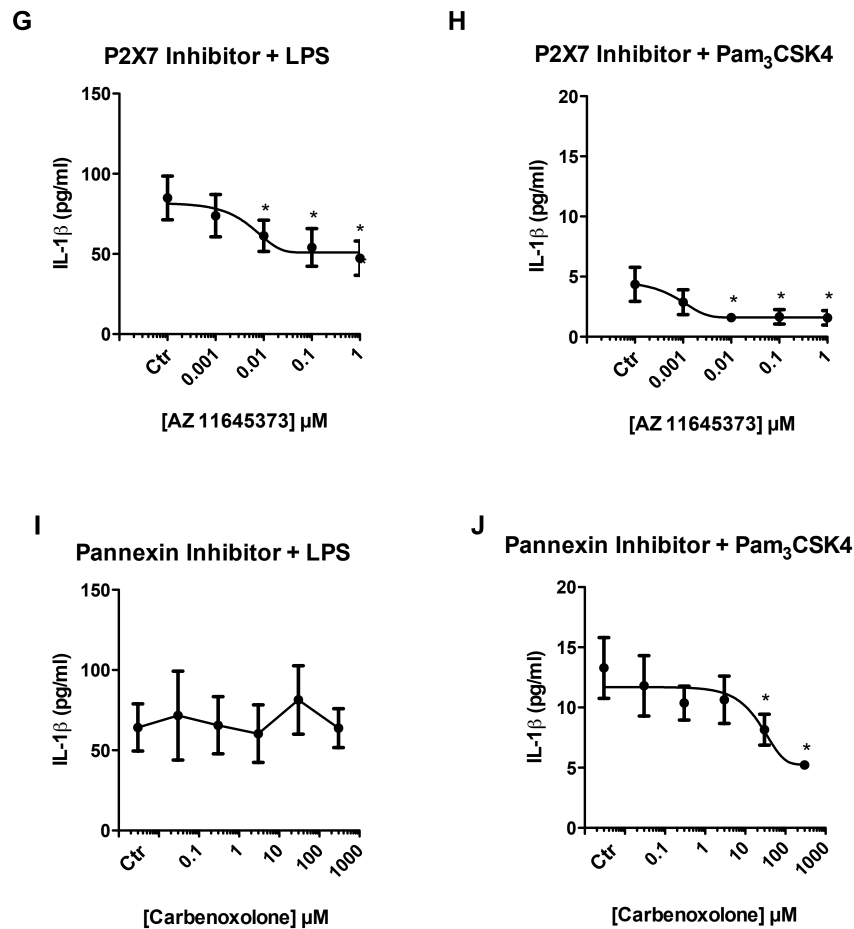
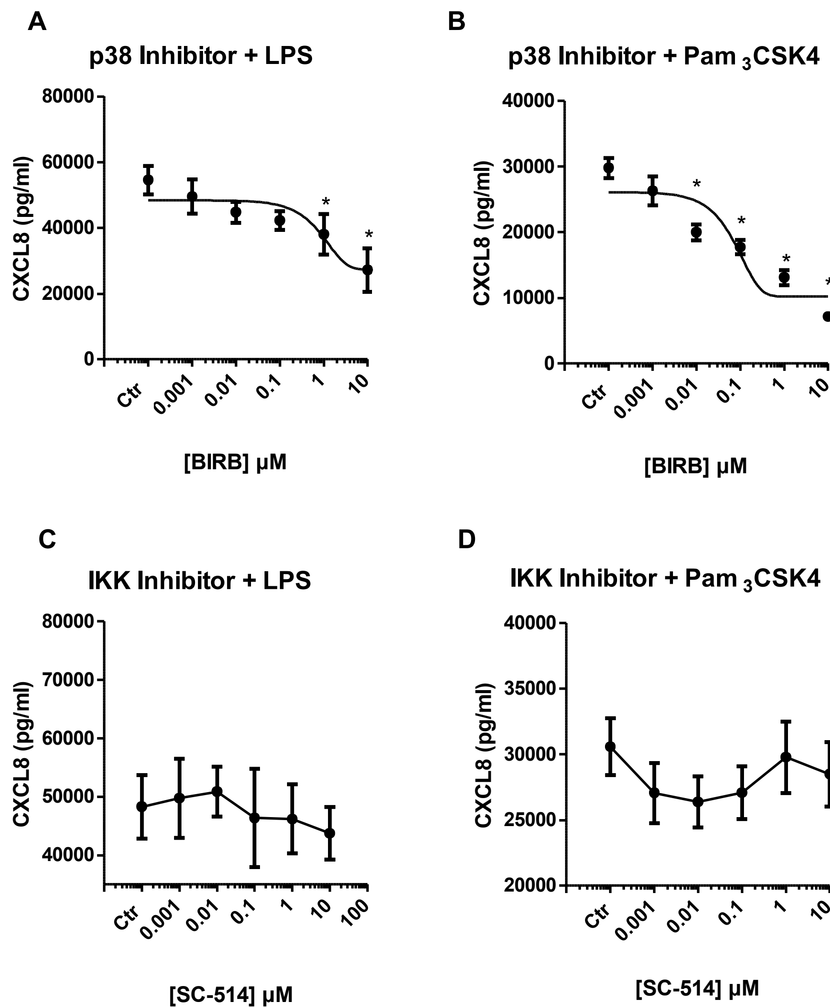


Figure 16. Effect of signalling inhibitors on TLR2 and TLR4 induced IL-1β release from PBMCs. Freshly isolated PBMCs were seeded in a 96 well plates at 1 x 10^5 cells per well in RPMI with 10% FCS and allowed to equilibrate for 12h. After this time period cells were treated with increasing concentration of BIRB (A,B), SC-514 (C,D), Z-VAD-FMK (E,F), AZ 11645373 (G,H) and Carbenoxolone (I,J). After 0.5h, LPS (1µg/ml) or Pam_3CSK4 (1µg/ml) were added. IL-1β release was measured by ELISA after 24h. Data represented are the mean ± SEM of n=12 replicates from 6 separate donors. *denotes $p \leq 0.05$ as assessed using a one-way ANOVA followed by a Dunnett's multiple comparison post-hoc test.

3.8. Effect of signalling inhibitors on TLR2 and TLR4-induced CXCL8 release by PBMCs.

CXCL8 release was decreased only after p38 inhibition (Fig. 17A,B). Surprisingly, we did not observe any reduction after pre-incubation with the IKK inhibitor (Fig. 17C,D). All other inflammasome inhibitors did not have any effect on CXCL8 release.



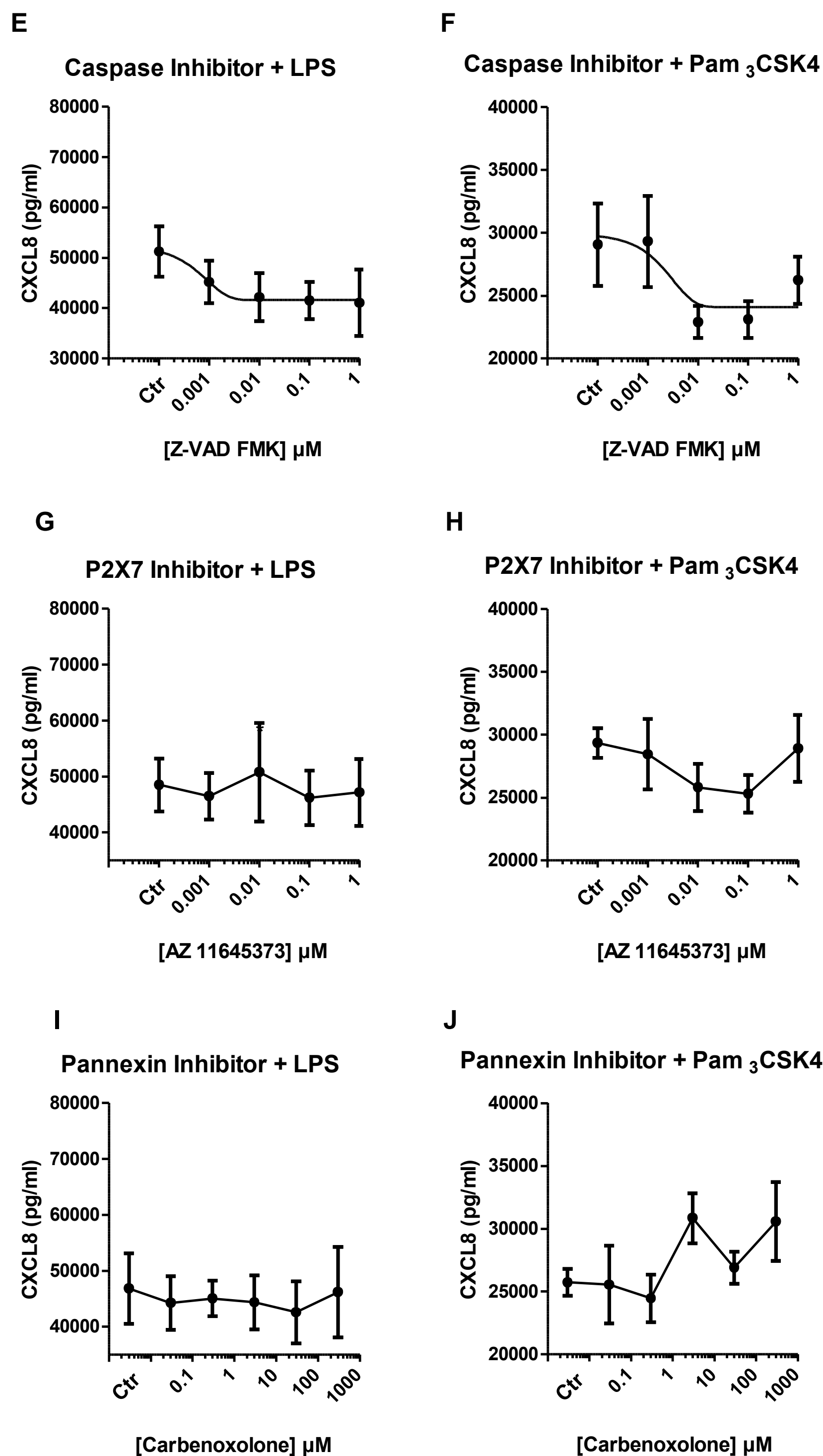


Figure 17. Effect of signalling inhibitors on TLR2 and TLR4 induced CXCL8 release from PBMCs. Freshly isolated PBMCs were seeded in a 96 well plates at 1 x 10^5 cells per well in RPMI with 10% FCS and allowed to equilibrate for 12h. After this time period cells were treated with increasing concentration of BIRB (A,B), SC-514 (C,D), Z-VAD-FMK (E,F), AZ 11645373 (G,H) and Carbenoxolone (I,J). After 0.5h, LPS (1µg/ml) or Pam_3CSK4 (1µg/ml) were added. CXCL8 release was measured by ELISA after 24h. Data represented are the mean ± SEM of n=12 replicates from 6 separate donors. *denotes p≤ 0.05 as assessed using a one-way ANOVA followed by a Dunnett's multiple comparison post-hoc

3.9. IL-1β release after P2X7 and pannexin-1 knock down in THP-1 cells.

Previous experiments showed that there is a difference between TLR2 and TLR4-induced IL-1β release, where the TLR2 but not the TLR4 pathway involved panx1. To verify our results we knocked down *panx1* and *P2X7* genes using siRNA. After 48h, Pam_3CSK4 and LPS were added to THP-1 cells, Il-1β levels were measured after 24h incubation. Gene knock down was confirmed using RT-PCR (Fig. 19A) and cell viability was measured using trypan blue exclusion (Fig. 19B). Our results were consistent with results we obtained using P2X7 and pannexin-1 inhibitors in PBMCs and THP-1 cells. We demonstrated that when we knock down *panx1* gene, levels of IL-1β decreased after TLR2 but not TLR4 stimulation, suggesting that TLR2-induced IL-1β release requires panx1 (Fig. 18B). IL-1β levels were reduced by 50%, which corresponded with a decrease in panx1 mRNA levels (Fig. 19A). There was no difference in LPS stimulated cells (Fig. 18A).

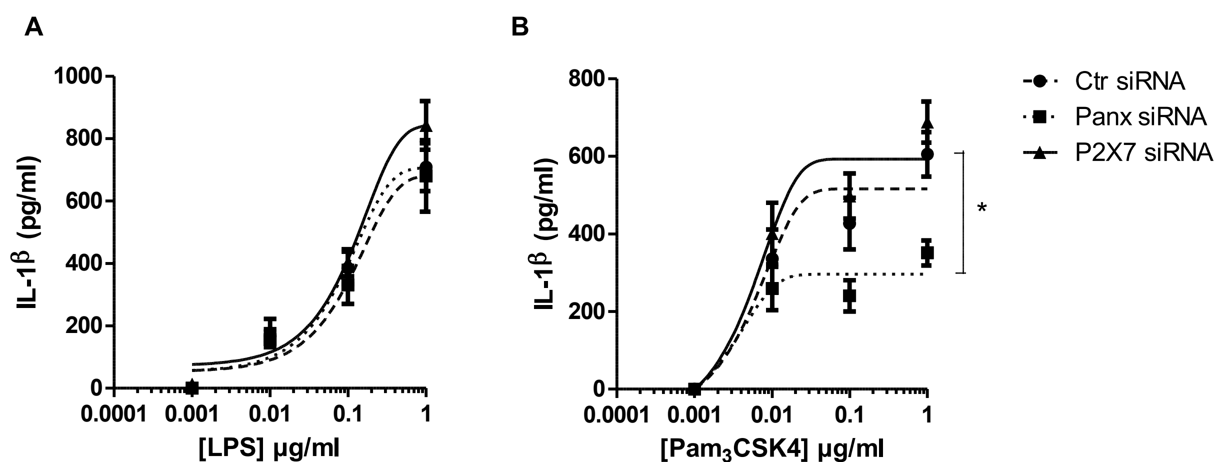


Figure 18. Effect of P2X7 and pannexin-1 knock down on TLR2 and TLR4 induced IL-1β release from THP-1 cells. THP-1 cells were transfected with 100µM of siRNA using Amaxa Nucleofector system. Cells were seeded in 96 well plates at 1 x 10^5 cells per well in RPMI with 10% FCS. After 48h LPS (A) and Pam_3CSK4 (B) were added to the cells. ELISA was performed after 24h incubation. Data represented are the mean ± SEM of n=15 replicates over 3 experimental days. *denotes $p \leq 0.05$ as assessed using a one-way ANOVA followed by a Dunnett's multiple comparison post-hoc test.

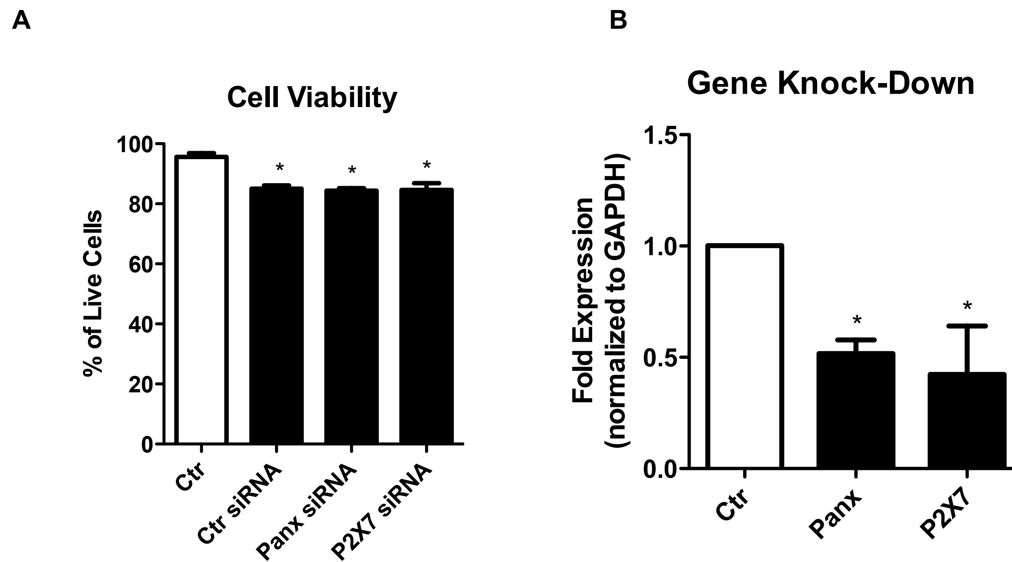


Figure 19. Cell viability and gene expression after siRNA transfection. THP-1 cells were transfected with 100μM of siRNA using Amaxa Nucleofector system. Cells were seeded in 96 well plates at 1 x 10^5 cells per well in RPMI with 10% FCS. After 48h cell viability was measured by the AlamarBlue assay (A) and RNA was extracted for future analysis. Gene expression was measured by RT-PCR (B). Data represented are the mean ± SEM of n=3 replicates over 3 experimental days. *denotes $p \leq 0.05$ as assessed using a one-way ANOVA followed by a Dunnett's multiple comparison post-hoc test.

3.10. IL-β release after activation of adaptor proteins in response to PAMPs.

TLR4 unlike TLR2 upon stimulation with PAMPs was shown to activate Myd88-dependent and TRIF-dependent signalling pathways. Stimulation of PBMCs with LPS (Fig. 20A) induced much higher response in IL-1β release than with Pam_3CSK4 (Fig. 20B). To investigate whether activation of TRIF can induce inflammasome assembly, we decided to stimulate Myd88, TRIF and both pathways together to measure IL-1β levels in supernatants. LPS (Fig. 20A) was the best agonist to induce IL-1β release, but Pam_3CSK4 (Fig. 20B), Poly I:C (Fig. 20C) and FSL-1 (Fig. 20D) activation also produced significant amounts of IL-1β when stimulated with the highest concentration of ligands. When we treated PBMCs with Pam_3CSK4 (activates Myd88) or FSL-1 (activates Myd88) together with Poly I:C (activates TRIF), we observed a significant increase in IL-1β production but the release was not as high as after stimulation with LPS alone (Fig. 20 E,F). My data suggests that in the presence of Poly I:C, LPS is a more potent activator of IL-1β release than either Pam3CSK4 or FSL-1. This could be due to differences in the signalling between TLR adaptor protein pathways for TLR4 and TLR2. For example, TLR4, but not TLR2, signalling is associated with TRAM.

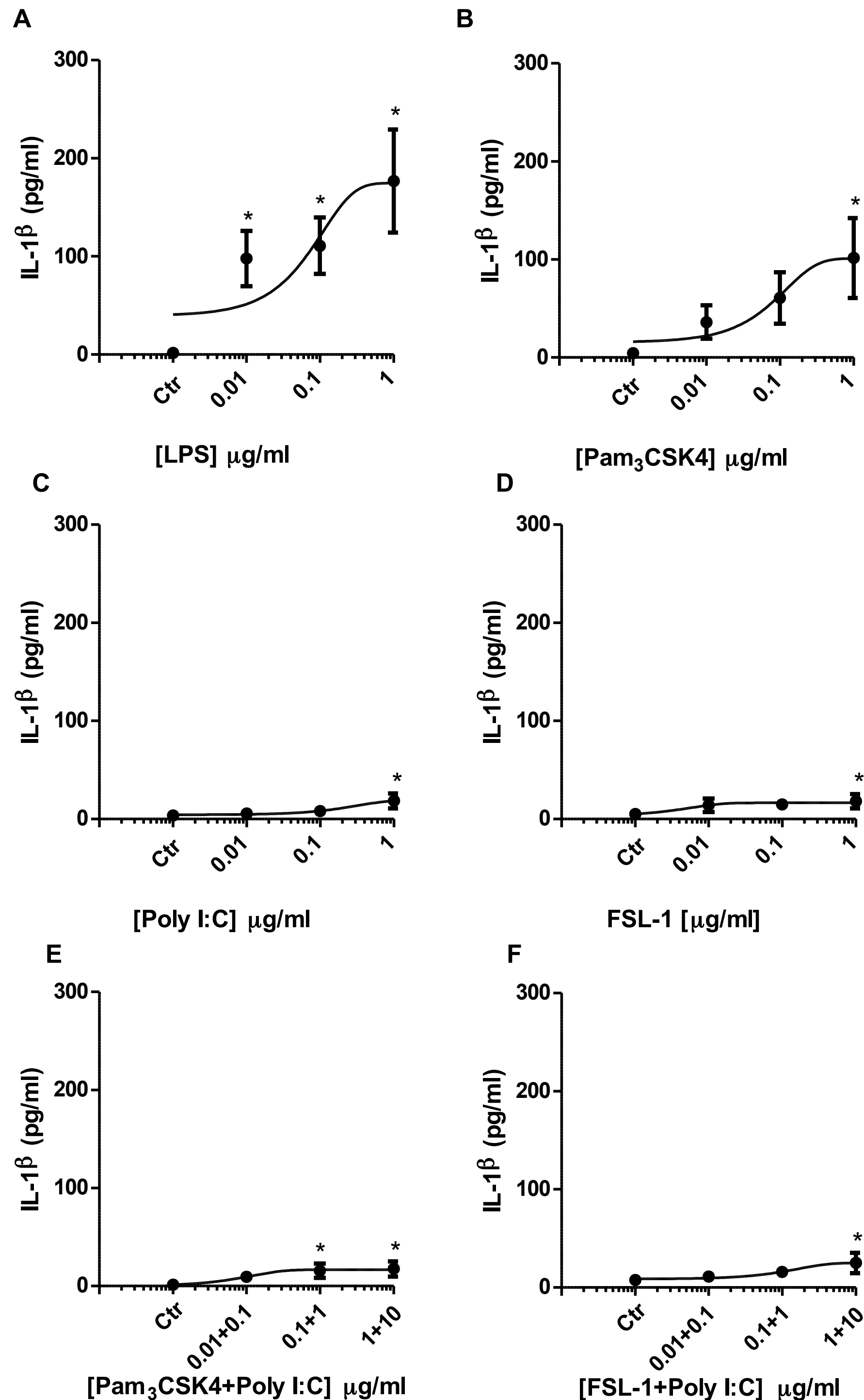


Figure 20. IL-β release after activation of Myd88 and TRIF in response to PAMPs. Freshly isolated PBMCs were seeded in a 96 well plates at 1 x 10^5 cells per well in RPMI with 10% FCS and allowed to equilibrate for 12h. After this time period cells were treated with LPS alone (A), Pam_3CSK4 alone (B), Poly I:C alone (C), FSL-1 alone (D), Pam_3CSK4+Poly I:C (E) and FSL-1+Poly I:C (F). IL-1β release was measured by ELISA after 24h. Data represented are the mean ± SEM of n=6 replicates from 3 separate donors. *denotes p≤ 0.05 as assessed using a one-way ANOVA followed by a Dunnett's multiple

3.11. CXCL8 release after activation of adaptor proteins, Myd88 and TRIF in response to PAMPs.

We also measured CXCL8 release and we observed significant increase after stimulation with LPS, Pam_3CSK4, FSL-1 and Poly I:C. Activation of Myd88 and TRIF pathways simultaneously by TLR2 and TLR3 agonists did not cause an additive or synergistic effect in CXCL8 release (Fig. 21).

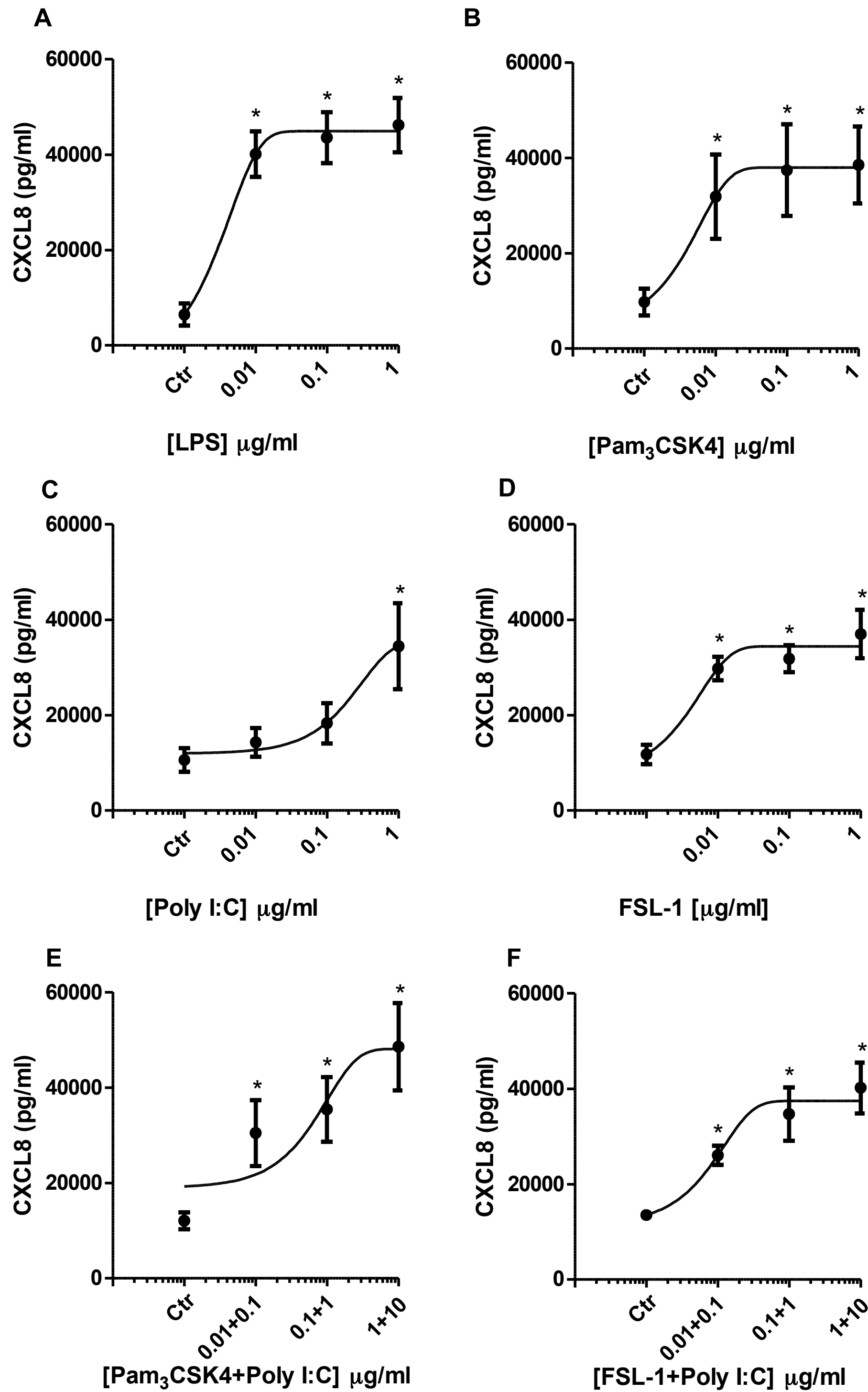


Figure 21. CXCL8 release after activation of Myd88 and TRIF in response to PAMPs. Freshly isolated PBMCs were seeded in a 96 well plates at 1 x 10^5 cells per well in RPMI with 10% FCS and allowed to equilibrate for 12h. After this time period cells were treated with LPS alone (A), Pam_3CSK4 alone (B), Poly I:C alone (C), FSL-1 alone (D), Pam_3CSK4+Poly I:C (E) and FSL-1+Poly I:C (F). CXCL8 release was measured by ELISA after 24h. Data represented are the mean ± SEM of n=6 replicates from 3 separate donors. *denotes $p \leq 0.05$ as assessed using a one-way ANOVA followed by a Dunnett's multiple comparison post-hoc test.

3.12. IL-1β release after inhibition of Myd88 and TRIF adaptor proteins.

To investigate further the role of adaptor proteins in inflammasome activation and IL-1β release, we used bb loop inhibitory peptides to block Myd88 and TRIF adaptor proteins. TIR domains of TLRs are composed of five β-strands alternating with five α-helices. A functionally important proline-glycine combination that is highly conserved among TIR domains is located in the loop connecting the second β-strand with the second helix, the "BB loop". In our study we used Myd88 and TRIF BB loop inhibitory peptides which block MyD88 signalling by inhibiting its homodimerization through binding, and blocks TRIF signalling by interfering with TLR-TRIF interaction.

After 6 hours we treated PBMCs with LPS, Pam_3CSK4, Poly I:C and cigarette smoke extract (CSE) and we measured IL-1β, CXCL8 and CXCL10 release 24h later. We observed a significant increase in IL-1β release after treatment with LPS and Pam_3CSK4. Poly I:C and CSE did not have any effect on cytokine production (Fig. 22A). Pre-treatment of cells with 10μM of Myd88, TRIF and both inhibitors together reduced LPS-induced IL-1β production (Fig. 22C). When we treated PBMCs with Pam_3CSK4 after pre-incubation with inhibitors, we observed a reduction in IL-1β production only when we used Myd88 and TRIF peptides together (Fig. 22D). Pre-treatment of PBMCs did not have any effect on Poly I:C and CSE-induced IL-1β release (Fig. 22E, F).

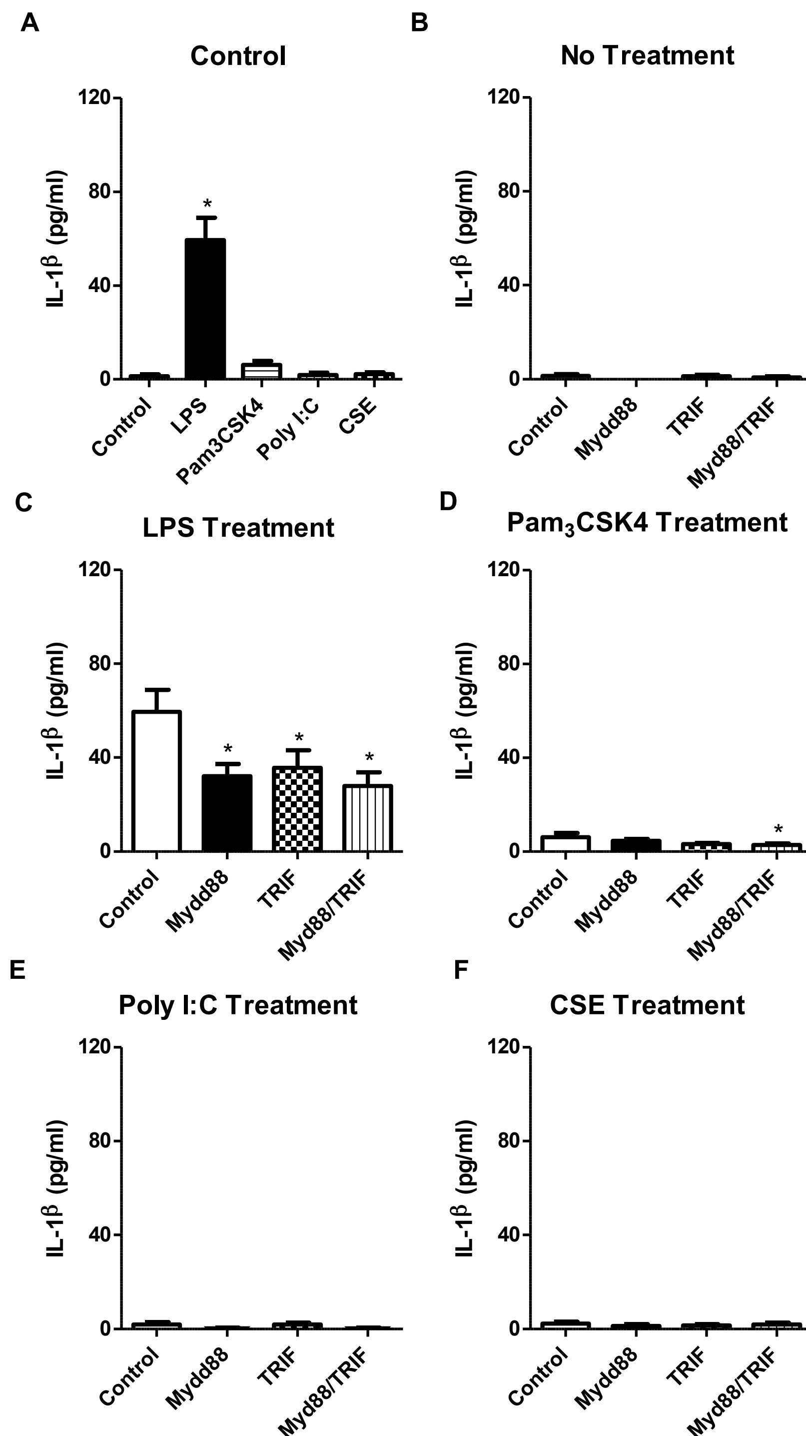


Figure 22. IL-1β release after inhibition of Myd88 and TRIF adaptor proteins. Freshly isolated PBMCs were seeded in a 96 well plates at 1 x 10^5 cells per well in RPMI with 10% FCS and allowed to equilibrate for 12h. After this time period cells were treated with 10μM of inhibitors. After 6h LPS (C), Pam_3CSK4 (D), Poly I:C (E) and CSE (F) were added. IL-1β release was measured by ELISA after 24h. Data represented are the mean ± SEM of n=6 replicates from 3 separate donors. *denotes $p \leq 0.05$ as assessed using a one-way ANOVA followed by a Dunnett's multiple comparison post-hoc test. (A) treatment with TLR agonists only, (B) treatment with inhibitors only.

Similar to IL-1β release only LPS and Pam_3CSK4 significantly induced CXCL8 production while Poly I:C and CSE did not cause any effect (Fig. 23A). When we treated cells with LPS after pre-incubation with inhibitors we detected a decrease in CXCL8 production under all conditions used (Fig. 23E,C). Surprisingly, Pam_3CSK4-induced CXCL8 release, which is not TRIF dependent, was reduced after pre-incubation with TRIF inhibitory peptide, suggesting that these inhibitors might be non-selective (Fig. 23D). These inhibitors had no effect on Poly I:C and CSE treated cells (Fig. 23E,F).

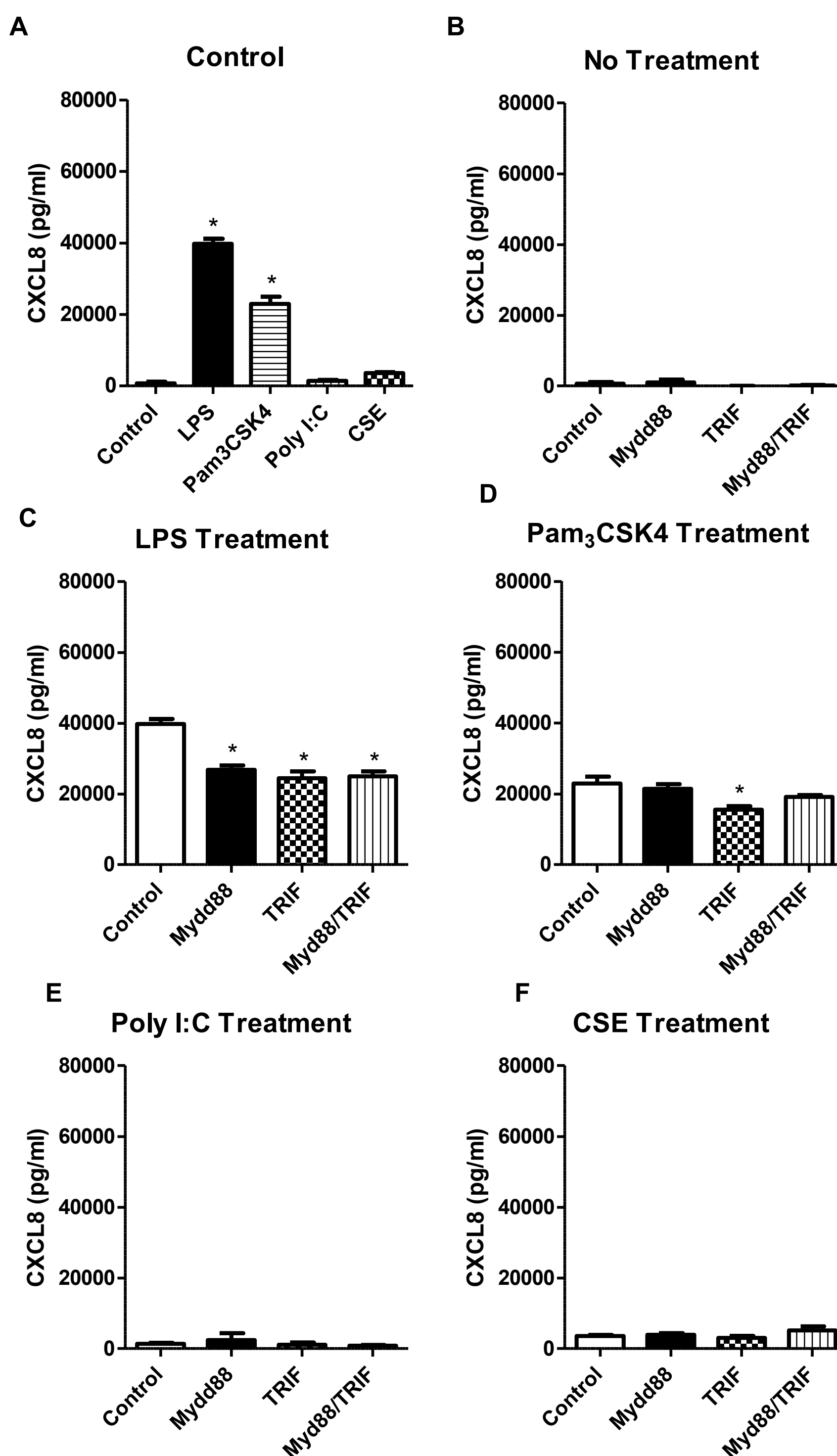


Figure 23. CXCL8 release after inhibition of Myd88 and TRIF adaptor proteins. Freshly isolated PBMCs were seeded in a 96 well plates at 1 x 10^5 cells per well in RPMI with 10% FCS and allowed to equilibrate for 12h. After this time period cells were treated with 10μM of inhibitors. After 6h LPS (C), Pam_3CSK4 (D), Poly I:C (E) and CSE (F) were added. CXCL8 release was measured by ELISA after 24h. Data represented are the mean ± SEM of n=6 replicates from 3 separate donors. *denotes $p \leq 0.05$ as assessed using a one-way ANOVA followed by a Dunnett's multiple comparison post-hoc test. (A) treatment with TLR agonists only, (B) treatment with inhibitors only.

3.14. CXCL10 release after inhibition of Myd88 and TRIF adaptor proteins.

Only Poly I:C induced significant release of CXCL10 (Fig. 24A) but pre-incubation with inhibitory peptides did not have any effect on this cytokine production. We observed an increase in CXCL10 after pre-incubation with Myd88 inhibitor after LPS and CSE treatment (Fig. 24C,D).

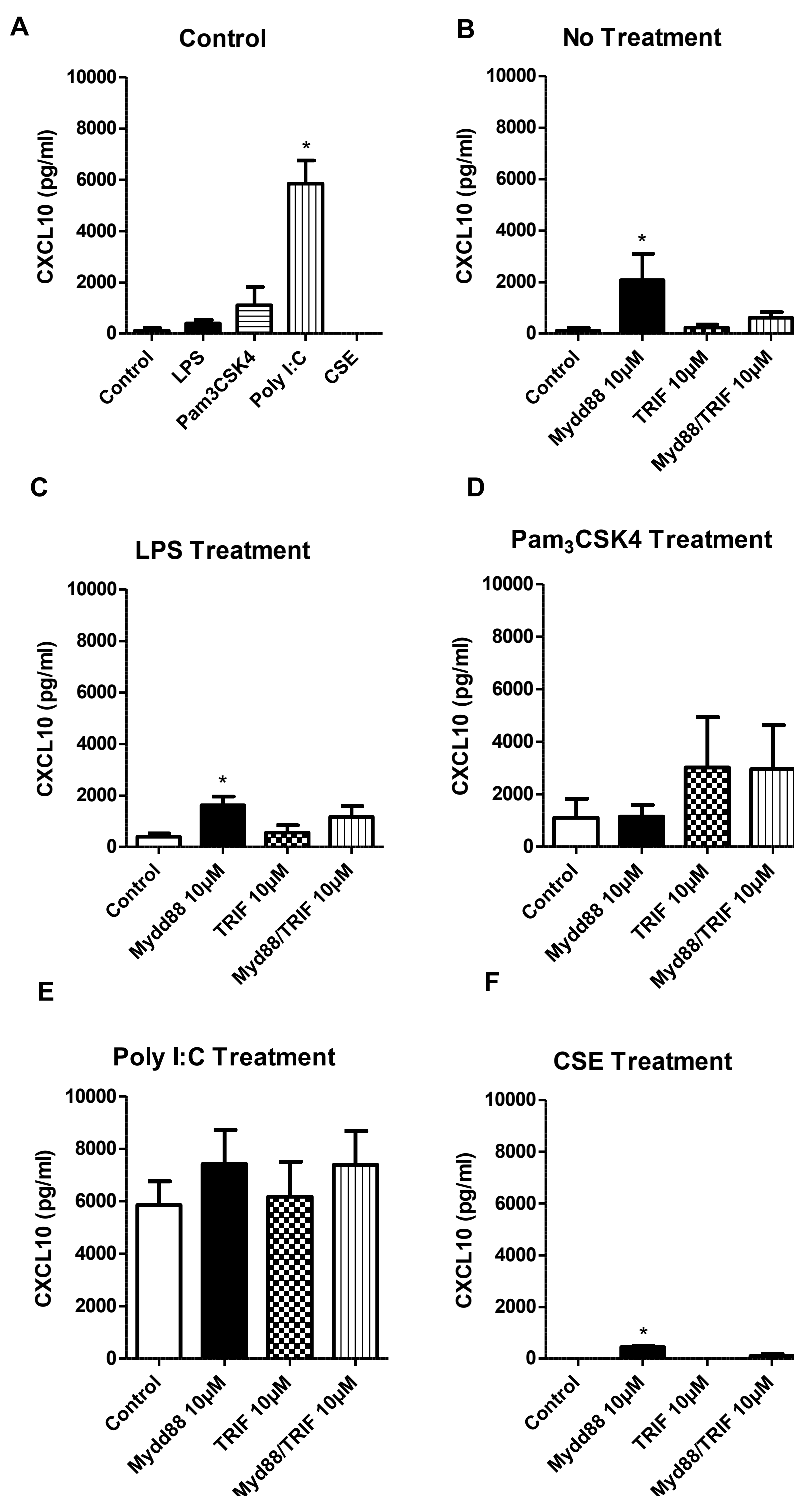


Figure 24. CXCL10 release after inhibition of Myd88 and TRIF adaptor proteins. Freshly isolated PBMCs were seeded in a 96 well plates at 1 x 10^5 cells per well in RPMI with 10% FCS and allowed to equilibrate for 12h. After this time period cells were treated with 10µM of inhibitors. After 6h LPS (C), Pam_3CSK4 (D), Poly I:C (E) and CSE (F) were added. CXCL10 release was measured by ELISA after 24h. Data represented are the mean ± SEM of n=6 replicates from 3 separate donors. *denotes $p \leq 0.05$ as assessed using a one-way ANOVA followed by a Dunnett's multiple comparison post-hoc test. (A) treatment with TLR agonists only, (B) treatment with inhibitors only.

Treatment	Concentration	Mean Absorbance	SEM	n	p
[Prednisone] µM	0	0.252	0.011	4	
	0.1	0.232	0.006	4	ns
	1	0.246	0.018	4	ns
	10	0.232	0.008	4	ns
[SC-514] µM	0	0.328	0.054		
	0.1	0.253	0.002	4	ns
	1	0.303	0.036	4	ns
	10	0.263	0.006	4	ns
[5Z-7] µM	0	0.339	0.050		
	0.01	0.128	0.086	4	ns
	0.1	0.288	0.074	4	ns
	1	0.312	0.033	4	*
[BIRB] µM	0	0.286	0.045		
	0.1	0.247	0.007	4	ns
	1	0.252	0.021	4	ns
	10	0.242	0.007	4	ns
[Z-VAD-FMK] µM	0	0.289	0.023		
	0.001	0.205	0.015	4	*
	0.01	0.218	0.019	4	*
	0.1	0.226	0.006	4	*
	1	0.222	0.005	4	*
	10	0.225	0.019	4	*
[AZ 11645373] µM	0	0.270	0.004		
	0.001	0.168	0.033	4	ns
	0.01	0.212	0.009	4	ns
	0.1	0.260	0.056	4	ns
	1	0.256	0.009	4	ns
	10	0.294	0.049	4	ns
[Carbenoxolone] µM	0	0.253	0.005		
	0.03	0.265	0.011	4	ns
	0.3	0.216	0.015	4	ns
	3	0.248	0.011	4	ns
	30	0.220	0.026	4	ns
	300	0.231	0.01433	4	ns

Table 2. Effect of signalling inhibitors on cell viability/respiration. THP-1 cells were seeded in a 96 well plates at 1 x 10^5 cells per well in RPMI with 10% FCS and allowed to equilibrate for 12h. After this time period cells were treated with increasing concentration of inhibitors. Cell viability was measured by the AlamarBlue assay after 24h. Data represented are the mean ± SEM of n=6 replicates from 3 separate experiments *denotes $p \leq 0.05$ as assessed using a one-way ANOVA followed by a Dunnett's multiple comparison post-hoc test.

- **PBMCs**

Treatment	Concentration	Mean Absorbance	SEM	n	p
[SC-514] µM	0	0.470	0.021		
	0.001	0.427	0.045	6	ns
	0.01	0.467	0.014	6	ns
	0.1	0.493	0.010	6	ns
	1	0.477	0.021	6	ns
	10	0.464	0.025	6	ns
[BIRB] µM	0	0.463	0.004		
	0.001	0.430	0.009	6	ns
	0.01	0.446	0.017	6	ns
	0.1	0.453	0.011	6	ns
	1	0.418	0.008	6	ns
	10	0.403	0.021	6	*
[Z-VAD-FMK] µM	0	0.440	0.019		
	0.001	0.452	0.006	6	ns
	0.01	0.472	0.026	6	ns
	0.1	0.453	0.015	6	ns
	1	0.477	0.018	6	ns
	10	0.332	0.036	6	*
[AZ 11645373] µM	0	0.460	0.022		
	0.001	0.486	0.019	6	ns
	0.01	0.467	0.007	6	ns
	0.1	0.463	0.020	6	ns
	1	0.434	0.048	6	ns
	10	0.362	0.049	6	ns
[Carbenoxolone] µM	0	0.450	0.022		
	0.03	0.436	0.022	6	ns
	0.3	0.470	0.014	6	ns
	3	0.461	0.023	6	ns
	30	0.472	0.008	6	ns
	300	0.542	0.012	6	*

Table 3. Effect of signalling inhibitors on cell viability/respiration. Freshly isolated PBMCs were seeded in a 96 well plates at 1 x 10^5 cells per well in RPMI with 10% FCS and allowed to equilibrate for 12h. After this time period cells were treated with increasing concentration of inhibitors. Cell viability was measured by the AlamarBlue assay after 24h. Data represented are the mean ± SEM of n=6 replicates from 3 separate experiments. *denotes $p \leq 0.05$ as assessed using a one-way ANOVA followed by a Dunnett's multiple comparison post-hoc test.

Discussion

Aberrant inflammasome activation has been linked to a number of hereditary cryopyrinopathies such as familial cold autoinflammatory syndrome, Muckle-Wells syndrome and chronic infantile neurologic cutaneous articular syndrome. Blood monocytes from patients with these disorders release more IL-1β than monocytes from unaffected controls and mutation of NLRP3 gene has also been found [152]. Recent study by Eltom et al. using either a selective P2X7 inhibitor or P2X7 knockout mice has also shown a temporal correlation between markers of the P2X7/inflammasome pathway activation and airway inflammation [111]. Thus, there is an increased interest in the biology of IL-1β as a potential target for inflammatory disease. The role of TLR receptors in inflammasome activation has been described previously (Chapter 1, section 1.5) but the exact mechanism of inflammasome activation is not fully understood. A number of questions remain surrounding the events that occur upstream of NLRP activation and it is important to answer them for future development of drugs that will make them more effective and specific for the treatment of inflammation. In the current study we have confirmed the work of others by demonstrating that LPS, Pam_3CSK4 and FSL-1 activate human THP-1 monocytes to release IL-1β. IL-1β release by these agonists occurred independently of additional ATP. However, IL-1β levels could be elevated further when ATP was added exogenously. This is wholly consistent with what is known for LPS-induced IL-1β release from these cells [151]. As with other cytokines, including CXCL8 [153] we show here that TLR2 and TLR4 signalling also induce CXCL8 release over 24h. We also found that activation of TLR3 with Poly I:C did not induce IL-1β and CXCL8 release from THP-1 monocytes.

To investigate TLR2 vs TLR4-induced IL-1β and CXCL8 release, we used TLR signalling pathway inhibitors and measured cytokine release after 24h. We observed a decrease in CXCL8 levels after inhibition of IKK, TAK-1 and p38, suggesting that all these proteins are

involved in the release of this cytokine. Prednisone which inhibits transcription of inflammatory mediators also reduced CXCL8 release form THP-1 cells. However, there was no difference between TLR2 and TLR4 pathways. Inflammasomes are not involved in CXCL8 production and it was not surprising that we did not observed any decrease after treatment of THP-1 cells with inhibitors of components of the inflammasome (caspase-1, P2X7 and pannexin-1). We obtained different results after IL-1β measurements. Again there was a decrease in IL-1β release after inhibition of TLR signalling pathway (IKK, TAK-1, p38) and after steroid treatment and there was no difference between TLR2 and TLR4. IL-1β levels were reduced after caspase-1 inhibition, which is responsible for the cleavage of pro-IL-1β to mature IL-1β. P2X7 receptor inhibition also caused a decrease after TLR2 stimulation, whereas only the highest dose of this inhibitor reduced TLR4 stimulation with LPS. The most interesting results we obtained after inhibition of pannexin-1, where there was a reduction of IL-1β levels after TLR2 but not after TLR4 activation. We decided to perform the same experiment on primary mononuclerar cells freshly isolated from blood as there are sometimes differences in the responses from cell lines and primary cells. In terms of CXCL8 release, we obtained the same results as was seen in THP-1 cells after stimulation with TLR2 and TLR4 agonist followed by inhibition with components of the TLR signalling pathway. The only difference seen was after IKK inhibition where we did not observe a reduction in CXCL8 release. One possible reason for this might be that the release of CXCL8 is also under the regulation of the transcription factor AP-1. It is interesting to note that the signalling pathways leading to NF-κB and AP-1 activation overlap and both are involved in the induction and regulation of cytokines [154]. NF-κB and AP-1 transcription factors binding sites have been identified in the promoter region of CXCL8 [155] and the effect we have seen in PBMCs might be due to the activation of AP-1 [146]. As with THP-1 cells there was no reduction in levels of CXCL8 after inhibition of inflammasome assembly components in PBMCs. Regarding IL-

1β release, we observed a reduction after p38 inhibition, however no differences between TLR2 and TLR4 activation were observed. However, when we blocked IKK with SC-514, we observed a decrease after TLR2 but not TLR4 stimulation. In comparison to early activation, late activation of NF-κB and the induction of type I IFNs following TLR4 activation with LPS, have been demonstrated to utilize different adaptor proteins. TRIF was identified to be the adaptor protein responsible for MyD88-independent pathway activation by TLR4 [24]. Importantly TRIF also has the ability to activate NF-kB and the IFNβ promoter. The difference between TLR2 and TLR4-induced IL-1β release might be explained by late NF-κB activation after TRIF recruitment by TLR4 [156]. After inhibition of inflammasome activation pathway components, a decrease in IL-1β release was observed after caspase-1 and P2X7R blockage. Once again the most interesting effect was seen after pannexin-1 inhibition with carbenoxolone. Our results showed that similarly to THP-1 cells, there was a reduction in IL-1β levels after TLR2 but not TLR4 activation. To confirm our results by a different approach we used siRNA to knock down *P2X7R* and *panx1* genes in THP-1 cells. This also resulted in a reduction in IL-1β release after TLR2 but not TLR4 activation. P2X7 receptor is an ion channel gated by high concentrations of extracellular ATP which are known to be present at sites of injury and inflammation. Its activation not only opens a 'typical' ion channel selective for small cations but also leads to the gradual opening of a larger pore that allows passage of molecules up to 900 Da [149]. Pannexin-1 is one of three vertebrate pannexins that show homology to gap junction-forming invertebrate innexins [157]. Pannexin-1 is expressed broadly, whereas pannexin-2 is expressed largely in brain, and pannexin-3 is in skin and connective tissues [158]. A study by Pelegrin et al. showed that panx1 was highly expressed in human and mouse macropgahes and it was upregulated by LPS. It also co-immunoprecipitated with P2X7 protein. Inhibition of panx1 by siRNA and inhibitory peptide, reduced P2X7R-mediated dye uptake without altering P2X7 protein expression. It also blocked caspase-1 cleavage as well as IL-1β processing and release from LPS-primed mouse and human

macrophages [150]. Pannexin-1 dependent pore formation has been suggested as a mechanism for IL-1β secretion after cleavage of pro-IL-1β by caspase [150, 159], as IL-1β lacks signal sequences for export via the classical endoplasmic reticulum/Golgi dependent secretory pathway [160]. It should be noted that most of the investigators studying the inflammasome accelerate the cleavage of the IL-1β precursor by shocking cells with millimolar concentrations of ATP. Primary monocytes from patients with Still's disease or neonatal onset multi-inflammatory disease secrete significantly more IL-1β whether in a resting state, or after endotoxin stimulation during a 24h culture without the addition of ATP [161, 162]. ATP can also accumulate in the extracellular space at sites of inflammation where it is thought to activate pannexin-1. The role of pannexin-1 in IL-1β release in the absence of exogenous ATP has yet to be established in primary human monocytes [163]. Our results showed that IL-1β release is enhanced by exogenous ATP, but ATP is not required in this system for IL-1β release. In the absence of exogenous ATP, TLR2 but not TLR4 pathways require pannexin-1 membrane protein for the release of mature IL-1β. Study by Qu et al. using murine bone marrow derived macrophages *(*BMDMs) lacking pannexin-1 demonstrated that neither caspase-1 activation nor IL-1β secretion mediated by either NLRC4 or NLRP3 was compromised by pannexin-1 loss after LPS stimulation [92]. This is in-line with our data, where we observed the same effect after TLR4 stimulation by LPS but not after TLR2/1 stimulation with Pam_3CSK3 and TLR2/6 stimulation with FSL-1. The difference between TLR4 and TLR2 signalling pathway is that TLR4 can activate Myd88 dependent and Myd88-indepented signalling responses. The independent pathway is mediated by the TRIF adaptor protein and also leads to proinflammatory responses. To take our study further we decided to investigate the role of TRIF in inflammasome activation. Previous results by Saitoh et al. showed that TRIF was linked to NLRP3 signalling in situations in which the autophagy machinery was blocked or depleted [164]. Another study on Kupffer cells showed that despite their normal production of pro-IL-1β and pro-IL-18, $Trif^{-/-}$ Kupffer cells could not release IL-1β or IL-18

due to their inability to activate caspase-1 [165, 166]. To investigate the role of adaptor molecules in inflammasome activation and IL-1β release in our model, we activated adaptor proteins with different TLR ligands. Our previous results showed that LPS induced much higher IL-1β release in PBMCs than Pam_3CSK4 and we wanted to investigate whether TRIF activation was responsible for this response. Since TLR4 activates TRIF and Myd88, TLR3 activates TRIF, and TLR2 activates Myd88 (Fig. 25), we treated PBMCs with different TLR ligands to investigate whether separate activation of TRIF and Myd88 pathways by different ligands can induce IL-1β levels comparable to LPS treatment.

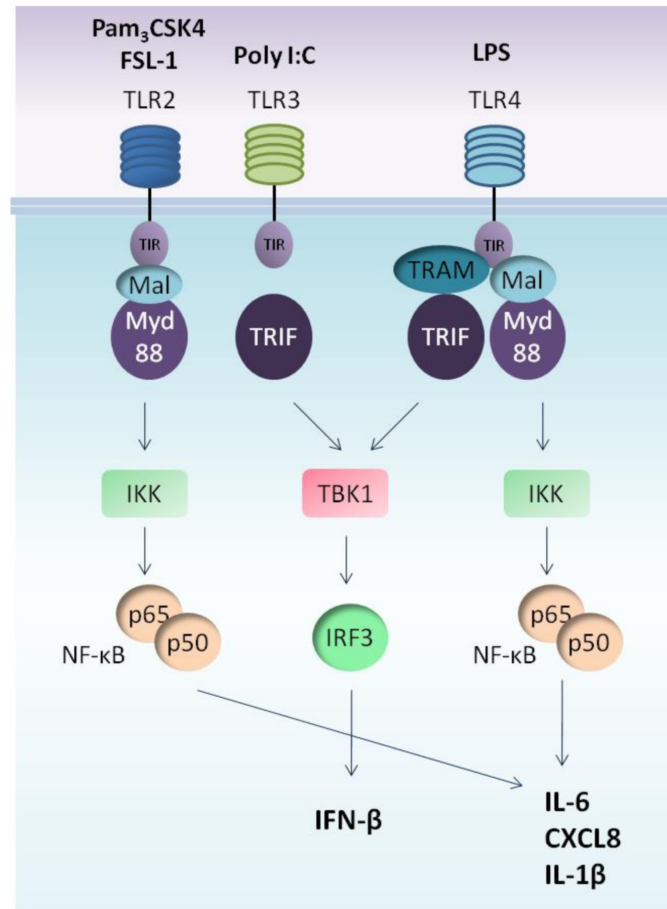


Figure 25. TLR adaptor proteins. Upon activation of TLRs, the adaptor proteins Mal, MyD88, TRIF and TRAM are differentially recruited to the various receptors. MyD88 is utilised by all the TLRs with the exception of TLR3. TRIF is activated downstream of TLR3 and TLR4. Mal and TRAM act as bridging proteins, recruiting MyD88 and TRIF, respectively to TLR4. The recruitment of the adaptor proteins leads to the activation of signalling pathways and production of proinflammatory cytokines and IFNs.

Our results showed that when we treated cells with Poly I:C (activates TRIF) together with Pam_3CSK4 (activates Myd88) or FSL-1 (activates Myd88), IL-1β levels were still much lower that after LPS treatment (activates TRIF and Myd88). CXCL8 levels were similar after all treatments. These results may be explained by the fact that the signalling mechanisms involved in TLR3 activation of TRIF are different than those of TLR4, where the additional adaptor TRAM is required. It was initially believed that TRAM acted as an

anchor protein for TRIF after activation of TLR4, however recent data shows that TRAM induces unique signalling events, which are specific to this adaptor protein [167].

To investigate further the role of adaptor proteins in inflammasome activation and IL-1β release, we used blocking peptides (BPs) comprised of 14 aa that correspond to the sequences of the BB loops of the two TIR domain-containing adaptor proteins, Mydd88 and TRIF. After treating PBMCs with these inhibitors we stimulated cells with LPS, Pam_3CSK4, FSL-1 and cigarette smoke extract (CSE), which has been shown to activate TLRs [64]. Our results showed that there was a decrease in IL-1β release in PBMCs stimulated with LPS after inhibition of Myd88, TRIF and both together. However, after Pam_3CSK4 stimulation we saw a reduction only after Mydd88/TRIF inhibition. Similar results were obtained after CXCL8 measurement. Pam_3CSK4 stimulation caused a decrease in cytokine levels only after TRIF inhibition even though TRIF does not take part in TLR2 signalling pathway. We expected a reduction in CXCL10 levels in cells stimulated with Poly I:C after inhibition of TRIF but surprisingly this inhibitor did not have any effect on CXCL10 release in these cells. A possible explanation for our results is described in a paper by Toshchakov et al. where BPs showed little specificity with respect to genes induced by LPS via Myd88-dependent and Myd88-indepentent pathways. For example, the Myd88 inhibitory peptide did not preferentially block IL-1β gene induction, an event that is reliant on Myd88 signalling. It was suggested that the stability of the TLR4 signalling platform is disrupted or fails to assemble correctly as a consequence of BP-target protein interactions. In addition, these BPs were much weaker inhibitors of TLR2-mediated signalling and did not affect TLR2-induced activation of JNK and ERK, and had much weaker effect on IL-1β expression suggesting that that the architecture of receptor-adaptor complexes differs between TLR2 and TLR4. The idea that adaptors shared by different TLRs can interact with individual receptors in a fundamentally different ways supports the existence of asymmetrical models of TIR-TIR complex formation [168]. A

recent study by Dunne et al. predicted that TLR4 and TLR2 bind Myd88 through different non-overlapping sites [169].

While I was writing this thesis there was a paper published in August by Rathinam et al. demonstrating that in mouse model TRIF licensed caspase-11 dependent NLRP3 activation by Gram-negative bacteria. In this study it was shown that TRIF activates caspase-11 via type I IFN signalling. Caspase-11 subsequently synergized with NLRP3 inflammasome to regulate caspase-1 activation. These events occurred specifically during infection with Gram-negative but not Gram-positive bacteria [170]. In Our study LPS induced higher IL-1β release than Pam_3CSK4, and it did not required pannexin-1 for IL-1β cleavage. TLR2-induced IL-1β release required second signal for the inflammasome assembly and it was under regulation of pannexin-1, which form large pores in the membrane allowing the passage of substances with a molecular weight of up to about 900 Da. The last remaining question is what is the second signal required for the inflammasome activation after TLR2 stimulation and exactly how this stimulant causes assembly of the inflammasome. NLRP3 inflammasome is activated by a diverse series of endogenous and exogenous agonists but there is no evidence of direct ligand binding, thus challenging the concept that this is a receptor. However, it might be possible that after large pore formation by activated pannexin-1 there is an influx to the cytoplasm of pathogen-associated molecular patterns and other factors like ATP, uric acid, hyaluronan, and many more molecules that could activate the inflammasome [91, 171, 172]. The possible explanation of our results is shown in Figure 26.

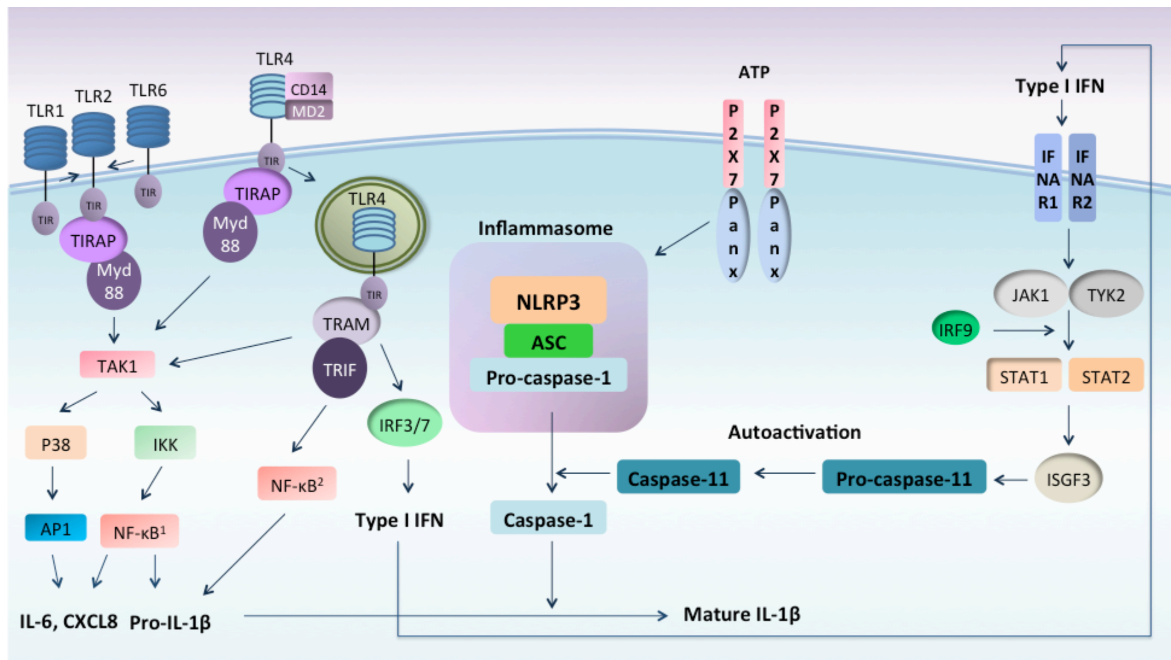


Figure 26. Mechanism of IL-1β release. Release of IL-1β requires two well-defined signals: a "priming" signal to induce pro-IL-1β synthesis, and a signal to trigger assembly of the NLRP3 inflammasome, which culminates in the processing and release of mature IL-1β. TLR2 and TLR4 activation leads to NF-kB activation and induction of pro-IL-1β via MyD88-dependent mechanism. TLR2-induced IL-1β release requires second signal like ATP which is believed to activate the inflammasome by binding to P2X7 receptors and opening a cation channel and a large pore through pannexin-1. Triggering K^+ efflux and Ca^{2+} influx activates inflammasome assembly through as yet unconfirmed mechanisms. Moreover, TLR4 ligation also activates TRIF which licenses NLRP3 inflammasome activation via the type I IFN pathway. Type I IFNs upregulate caspase-11 expression, an event that is both necessary and sufficient to enable caspase-11 autoactivation. Caspase-11 activation via the TLR4-TRIF-IFNβ pathway synergizes with NF-κB activation to orchestrate caspase-1-dependent IL-1β processing and secretion without the need of P2X7R and pannexin-1 recruitment.

Chapter summary

To summarize our results, we demonstrated that (i) THP-1 cells and PBMCs released IL-1β and CXCL8 after stimulation with TLR4 and TLR2 ligands, (ii) ATP further enhanced IL-1β release, (iii) in the absence of exogenous ATP, TLR2 but not TLR4 pathways require pannexin-1 hemichannel protein. This data suggests an important dissociation in inflammasome activation induced by TLR4 versus TLR2 and could be important in our

understanding of both bacterial and sterile inflammation in man. It also can help in the future to design more efficacious and more specific drugs to treat inflammatory conditions.

Chapter 4

The effect of TLR and NOD1 agonists on IL-1β and CXCL8 release in patients with respiratory disease.

Rationale

Chronic pulmonary obstructive disease (COPD) affects 200 million people worldwide and is currently the fourth leading cause of death in industrialized countries. The most important risk factor for COPD is smoking tobacco, although it is known that other environmental and genetic factors also contribute to the development of COPD. COPD is defined by a combination of three clinical manifestations; chronic bronchitis, small airways obstruction and emphysema, and is associated with a decrease in quality of life and premature death [173]. The lower airway inflammation present in COPD patients involves the recruitment and activation of inflammatory cells and results in changes in the structure of the lung. Increased secretion of cytokines, chemokines, growth factors and adhesion molecules is observed and in most cases increased expression of these molecules in associated with enhanced gene transcription [174]. Exacerbation of COPD is a sudden worsening of COPD symptoms. They are usually triggered by bacterial and viral infections and are a major cause of hospital admissions and mortality. As a results there is a need for developing effective treatment and vaccines to improve the health status of COPD patients [117]. Conserved germ-line pathogen associated molecular patterns (PAMPs) present on or within bacteria, viruses and fungi are recognised by numerous pathogen recognition receptors (PRRs), which include TLRs and NLRs [175]. In addition, recent evidence from our group and others have shown that these receptors also act as danger recognitions receptors (DRRs) and are activated by structural proteins, such as fibrinogen hyaluronan and oxidants [105]. These molecules are present only when cells are damaged and undergo necrosis during bouts of inflammation, infection or trauma. We therefore hypothesised that these PRRs may be dysregulated or sensitised during chronic inflammatory pathologies, thereby playing a major role in controlling inflammation

observed in COPD. In this chapter we hypothesised that the blood of COPD patients is more sensitive, than that of age matched control donors, to bacterial and viral TLR ligands. In this chapter we measured basal levels of IL-1β and CXCL8 levels in blood serum of COPD patients and healthy volunteers. To assess if COPD patients have a greater susceptibility to infection, we measured their innate immune response to IL-1R, TLR2/1, TLR2/6, TLR3, TLR4 and NOD1 agonists in serum and compared our data to that obtained from healthy controls. Since pulmonary hypertension (PAH) and pulmonary vascular remodelling is a common complication of COPD [176], we also measured cytokine release profile in patients with PAH and healthy controls to see whether any changes in sensitivity to agonists are specific for COPD and not just related to a general inflammatory condition in the lung.

Methods

Patients with chronic obstructive pulmonary disease (COPD) and pulmonary hypertension (PAH) were invited to participate. 12mls of whole blood was collected into 2.7mls vacutainers containing sodium citrate. 190mls of blood was aliquoted onto 96 well plates and incubated with TLR (LPS, FSL-1, Pam_3CSK4, Poly I:C), NOD1 (C12-IE-DAP) and IL-1R (IL-1β) agonists (as described in chapter 2.3.2). Whole blood was incubated at 37°C containing 5% CO_2 for 24h. Plates were centrifuged at 1000xg at room temperature for 5 min with very low breaking. Serum was carefully transferred to the dummy plate and frozen at -20ºC for future analysis of IL-1β and CXCL8 release by ELISA (as described in chapter 2.2.2).

ID No	Age	GOLD Stage	FEV_1%	FEV_1/FVC%	Smoking Status	Pack/Yrs	Gender
COPD1	84	I	81	60	ex	10	F
COPD2	72	IV	24	21	ex	114	M
COPD3	70	II	56.9	39.6	ex	26	F
COPD4	74	II	60	48	ex	10	M
COPD5	74	I	88	69	ex	63	M
COPD6	68	III	47	51	ex	5	M
AMC1	60	N/A	N/A	N/A	non	N/A	F
AMC2	63	N/A	N/A	N/A	non	N/A	M
AMC3	68	N/A	N/A	N/A	non	N/A	M
AMC4	60	N/A	N/A	N/A	non	N/A	M
AMC5	79	N/A	N/A	N/A	ex	33	F
AMC6	67	N/A	N/A	N/A	non	N/A	F
AMC7	76	N/A	N/A	N/A	ex	2.5	F
AMC8	73	N/A	N/A	N/A	non	N/A	F

Table 4. Clinical assessment of COPD patients (COPD) and age-matched controls (AMC)

ID No	Age	Diagnosis	Disease Severity	Smoking Status	Pack/Yrs	Gender
PH1	31	iPAH	IV	non	N/A	F
PH2	69	CTD-PAH	III	non	N/A	F
PH3	32	iPAH	III	non	N/A	F
PH4	43	iPAH	III	non	N/A	M
PH5	47	iPAH	II	non	N/A	F
PH6	42	iPAH	III	non	N/A	M
PH7	63	severe PAH	no data	ex	5	F
PHC1	38	N/A	N/A	non	N/A	F
PHC2	46	N/A	N/A	non	N/A	M
PHC3	27	N/A	N/A	non	N/A	F
PHC4	68	N/A	N/A	non	N/A	F
PHC5	61	N/A	N/A	non	N/A	M
PHC6	31	N/A	N/A	non	N/A	M
PHC7	41	N/A	N/A	non	N/A	M

Table 5. Clinical assessment of pulmonary hypertension patients (PAH) and age-matched controls (PHC). iPAH-Idiopathic Pulmonary Arterial Hypertension, CTD-PAH-Pulmonary Arterial Hypertension associated with Connective Tissue Diseases.

	COPD Patients	Age-Matched Controls
N	6	8
Age, Years (mean+SEM)	73.6±2.2	68.2±2.5
Gender F/M	2/4	5/3
Smoking Status (non/ex/current)	0/6/0	6/2/0
Pack/Years	38±17.5	4.4±4
FEV_1% (mean+SEM)	59.4±9.4	N/A
FEV_1/FVC% (mean+SEM)	53.1±8.4	N/A

Table 6. Clinical assessment COPD patients vs controls.

	PAH Patients	Age-Matched Controls
N	7	7
Age, Years (mean+SEM)	46.71(5.4)	44.57 (5.7)
Gender F/M	5/2	3/4
Smoking Status (non/ex/current)	0/0/1	0/0/1
Pack/Years	0.71±0.71	N/A
FEV_1% (mean+SEM)	N/A	N/A
FEV_1/FVC% (mean+SEM)	N/A	N/A

Table 7. Clinical assessment PAH patients vs controls.

Patient ID	White Blood Cell Count (x 10^9 cells/liter)	Normal Range (4.8-10.8)
COPD2	7.4	normal
COPD3	7.7	normal
COPD4	8.4	normal
COPD6	7.1	normal

Table 8. COPD patients' white blood cell count

Results

4.1. Assessment of control groups – IL-1β release.

The aged immune system decreases its responsiveness to infections and increases the sensitivity to systemic inflammation and autoimmune disease [177]. As it was mentioned before, to investigate whether any changes in cytokines release were caused by disease or were associated with aging, we divided our control subjects into three groups:

- Young controls (<30yrs)
- Medium-age controls (30-50yrs)
- Older controls (>50yrs)

We found that each control group that we used demonstrated differences in sensitivity to TLR and NOD1 agonists. Looking at IL-1β release (Fig. 27), we observed that older control groups (43.7 yrs and 68.2 yrs) produced generally higher levels of the cytokine compared with younger group. Poly I:C induced higher levels of IL-1β in blood of younger versus older control volunteers.

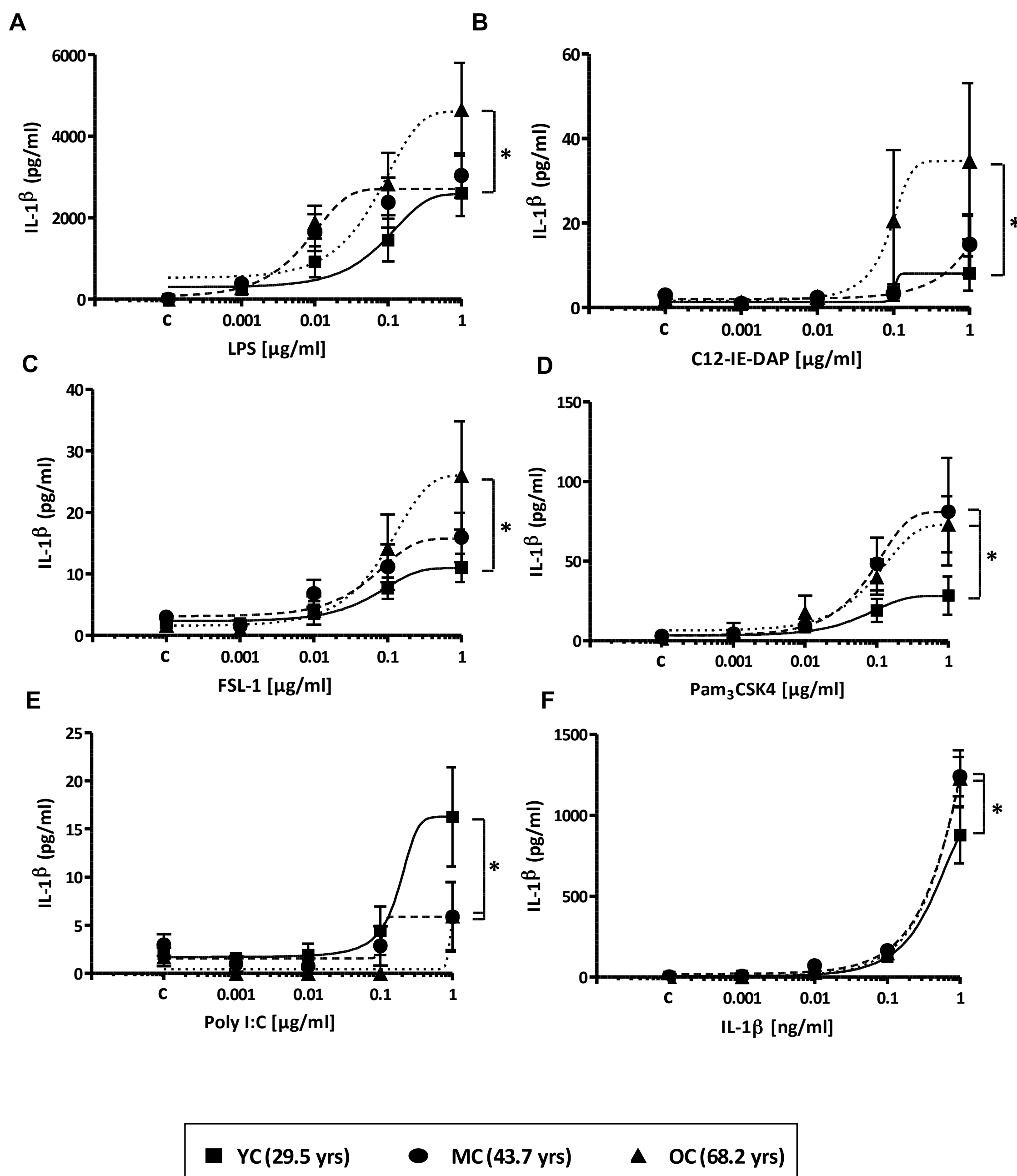


Figure 27. IL-1β release from whole blood after TLR and NOD1 stimulation. Blood from participants was collected and treated with LPS (A), C12-IE-DAP (B), FSL-1 (C), Pam_3CSK4 (D), Poly I:C (E) and IL-1β (F). After 24h, serum was separated from whole blood by centrifugation at 1000xg for 5min and stored at -20ºC. Levels of IL-1β were measured by ELISA. Data are the mean ± SEM of n=7 for YC (young controls), n=7 for MC (medium-age controls) and n=8 for OC (older controls). *denotes $p<0.05$ two-way ANOVA followed by Bonferroni post test.

4.2. Assessment of control groups – CXCL8 release.

CXCL8 levels (Fig. 28) were higher in younger control group except for C12-IE-DAP treatment where medium-age control group released more cytokine. Control group (44 yrs) had the lowest levels of CXCL8 after stimulation with all agonists. Thus, for the experiments in this chapter we calculated the mean age of COPD and PAH patients and chose the volunteers for the control group to give us as similar an age range as possible.

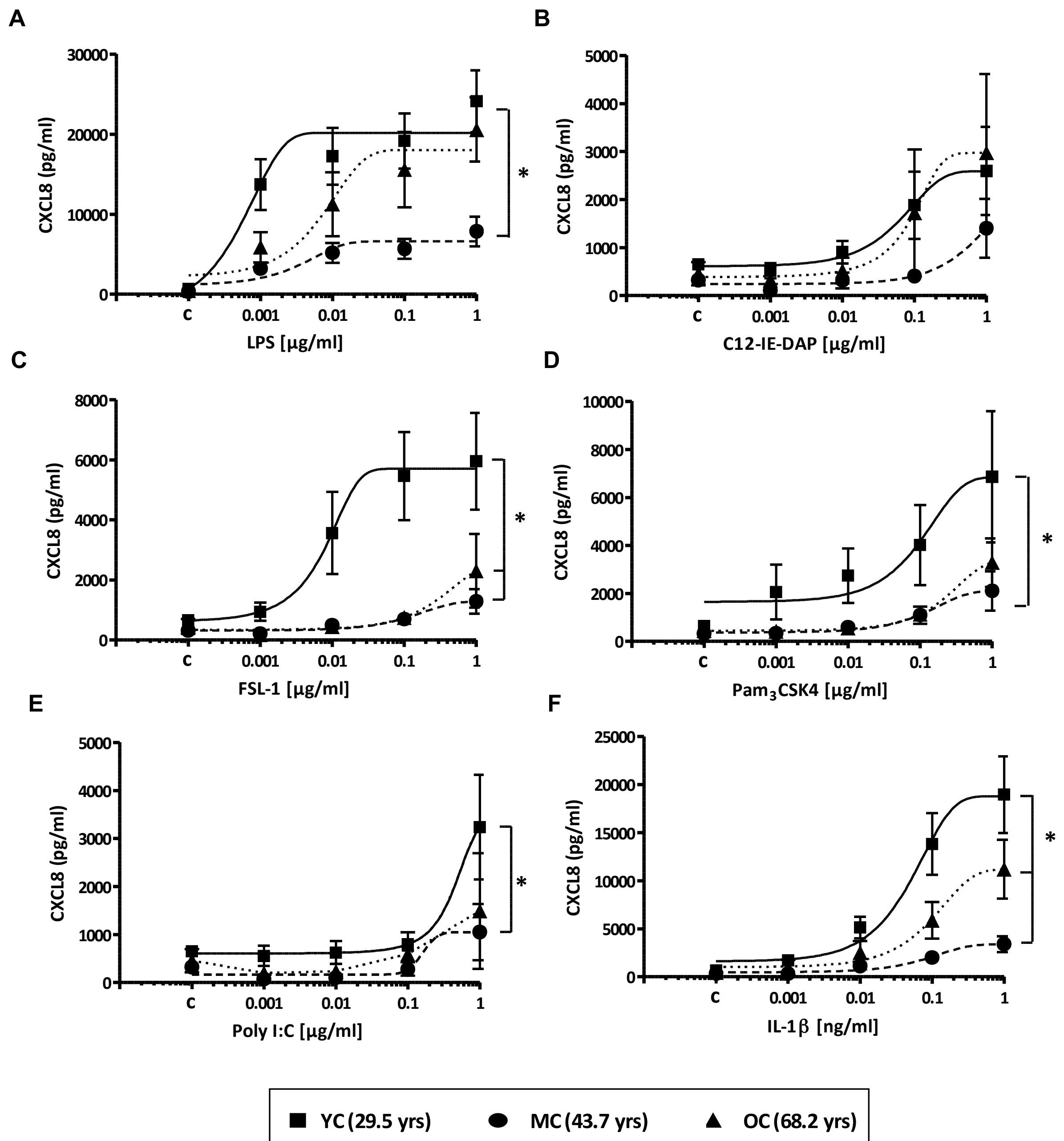


Figure 28. CXCL8 release from whole blood after TLR and NOD1 stimulation. Blood from participants was collected and treated with LPS (A), C12-IE-DAP (B), FSL-1 (C), Pam_3CSK4 (D), Poly I:C (E) and IL-1β (F), After 24h, serum was separated from whole blood by centrifugation at 1000xg for 5min and stored at -20ºC. Levels of CXCL8 were measured by ELISA. Data are the mean ± SEM of n=7 for YC (young controls), n=7 for MC (medium-age controls) and n=8 for OC (older controls). *denotes $p<0.05$ two-way ANOVA followed by Bonferroni post test.

4.3. Basal levels of IL-1β and CXCL8 in whole blood.

Basal levels of IL-1β (Fig. 29A) were higher in COPD patients than in age-matched controls but the difference did not reach significance. Only 2 out of 6 patients had significantly higher levels of IL-1β which was not associated with disease severity. Similar results were seen after CXCL8 measurement, where cytokine levels (Fig. 29B) were significantly increased in COPD patients compared with controls but there was also no correlation between plasma cytokine levels and disease severity according to GOLD status (Fig. 30A,B).

In PAH patients, we did not observe any difference in basal IL-1β and CXCL8 levels compared to control group. (Fig. 29C,D). There was also no correlation between plasma cytokines levels and disease severity as stated in Table 5 (Fig. 30C,D).

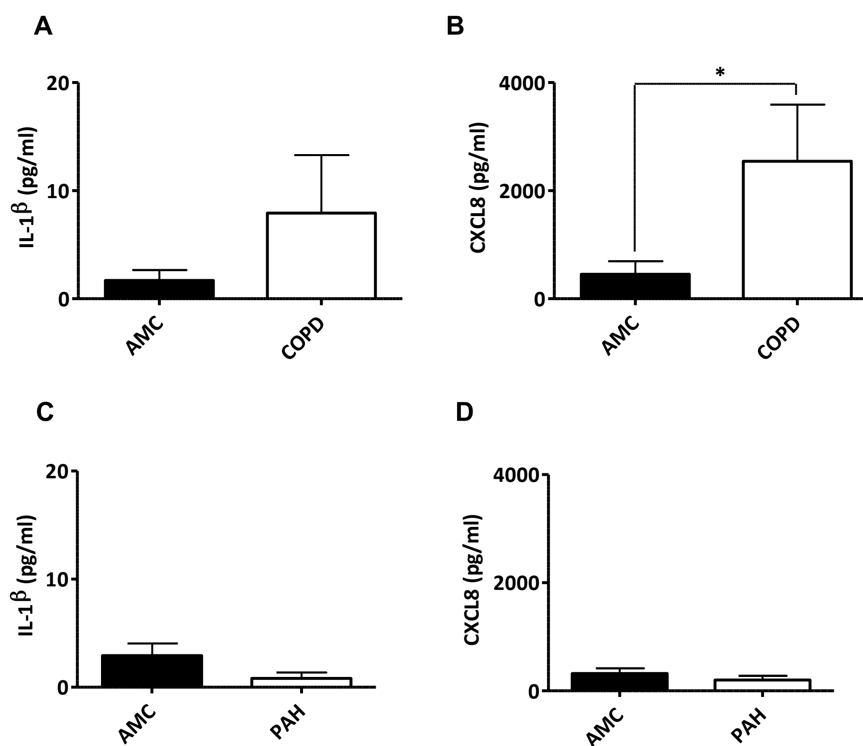


Figure 29. Basal levels of IL-1β and CXCL8 in blood plasma. Plasma was separated from whole blood by centrifugation at 1000xg for 5min and stored at -20ºC. Levels of IL-1β (A,C) and CXCL8 (B,D) were measured by ELISA. Data are the mean ± SEM of n=6 for COPD patients and n=8 for age-matched controls (A,B), n=7 for PAH patients and their controls (C,D). *denotes $p<0.05$ one-way ANOVA followed by Bonferroni post test (3A and 3B) and two-tailed t-test (3C and 3D). AMC – age-matched controls, COPD – COPD patients, PAH- pulmonary hypertension patients.

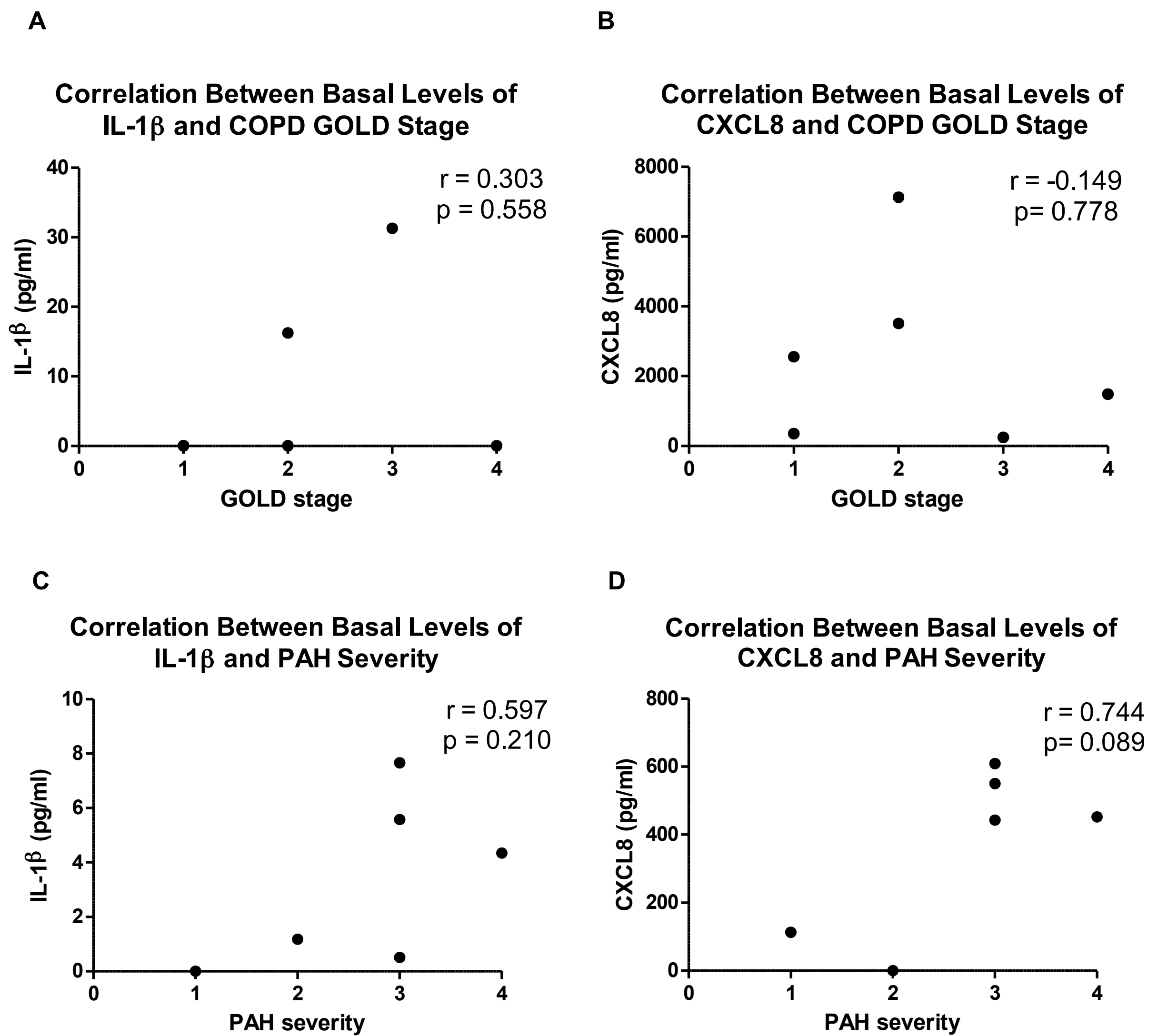


Figure 30. Correlation between plasma levels of IL-1β (A,C) and CXCL8 (B,D) and disease severity. COPD – COPD patients (A,B), PAH- pulmonary hypertension patients (C,D).

4.4. IL-1β and CXCL8 release in response to Gram-negative bacterial ligands in COPD and PAH patients.

IL-1β release in response to the TLR4 agonist, LPS was similar in COPD patients (Fig. 31A) and the age-matched healthy control group. In blood of PAH patients (Fig. 31B), we observed a significantly higher release of IL-1β at the top concentration of LPS used when compared with their age-matched controls.

In contrast, CXCL8 levels were significantly higher in the COPD patient group than in age-matched controls with no difference seen between PAH patients and their controls.

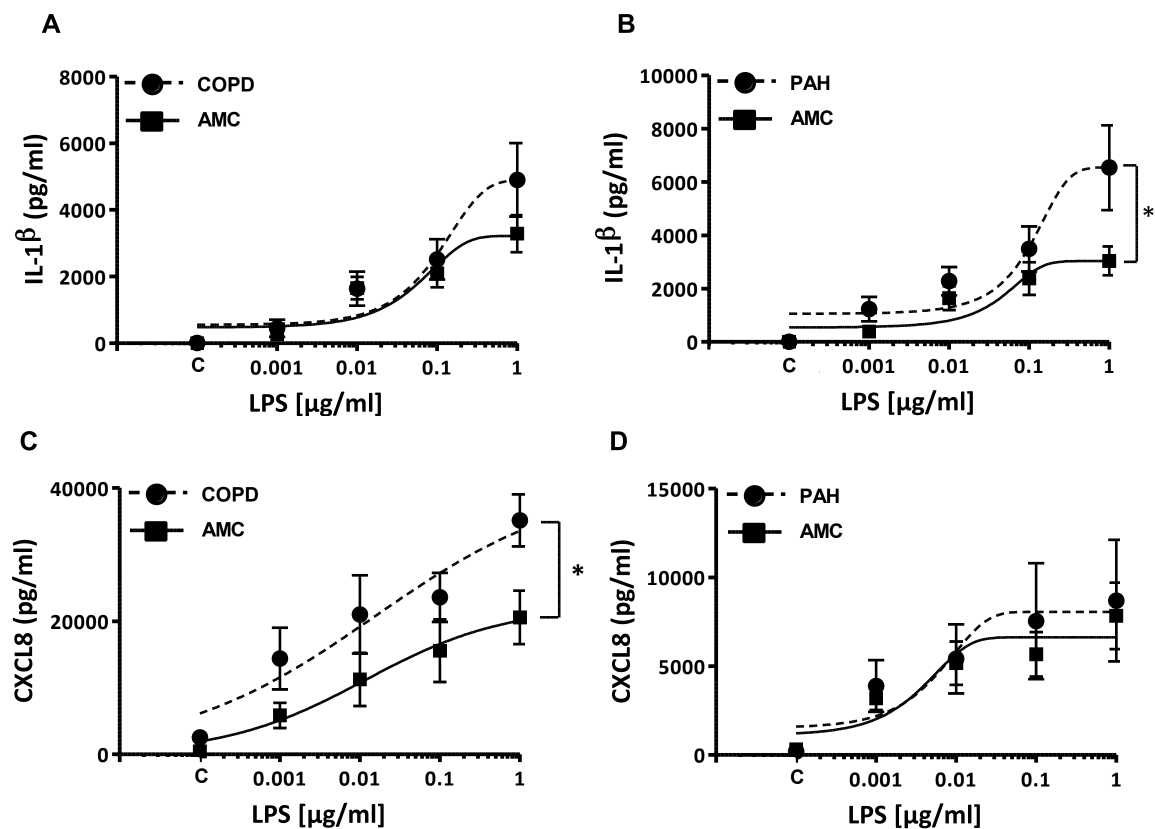


Figure 31. IL-1β and CXCL8 release from whole blood after LPS stimulation. Blood from participants was collected and treated with LPS (0.001-1µg/ml). After 24h, plasmawas separated from whole blood by centrifugation at 1000xg for 5min and stored at -20ºC. Levels of IL-1β (A,B) and CXCL8 (C,D) were measured by ELISA. Data are the mean ± SEM of n=6 for COPD patients and n=8 for age-matched controls (A,C), n=7 for PAH patients and their controls (B,D). *denotes $p<0.05$ two-way ANOVA followed by Bonferroni post test. AMC – age-matched controls, COPD – COPD patients, PAH-pulmonary hypertension patients.

The NOD1 agonist, C12-IE-DAP induced very low levels of IL-1β (Fig 32A, B) with no difference observed between patient groups and their controls.

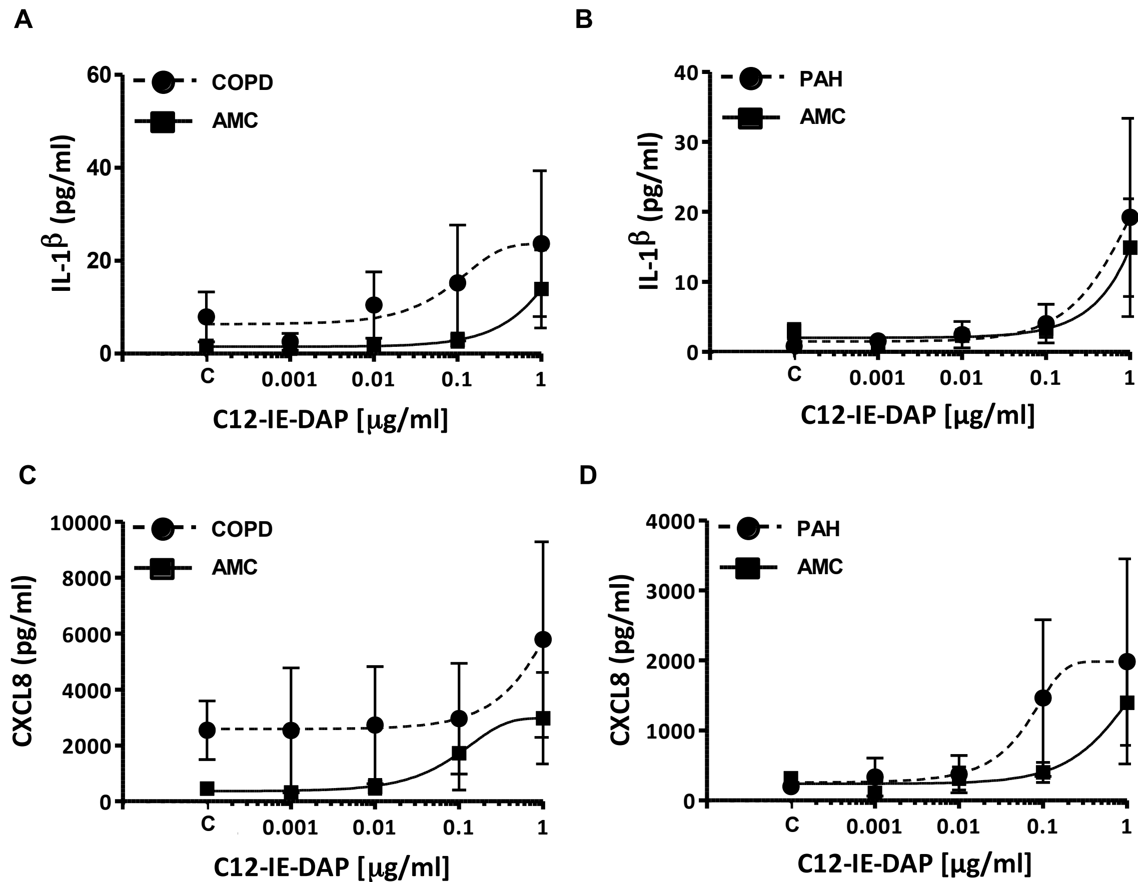


Figure 32. IL-1β and CXCL8 release from whole blood after C12-IE-DAP stimulation. Blood from participants was collected and treated with C12-IE-DAP (0.001-1μg/ml). After 24h plasma was separated from whole blood by centrifugation at 1000xg for 5min and stored at -20ºC. Levels of IL-1β (A,B) and CXCL8 (C,D) were measured by ELISA. Data are the mean ± SEM of n=6 for COPD patients and n=8 for age-matched controls (A,C), n=7 for PAH patients and their controls (B,D). *denotes p<0.05 two-way ANOVA followed by Bonferroni post test. AMC – age-matched controls, COPD – COPD patients, PAH-pulmonary hypertension patients.

4.5. IL-1β and CXCL8 release in response to Gram-positive bacteria ligands in COPD and PAH patients.

There was no significant difference in IL-1β levels between groups after FSL-1 (TLR2/6 agonist) treatment (Fig. 33A,B).

CXCL8 secretion after FSL-1 stimulation (Fig. 33C,D) was slightly higher in COPD group than in age-matched control group but this did not reach significance. Similarly, there was no difference between PAH patients and their controls.

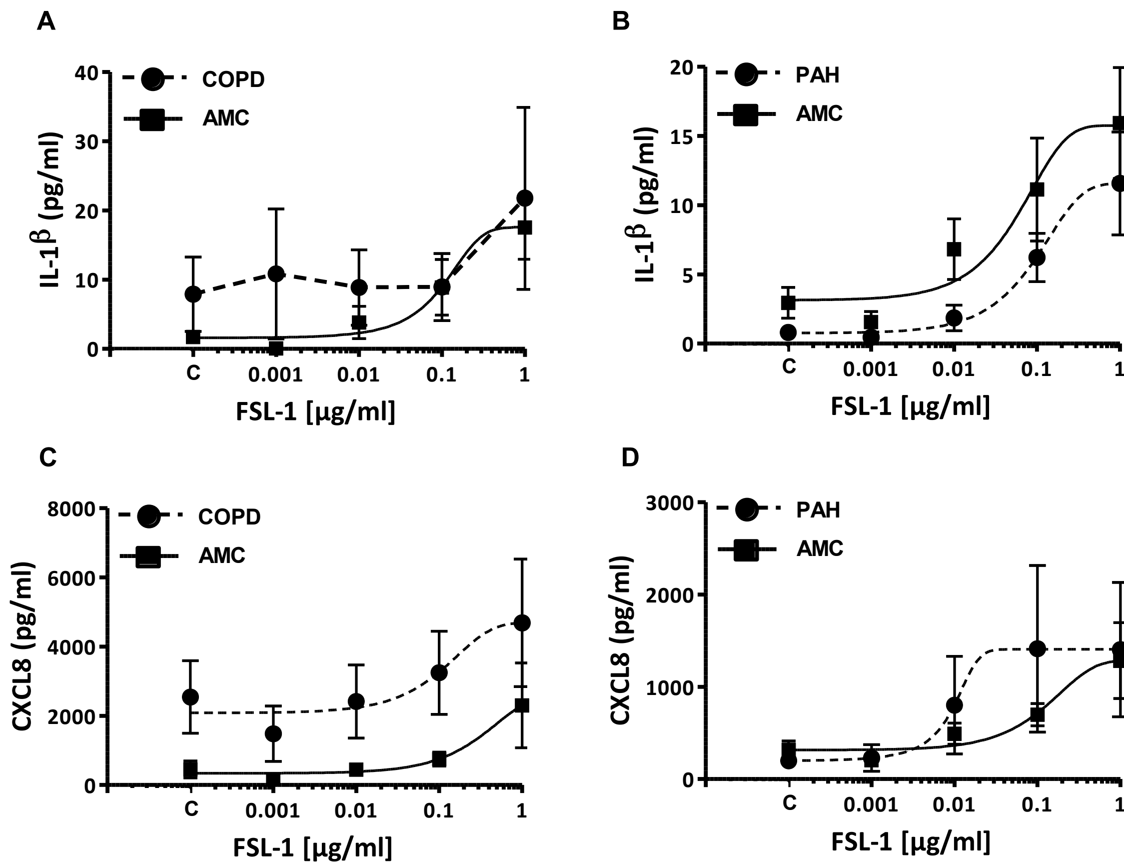


Figure 33. IL-1β and CXCL8 release from whole blood after FSL-1 stimulation. Blood from participants was collected and treated with FSL-1 (0.001-1µg/ml). After 24h incubation plasma was separated from whole blood by centrifugation at 1000xg for 5min and stored at -20ºC. Levels of IL-1β (A,B) and CXCL8 (C,D) were measured by ELISA. Data are the mean ± SEM of n=6 for COPD patients and n=8 for age-matched controls (A,C), and n=7 for PAH patients and their controls (B,D). *denotes $p<0.05$ two-way ANOVA followed by Bonferroni post test. AMC – age-matched controls, COPD – COPD patients, PAH- pulmonary hypertension patients.

Pam_3CSK4 (TLR2/1 agonist) in general released higher levels of IL-1β and CXCL8 than the TLR2/6 agonist, FSL-1.

Levels of IL-1β after Pam3CSK4 stimulation (Fig. 34A) at the top concentration were significantly higher in blood of COPD patients than in the age-matched control subjects.

No difference in levels of IL-1β was seen between responses of blood from PAH patients and their controls (Fig. 34B).

Similarly, COPD patients released higher levels of CXCL8 after TLR2/1 stimulation (Fig. 34C) than their controls and they were significantly different compared with age-matched controls. Once again, there was no difference between PAH patients and age-matched controls (Fig. 34D).

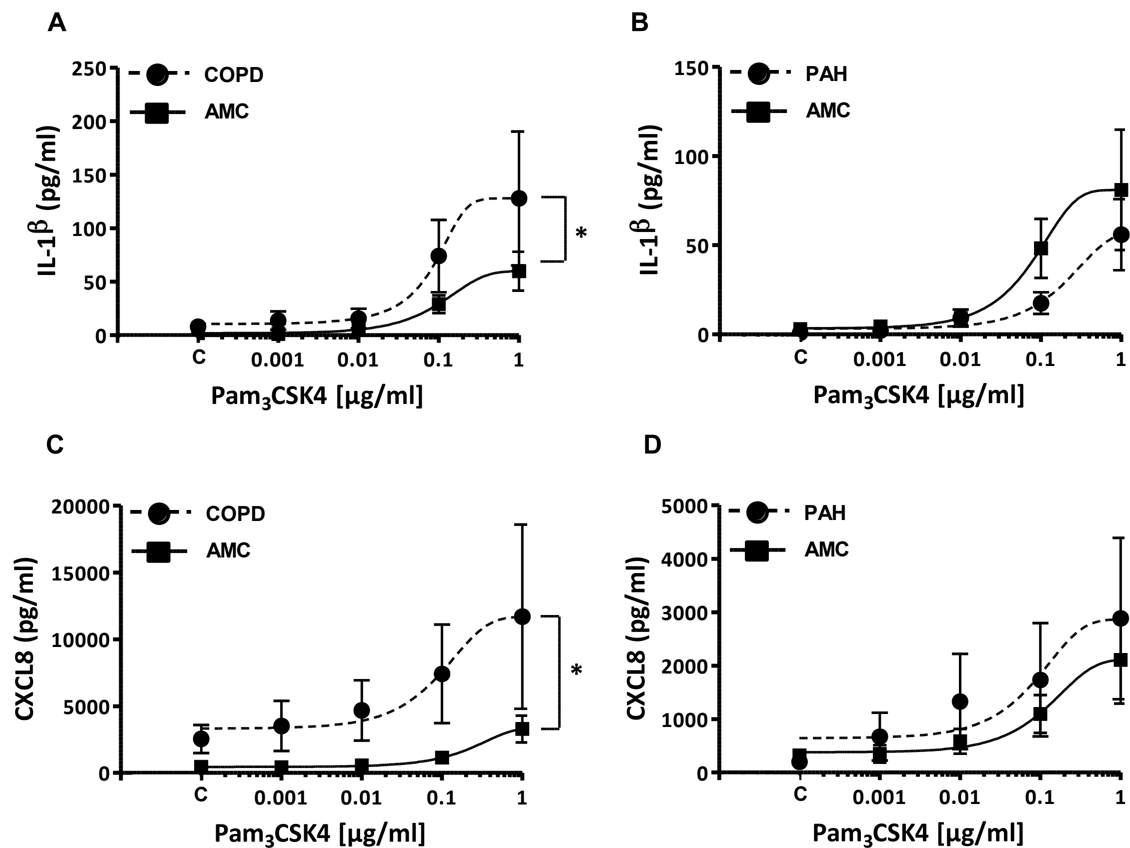


Figure 34. IL-1β and CXCL8 release from whole blood after Pam$_3$CSK4 stimulation. Blood from participants was collected and treated with Pam$_3$CSK4 (0.001-1µg/ml). After 24h, plasma was separated from whole blood by centrifugation at 1000xg for 5min and stored at -20ºC. Levels of IL-1β (A,B) and CXCL8 (C,D) were measured by ELISA. Data are the mean ± SEM of n=6 for COPD patients and n=8 for age-matched controls (A,C), n=7 for PAH patients and their controls (B,D). *denotes $p<0.05$ two-way ANOVA followed by Bonferroni post test. AMC – age-matched controls, COPD – COPD patients, PAH-pulmonary hypertension patients.

4.6. IL-1β and CXCL8 release in response to viral ligand in COPD and PAH patients.

Poly I:C induced significantly higher levels of IL-1β in COPD patients than in the age-matched control group (Fig. 35A). Although, there was no significant difference between PAH patients and their controls. (Fig. 35B). After the measurement of CXCL8 secretion, we observed that COPD patients induced significantly higher levels of CXCL8 in response to viral ligands compared with their controls (Fig. 35C,D). By contrast, blood of PAH patients did not release IL-1β after stimulation with viral ligands. However, blood of aged matched control donors released IL-1β after stimulation with Poly I:C. This suggests that TLR3 signalling may be compromised in patients with PAH.

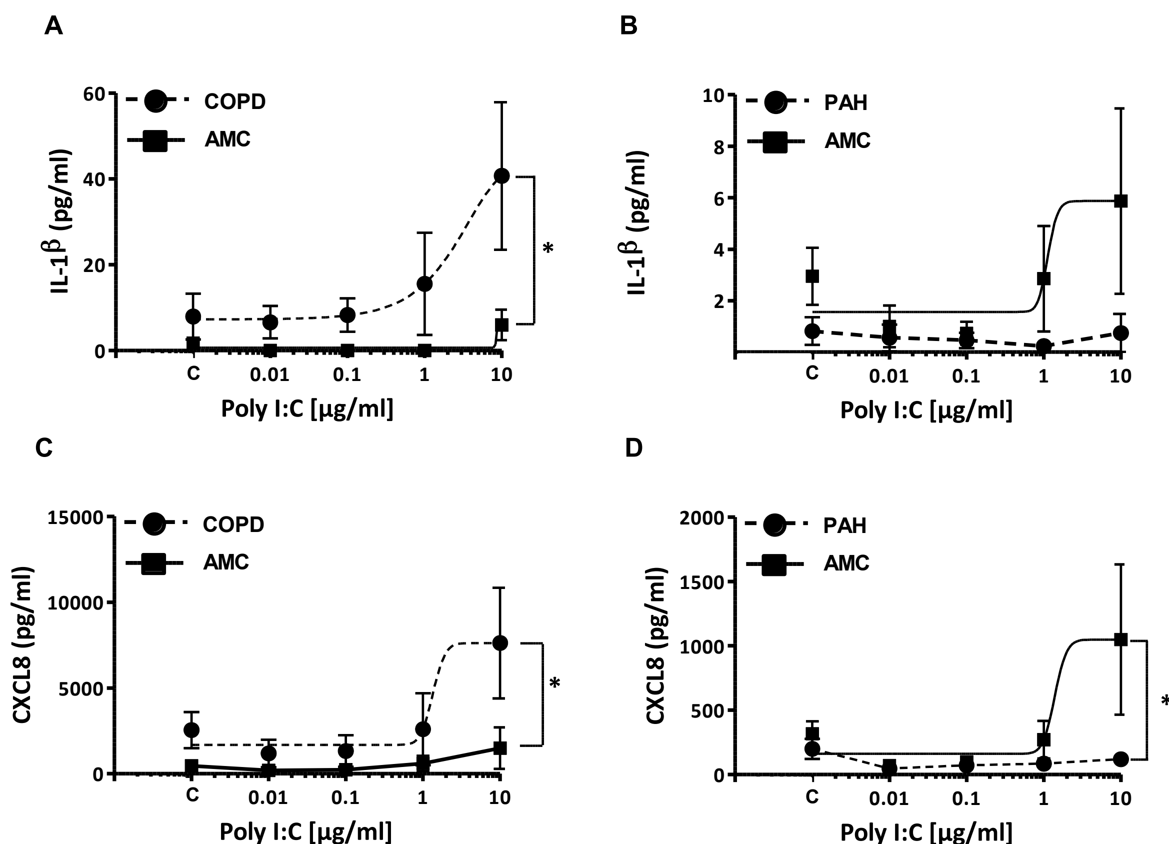


Figure 35. IL-1β and CXCL8 release from whole blood after Poly I:C stimulation. Blood from participants was collected and treated with Poly I:C (0.01-10µg/ml). After 24h, plasma was separated from whole blood by centrifugation at 1000xg for 5min and stored at -20°C. Levels of IL-1β (A,B) and CXCL8 (C,D) were measured by ELISA. Data are the mean ± SEM of n=6 for COPD patients and n=8 for age-matched controls (A,C), n=7 for PAH patients and their controls (B,D). *denotes $p<0.05$ two-way ANOVA followed by Bonferroni post test. AMC – age-matched controls, COPD – COPD patients, PAH- pulmonary hypertension patients.

4.7. IL-1β and CXCL8 release in response to interleukin-1 receptor (IL-1R) agonist.

TLRs and IL-1R-family members share the same adaptor and signalling components such as Myd88, IRAK and TRAF6 and both can activate NF-κB and MAP kinases. We used the IL-1R agonist, IL-1β to control for possible differences or perturbations in the TLR

signalling pathway. It was difficult to analyse the data from the IL-1β ELISA, where IL-1β was used as a stimulus due to reactivity with the IL-1β antibodies (data not shown).

Looking at CXCL8 release there was significantly higher release in COPD patients that in age-matched controls (Fig. 36A) with no difference between PAH patients and their controls (Fig. 36B).

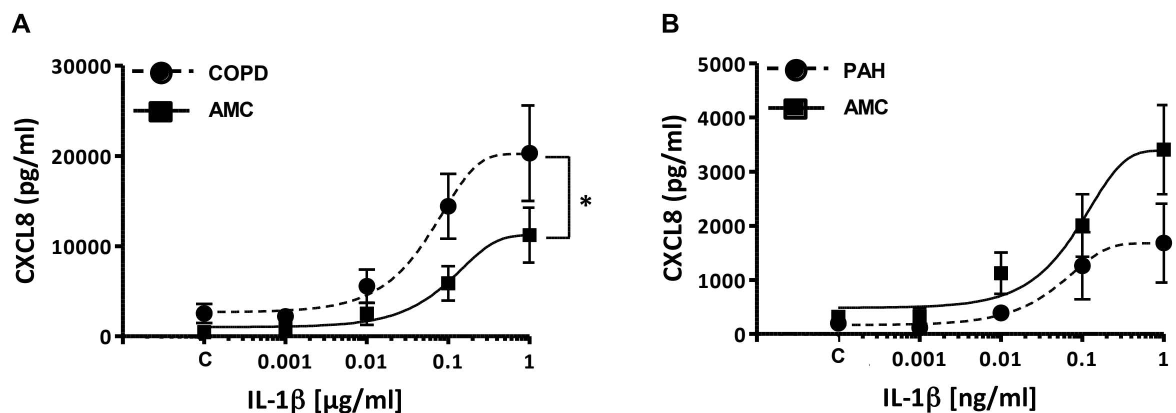


Figure 36. CXCL8 release from whole blood after IL-1β stimulation. Blood from participants was collected and treated with IL-1β (0.001-1ng/ml). After 24h, plasma was separated from whole blood by centrifugation at 1000xg for 5min and stored at -20ºC. Levels of CXCL8 were measured by ELISA. Data are the mean ± SEM of n=6 for COPD patients and n=8 for age-matched controls (A), n=7 for PAH patients and their controls (B). *denotes $p<0.05$ two-way ANOVA followed by Bonferroni post test. AMC – age-matched controls, COPD – COPD patients, PAH- pulmonary hypertension patients.

4.8. Correlation between cytokines release and disease severity.

To investigate whether there is any relationship between cytokine levels and disease severity, we performed correlation studies where we quantified the association between cytokine release and COPD GOLD stage for each patient. Our results demonstrated that there was negative correlation between CXCL8 levels and COPD GOLD stage after LPS treatment. We found that blood of patients with a lower GOLD status released higher amounts of CXCL8 after TLR4 stimulation (Fig. 37C). We did not find any correlation between cytokine levels and PAH severity.

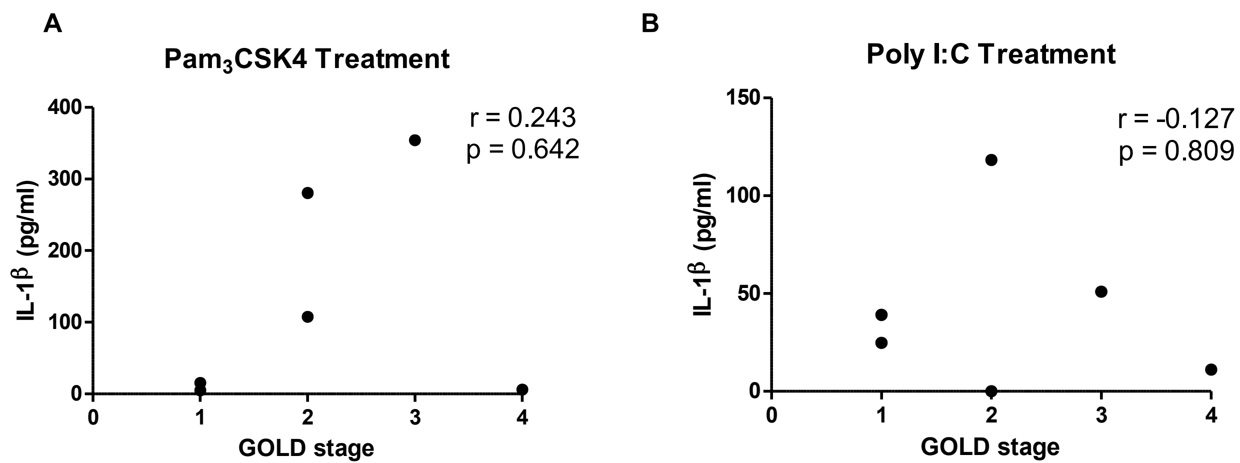


Figure 37. Correlation between IL-1β levels and COPD severity after Pam_3CSK4 (A) and Poly I:C (B) treatment.

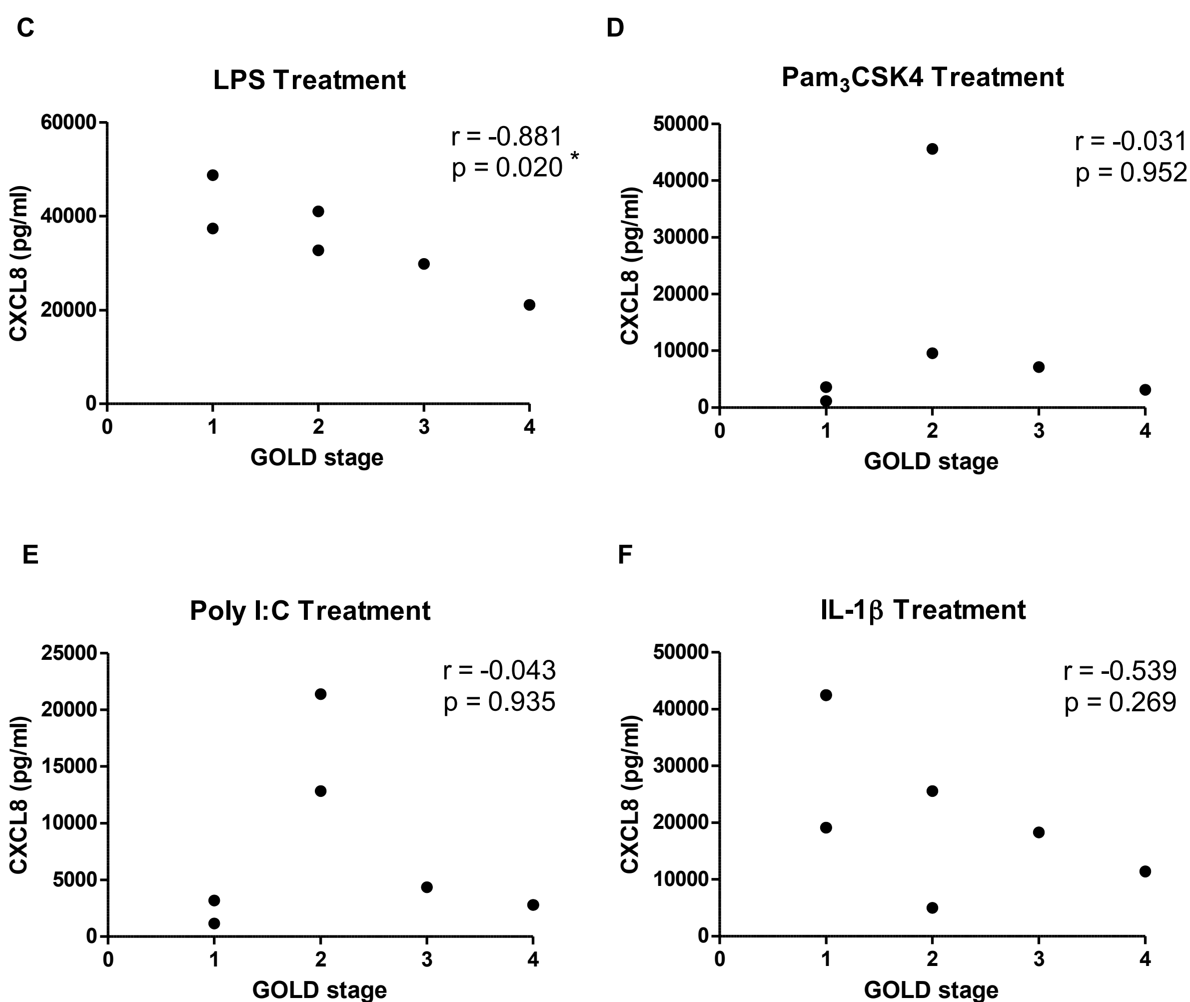


Figure 38. Correlation between CXCL8 levels and COPD severity after LPS (A), Pam₃CSK4 (B), Poly I:C (C) and IL-1β (D) treatment.

Correlation Between CXCL8 Levels and PAH Severity

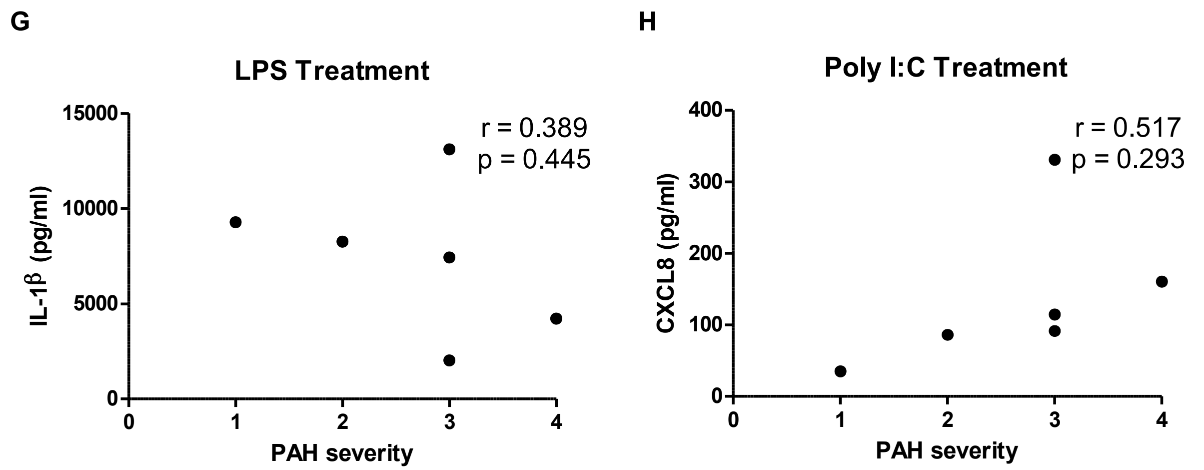


Figure 39. Correlation between levels of cytokines and PAH severity after LPS (A) and Poly I:C (B) treatment.

Discussion

In this study we assessed whether there was a dysfunction in the immune system of COPD patients making their blood more/less sensitive to bacterial and viral PAMPs. Responses of blood from patients with a different chronic lung condition, namely PAH, were compared. Since PAH is a disease of pulmonary vessels rather than airways, this comparison enabled us to expand our discussions into a clinical setting.

COPD is a disease where chronic inflammation plays a central role. The predominant cause of COPD is cigarette smoking triggering an inflammatory response and alveolar destruction. Inflammation is further amplified by oxidative stress. Increased numbers of neutrophils and macrophages are found in the BAL fluid and sputum of COPD patients and in the small airways, where there are increased numbers of $CD68^+$ macrophages and $CD8^+$ T-cells [114]. Neutrophils have also been associated with tissue damage in COPD through the release of a number of enzymes such as elastases and matrix metalloproteinases (MMPs) [178]. Cytokines are known to orchestrate the inflammatory responses in COPD. Increased levels of IL-6, IL-1β, TNF-α and CXCL8 have been observed in sputum of COPD patients [179]. Release of CXCL8, IL-1β, TNF-α and CXCL10 from alveolar macrophages of COPD patients has also been observed [180]. Our results showed that basal levels of IL-1β were not significantly increased in the plasma of COPD patients as compared to controls. Only 2 of the 6 patients sampled had higher levels of IL-1β in plasma and there was no association with disease severity. However, CXCL8 levels were significantly higher in COPD patients than in controls. CXCL8 is a chemokine which acts as a neutrophil chemoattractant and activator. CXCL8 recruits PMNs to sites of inflammation, where they can have an anti-pathogenic effect via the release of free radicals and enzymes, which if persistent can initiate tissue damage. CXCL8 has been shown to play a central role in neutrophilic inflammation, mucus

hypersecretion and destruction of alveoli [181], and can be one of the many factors that contribute to the COPD pathogenesis.

Bacterial and viral infections have been implicated in exacerbations of COPD. They are often associated with a decline in lung function and disease progression resulting in reduced physical activity, poorer quality of life and increased risk of death. Exacerbations are important outcomes in clinical trials and their prevention is a key component of COPD management strategies. However, little is known about their incidence, determinants and their effects in COPD at various levels of severity. COPD with frequent exacerbations is a distinct phenotype that is seen in moderate and severe stages of disease and that the incidence of frequent exacerbations increases with increasing disease severity [16]. It can therefore be hypothesised that TLRs are important in COPD because of their role in activating the immune system against bacterial and viral infections. TLRs can not only be found on immune cells as they are also expressed on epithelial and endothelial cells of the lung where their activation may play a pivotal role in disease pathology. [112]. A recent study by Nadigel et al. showed that COPD patients had increased expression of TLR4 and TLR9 on lung CD8+ cells. However, the overall expression of these receptors in the lung tissue and peripheral blood of COPD patients was not increased [182]. TLR2 has also been demonstrated to be up-regulated in peripheral blood monocytes [120] and peripheral blood neutrophils [183] but downregulated in alveolar macrophages [119]. A recent study by Wang et al. demonstrated that TLR5 at the level of mRNA, protein, and function levels was downregulated in airway epithelium of healthy smokers and in smokers with COPD. [123]. Moreover, single nucleotide polymorphisms (SNPs) in TLR genes have been previously described in COPD patients. Asp299Gly is a SNP in the TLR4 gene, which is associated with decreased LPS signalling and has been shown to have a lower prevalence in patients with COPD than in aged-matched controls [184, 185]. Another study by Budulac et al. showed that tagging SNP in *TLR2* and *TLR4* were

associated with the decline of lung function as well as with inflammatory cell numbers in induced sputum in COPD patients [186]. However, some other studies focusing on *TLR2* showed that Arg677Trp and Arg753Gln were not associated with either the onset or the course of COPD [187]. TLRs also recognize DAMPs such as heat shock proteins, hyaluronic acid, fibronectin and ROS. DAMPs are increased during inflammation and can propagate 'sterile inflammation' via TLRs. This kind of sterile inflammation may also contribute to the alveolar destruction and the pathology seen in COPD. This may also explain why the lung function of patients with COPD tends to stabilise after smoking cessation. DAMPs are found in abundance in cigarette smoke and play a significant role in cigarette smoke-induced lung inflammation [64]. Cigarette smoke has been shown to decrease the expression of TLR4 and TLR9 on the lining of epithelial cells of human lung [122] [110]. To investigate the role of TLRs in the immune response of COPD patients, we examined cytokine release in whole blood after stimulation with TLR, NOD1 and IL-1R ligands and compared these results to those obtained using blood of aged-matched controls. Our results showed that after TLR4 stimulation with LPS, CXCL8 levels were significantly higher in the blood of COPD patients suggesting the importance of this cytokine in disease pathogenesis. We found no effect on IL-1β secretion. Although the relative expression of TLR4 on blood cell components was not assessed in this study, the difference in effects observed in CXCL8 and IL-1β suggest that the sensitivity to LPS is probably not at the levels of the receptor. In contrast, we observed that there was an increase in IL-1β release after LPS treatment in patients with PAH. IL-1β release requires inflammasome activation to cleave pro-ILβ to the mature, active form. Inflammasomes have been implicated in a number of inflammatory and heritable disorders. Increased levels of IL-1β in PAH patients after LPS stimulation may suggest that IL-1β-mediated inflammation plays a significant role in the pathogenesis of PAH but not COPD. Stimulation with NOD1 and a TLR2/6 agonist did not show any increase in cytokine

production but activation of TLR2/1 with Pam_3CSK4 significantly increased IL-1β and CXCL8 levels in COPD patients. We obtained interesting results after stimulation with the viral ligand, Poly I:C where both cytokines were significantly increased in COPD patients compared with controls suggesting the importance of viral induced exacerbations in pathogenesis of this disease. CXCL8 levels were also significantly increased in PAH patients. The major causes of exacerbations in COPD are respiratory infections caused by bacteria and viruses. TLR activation could contribute to the inflammation observed during exacerbations and may be partially responsible for the deterioration of lung function [188] Our results showing that COPD patients released more cytokines than controls after treatment with Gram-negative bacteria and viral ligands can explain why these patients are more sensitive to infections and that these exaggerated responses contribute even further to the pathogenesis of COPD. Although excessive mucus secretion and an increase in its viscosity are directly involved in the clearance of bacteria and viruses from the airways.

Looking at the association of cytokines levels with disease severity, we found a negative correlation between CXCL8 levels after TLR4 activation and COPD GOLD stage. It is consistent with the results from MacRedmont et al. where it was shown that severe COPD was associated with reduced TLR4 expression compared to less severe disease, with good correlation between nasal and tracheal expression [122]. These findings can contribute in the future to developing a novel biomarker tool in COPD to divide patients into different phenotypic groups to identify those patients who are at a higher risk of repeated exacerbations and therefore may suffer a more rapid deterioration in lung function [189]. Because COPD is a complex disease with pulmonary and extra-pulmonary manifestations, the identification and prospective validation of specific clinical phenotypes is a key for the development of novel and more effective therapies. Although lung function remains a useful guide in assessing disease severity in COPD, there is considerable

patient 'quality of life' variability within a given GOLD Stage. For example, there are patients with a low FEV1 and fewer exacerbations and a better quality of life than those with a higher FEV1 and more frequent exacerbations. Therefore, COPD patients should be categorised by FEV1, QOL (symptom scores) and exacerbation frequency because there might be more systemic inflammation in the high symptom group patients with preserved lung function and more exacerbations. Patients who demonstrate a higher level of inflammation may therefore constitute a novel distinct phenotype within the larger group of patients with COPD and could be the target of novel therapeutic strategies [190]. Our results showing that COPD patients are more sensitive to some bacterial and viral ligands may give us insight into why some individuals exacerbate more frequently.

It is also worth noting that white blood cell counts were in the normal range in all of the COPD patients but unfortunately we did not obtain these data for healthy controls and so were unable to make comparisons. Only selective agonists affected CXCL8 and IL-1β levels in the blood suggesting that small differences in the overall cellular composition of the blood had little effect on the overall responses to PAMPs. Analysing COPD study results may be limited because of the effect of inhaled corticosteroids and long-acting β_2 agonists which are commonly prescribed for COPD. This type of therapy can control the symptoms of COPD and lower mortality risk [121]. These drugs together affect a broad range of physiological processes which are central to inflammation. They can inhibit proliferation of airway smooth muscle cells [191], abolish TNF-α-induced eotaxin release [192], inhibit NF-κB –regulated gene expression [193] and TNF-α-induced CXCL8 release [194]. All our patients were on different combinations of therapies, however an inflammatory response was still observed after TLR or NLR activation. Another limitation is the number of patients used in this study. Since this is a pilot study, more subjects are required to draw concrete conclusions from the data. However, our results are promising

and may lead to a tool for stratification of COPD patients and subsequent improvements in their clinical management.

Chapter summary

To summarize our results, we demonstrated that (i) levels of CXCL8 are higher in COPD patients than controls, (ii) COPD patients showed increased response in CXCL8 production after TLR4 stimulation with LPS and TLR2 stimulation with Pam_3CSK4 (iii) after stimulation with a viral ligand, COPD patients had significantly increased release of CXCL8 and IL-1β in plasma. These findings suggest that CXCL8 is an important cytokine in COPD pathogenesis and can contribute to disease progression by recruitment, activation and survival of inflammatory cells, and can increase susceptibility to bacterial and viral infections of COPD patients. Together our results may give us an insight into why patients with COPD experience repeated infective exacerbations and help us to understand why numerous exacerbations lead to worsening lung function.

Chapter 5

The Role of TLRs in lung cancer cell proliferation and survival.

Rationale

Toll-like receptors (TLRs) are type I transmembrane receptors that recognize structurally conserved pathogen associated molecular patterns (PAMPs) and non-pathogenic self-danger molecules. They participate in the first line of defence and play a significant role in inflammation, immune cell regulation, survival and proliferation [195]. Most of the evidence for TLRs and proliferation in noncancerous cells has been shown in vascular smooth muscle cells (VSMC) and cardiovascular models [196] [127] but the mechanisms responsible for the control of these outcomes are poorly defined. Chronic infection and inflammation are widely known as risk factors for tumorigenesis. Immune system cells are present at tumor sites and in the supporting stroma. Damage caused by invading pathogens and danger molecules can result in excessive tissue remodeling, loss of tissue and DNA mutations [197]. Activated TLRs on cancer cells may promote cancer progression, anti-apoptotic activity and resistance to host immune response. Ligation of TLRs increases production of pro-inflammatory cytokines and chemokines These TLR-dependent mechanisms maintain the tumor microenvironment and promote cancer progression [128] [129]. Cigarette smoke-induced oxidative stress has been shown to cause DNA damage in the form of strand breaks and oxidative modifications. Through the activation of survival signaling cascades like EGFR/NF-κB/Akt, oxidative stress causes uncontrolled cell proliferation and cell transformation [198]. Also the airway epithelium in chronic bronchitis and COPD often shows squamous metaplasia, which may result from increased proliferation of airway epithelial cells. Proliferation in basal airway epithelial cells, measured by proliferative cell nuclear antigen, is increased in some normal smokers but is markedly increased in patients with chronic bronchitis and correlates with the degree of squamous metaplasia [199].

Recently, the expression or up-regulation of TLRs has been detected in many tumor cell lines or tumors, especially epithelial derived cancers. NF-κB activation leads to the transcription of many anti-apoptotic genes such as BCL2A1 [83]. Because TLRs are potent activators of NF-κB pathway there is growing evidence that they provide a signal to promote the survival of epithelial cells under stress conditions [22]. Recent studies showed that activation of TLRs resulted in resistance to apoptosis [136] [200] [119] and better understanding of function and regulation of TLRs is essential for developing TLR-based therapies inhibiting cell proliferation, tumor growth and progression. Therefore in this chapter we hypothesised that activation of TLRs on lung cancer cells will induce cell proliferation and resistance to apoptosis. In the present study, we stimulated the epithelial lung cancer cell line (A549) with TLR ligands and measured cytokine release to demonstrate that TLRs were active in these cells. To assess apoptosis in A549 cells, cell viability/respiration was measured by AlamarBlue and expression of annexin V was measured by FACS.

Methods

A549 cells were cultured in 96 well or 6 well plates as described in chapter 2. Cells were treated with TLR agonists for 24, 48 and 72h. Proliferation was assessed by a few different methods described in the results section. A549 cells cycle profile was analyzed by Cellomics. CXCL8 and CXCL10 levels were measured by ELISA. For cell viability experiments A549 were treated with apoptotic agents 4h prior to TLRs activation. Apoptosis resistance was measured by AlamarBlue assay and FACS after 24h. More details about methods can be found in chapter 2 and in the figure legends.

Results

5.1. Stimulation of A549 cells with TLR ligands.

TLR ligation can stimulate immune cells to secrete many kinds of proinflammatory cytokines such as CXCL8, TNF-α and IL-1β. CXCL8 is a proangiogenic chemokine that has anti-apoptotic properties and is secreted by tumor cells [136]. Hence, CXCL8 can promote cancer metastasis and apoptosis resistance in malignant cells. To investigate whether TLRs expressed on lung cancer cells are functionally active, we performed ELISA assays to measure secreted CXCL8 levels after TLR stimulation. Thus, A549 cells were cultured in either 0 or 10% FCS and stimulated with bacterial (Fig. 40) or viral (Fig. 41) ligands for 24, 48 and 72h.

Stimulation of TLR2/1 with Pam_3CSK4 or TLR2/6 with FSL-1 or TLR4 with LPS resulted in a concentration dependent increase in CXCL8 production after 24, 48 and 72h. This was true regardless of the presence or absence of serum. FSL-1 in all cases caused a greater increase in CXCL8 levels than Pam_3CSK4 and LPS. Our results demonstrated that TLR2 heterodimers and TLR4 homodimers are functionally active on this cancer cell line.

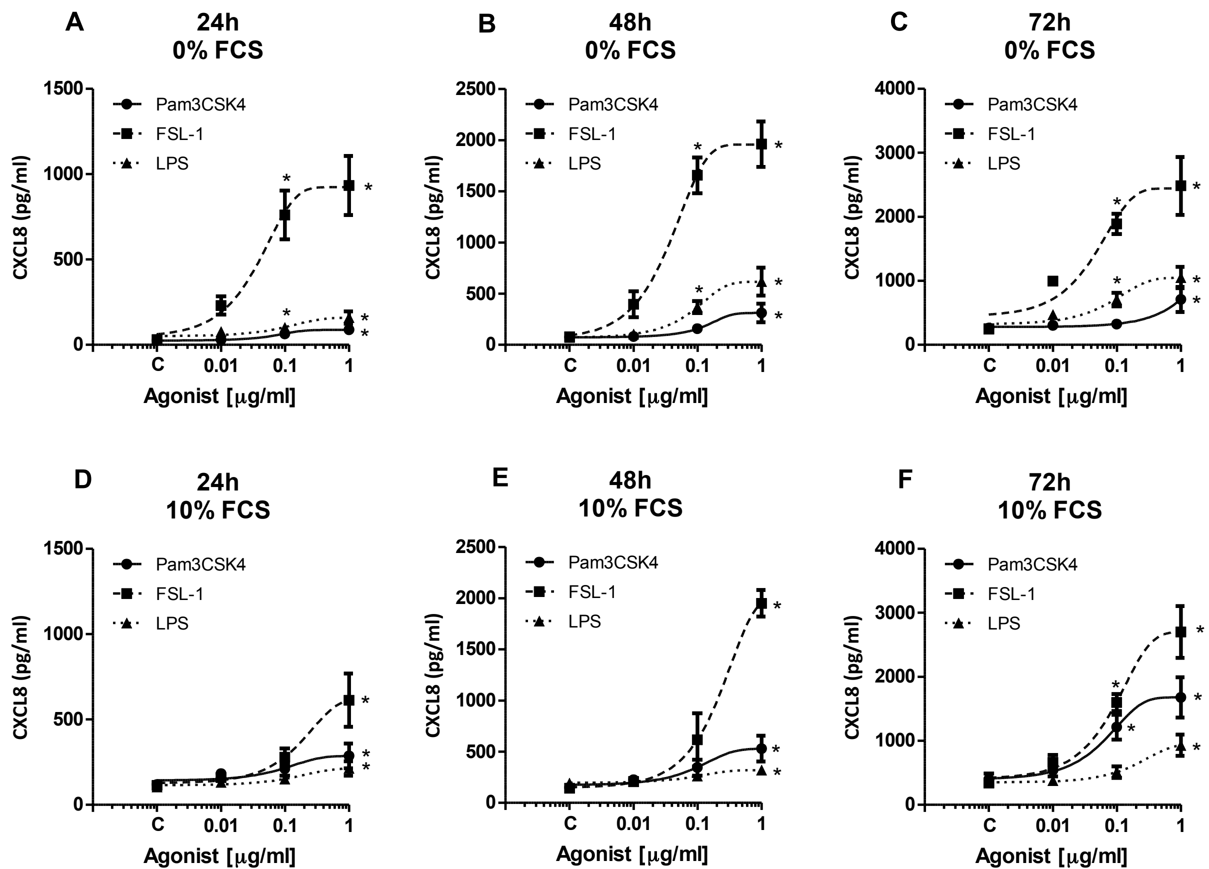


Figure 40. The effect of bacterial ligands on the activation of A549 cells. A549 cells were seeded in a 96 well plates at 1 x 10^4 cells per ml in DMEM with 10% FCS and allowed to equilibrate for 12h. After this time period, media was replaced with DMEM containing either 0 (A-C) or 10% FCS (D-F). Cells were treated with the ligands and incubated for 24 (A,D), 48 (B,E), 72h (C,F) after which CXCL8 levels were measured in the supernatants by specific ELISA. Data represented are the mean ± SEM of n=9 replicates measured over 3 separate experimental days. *denotes $p \leq 0.05$ as assessed using a one-way ANOVA followed by a Dunnett's multiple comparison post-hoc test.

Production of CXCL8 after viral ligand stimulation was less clear (Fig. 41). We observed significant increase after TLR8 stimulation with ssRNA40/Lyovec in all condition we used but only the highest concentration of this ligand induced a significant release. Poly I:C which activates TLR3 also caused CXCL8 production in all conditions we used except 48h time point in the absence of serum (Fig. 41B). The TLR7 agonist (Imiquimod) was not a potent inducer of CXCL8 as we only observed significant release after 72h treatment. Generally, levels of CXCL8 were much lower after viral ligand stimulation, but they usually require an additional activation with IFNs to produce greater levels of this chemokine. We also measured CXCL10 release, which is a more appropriate cytokine to measure viral driven host immune responses, but levels of this cytokine were below the detection limit of the assay (Tab.9).

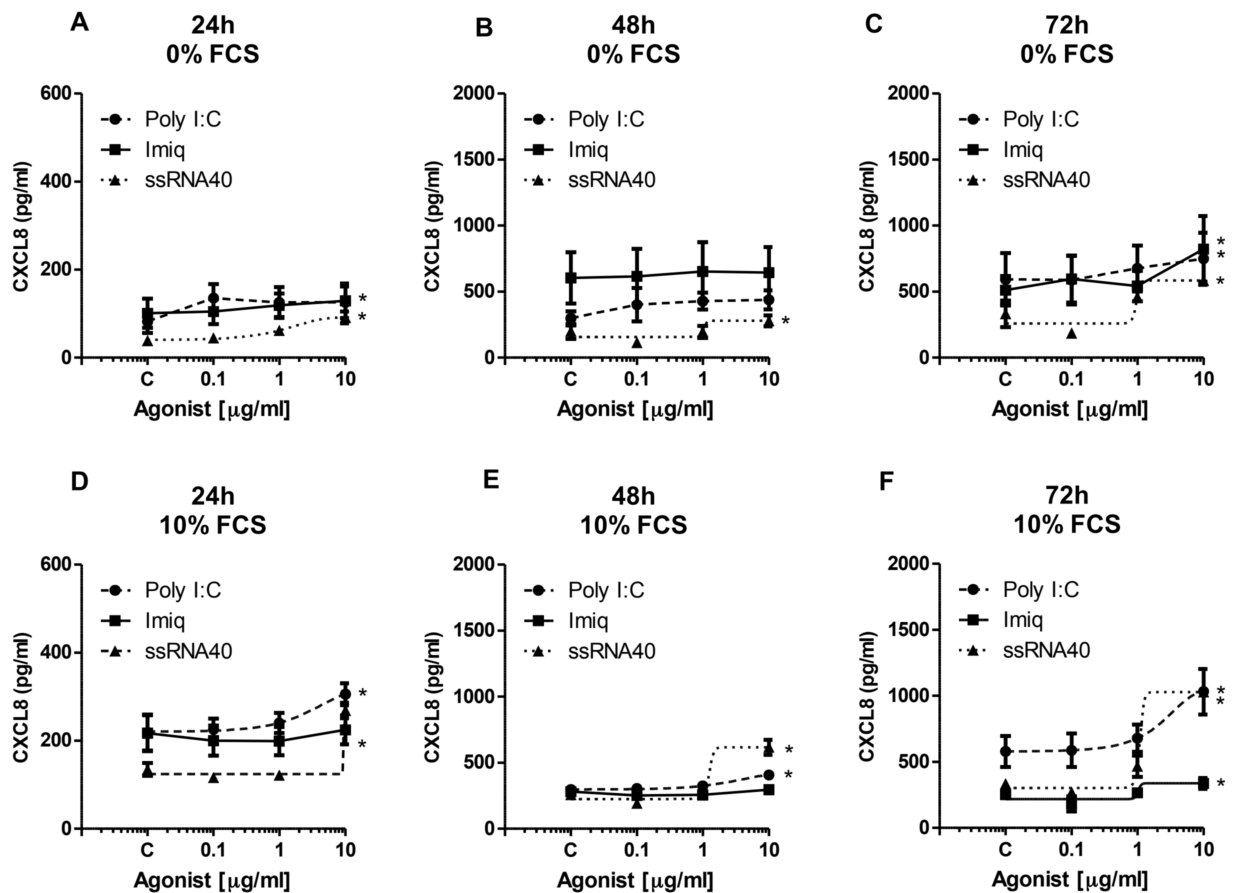


Figure 41. The effect of viral ligands on the activation of A549 cells. A549 cells were seeded in a 96 well plates at 1 x 10^4 cells per ml in DMEM with 10% FCS and allowed to equilibrate for 12h. After this time period, media was replaced with DMEM containing either 0 (A-C) or 10% FCS (D-F). Cells were treated with the ligands and incubated for 24 (A,D), 48 (B,E), 72h (C,F) after which CXCL8 levels were measured in the supernatants by specific ELISA. Data represented are the mean ± SEM of n=9 replicates measured over 3 separate experimental days. *denotes p≤ 0.05 as assessed using a one-way ANOVA followed by a Dunnett's multiple comparison post-hoc test.

A

CXCL10 Release (0%FCS)									
	Ctr	Poly I:C (μg/ml)			Imiquimod (μg/ml)			ssRNA40 (μg/ml)		
		0.1	1	10	0.1	1	10	0.1	1	10
24h	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
48h	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
72h	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

B

CXCL10 Release (10%FCS)										
	Ctr	Poly I:C (μg/ml)			Imiquimod (μg/ml)			ssRNA40 (μg/ml)		
		0.1	1	10	0.1	1	10	0.1	1	10
24h	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
48h	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
72h	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

Table 9. The effect of viral ligands on the activation of A549 cells. A549 cells were seeded in a 96 well plates at 1 x 10^4 cells per ml in DMEM with 10% FCS and allowed to equilibrate for 12h. After this time period, media was replaced with DMEM containing either 0 (A) or 10% FCS (B). Cells were treated with the ligands and incubated for 24, 48, 72h an after which CXCL10 levels were measured in the supernatants by specific ELISA. Data represented are the mean ± SEM of n=9 replicates measured over 3 separate experimental days. *denotes p≤ 0.05 as assessed using a one-way ANOVA followed by a Dunnett's multiple comparison post-hoc test. (nd-not detected)

5.2. Effect of seeding density on A549 proliferation.

It has been previously described that ligation of TLR2, TLR4, TLR5 and TLR9 can induce proliferation of tumor cells, which include ovarian, breast, prostate, and gastric cancers [119, 136, 179, 180, 201, 202]. To test what seeding density would be best to study A549 cell proliferation over a 72h period, we seeded cells at 1x10^4 and 2.5x10^4cells/well. A549 cells were grown with either 0 or 10% FCS and treated with 0.1μg/ml and 1μg/ml of Pam_3CSK4 or FSL-1. We harvested cells 24, 48, 72h after stimulation and counted them using the haemocytometer.

After Pam3CSK4 stimulation we observed a significant increase in proliferation at the 72h time point with or without serum and at the lowest seeding density (Tab. 10A,C). Similarly,

FSL-1 caused an increase in proliferation at 72h in the absence of serum. When we seeded A549 cells at density $2.5X10^4$ cells/well there was an increase in proliferation after Pam_3CSK4 and FSL-1 stimulation in the presence of serum at 48h (Tab. 10D).

A549 cells are a cancerous cell line and proliferate rapidly. We observed that these cells seeded at a density of 2.5x104 cells/well became fully confluent after 48h. When we seeded A549 cells at this density and stimulated them with Pam3CSK4 and FSL-1 in the presence of serum, an increase in proliferation was observed after 48h (Tab. 10D). For that reason, we decided to use the lower seeding density for further experiments.

A

0% FCS Seeding Density – $1x10^4$ cells/well									
	Control	**Pam$_3$CSK4**				**FSL-1**			
		0.1 µg/ml		**1µg/ml**		**0.1 µg/ml**		**1µg/ml**	
24h	10.8±2.5	7.0±1.4	ns	8.6±1.4	ns	10.1±2.2	ns	10.3±1.6	ns
48h	27.5±5.9	25.9±6.4	ns	27.0±5.3	ns	28.9±5.7	ns	25.6±5.6	ns
72h	26.6±4.6	41.3±5.7	ns	33.9±4.0	*	36.0±5.7	ns	31.8±6.0	*

B

0% FCS Seeding Density – $2.5x10^4$ cells/well									
	Control	**Pam$_3$CSK4**				**FSL-1**			
		0.1 µg/ml		**1µg/ml**		**0.1 µg/ml**		**1µg/ml**	
24h	29.6±4.8	29.5±3.6	ns	24.2±3.0	ns	32.2±5.9	ns	29.1±4.4	ns
48h	61.3±13.3	68.6±11.7	ns	67.0±8.9	ns	56.2±8.9	ns	51.5±13.1	ns
72h	65.3±7.8	72.7±7.0	ns	77.0±7.7	ns	66.2±9.4	ns	68.6±7.2	ns

C

10% FCS Seeding Density – $1x10^4$ cells/well									
	Control	**Pam$_3$CSK4**				**FSL-1**			
		0.1 µg/ml		**1µg/ml**		**0.1 µg/ml**		**1µg/ml**	
24h	14.5±1.0	13.5±1.0	ns	13.1±0.5	ns	13±1.4	ns	12.8±1.8	ns
48h	40.9±5.6	47.4±6.9	ns	46.0±6.9	ns	48.8±6.9	ns	47.1±5.8	ns
72h	53.1±12.8	71.6±7.5	*	55.6±13.8	ns	64.2±11.0	ns	58.4±9.3	ns

D

10% FCS Seeding Density – $2.5x10^4$ cells/well									
	Control	**Pam$_3$CSK4**				**FSL-1**			
		0.1 µg/ml		**1µg/ml**		**0.1 µg/ml**		**1µg/ml**	
24h	43.3±4.4	39.6±2.1	ns	44.5±3.9	ns	43.5±4.5	ns	35.0±4.7	ns
48h	71.0±9.8	84.0±8.0	*	84.3±8.7	*	88.4±9.2	ns	90.6±8.1	*
72h	91.3±9.8	96.3±14.5	ns	92.6±10.7	ns	94.6±12.0	ns	77.5±12.5	ns

Table 10. Effect of seeding density on A549 proliferation. A549 cells were seeded in a 96 well plates at 1 x 10^4 (A,C) or 2.5 x 10^4 (B,D) cells per well in DMEM with 10% FCS and allowed to equilibrate for 12h. After this time period media was replaced with DMEM containing either 0 (A,B) or 10 % FCS (C,D). Cells were treated with the ligands and incubated for 24, 48 and 72h after which proliferation was assessed by direct cell counting. Data represented are the mean ± SEM of n=9 replicates measured over 3 separate experimental days. *denotes $p \leq 0.05$ as assessed using one-way ANOVA followed by Dunnett's multiple comparison post-hoc test.

5.3. Concentration-dependent effect of Pam_3CSK4 on the proliferation of A549 cells.

The previous experiment, using direct cell counting, indicated that Pam_3CSK4 activated TLR2/1 heterodimer and stimulated the proliferation of A549 cells; although this appeared to be inversely related to concentration. It was therefore important to perform additional experiments using a more extensive range of concentrations of the agonist. To determine whether these cells proliferated to Pam_3CSK4 in a concentration dependent manner the experiment was repeated using a range of concentrations from 0.001µg/ml to 3µg/ml. We chose a 72h time point, as this was where we observed the significant increase in cell number. Cells were seeded at the density $1x10^4$ cell/well and counted using the haemocytometer.

Figure 42 shows that activation of TLR2 increased A549 proliferation in the presence of 10% serum. All doses of the ligand significantly increased cell growth, however the biggest effect was observed with the lowest concentration of Pam_3CSK4, where there was 65% increase compared with the control (Fig. 42B).

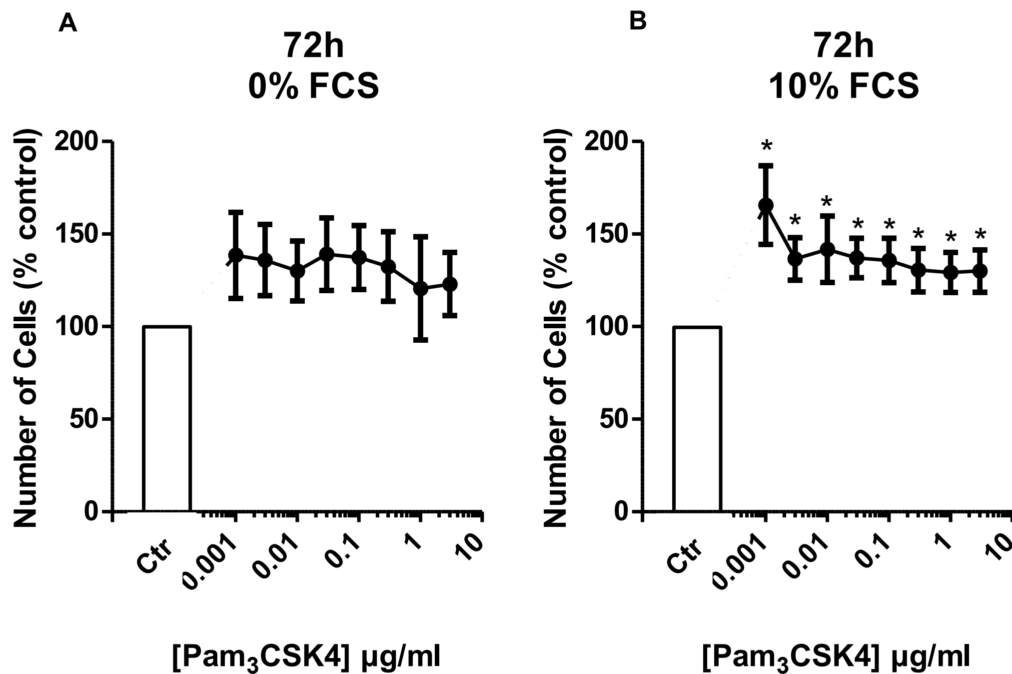


Figure 42. Effect of Pam_3CSK4 concentration on the proliferation of A549 cells after 72h. A549 cells were seeded in a 96 well plates at 1 x 10^4 cells per ml in DMEM with 10% FCS and allowed to equilibrate for 12h. After this time period media was replaced with DMEM containing either 0% FCS (A) or 10% FCS (B). Cells were treated with the ligands and incubated for 72h after which proliferation was assessed by direct cell counting. Data represented are the mean ± SEM of n=9 replicates measured over 3 separate experimental days. *denotes p≤ 0.05 as assessed using column statistics followed by one sample t-test of normalised data.

5.4. TLR2/1 stimulation and cell cycle profile.

The increase in cellular proliferation in response to the TLR2/1 agonist, Pam_3CSK4, may be a result of a change in the cell cycle profile induced in A549 cells. Therefore, to test this cell cycle profile was assessed using a Cell Cycle BioApplication protocol (Cellomics). Fluorescent images of A549 cells stained with DAPI were captured by a high-resolution camera. Image analysis was performed on DAPI positive cellular bodies, which allowed identification of chromatin staining within individual cells and subsequent characterisation

of the cell cycle stage. Briefly, A549 cells were seeded in 96-well plates either with 1 or 10% FCS and stimulated with Pam_3CSK4 for 6, 24, 48 and 72h.

Looking at 6 at 24h time points more closely (Fig. 43A-D) we observed that a reduction in cell number in the G0/G1 phase corresponded to an increase in the S phase. The results showed significant changes in the G0/G1 and S phase only after 6 and 48h in cells grown with 1% FCS, but not in the G2/M phase. All three concentration of Pam_3CSK4 induced small modification of DNA profile compared with the control. We were not able to analyse cells stimulated for 72h as A549 cells were too confluent therefore making it impossible for the camera to distinguish the boundaries of individual cells.

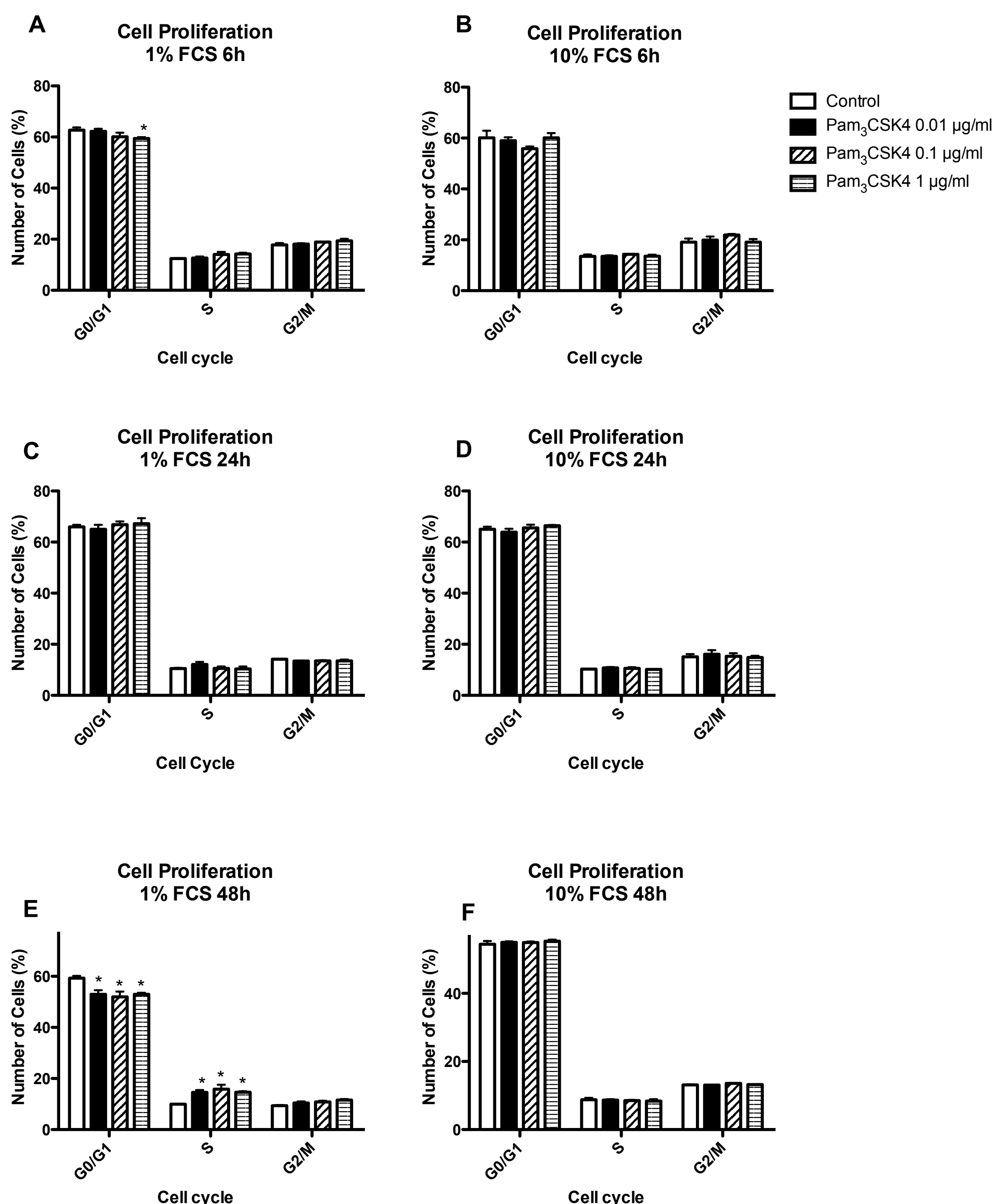


Figure 43. Effect of TLR2/1 stimulation on A549 cell cycle profile. A549 cells were seeded in a 96 well plates at 1 x 10^4 cells per ml in DMEM with 10% FCS and allowed to equilibrate for 12h. After this time period media was replaced with DMEM containing 1 or 10% FCS. Cells were treated with the ligands and incubated for 6 (A,B), 24 (C,D), 48 (E,F) after which DNA profile was assessed by Cell Cycle BioApplication (Cellomics). Data represented are the mean ± SEM of n=3 replicates. * denotes $p \leq 0.05$ as assessed using two-way ANOVA followed by Bonferroni post-test.

5.5. Effect of TLRs stimulation on A549 proliferation/viability.

TLR2/1 activation induced a small increase in A549 proliferation. Thus, we decided to check whether this is true for other TLR ligands. Again we stimulated cells with bacterial and viral ligands and measured cell proliferation using AlamarBlue assay. AlamarBlue is a permeable and non-toxic cell health indicator. It uses the reducing power of living cells to convert resazurin (blue and non-fluorescent) to resorufin (red in color and highly fluorescent). Viable cells continuously convert resazurin to resorufin, increasing the overall fluorescence and color of the media surrounding cells, thereby making it easy to quantitatively measure proliferation, using the assumption that more cells produce a greater colour over the same period of time. Direct cell counting using the haemocytometer is time-consuming and can be affected by human bias and sampling error. Thus, the AlamarBlue assay was used to measure A549 proliferation/viability.

Although, we saw an increase in cell proliferation of A549 cells using direct cell counting after Pam3CSK4 treatment, using the AlamarBlue assay we did not observe any significant difference in cellular proliferation after stimulation of TLR2/1, TLR2/6, TLR4 (Fig. 44) and TLR3 (Fig. 45). TLR7 and TLR8 agonist induced cell death at 24h, 48h and 72h in the presence and absence of serum (Fig. 45).

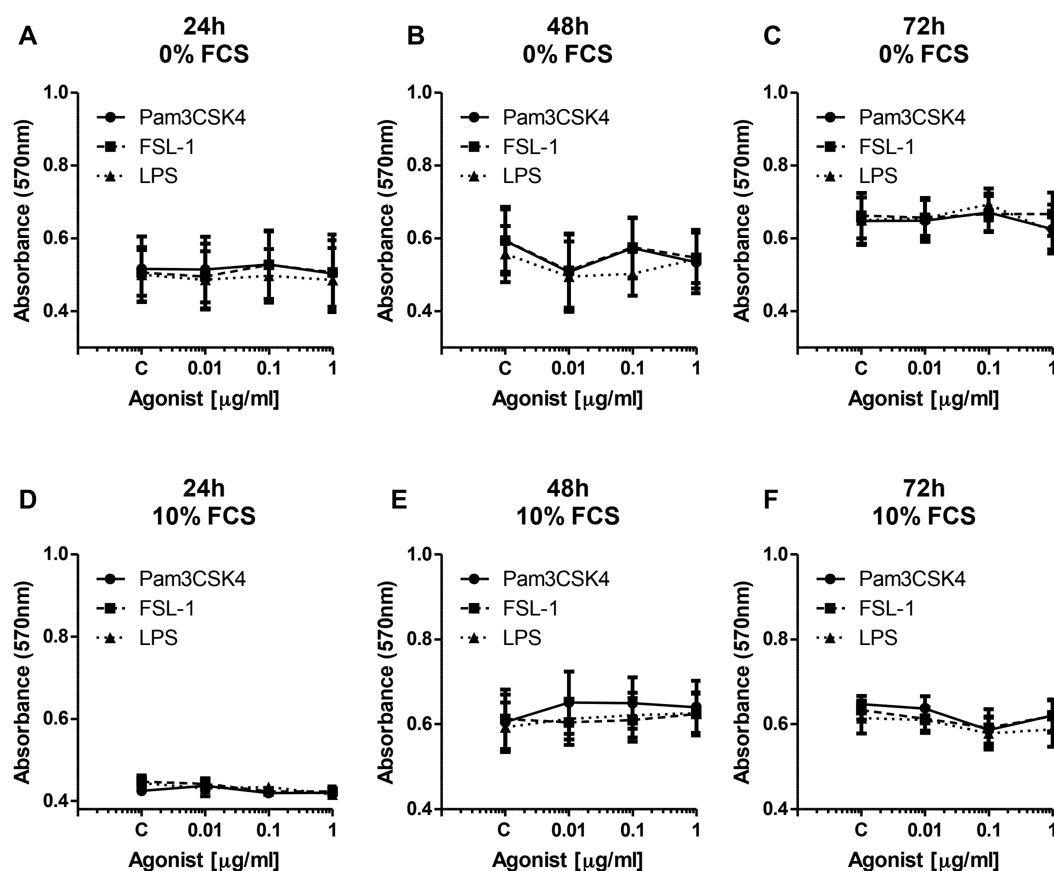


Figure 44. Effect of bacterial ligands on the proliferation of A549 cells over 72 hours. A549 cells were seeded in a 96 well plates at 1 x 10^4 cells per ml in DMEM with 10% FCS and allowed to equilibrate for 12h. After this time period media was replaced with DMEM containing 0 (A-C) or 10% FCS (D-F). Cells were treated with increasing concentrations of agonists. Cells were then incubated for 24 (A,D), 48 (B,E) and 72h (C,F) after which proliferation was assessed using the AlamarBlue assay. Data represented are the mean ± SEM of n=6 replicates measured over 3 separate experimental days. *denotes $p \leq 0.05$ as assessed using a one-way ANOVA followed by a Dunnett's multiple comparison post-hoc test.

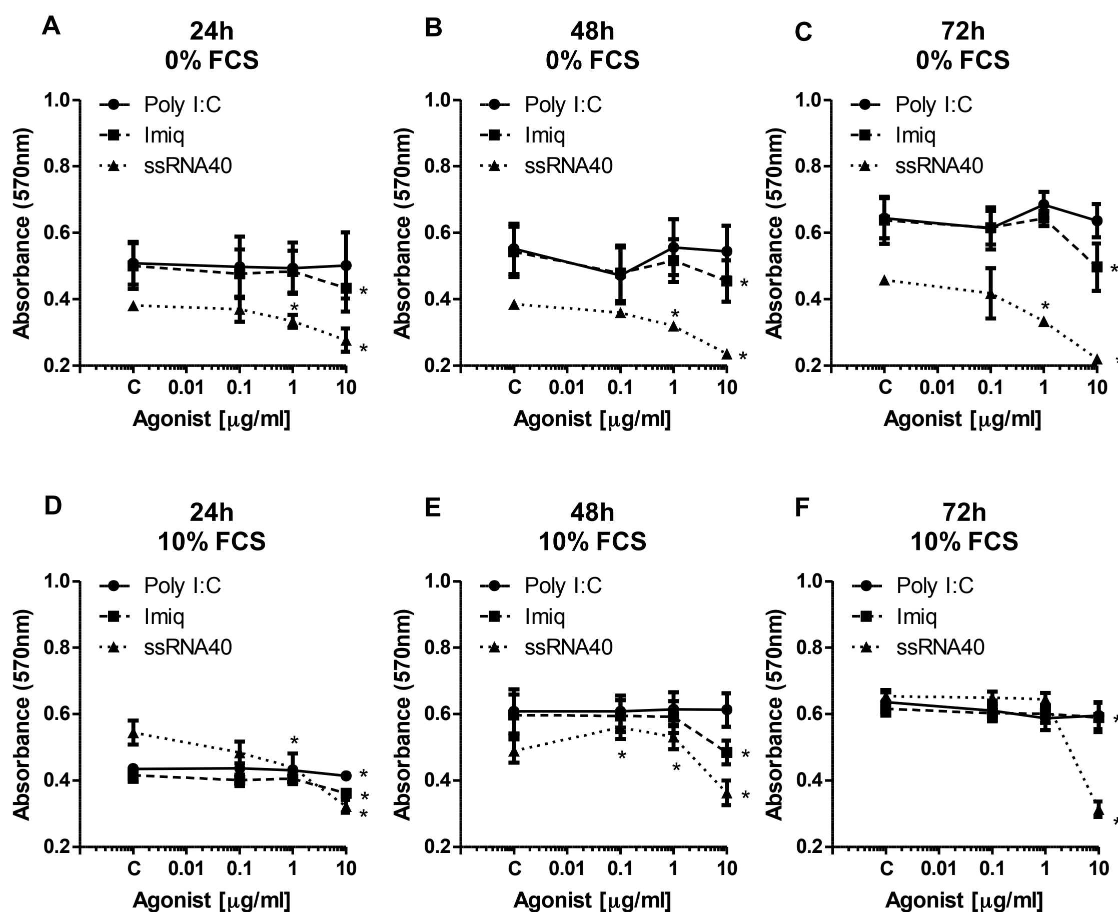


Figure 45. The effect of viral ligands on the proliferation of A549 cells over 72 hours. A549 cells were seeded in a 96 well plates at 1 x 10^4 cells per ml in DMEM with 10% FCS and allowed to equilibrate for 12h. After this time period media was replaced with DMEM containing 0 (A-C) or 10% FCS (D-F). Cells were treated with increasing concentrations of agonists. Cells were then incubated for 24 (A,D), 48 (B,E) and 72h (C,F) after which proliferation was assessed using the AlamarBlue assay. Data represented are the mean ± SEM of n=6 replicates measured over 3 separate experimental days. *denotes $p \leq 0.05$ as assessed using a one-way ANOVA followed by a Dunnett's multiple comparison post-hoc test.

5.6. A549 proliferation in response to serum

AlamarBlue monitors cellular respiration as indicator of cell viability and as such is an indirect measure of proliferation. In order to validate observations using AlamarBlue, DNA was quantified using the CyQuant assay. This assay is a fluorescence-based method that utilises 'GR-CyQuant' dye, which binds to the nucleic acid within the cell. The DNA content of a cell is constant therefore; measuring cell number using DNA provides an

accurate indication of relative cell number. Firstly, we assessed the proliferation potential of A549 cells and the suitability of the CyQuant assay as readout for proliferation. This was achieved by growing A549 cells in different concentrations of FCS (0-10%) over a 72h time period. For each experiment, a DNA standard curve was prepared from different dilutions of bacteriophage λ DNA that was provided with the kit. The DNA content of samples was calculated using the standard curve generated each time. Increased DNA content in our experiments represented an increase in a relative number of cells.

Serum caused a concentration-dependent increase in A549 cells at 24, 48 and 72h (Fig. 46). It is also worth noting that A549 cells can still proliferate after serum withdrawal. This is a property that is common in nontransformed cancer cell lines and distinguish them from primary cells [203]. After 24h DNA content in the 0% FCS was 581ng/ml, this was also similar at 48h (551ng/ml) and increased to 868ng/ml after 72h. However, in the presence of 10% FCS the level of DNA present in A549 cells was 913ng/ml at 24h and increased to 2152ng/ml after 72h.

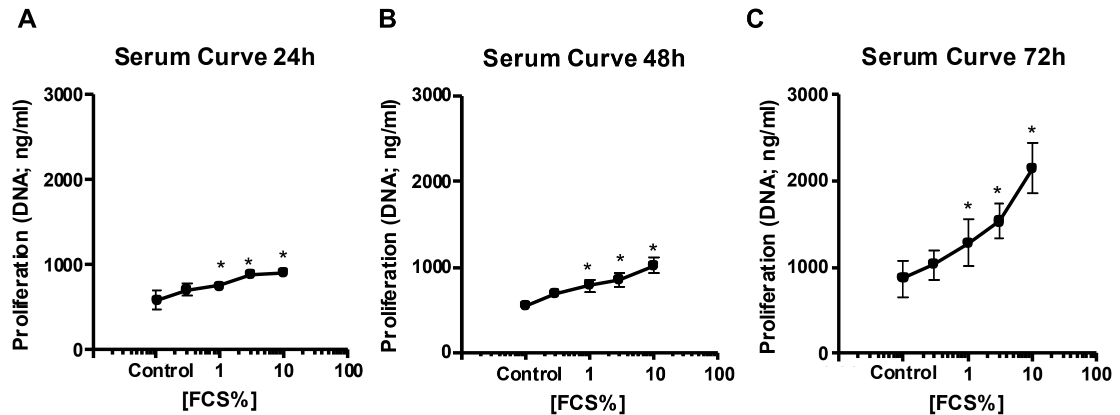


Figure 46. Effect of serum on the proliferation of A549 cells over 72 hours. A549 cells were seeded in a 96 well plates at 1 x 10^4 cells per ml in DMEM with 10% FCS and allowed to equilibrate for 12h. After this time period media was replaced with DMEM containing 0, 0.3, 1, 3 and 10% FCS. Cells were then incubated for 24 (A), 48 (B) and 72h (C) after which proliferation was assessed by DNA content using the CyQuant assay. Data represented are the mean ± SEM of n=9 replicates measured over 3 separate experimental days. *denotes $p \leq 0.05$ as assessed using a one-way ANOVA followed by a Dunnett's multiple comparison post-hoc test.

5.7. A549 proliferation after TLRs stimulation assessed using the the CyQuant assay.

In order to perform the CyQuant assay, we chose TLR agonists, which were the most potent inducers of CXCL8 release. We also used ssRNA40/Lyovec as we saw a decrease in cell survival after TRL8 stimulation. Briefly, A549 cells were treated for 24, 48 and 72h with the TLR2/1, TLR2/6, TLR4 and TLR8 agonists and subjected to CyQuant assay.

After 24 hours Pam_3CSK4 at 0.1μg/ml and 1 μg/ml significantly induced proliferation (Fig. 47A). By contrast, we observed small decrease in DNA content after 48h in 0.3% FCS (Fig. 47B). After 72h there was increase in A549 cell number when media with 10% of FCS was used (Fig. 47C).

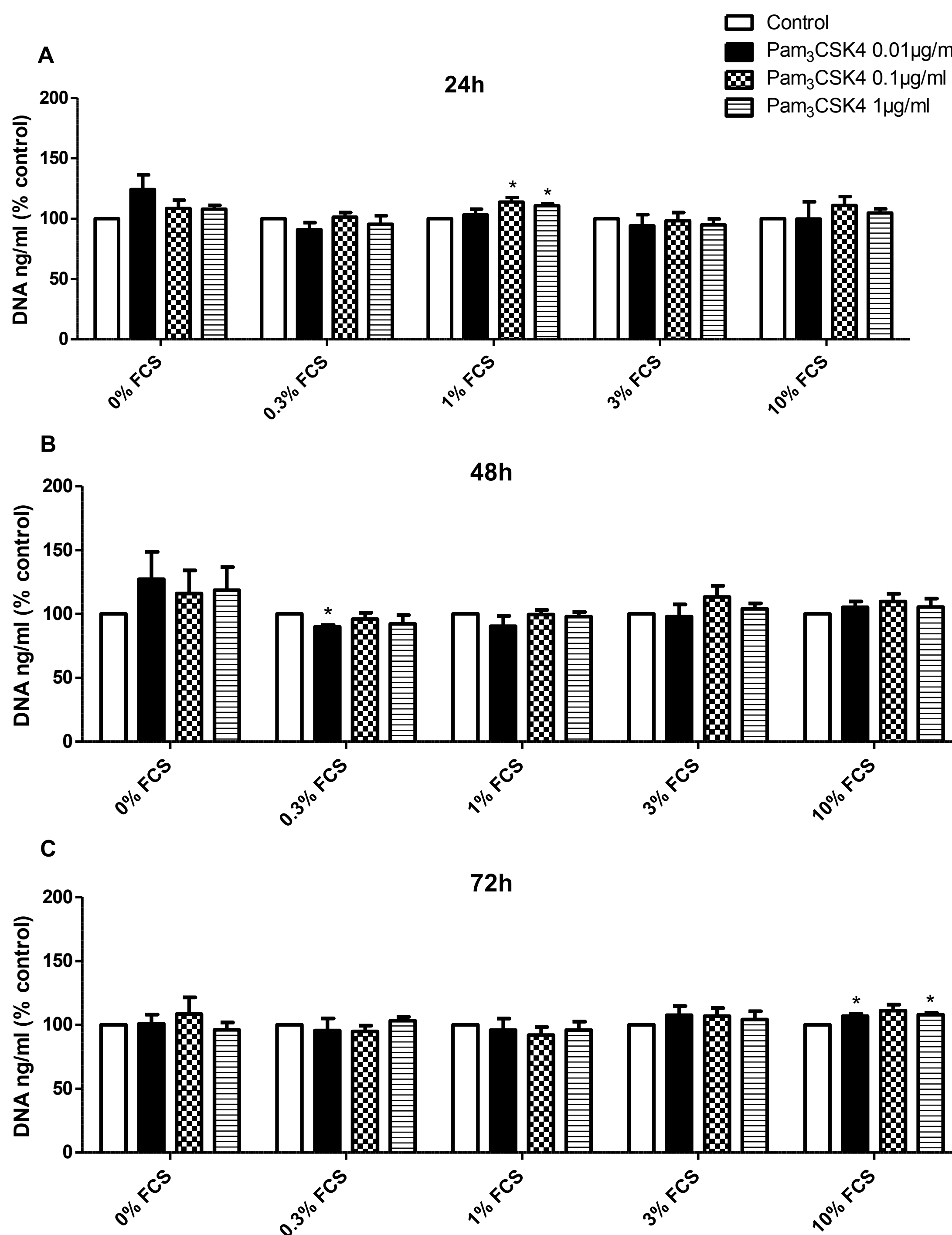


Figure 47. Effect of TLR2/1 stimulation on A549 proliferation grown in different concentration of FCS over 72h. A549 cells were seeded in a 96 well plates at 1 x 10^4 cells per ml in DMEM with 10% FCS and allowed to equilibrate for 12h. After this time period media was replaced with DMEM containing 0, 0.3, 1, 3 and 10% FCS. Cells were treated with Pam_3CSK4 and incubated for 24h (A), 48h (B) and 72h (C) after which proliferation was assessed by DNA content using the CyQuant assay. Data represented are the mean ± SEM of n=6 replicates measured over 3 separate experimental days. *denotes $p \leq 0.05$ as assessed using a one-way ANOVA followed by one sample t-test of normalised data.

After FSL-1 treatment we observed significant increase in cell number when media with 10% FCS was used (Fig. 48A), but there was a decrease after 48h (0.3% and 1% FCS) (Fig. 48B). At 72h time point again we observed a small increase with 3% FCS (Fig. 48C).

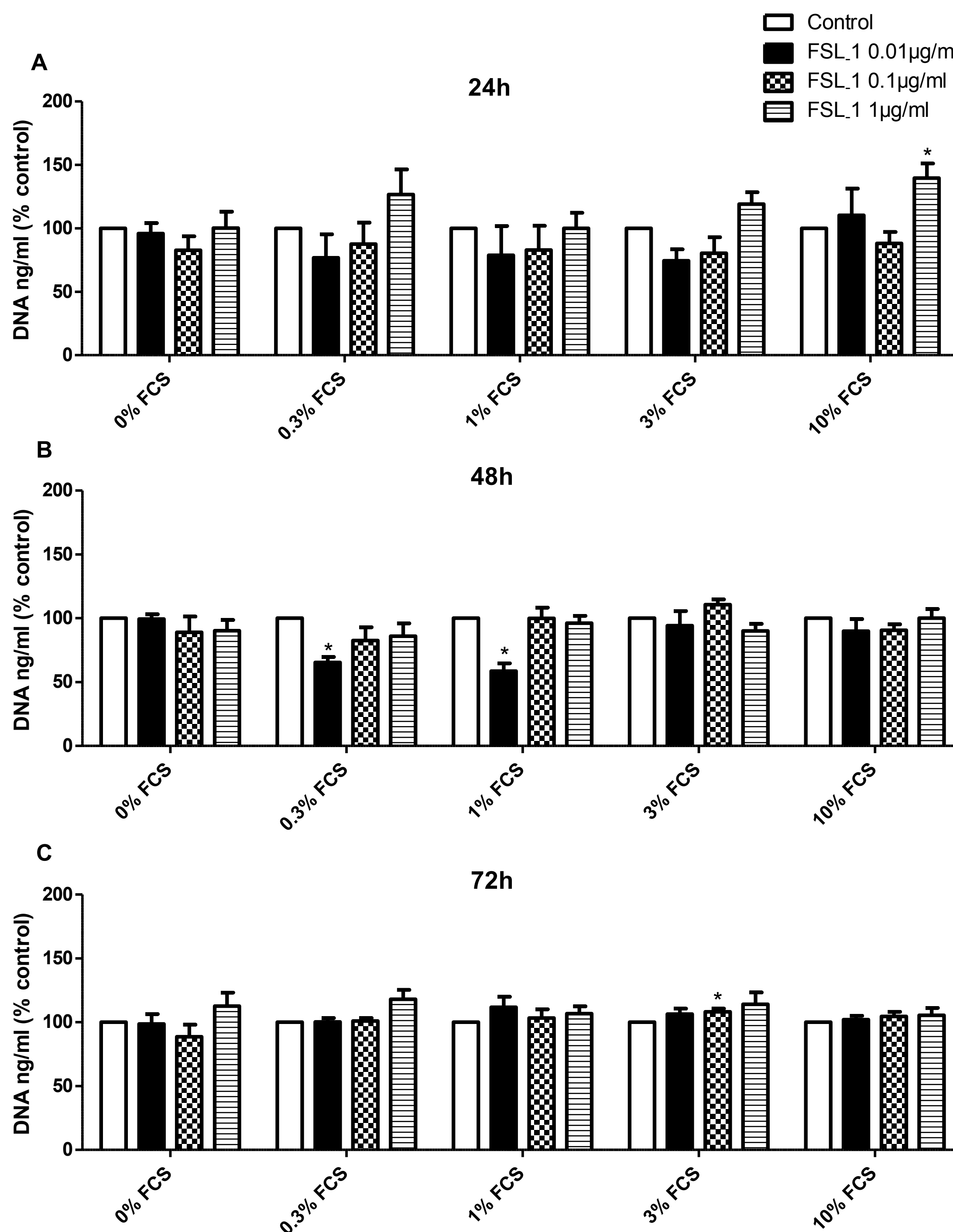


Figure 48. Effect of TLR2/6 stimulation on A549 proliferation grown in different concentration of FCS over 72h. A549 cells were seeded in a 96 well plates at 1 x 10^4 cells per ml in DMEM with 10% FCS and allowed to equilibrate for 12h. After this time period media was replaced with DMEM containing 0, 0.3, 1, 3 and 10% FCS. Cells were treated with FSL-1 and incubated for 24h (A), 48h (B) and 72h (C) after which proliferation was assessed by DNA content using the CyQuant assay. Data represented are the mean ± SEM of n=6 replicates measured over 3 separate experimental days. *denotes $p \leq 0.05$ as assessed using a one-way ANOVA followed by one sample t-test of normalised data.

Our results after activation of TLR4 with LPS were not consistent. It induced a decrease in DNA content (Fig. 49) as well as an increase, suggesting that the effect of TLR agonism on cellular proliferation may be more complex than first envisaged.

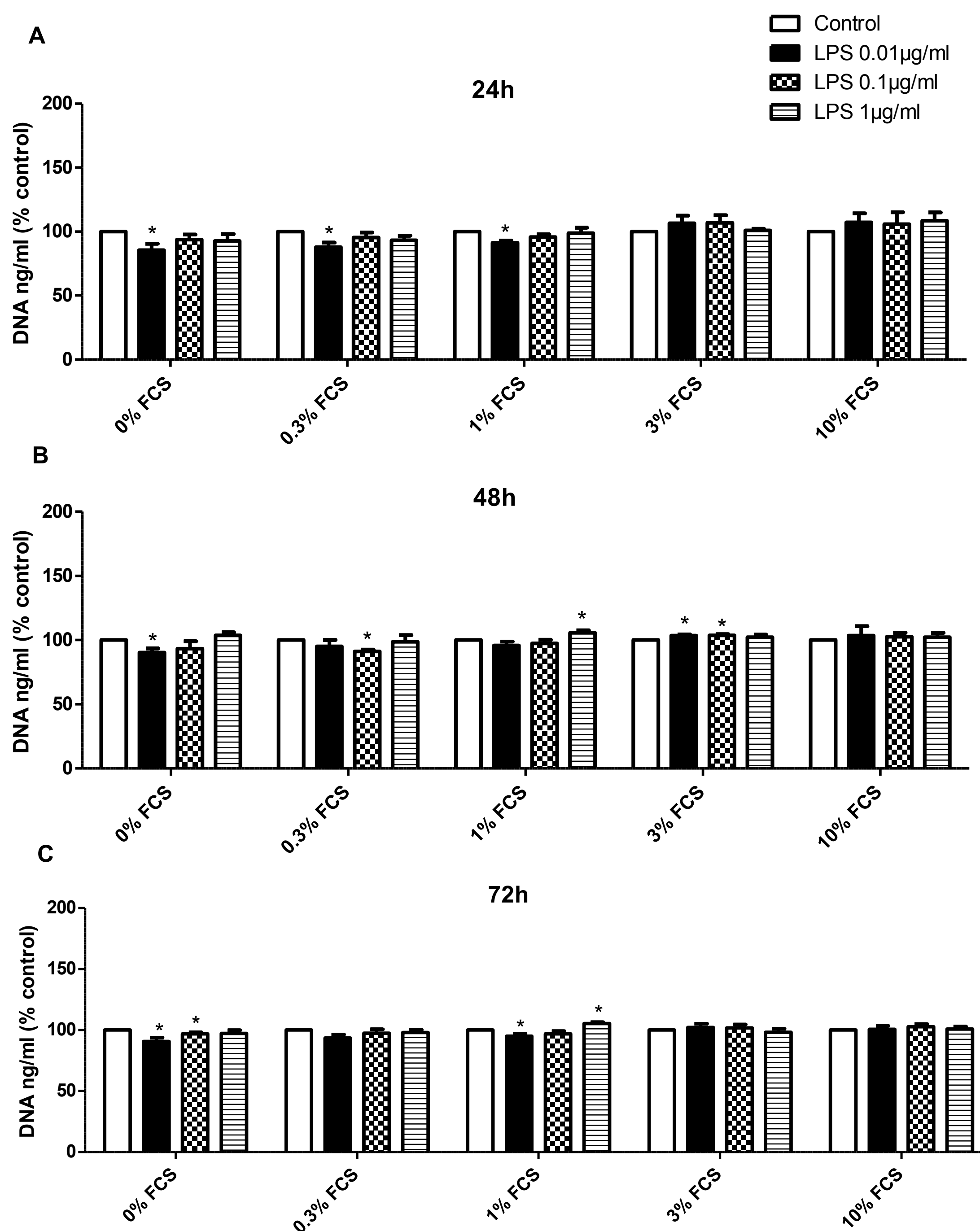


Figure 49. Effect of TLR4 stimulation on A549 proliferation grown in different concentration of FCS over 72h. A549 cells were seeded in a 96 well plates at 1 x 10^4 cells per ml in DMEM with 10% FCS and allowed to equilibrate for 12h. After this time period media was replaced with DMEM containing 0, 0.3, 1, 3 and 10% FCS. Cells were treated with LPS and incubated for 24h (A), 48h (B) and 72h (C) after which proliferation was assessed by DNA content using the CyQuant assay. Data represented are the mean ± SEM of n=6 replicates measured over 3 separate experimental days. *denotes p≤ 0.05 as assessed using a one-way ANOVA followed by one sample t-test of normalised data.

The most interesting results were obtained after TLR8 stimulation. Our results showed that the lowest concentration of this ligand (0.1μg/ml) induced increase in DNA content but at the highest concentration (10μg/ml) induced cell death (Fig. 50).

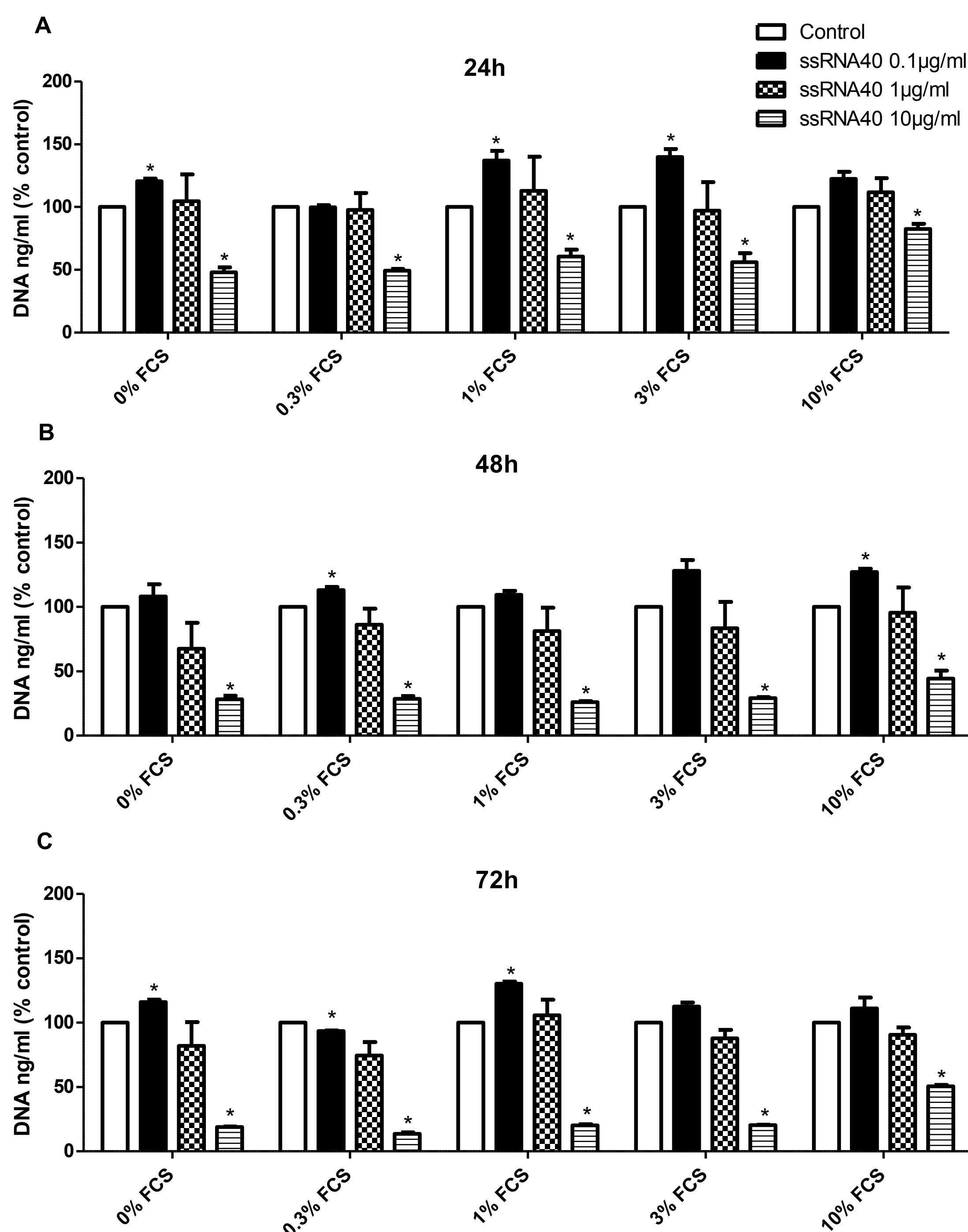


Figure 50. Effect of TLR8 stimulation on A549 proliferation grown in different concentration of FCS over 72h. A549 cells were seeded in a 96 well plates at 1 x 10^4 cells per ml in DMEM with 10% FCS and allowed to equilibrate for 12h. After this time period media was replaced with DMEM containing 0, 0.3, 1, 3 and 10% FCS. Cells were treated with ssRNA40/Lyovec and incubated for 24h (A), 48h (B) and 72h (C) after which proliferation was assessed by DNA content using the CyQuant assay. Data represented are the mean ± SEM of n=6 replicates measured over 3 separate experimental days. *denotes $p \leq 0.05$ as assessed using a one-way ANOVA followed by one sample t-test of normalised data.

5.8. Effect of apoptotic agents on A549 cell survival.

Previous results by other groups showed that activation of TLR4 can induce resistance to apoptosis [11]. To determine whether it is true for our cell line and other TLRs, we performed AlamarBlue assay after treatment with apoptotic agents. Firstly, to choose the best apoptotic agent, we stimulated A549 cells with TNF-α+cycloheximide, cycloheximide alone, TNF-α alone and H2O2 alone.

Figure 51 shows that all agents except TNF-α induced cell death. The death rate for TNF-α+ cycloheximide was 25% and 24% for cells grown with 1% and 10% FCS respectively, 25% and 40% for cycloheximide, and 24% and 32% for H_2O_2.

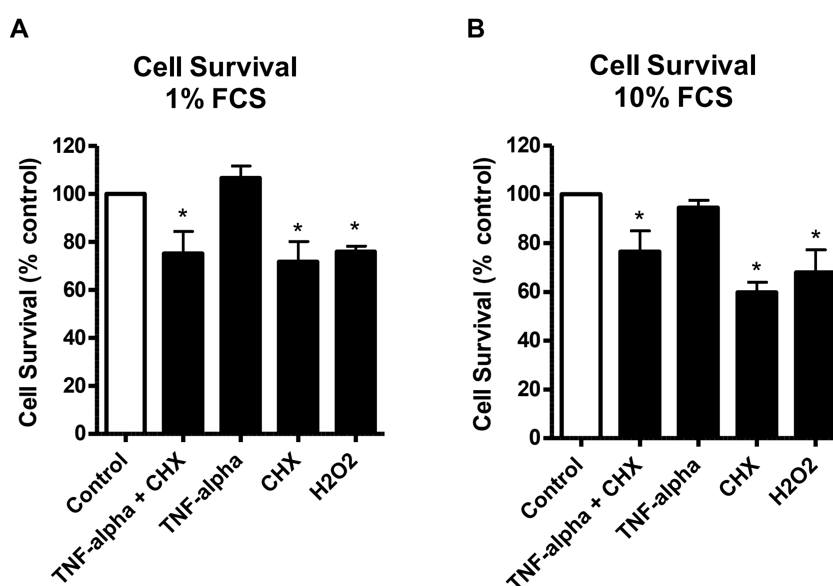


Figure 51. Effect of apoptotic agents on A549 survival. A549 cells were seeded in a 96 well plates at 1 x 10^4 cells well in DMEM with 10% FCS and allowed to equilibrate for 12h. After this time period media was replaced with DMEM containing 1 (A) or 10% FCS (B). Cells were treated with TNF-α (3ng/ml)+CHX (3µg/ml), CHX (3µg/ml), TNF-α (3ng/ml) and H_2O_2 (0.6mM). After 24h incubation cell viability was measured by the AlamarBlue assay. Data represented are the mean ± SEM of n=8 replicates measured over 4 separate experimental days.

To ascertain whether activation of TLRs can induce apoptosis resistance, we treated A549 cells with increasing concentrations of Pam_3CSK4 in 96 well plates. These conditions were chosen based on our preliminary data. After 4h, the cell death agents were added and cells were incubated for a further 24h.

Our results showed that when A549 cells were stimulated with Pam_3CSK4 (1µg/ml) in 10% FCS prior to treatment with cycloheximide, cell viability was increased from 62% to 77%, and appeared to be dose dependent (Fig. 52D). These findings did not hold true for treatment with TNF-α+cycloheximide and H_2O_2 (Fig. 52A,B,E,F).

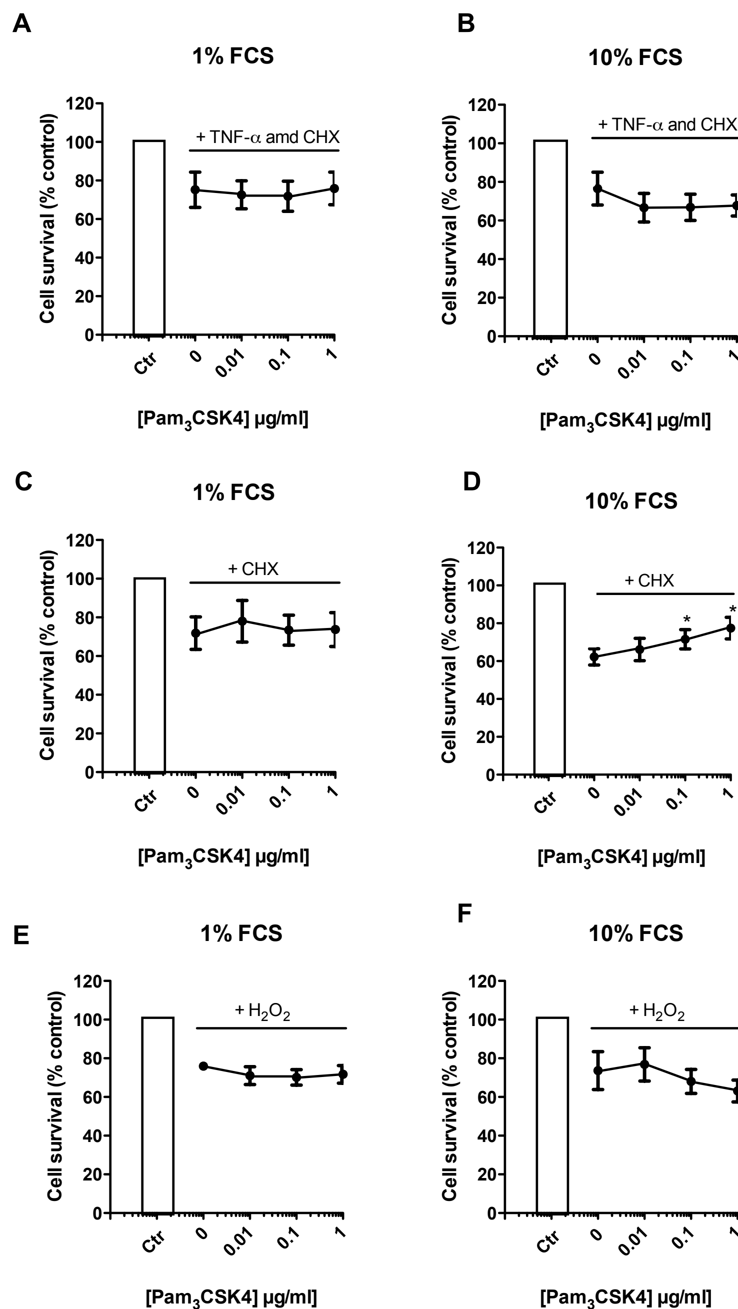


Figure 52. Effect of TLR2/1 stimulation on A549 survival. A549 cells were seeded in a 96 well plates at 1 x 10^4 cells per well in DMEM with 10% FCS and allowed to equilibrate for 12h. After this time period media was replaced with DMEM containing 1 (A,C,E) or 10% FCS (B,D,F). 4h after adding Pam_3CSK4 cells were treated with TNF-α+CHX (A,B), CHX alone (C,D) and H_2O_2 (E,F). After 24h incubation cell viability was measured by the AlamarBlue assay. Data represented is the mean ± SEM of n=8 replicates measured over 4 separate experimental days. * denotes $p \leq 0.05$ as assessed using one-way ANOVA followed by one-sample t-test of normalized data.

5.9. Flow cytometric analysis of apoptosis in A549 cells after TLR stimulation.

For analysis of apoptosis in the A549 cell line we used annexin V and propidium iodide (PI) staining. FITC annexin V staining precedes the loss of membrane integrity which accompanies the latest stages of cell death resulting from either apoptotic or necrotic processes. Therefore, staining with FITC annexin V is typically used in conjunction with a vital dye such as propidium iodide (PI) to allow the investigator to identify early apoptotic cells. We used four different apoptotic agents: cycloheximide, TRAIL, and cycloheximide combined with TNF-α. Firstly, we analysed cells health status by looking at them under the microscope. Thus, we seeded A549 cells in 6 well plates at $3x10^3$ cell/well and treated them with apoptotic agents. After 24h we took the pictures of these cells using Cannon Coolpix™ digital camera (5 mega pixels), representative pictures are shown in Fig. 53-56.

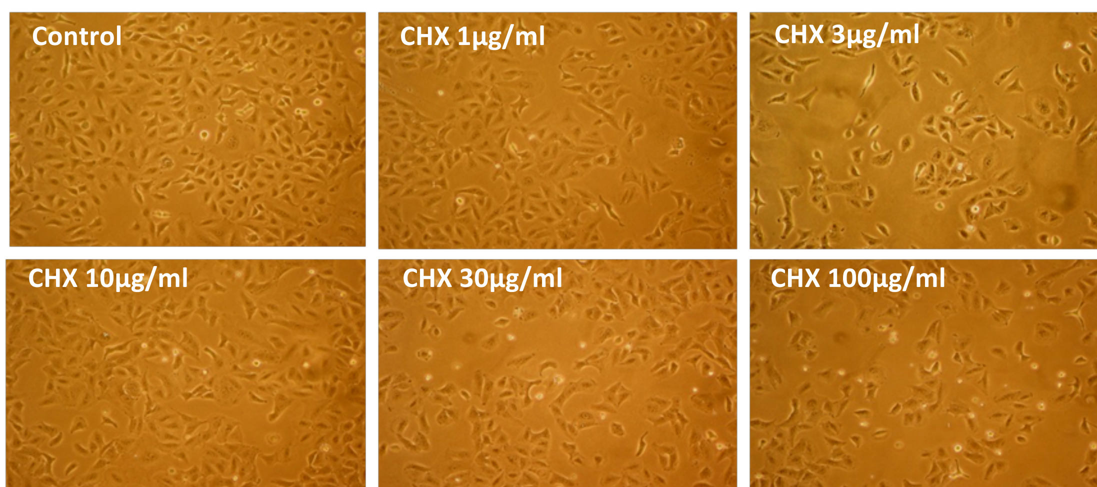


Figure 53. Effect of cycloheximide on A549 cells. A549 cells were seeded in a 6 well plates at 3 x 10^5 cells per well in DMEM with 10% FCS and allowed to equilibrate for 12h. After this time period cells were treated with increasing concentration of cycloheximide. After 24h pictures were taken with Cannon Coolpix™ digital camera (5 mega pixels) with 100x magnification.

Figure 54. Effect TRAIL on A549 cells. A549 cells were seeded in a 6 well plates at 3 x 10^5 cells per well in DMEM with 10% FCS and allowed to equilibrate for 12h. After this time period cells were treated with increasing concentration of TRAIL. After 24h pictures were taken with Cannon Coolpix™ digital camera (5 mega pixels) with 100x magnification.

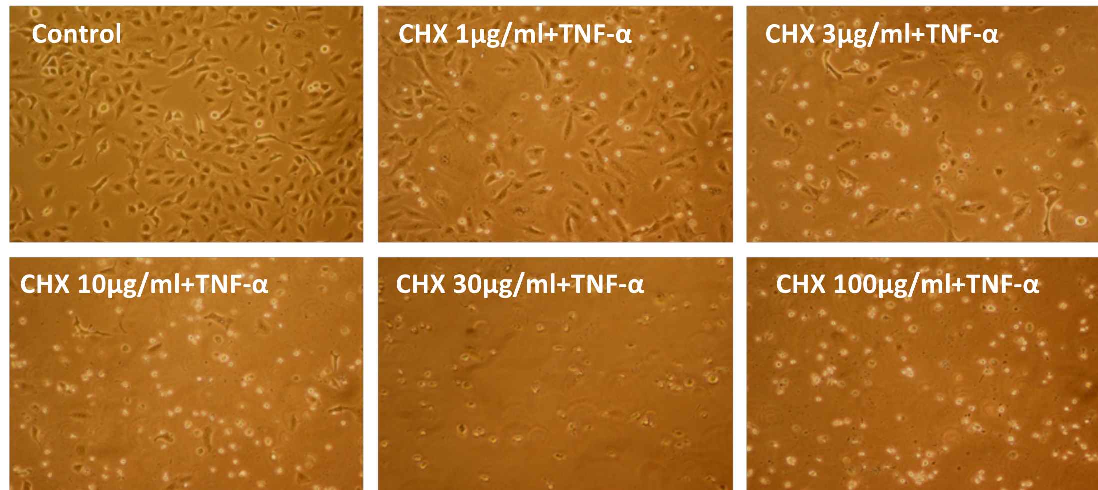


Figure 55. Effect of cycloheximide and TNF-α on A549 cells. A549 cells were seeded in a 6 well plates at 3 x 10^5 cells per well in DMEM with 10% FCS and allowed to equilibrate for 12h. After this time period cells were treated with increasing concentration of cycloheximide and TNF-α (15ng/ml). After 24h pictures were taken with Cannon Coolpix^{TM} digital camera (5 mega pixels) with 100x magnification.

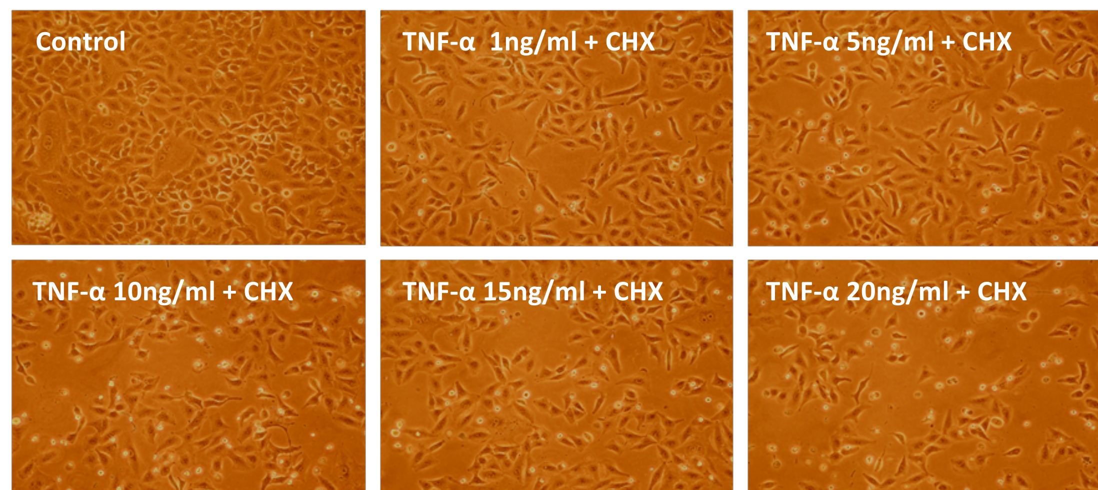


Figure 56. Effect of TNF-α and cycloheximide on A549 cells. A549 cells were seeded in a 6 well plates at 3 x 10^5 cells per well in DMEM with 10% FCS and allowed to equilibrate for 12h. After this time period cells were treated with increasing concentration of TNF-α and cycloheximide (1µg/ml). After 24h pictures were taken with Cannon Coolpix^{TM} digital camera (5 mega pixels) with 100x magnification.

To analyse apoptosis in more detail using FACS, A549 cells were harvested after treatment and stained with annexin V-FITC and PI. Cell were analysed within an hour.

TRAIL (TNF-related apoptosis-inducing ligand) is a type II transmembrane protein with homology to other members of the TNF family. It binds to the death receptors, DR4 and DR5. The process of apoptosis is caspase-8-dependent. This protein preferentially induces apoptosis in transformed and tumor cells, but does not appear to kill normal cells although it is expressed at a significant level in most normal tissues. Our results showed that TRAIL induced apoptosis from the concentration of 500ng/ml (Fig. 57A,B).

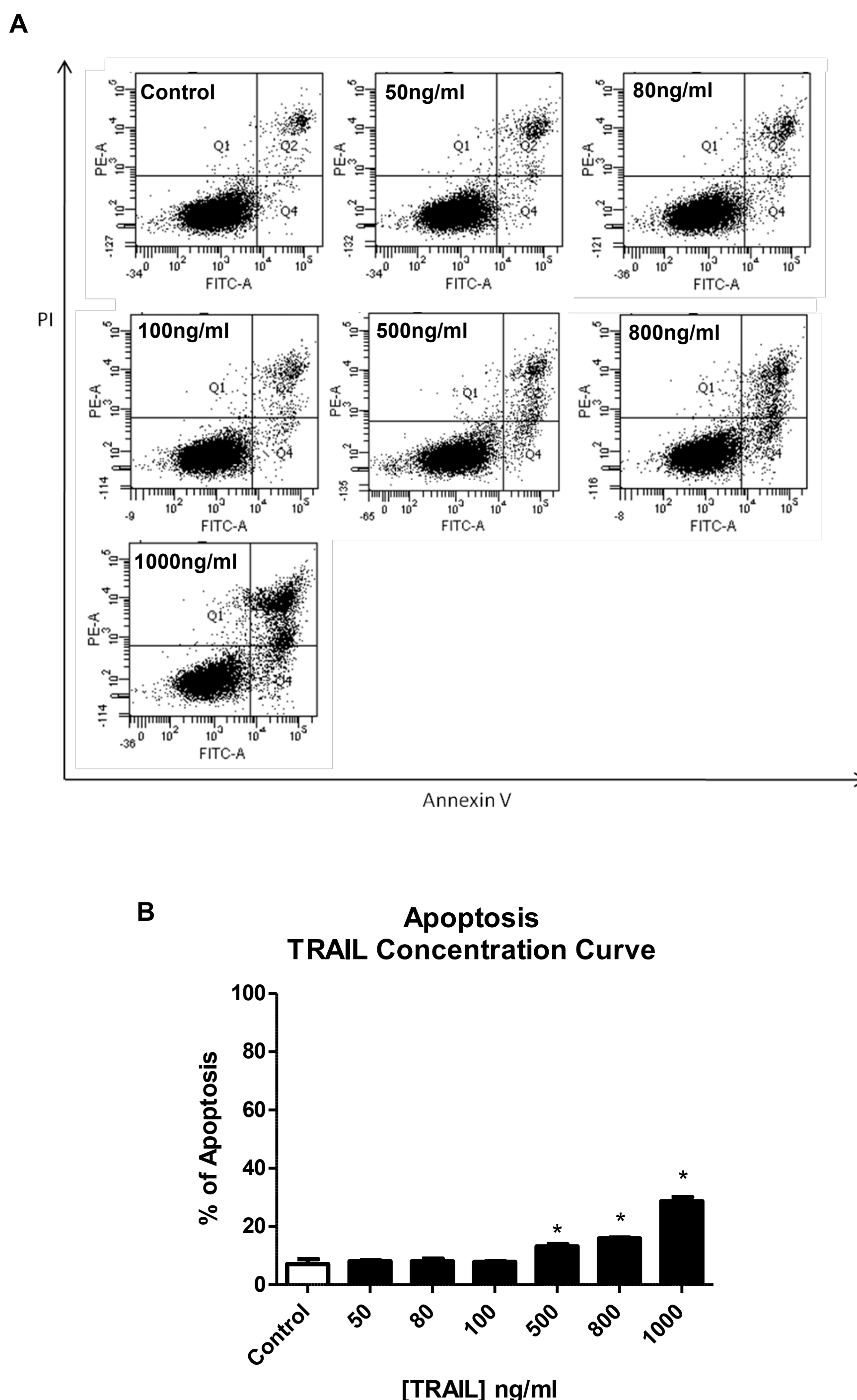


Figure 57. Effect of TRAIL on A549 cells viability. A549 cells were seeded in a 6 well plates at 3 x 10^5 cells per well in DMEM with 10% FCS and allowed to equilibrate for 12h. After this time period cells were treated with increasing concentration of TRAIL and incubated for 24h at 37°C. Cells were harvested and stained with annexin V and PI and analysed by FACS. Representative scatter plot of PI and annexin V staining (A). The % of dead cells from the scatter plot Q1, Q2 and Q4 (B). Data represented are the mean ± SEM of n=3 replicates measured over 3 separate experimental days. *denotes p≤0.05 as assessed using one-way ANOVA followed by Dunnett's multiple comparison test. Q1 – dead cells (annexin V negative, PI positive), Q2- late apoptotic cells (annexin V and PI positive), Q3- live cells (annexin V and PI negative), Q4- early apoptotic cells (annexin V positive, PI negative).

Cycloheximide (CHX) is an antibiotic produced by *S. griseus*. Its main biological activity is translation inhibition in eukaryotes resulting in cell growth arrest and cell death. It induced apoptosis in 13% of A549 cells when a concentration of 100μg/ml was used (Fig. 58A,B).

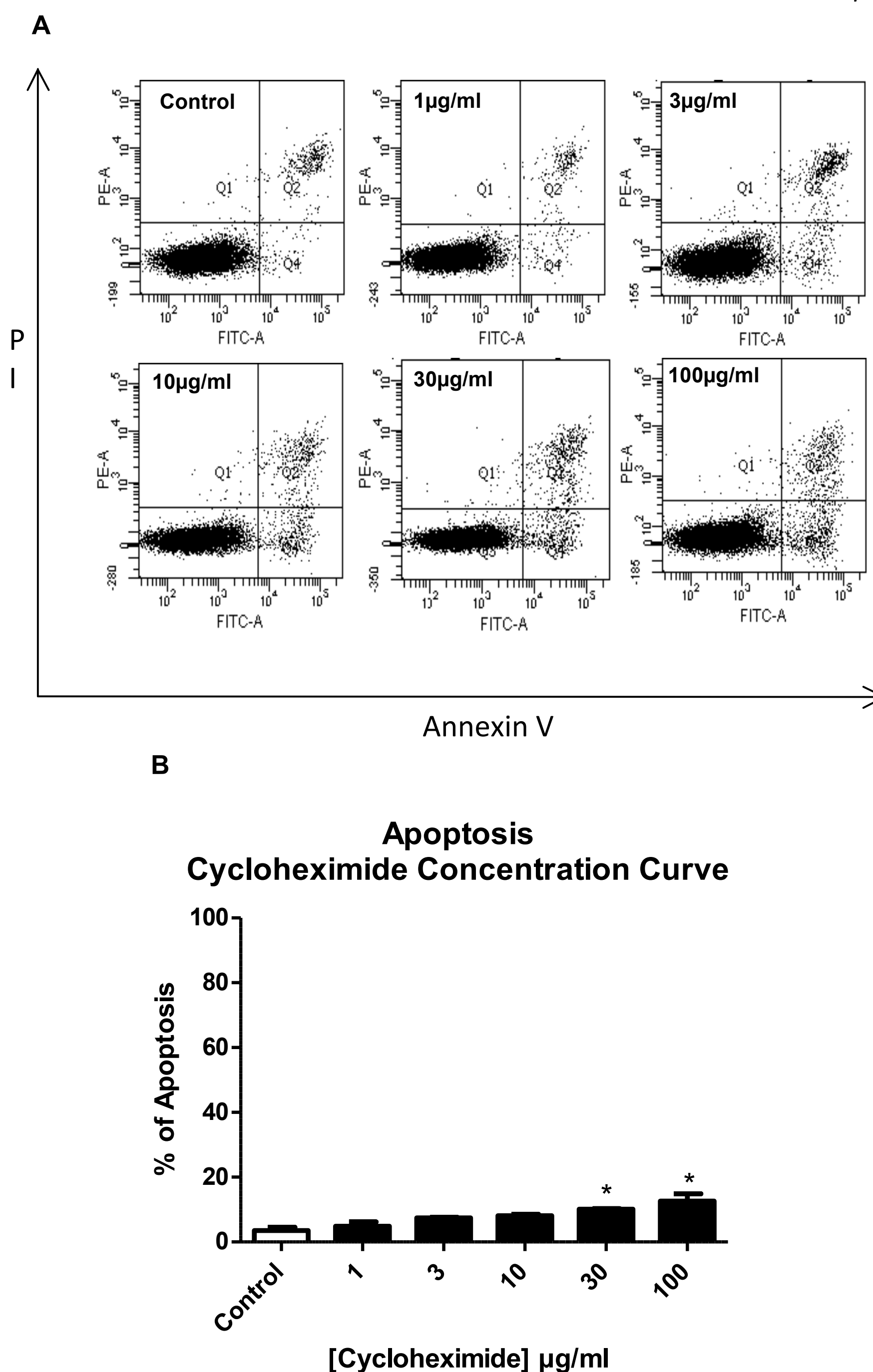
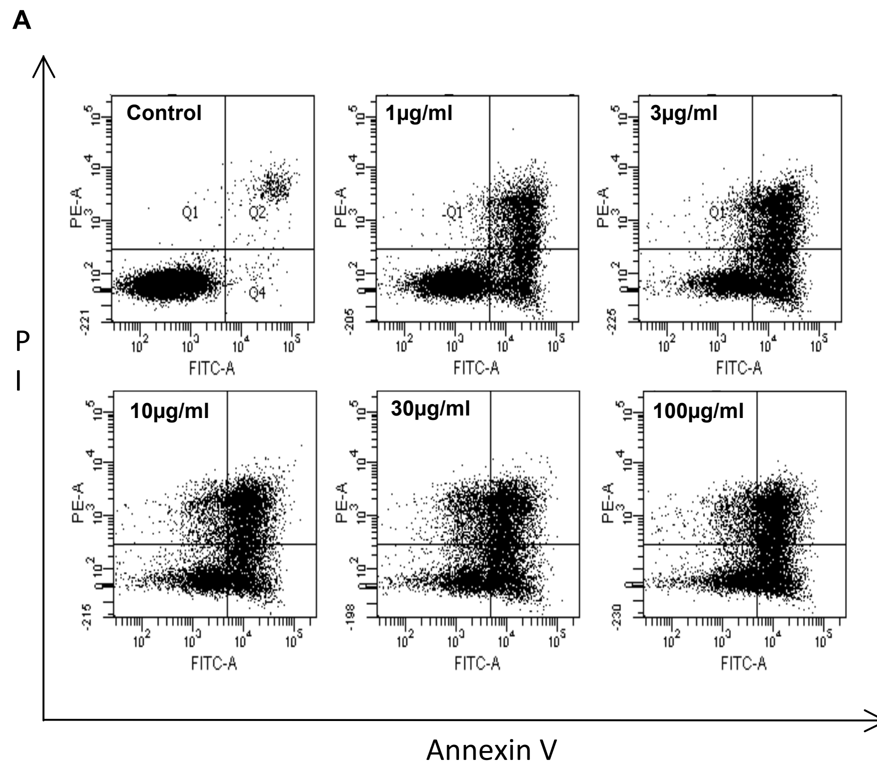


Figure 58. Effect of TRAIL on A549 cells viability. A549 cells were seeded in a 6 well plates at 3 x 10^5 cells per well in DMEM with 10% FCS and allowed to equilibrate for 12h. After this time period cells were treated with increasing concentration of CHX and incubated for 24h at 37°C. Cells were harvested and stained with annexin V and PI and analysed by FACS assay. Representative scatter plot of PI and annexin V staining (A). The % of dead cells from the scatter plot Q1, Q2 and Q4 (B). Data represented are the mean ± SEM of n=3 replicates measured over 3 separate experimental days. *denotes $p \leq 0.05$ as assessed using one-way ANOVA followed by Dunnett's multiple comparison test. Q1 – dead cells (annexin V negative, PI positive), Q2- late apoptotic cells (annexin V and PI positive), Q3- live cells (annexin V and PI negative), Q4- early apoptotic cells (annexin V positive, PI negative).

TNF-α alone did not cause apoptosis of A549 cells in our model. CHX has been previously shown to sensitize cells to TNF-α-induced cell death by lowering FLICE-like inhibitory protein (FLIP) levels. Thus, we modified our approach by combining TNF-α and CHX [2]. Therefore, we used one concentration of TNF-α (15ng/ml) and increasing concentration of CHX. Figure 59 shows that cells were very sensitive for these agents and all concentrations of CHX caused apoptosis of A549 cells.

A



B

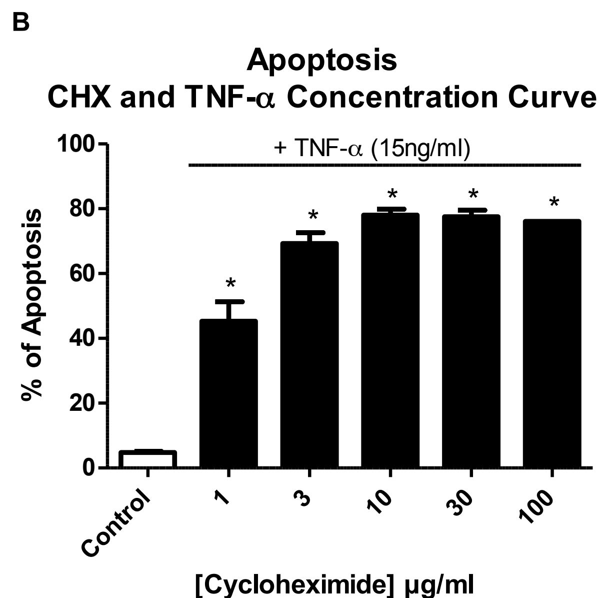
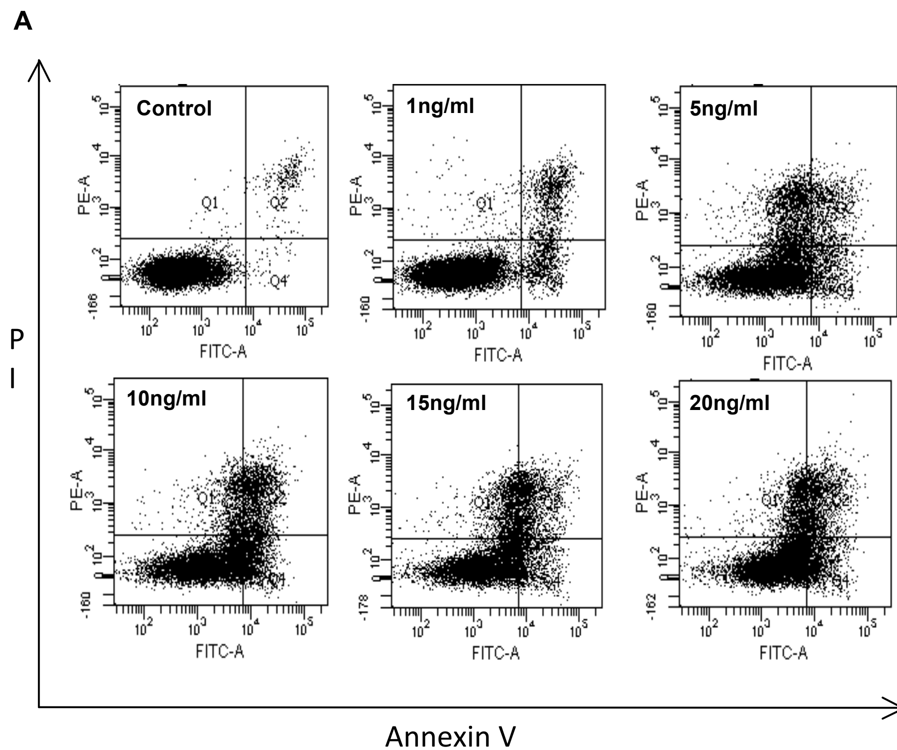


Figure 59. Effect of TRAIL on A549 cells viability. A549 cells were seeded in a 6 well plates at 3 x 10^5 cells per well in DMEM with 10% FCS and allowed to equilibrate for 12h. After this time period cells were treated with increasing concentration of CHX + TNF-α (15ng/ml) and incubated for 24h at 37°C. Cells were harvested and stained with annexin V and PI and analysed by FACS. Representative scatter plot of PI and annexin V staining (A). The % of dead cells from the scatter plot Q1, Q2 and Q4 (B). Data represented are the mean ± SEM of n=3 replicates measured over 3 separate experimental days. *denotes p≤0.05 as assessed using one-way ANOVA followed by Dunnett's multiple comparison test. Q1 – dead cells (annexin V negative, PI positive), Q2- late apoptotic cells (annexin V and PI positive), Q3- live cells (annexin V and PI negative), Q4- early apoptotic cells (annexin V positive, PI negative).

The effects of combining increasing concentrations of TNF-α with CHX (1μg/ml) had no significant effect on the amount of apoptosis seen. In all conditions used, we observed a concentration dependent apoptosis of A549 cells (Fig. 60).

For future experiments, we decided to use TRAIL at a concentration of 1μg/ml and TNF-α+CHX at concentrations 15ng/ml and 1μg/ml respectively, which gave us approximately 40% apoptosis.



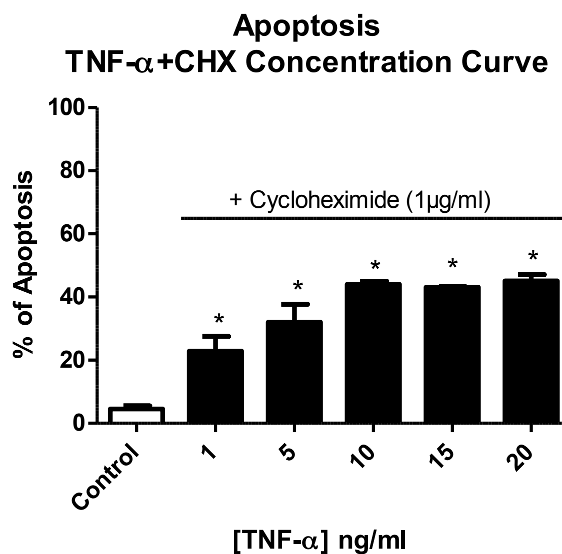
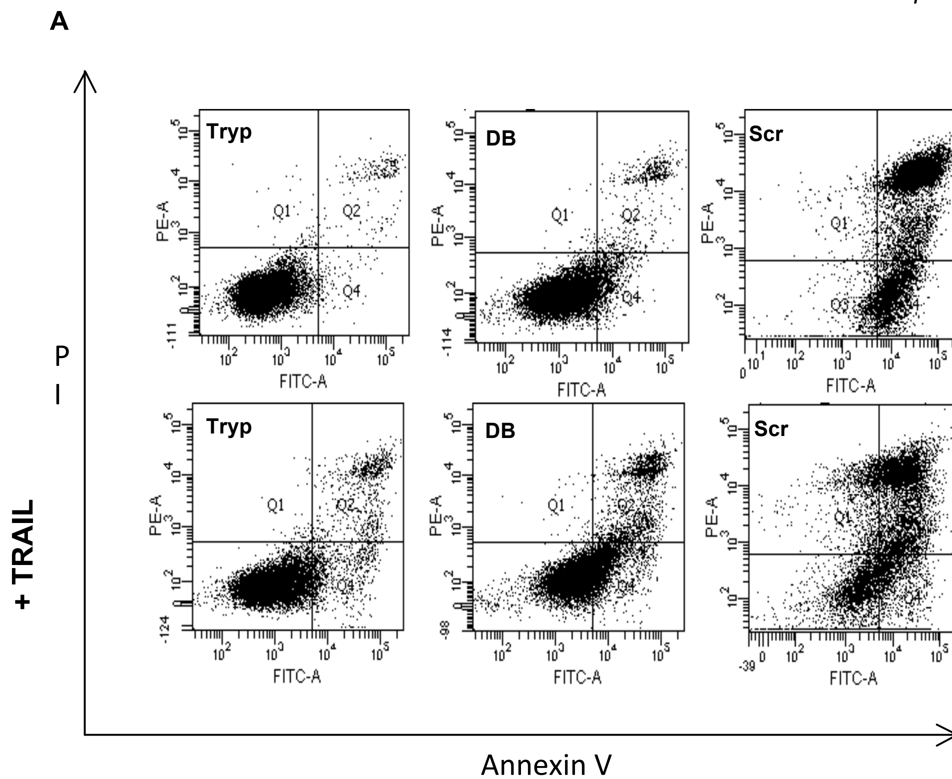


Figure 60. Effect of TRAIL on A549 cells viability. A549 cells were seeded in a 6 well plates at 3 x 10^5 cells per well in DMEM with 10% FCS and allowed to equilibrate for 12h. After this time period cells were treated with increasing concentration of TNF-α + CHX (1μg/ml) and incubated for 24h at 37°C. Cells were harvested and stained with annexin V and PI and analysed by FACS. Representative scatter plot of PI and annexin V staining (A). The % of dead cells from the scatter plot Q1, Q2 and Q4 (B). Data represented are the mean ± SEM of n=3 replicates measured over 3 separate experimental days. *denotes $p \leq 0.05$ as assessed using one-way ANOVA followed by Dunnett's multiple comparison test. Q1 – dead cells (annexin V negative, PI positive), Q2- late apoptotic cells (annexin V and PI positive), Q3- live cells (annexin V and PI negative), Q4- early apoptotic cells (annexin V positive, PI negative).

A549 is an adherent cell line and specific membrane damage may occur during cell detachment or harvesting. Thus, we tried three different methods of harvesting to avoid cell damage. We used (i) standard trypsin detachment, (ii) dissociation buffer (DB) which is designed for gentle dissociation of mammalian cells from each other and the culture plate, and (iii) a scraping method. Our results showed that using trypsin was the best way to dissociate the cells and caused less apoptosis than scraping (Fig. 61).



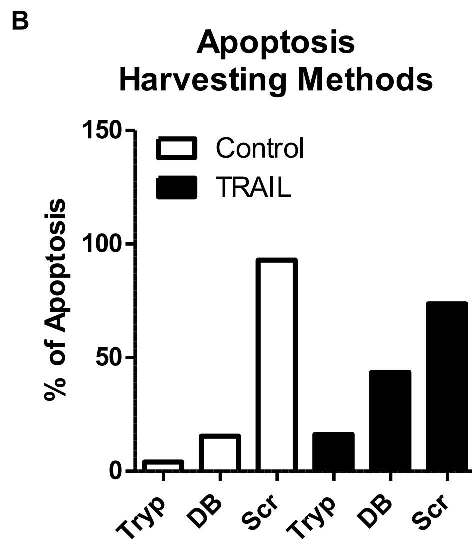


Figure 61. Effect of TRAIL on A549 cells viability. A549 cells were seeded in a 6 well plates at 3 x 10^5 cells per well in DMEM with 10% FCS and allowed to equilibrate for 12h. After this time period cells were treated with TRAIL (1µg/ml) and incubated for 24h at 37°C. Cells were harvested and stained with annexin V and PI and analysed by FACS. Representative scatter plot of PI and annexin V staining (A). The % of dead cells from the scatter plot Q1, Q2 and Q4 (B). Data represented are the mean ± SEM of n=3 replicates measured over 3 separate experimental days. *denotes $p \leq 0.05$ as assessed using one-way ANOVA followed by Dunnett's multiple comparison test. Q1 – dead cells (annexin V negative, PI positive), Q2- late apoptotic cells (annexin V and PI positive), Q3- live cells (annexin V and PI negative), Q4- early apoptotic cells (annexin V positive, PI negative).

The amount of purified recombinant annexin V required to saturate binding sites may vary according to cell type and stage of apoptosis so titration of annexin V and PI is required to obtain optimal results. Figure 62 shows that all concentrations of annexin V and PI that we used were equally effective at detecting apoptosis of the cells. We therefore used a concentration of 2.5μl for future experiments as this was the lowest concentration that produced maximal detection of apoptosis.

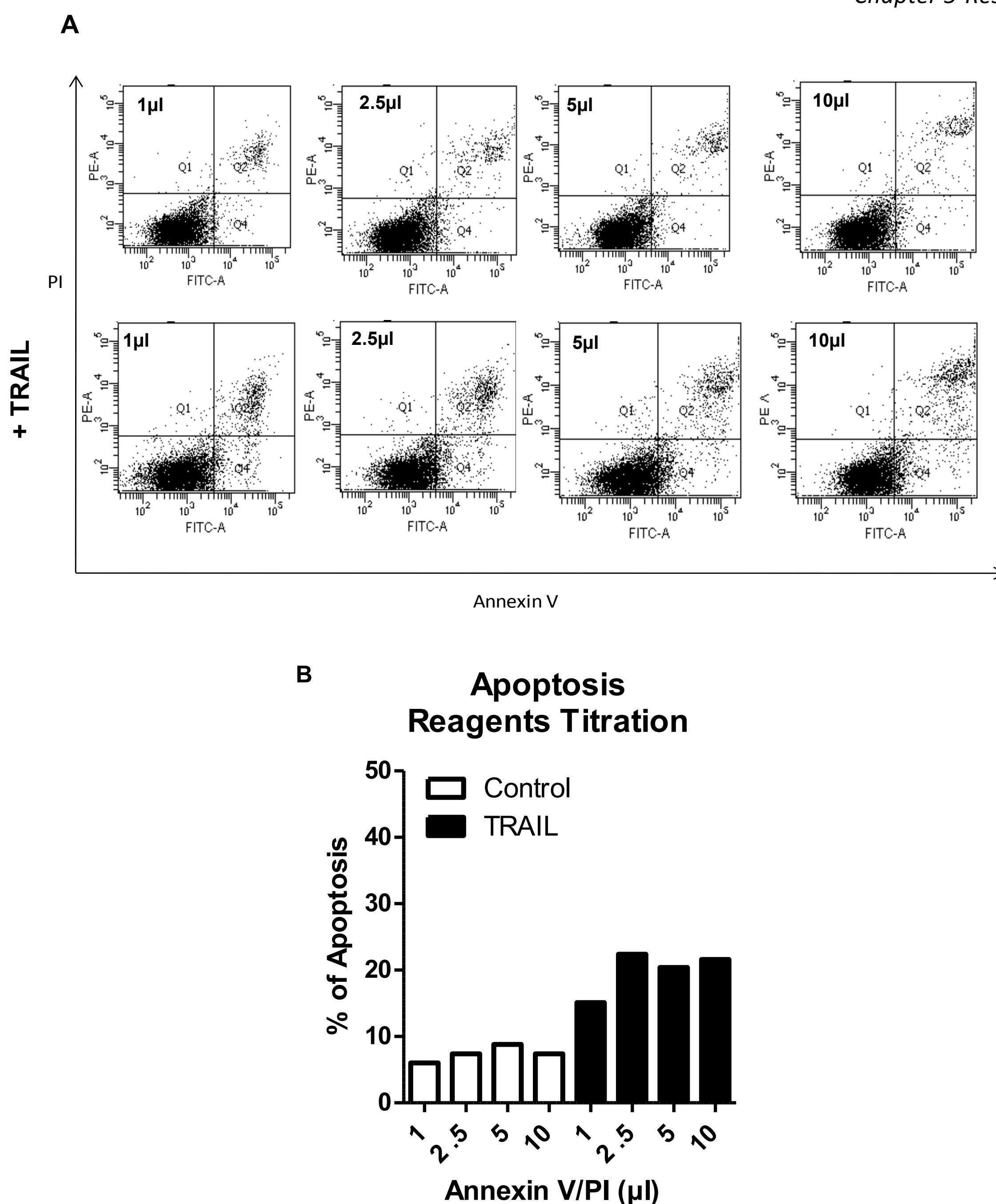


Figure 62. Effect of TRAIL on A549 cells viability. A549 cells were seeded in a 6 well plates at 3 x 10^5 cells per well in DMEM with 10% FCS and allowed to equilibrate for 12h. After this time period cells were treated with TRAIL (1µg/ml) and incubated for 24h at 37°C. Cells were harvested and stained with annexin V and PI and analysed by FACS. Representative scatter plot of PI and annexin V staining (A). The % of dead cells from the scatter plot Q1, Q2 and Q4 (B). Data represented are the mean ± SEM of n=3 replicates measured over 3 separate experimental days. *denotes p≤0.05 as assessed using one-way ANOVA followed by Dunnett's multiple comparison test. Q1 – dead cells (annexin V negative, PI positive), Q2- late apoptotic cells (annexin V and PI positive), Q3- live cells (annexin V and PI negative), Q4- early apoptotic cells (annexin V positive, PI negative).

After optimization of the FACS protocol we performed further experiments to determine whether activation of TLRs on A549 cells could induce apoptosis resistance. We seeded cells in 6 well plates and treated them with TLR agonists 4 hours prior to TNF-α+CHX and TRAIL treatment. We measured apoptosis 24h later. TRAIL was not a good agent to induce apoptosis. It worked in the first experiment but was not effective later on. TNFα+CHX worked better and induce around 20% of cell death.

Figures 63, 64 and 65 show that stimulation of TLR2/1, TLR2/6 and TLR4 with Pam$_3$CSK4, FSL-1 and LPS respectively did not have any effect on apoptosis resistance in A549 cell line. Similarly, the TLR3 ligand, Poly I:C also did not have any effect (Fig. 66).

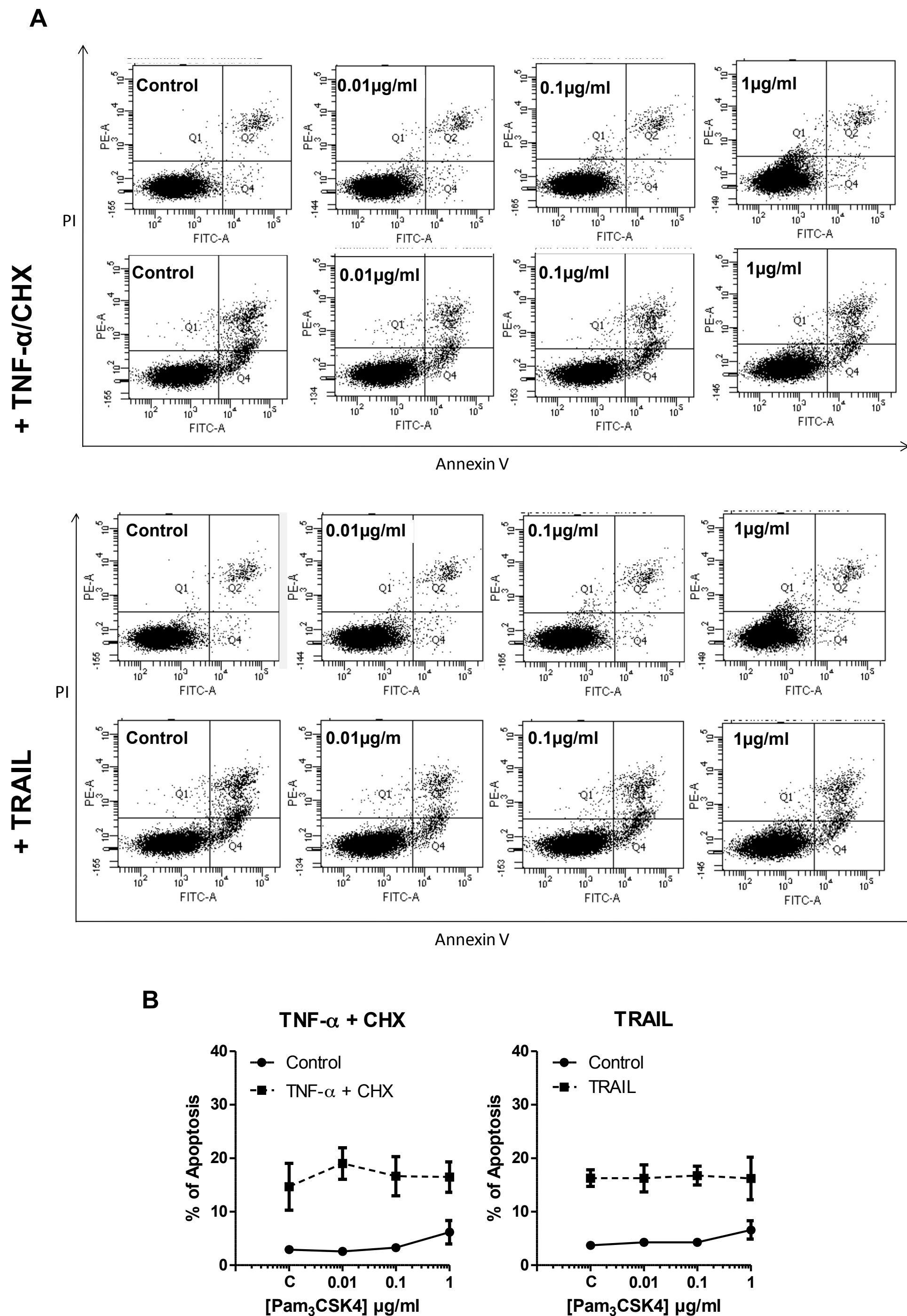


Figure 63. Effect of TLR2/1 agonist on A549 cells viability. A549 cells were seeded in a 6 well plates at 3 x 10^5 cells per well in DMEM with 10% FCS and allowed to equilibrate for 12h. After this time period cells were treated with TNF-α (15ng/ml)+CHX (1µg/ml) or TRAIL (1µg/ml) 4h prior Pam_3CSK4 treatment. After 24h cells were harvested and stained with annexin V and PI and analysed by FACS. Representative scatter plot of PI and annexin V staining (A). The % of dead cells from the scatter plot from Q1, Q2 and Q4 (B). Data represented are the mean ± SEM of n=3 replicates measured over 3 separate experimental days. *denotes $p \leq 0.05$ as assessed using one-way ANOVA followed by Dunnett's multiple comparison test. Q1 – dead cells (annexin V negative, PI positive), Q2- late apoptotic cells (annexin V and PI positive), Q3- live cells (annexin V and PI negative), Q4- early apoptotic cells (annexin V positive, PI negative).

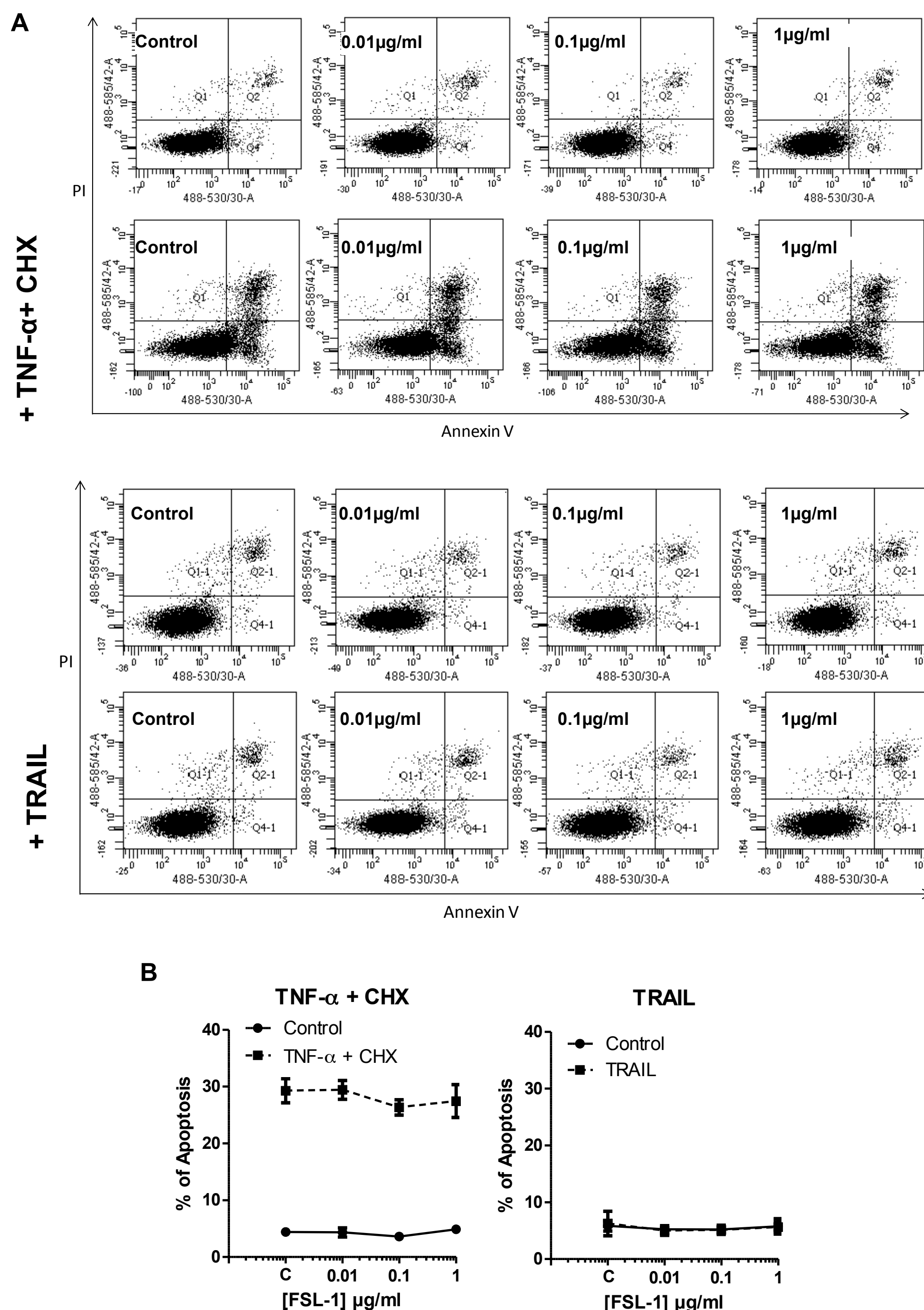


Figure 64. Effect of TLR2/6 agonist on A549 cells viability. A549 cells were seeded in a 6 well plates at 3 x 10^5 cells per well in DMEM with 10% FCS and allowed to equilibrate for 12h. After this time period cells were treated with TNF-α (15ng/ml)+CHX (1μg/ml) or TRAIL (1μg/ml) 4h prior FSL-1 treatment. After 24h cells were harvested and stained with annexin V and PI and analysed by FACS. Representative scatter plot of PI and annexin V staining (A). The % of dead cells from the scatter plot from Q1, Q2 and Q4 (B). Data represented are the mean ± SEM of n=3 replicates measured over 3 separate experimental days. *denotes p≤0.05 as assessed using one-way ANOVA followed by Dunnett's multiple comparison test. Q1 – dead cells (annexin V negative, PI positive), Q2- late apoptotic cells (annexin V and PI positive), Q3- live cells (annexin V and PI negative), Q4- early apoptotic cells (annexin V positive, PI negative).

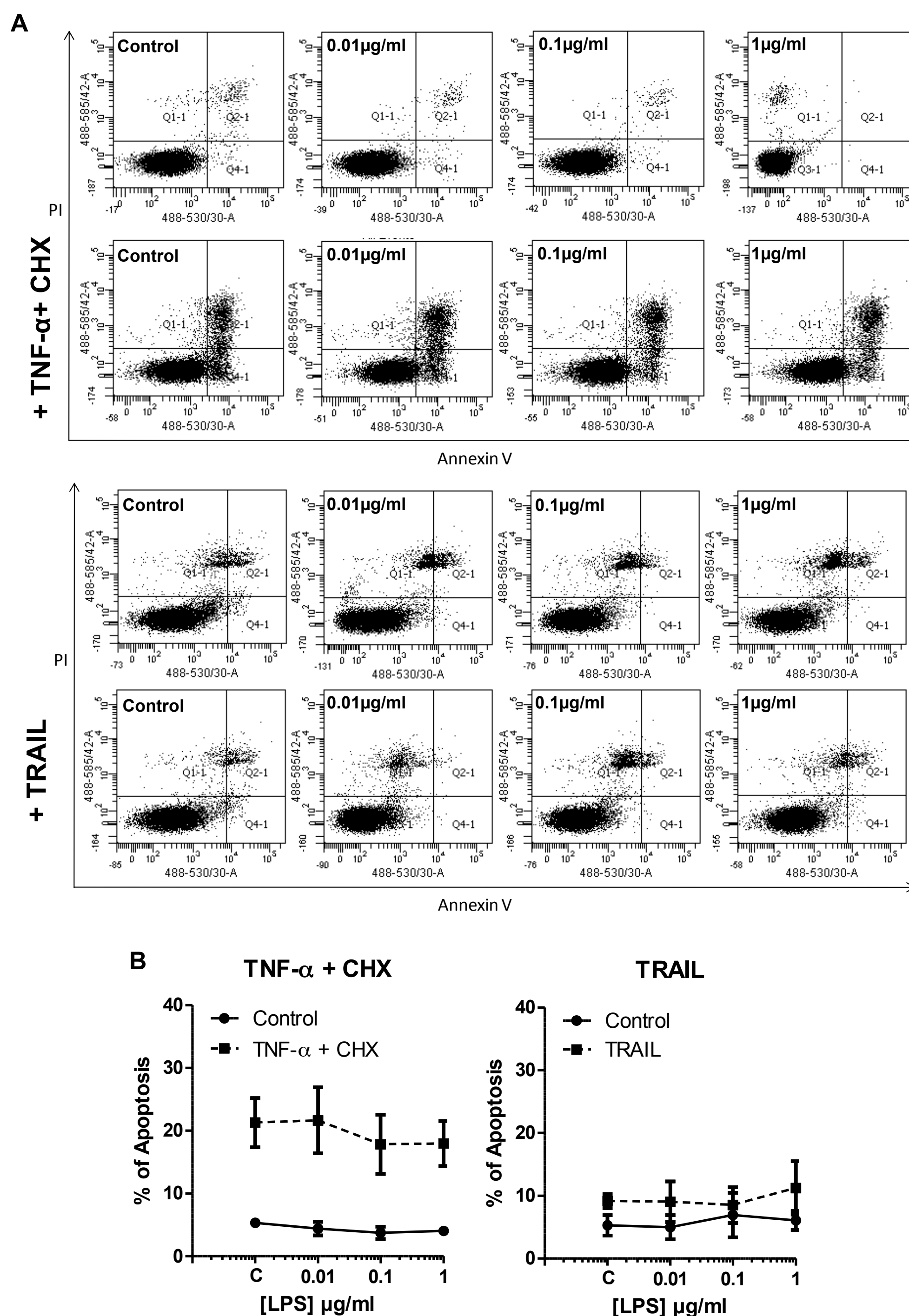


Figure 65. Effect of TLR4 agonist on A549 cells viability. A549 cells were seeded in a 6 well plates at 3 x 10^5 cells per well in DMEM with 10% FCS and allowed to equilibrate for 12h. After this time period cells were treated with TNF-α (15ng/ml)+CHX (1µg/ml) or TRAIL (1µg/ml) 4h prior LPS treatment. After 24h cells were harvested and stained with annexin V and PI and analysed by FACS. Representative scatter plot of PI and annexin V staining (A). The % of dead cells from the scatter plot from Q1, Q2 and Q4 (B). Data represented are the mean ± SEM of n=3 replicates measured over 3 separate experimental days. *denotes $p \leq 0.05$ as assessed using one-way ANOVA followed by Dunnett's multiple comparison test. Q1 – dead cells (annexin V negative, PI positive), Q2-late apoptotic cells (annexin V and PI positive), Q3- live cells (annexin V and PI negative), Q4- early apoptotic cells (annexin V positive, PI negative).

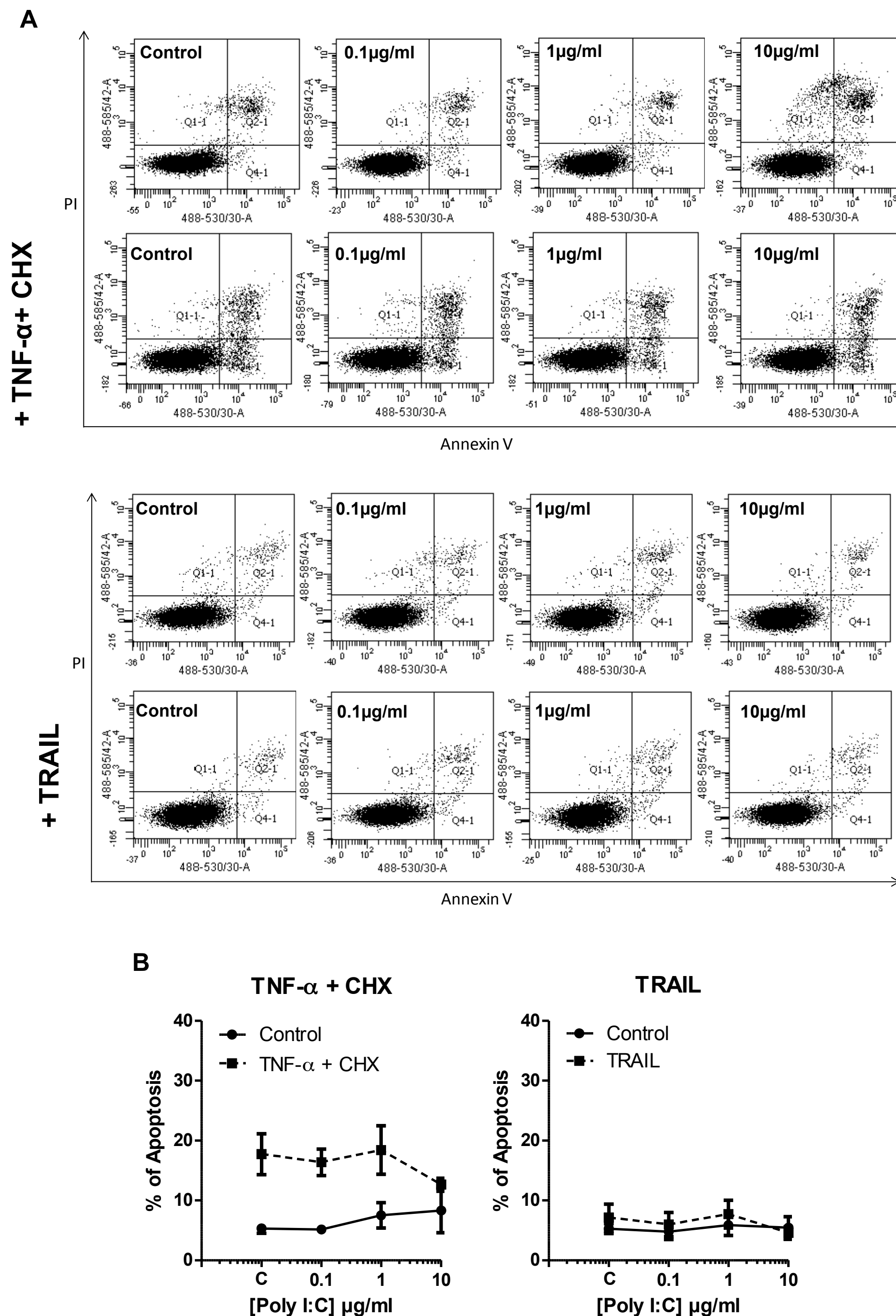


Figure 66. Effect of TLR3 agonist on A549 cells viability. A549 cells were seeded in a 6 well plates at 3 x 10^5 cells per well in DMEM with 10% FCS and allowed to equilibrate for 12h. After this time period cells were treated with TNF-α (15ng/ml)+CHX (1µg/ml) or TRAIL (1µg/ml) 4h prior Poly I:C treatment. After 24h cells were harvested and stained with annexin V and PI and analysed by FACS. Representative scatter plot of PI and annexin V staining (A). The % of dead cells from the scatter plot from Q1, Q2 and Q4 (B). Data represented are the mean ± SEM of n=3 replicates measured over 3 separate experimental days. *denotes $p \leq 0.05$ as assessed using one-way ANOVA followed by Dunnett's multiple comparison test. Q1 – dead cells (annexin V negative, PI positive), Q2- late apoptotic cells (annexin V and PI positive), Q3- live cells (annexin V and PI negative), Q4- early apoptotic cells (annexin V positive, PI negative).

Imiquimod, an agonist of TLR7, caused an increase in apoptosis when it was used together with TNF-α+CHX (Fig. 67A,D). Similar effects were observed with the TLR8 agonist, ssRNA40 (Fig. 68). This agonist alone was able to induce A549 apoptosis. This effect was even higher when it was used together with apoptotic agent, TNF-α+CHX (Fig. 67A,C).

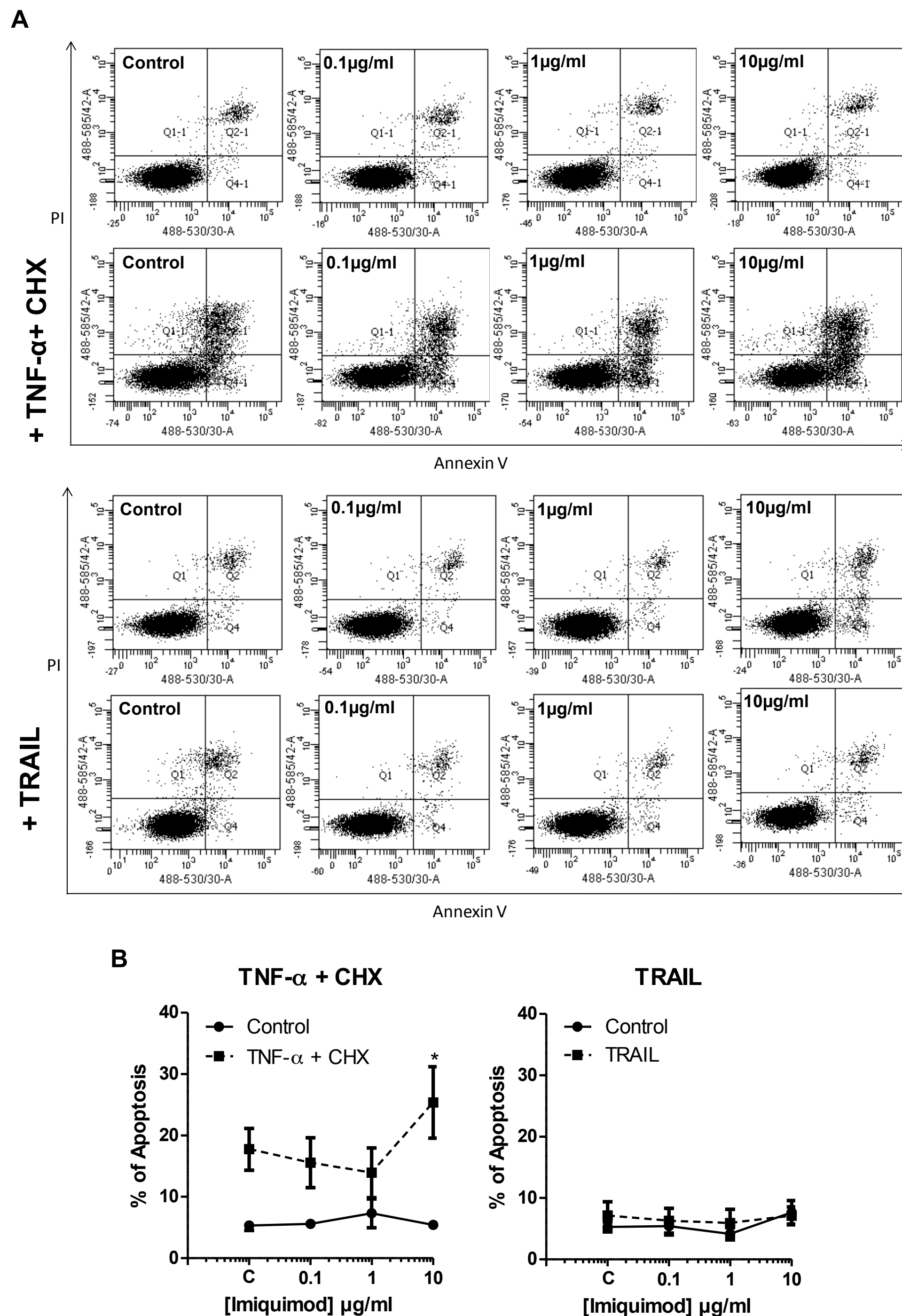


Figure 67. Effect of TLR7 agonist on A549 cells viability. A549 cells were seeded in a 6 well plates at 3 x 10^5 cells per well in DMEM with 10% FCS and allowed to equilibrate for 12h. After this time period cells were treated with TNF-α (15ng/ml)+CHX (1µg/ml) or TRAIL (1µg/ml) 4h prior Imiquimod treatment. After 24h cells were harvested and stained with annexin V and PI and analysed by FACS. Representative scatter plot of PI and annexin V staining (A). The % of dead cells from the scatter plot from Q1, Q2 and Q4 (B). Data represented are the mean ± SEM of n=3 replicates measured over 3 separate experimental days. *denotes $p \leq 0.05$ as assessed using one-way ANOVA followed by Dunnett's multiple comparison test. Q1 – dead cells (annexin V negative, PI positive), Q2- late apoptotic cells (annexin V and PI positive), Q3- live cells (annexin V and PI negative), Q4- early apoptotic cells (annexin V positive, PI negative).

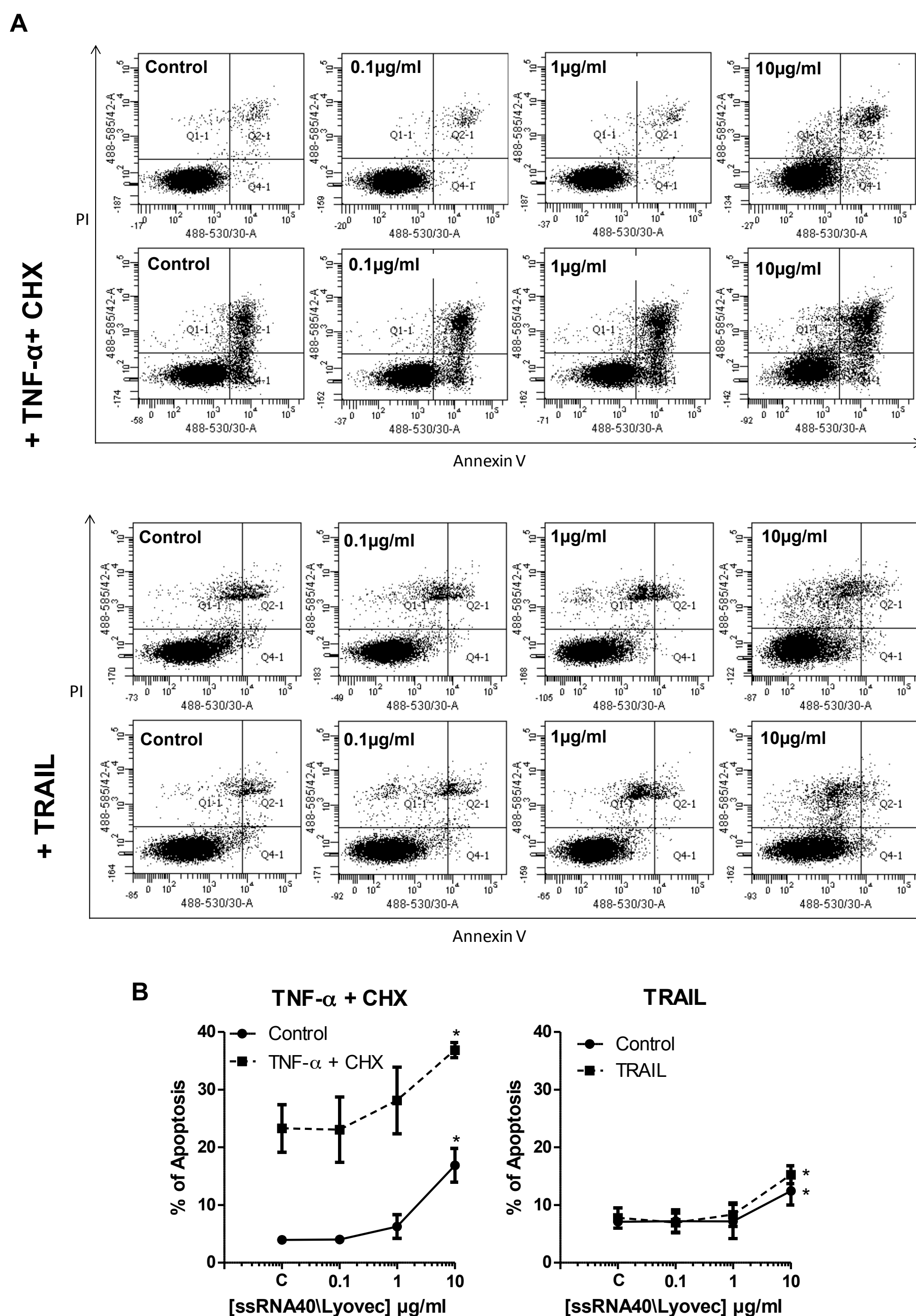


Figure 68. Effect of TLR8 agonist on A549 cells viability. A549 cells were seeded in a 6 well plates at 3 x 10^5 cells per well in DMEM with 10% FCS and allowed to equilibrate for 12h. After this time period cells were treated with TNF-α (15ng/ml)+CHX (1μg/ml) or TRAIL (1μg/ml) 4h prior ssRNA/Lyovec treatment. After 24h cells were harvested and stained with annexin V and PI and analysed by FACS. Representative scatter plot of PI and annexin V staining (A). The % of dead cells from the scatter plot from Q1, Q2 and Q4 (B). Data represented are the mean ± SEM of n=3 replicates measured over 3 separate experimental days. *denotes p≤0.05 as assessed using one-way ANOVA followed by Dunnett's multiple comparison test. Q1 – dead cells (annexin V negative, PI positive), Q2- late apoptotic cells (annexin V and PI positive), Q3- live cells (annexin V and PI negative), Q4- early apoptotic cells (annexin V positive, PI negative).

Discussion

Cigarette smoking is responsible for 90% of all lung cancers, which are the leading cause of cancer deaths in the world. It is also the major cause of COPD, the fourth leading cause of death worldwide. Cigarette smoke-induced chronic lung inflammatory microenvironment, oxidative stress and cell structural alterations such as the increase of cell proliferation, angiogenesis and apoptosis arrest are irreversible processes that are associated with lung tumor cell growth.

Our department has previously shown that TLRs are involved in the perception of inflammation in immune cells after acute cigarette smoke exposure and therefore may play a part in cellular processes such as increased cell proliferation and resistance to apoptosis found in lung cancer cells. To test this hypothesis we firstly looked to see if TLRs were functionally active on A549 cells. Agonists of TLRs resulted in a robust production of CXCL8, suggesting that TLRs are present and are functionally active. It is interesting to note that bacterial ligands were more potent in the release of CXCL8 compared with viral ligands. The secretion of CXCL8 in A549 cells was concentration dependent regardless of the presence or absence of serum.

We also demonstrated that TLRs were active on A549 cells, therefore we performed experiments to determine whether activation of these receptors could induce cell proliferation. It is suggested that carcinogenesis is an abnormal form of tissue repair, which requires proliferation. Molecules released during this process are important stimuli for TLR-induced cell proliferation [204]. In several types of cancer TLR stimulation led to increased cell proliferation and tumor metastasis. Three multiple myeloma cell lines, ANBL-6, RPMI-8226 and OH-2, also increased proliferation after Pam_3Cys and MALP-2 stimulation [205]. In addition, TLR2 also promoted migration and growth of cancer cells including U1810, HeLa and NCI-H292 [206]. However, two other studies on A549 cell line showed no increase in cell proliferation after TLR4, TLR7 and TLR8 stimulation [136, 200]. Activation of TLR2 on the oral squamous cell carcinoma cell line, YD-10B, also had

no effect on tumor progression [136]. In the present study we demonstrated that serum caused a concentration-dependent proliferation of A549 cells and that stimulation of TLRs with bacterial ligands induced a small increase in A549 proliferation under certain condition. However, the CyQuant assay which measures DNA content in the cell also showed some decrease in cell number suggesting that this effect was not biologically relevant. The The AlamarBlue assay showed that TLR7 and TLR8 ligands induced a decrease in cell viability/respiration, this was confirmed for a TLR8 agonist using the CyQuant assay, where we found decreased DNA content in A549 cells stimulated with ssRNA40/Lyovec. Our data from the cellomics assay showed that there was a small but significant increase in cellular proliferation at 48h after TLR2 activation. This was represented by a decrease in total cells in G0/G1 phase and a corresponding increase in cells in S phase. Although this data did not match the timings seen with the CyQuant assay, where a maximal effect was observed at 72h post TLR2 activation. Unfortunately the density of the cells as a monolayer was prohibitive for this technique to distinguish individual cells at 72h time point.

Since cancer cell survival and inflammation-induced chemoresistance have been linked to activation of NF-κB, upregulation of antiapoptotic proteins and inhibition of proapoptotic proteins [22], we wanted to verify whether TLR activation can induce apoptosis resistance in the A549 cell line. The AlamarBlue assay results showed that when A549 cells were stimulated with Pam3CSK4 prior to treatment with CHX cell viability was increased, with these effects being concentration dependent. Our results are similar to those obtained by He and colleagues where stimulation of TLR4 on A549 cells induced resistance to TRAIL-induced apoptosis [136]. Another study on a human lung cancer cell line showed that TLR7 and TLR8 agonism led to the activation of NF-κB and upregulation of the anti-apoptotic protein Bcl-2. This resulted in increased tumor survival and chemoresistance [200]. However, this was not an overall class effect of TLRs on cancer cells, as a TLR3 agonist, in combination with a type I IFN or a protein synthesis inhibitor, reduced cell

proliferation and induced tumor cell death in melanoma cells. This effect was found to be caspase dependent. Moreover, it was demonstrated that TLR3-mediated cell death involved the activation of caspases and engaged both extrinsic and intrinsic apoptotic pathways [207]. Our results from FACS analysis showed that activation of TLR2, TLR3 and TLR4 did not have any effect on resistance to apoptosis. However, again we observed increased cell death after TLR7 and TLR8 activation. The biggest effect was seen after TLR8 stimulation with ssRNA40/Lyovec, where we demonstrated that this agonist could induce A549 cell death. Taking all our findings into account, it is unlikely that bacterial ligands activating TLRs can induce cell proliferation and resistance to apoptosis. However CXCL8 released by these cells can contribute to cancer development and progression.

Both, cancer development and tissue repair processes, are characterized by high numbers of infiltrating immune cells. These cells produce high level of cytokines, growth factors and prostaglandins [22]. CXCL8 is a proangiogenic chemokine which has anti-apoptotic properties that promote tumor metastasis and death resistance. CXCL8 is found in high levels within the tumor microenvironment [208]. In addition to its chemotactic function, CXCL8 has been shown to promote tumor cell proliferation in breast cancer, melanoma and bronchogenic carcinoma [209-211]. Furthermore, levels of CXCL8 production correlated directly with the metastatic potential of tumors [122, 208, 212, 213]. Our data showed that secretion of CXCL8 in A549 cells was increased significantly by TLR ligation and may contribute to lung cancer cell escape from immune control but it does not affect cell proliferation. Our findings also showed that it is implausible that activation of TLR2, TLR3 and TLR4 can promote resistance to apoptosis. We found that only ligands for TLR7 and TLR8 induced cell death in A549 cells. Similar results were obtained by other groups where Ahn et al. demonstrated that Imiquimod inhibited effectively the growth of OSCC cells by inducing apoptosis and necrosis [214]. Moreover, TLR7/8 activation by imidazoquinolines also enhanced antitumor effects through inhibition

of angiogenesis, NK-mediated cytotoxicity and direct apoptosis of tumor cells [215-217]. Imiquimod formulated as imiquimod 5% cream (Aldara™) is indicated for the treatment of a variety of diseases ranging from human papillomavirus (HPV)-related external genital and perianal warts to primary skin malignancies or premalignant conditions (actinic keratosis). Treatment of superficial basal cell carcinoma (sBCC) with imiquimod 5% cream resulted in histological clearance rates of 79–82% in Phase III studies [218]. VTX-2337 (VentiRx Pharmaceuticals), a new TLR8 ligand is in phase I/II clinical trials in combination with current regimens for the treatment of lymphoma and various other solid tumors [219]. The antitumoral activities of TLR7/8 agonists have been associated with their ability to induce IL12p70 secretion by DC, enhancement of NK cell activation, as well as a decrease in Tregs. Their ability to induce a potent Th1-type immune response and decrease the number of Tregs could lead to a tumor microenvironment more favourable for the host immune system to respond strongly against the tumor [160].

The last remaining question is whether the cell death observed in A549 cells occurred because of a real TLR-mediated effect or whether the concentrations of the drugs we used were too high, resulting in cell death. When considering the use of these ligands in tumor therapy, it is important to consider the potential effect of these agonists on the entire organism as well. To answer this question a few additional experiments are required. It will be useful to extend the concentration range of these compounds, and also to try other TLR7 and TLR8 agonists to verify if this was a TLR class effect rather than a specific agonist effect. It would also be of use to try different approaches to capture apoptosis e.g. confocal microscopy with annexin V staining.

Some studies suggest that TLR activation can promote tumor growth and the escape of tumor cells from the host immune system while others claim the opposite effect. Thus, the choice between either agonist or antagonist for TLRs in cancer therapy may be very difficult. As TLR research is still in its infancy, more functional studies are required in

human diseases using both agonists and antagonists to give us definitive answers regarding the role of TLRs in inflammation and cancer.

Chapter summary

Our data showed that secretion of CXCL8 in A549 cells increased significantly after TLR ligation and may contribute to lung cancer cell escape from immune control, but it does not affect cell proliferation. Activation of TLR2, TLR3 and TLR4 did not promote resistance to apoptosis. Only the viral ligands, TLR7 and TLR8, had effects on A549 viability where we demonstrated that these agonists induced cell death.

Chapter 6

General Discussion

Cigarette smoke causes inflammation and is a major risk factor in the pathogenesis of many diseases including COPD, lung cancer and atherosclerosis. Oxidants, of which there are many in cigarette smoke, activate monocytes/macrophages and airway cells to release cytokines such as CXCL8 and IL-1β. Production of these cytokines can contribute to inflammatory responses observed in the lung and can result in chronic bronchitis, emphysema, airway wall remodelling, fibrosis and chronic inflammation. These chronic lung pathologies are known to be contributory risk factors for cancer. Work from our group has shown that when cells were stimulated with smoke *in vitro* there was an increase in the expression of some of the pro-inflammatory genes such as CXCL8, TNF-α as well as the anti-oxidant gene, HMOX1. Whilst we know that smoke is the causative agent in COPD we still don't know too much about how it induces inflammation. There are a great number of publications showing that TLRs mediate cigarette smoke-induced cytokine production and pro-inflammatory responses [64, 101, 106, 220] so the aim of this thesis was to study the role of TLRs in smoking related disease. The basic aims of my PhD have fallen into three areas. Firstly, I analysed the role of TLRs in inflammasome activation and cytokine release. Since TLRs and the inflammasome are now implicated in respiratory diseases such as COPD, I performed a mechanistic part to my PhD where I directly compared the signalling pathways between TLR4 and TLR2-induced release of IL-1β and CXCL8. Secondly, I have performed a clinical study to investigate how whole blood from patients with COPD, and later patients with PAH, respond to stimulation with specific TLR agonists. Thirdly, I analysed lung cancer cell proliferation and viability characteristics in response to TLR agonists.

Since inflammasome signalling in airway cells may play an important role in the pathogenesis of diseases like COPD [110], we wanted to investigate the contribution of

TLRs to inflammasome activation and cytokine release. In Chapter 3, we found that the activation of TLR4 and TLR2 resulted in the release of CXCL8 and IL-1β. ATP further enhanced IL-1β production. We found no difference between TLR2 and TLR4 mediated CXCL8 secretion. However, there were clear differences in the release of mature IL-1β after TLR2 versus TLR4 stimulation. In these experiments we found evidence to suggest that TLR2 and TLR4 induced IL-1β release proceeds via different signalling pathways where the TLR4-induced release did not involve pannexin-1, a hemichannel protein. We think that the TLR4 pathway, which activates TRIF, also activates inflammasome assembly. This was demonstrated in a paper by Rathinam et al. who showed that this TRIF-dependent activation of TLR4 was able to activate the inflammasome without the need for a second stimulus [170]. This is verified by the results that I obtained showing that TLR2, which doesn't activate TRIF pathway, required pannexin-1. Pannexin-1 is necessary in this process, it forms large pores in the cell membrane, which allows the passage of large molecules such as ATP, which can then stimulate inflammasome assembly by an undefined mechanism. Our data suggest an important dissociation in inflammasome activation induced by TLR4 versus TLR2 which could be important in our understanding of both bacterial and sterile inflammation in man. This is important as it may help us target diseases where TLR2 or TLR4-induced IL-1β release may be the underlying cause of pathology.

As it was mentioned before, cigarette smoke is the major cause in the development of COPD. TLRs play a role in oxidant sensing and there is growing evidence that activation of TLRs can contribute to disease pathogenesis and progression. COPD describes a heterogeneous group of disorders whereby some individuals suffer with frequent infective exacerbations, despite smoking cessation. These exacerbations are known to accelerate the destructive process and manifest in a more rapid decline in lung function. The "frequent exacerbator" phenotype has recently been elucidated [221] and is reflected in

the updated GOLD staging of COPD. However, little is known about what defines this group of individuals on a genetic or immunological basis. It is well established that patients with COPD are more susceptible to viral and bacterial airway infection, with a number of mechanisms proposed. We hypothesised that the systemic inflammation observed in patients with COPD may be regulated by TLRs and that TLR stimulation (by bacteria, viruses or DAMPs) will result in the release of inflammatory cytokines that play a role in alveolar destruction and systemic inflammation. This is important as it may help us target diseases where TLR2 or TLR4-induced IL-1β release may be the underlying cause of pathology. In Chapter 4, we tested this hypothesis using an *in vitro* system to model the effect of TLR stimulation of blood from COPD patients and measured the important inflammatory cytokines, CXCL8 and IL-1β. Among the mediators of neutrophil recruitment into the lung, the chemokine CXCL8 is considered to be a major player in COPD. We found that COPD patients had higher levels of CXCL8 in unstimulated blood. It was also demonstrated that the blood of COPD patients was more sensitive to some of the Gram-positive and Gram-negative bacterial ligands. In addition, elevated levels of CXCL8 and IL-1β were found in COPD subjects in response to the viral ligand, Poly I:C, compared to age-matched controls. Our data might help to understand better the relationship between responses to TLR activation in COPD and give us an insight into why patients with COPD experience repeated infective exacerbations leading to worsening lung function. A similar approach was taken when investigating the effects of TLRs in the blood of patients with PAH. Our results showed that LPS stimulation resulted in increases in levels of IL-1β suggesting an increased sensitivity to TLR4 activation in the blood of PAH patients. This very much fits into the hypothesis that patients with inflammatory lung disease are more susceptible to pathogen activation in immune cells.

In Chapter 5, we wanted to investigate the role of TLRs in a different disease associated with cigarette smoking, namely lung cancer. TLRs have been shown to regulate cancer

cell proliferation and survival, thus expanding the role of these immune cell receptors into the cancer field. The original idea that stimulation of TLRs has a positive role in tumorigenesis came from reports demonstrating that TLR ligands augment the growth of adoptively transferred tumors [222-224]. In our study, we wanted to investigate whether activation of TLRs on cancerous lung epithelial cell line A549 can contribute to increased cell proliferation and lung cancer progression. Firstly, we performed some preliminary experiments to choose appropriate conditions for our cell line. Then, we used different methods to establish proliferation characteristics of our cells and the effect of TLR agonism on this process. Our results showed that it is unlikely that TLR activation induces increased cell proliferation, however a decrease in cell viability was observed after stimulation of TLR8 with single-stranded RNA. To take our study further, we also wanted to verify whether activation of TLRs can induce apoptosis resistance rather than proliferation. Our FACS data indicated that activation of TLRs with bacterial ligands did not have any effect on cell survival. However, stimulation with the TLR8 agonist again caused an increase in A549 cell death and therefore TLR8 may act as a potential useful target in cancer therapy.

Since first being described in the fruit fly, Drosophila melanogaster, TLRs are seen as an attractive target to immunologists and investigators interested in the molecular basis of inflammation, as they directly sense PAMPs and DAMPs, and cause a robust inflammatory response [225]. There have been many attempts to use TLR manipulation for the treatment of infectious, allergic and autoimmune diseases, as well as cancer. These studies are in the early clinical phases, and results have not always been positive. TLRs recognize viral and bacterial PAMPs as well as DAMPs but the pathways they activate are complex and involve multiple genes and molecules. In addition, during the process of inflammation, there is a release of host breakdown products from the extracellular matrix, which are able to further stimulate TLRs. TLR activation occurs early

in the cascade of events that gives rise to inflammation, there might be an advantage in blocking them as they might be close to the initiating events that give rise to chronic inflammation. As it was mentioned before (Chapter 5) single stranded RNA-based TLR7 and TLR8 agonists are being developed for the treatment of cancer [225]. Early studies showed that the activation of TLR7 or TLR8 can reverse the suppressive function of Tregs. When combined with the ability of TLR7 and TLR8 to activate dendritic cells, this effect results in a strong antitumor response. However, some studies suggest that TLR activation can promote tumor growth, and tumor cell evasion of the host immune system. We therefore need more research on the functional aspects of TLRs in different cell types to establish whether an agonist or antagonist approach would be beneficial in specific clinical settings. Synthetic TLR activators are currently under clinical development for the treatment of asthma and allergies; these include; TLR3, TLR4, TLR5, TLR7, and TLR9 agonists [226]. TLR agonist or adjuvant therapy could be useful in the treatment of COPD exacerbations induced by bacteria or viruses. Effective defence mechanisms during infection together with a fast recovery will minimize the duration of disease worsening. Triggering of TLRs by using agonists may boost the protective inflammatory response that destroys pathogens and protects the host. On the other hand, antagonist therapy may be beneficial in chronic inflammation. Antagonist therapy could be achieved by blocking the interaction between the TLR and the disease-related ligand or by blocking downstream signalling molecules [116]. In a healthy person, there is a balance between the protective inflammatory host response and inhibition of an excessive inflammatory or autoimmune response. This balance might be disturbed during disease and is another reason for having a good understanding of the molecular mechanisms regarding TLR activation [227]. Disease may result from overactive TLR signalling triggered by harmless molecules or may result from insufficient TLR signalling during viral or bacterial infections leading to poor defence mechanisms and resolution. Both ways could contribute to disease

pathology, and for therapeutic implications, it is of huge importance to understand the causal relationships underlying disease symptoms [116].

TLRs offer promising therapeutic strategies for inflammatory diseases, which are caused by dysregulation of the immune system. However, targeting such a fundamental and complex pathway has to be carefully assessed [139]. There is a lot of data indicating that in the future TLRs will act as validated targets for inflammatory disorders. The aim of my thesis was to investigate role of TLRs in smoking-related disease. It is clear from my results that activation of these receptors has important effect on immune responses to their ligands. These findings could provide useful information about therapeutic potential of TLRs in clinical settings.

In conclusion, the work in my thesis shows that there is a distinct difference in the activation of the inflammasome and subsequent release of IL-1β by TLR2 and TLR4 pathways. This difference was identified to be at the level of pannexin-1 activation. Pannexin-1 makes large pores in the membrane, which allows the passive trafficking of molecules such as ATP, ROS and MSU into the cytoplasm of the cell. However, the specific function of this pore in the activation of the inflammasome is unclear. Further experiments are required in order to clarify how pannexin-1 specifically affects individual steps in the NLRP3 inflammasome assembly. Nevertheless, we can conclude that pannexin-1 is essential for the secretion of mature IL-1β after TLR2, but not TLR4, activation in human monocytes.

Using our whole blood assay we observed that blood of COPD patients was more sensitive to activation with LPS, Pam3CSK4 and Poly I:C. It is known clinically that COPD patients have a greater susceptibility to common colds and flu. One causative factor is that these patients suffer from excessive mucus secretion that is thicker, thereby reducing pathogen clearance in the airways leading to higher infection rates. Therefore the increased sensitivity to viral and bacterial PAMPs observed in the blood of patients, might

manifest itself in an overactive response to what would normally be considered threshold concentrations of PAMPs in healthy volunteers. Interestingly, our study highlighted a possible new biomarker for COPD, as we observed a negative correlation between TLR4-induced CXCL8 levels and GOLD stage of COPD severity. This observation may have therapeutic potential, although a larger patient cohort is required to make a definitive conclusion.

Considering the role of TLRs in lung cancer cell proliferation, our data showed that TLR8 caused an inhibition of proliferation by inducing apoptosis in A549 cells. These data identify TLR8 as a potential therapeutic target in lung cancer. Lung cancer has the poorest prognosis of all cancers; therefore the addition of TLR8 ligands to established chemotherapeutic regimes may increase the effectiveness of these agents thereby improving the prognosis. In this regard, a TLR7 agonist has been successfully used for the treatment of basal cell carcinoma in the skin. The specificity of these agents may also be improved with the use of nano-cages, which are reported to target chemotherapeutic agents to cancer cells [228]

My thesis therefore shows that TLRs contribute to the inflammation and pathology of respiratory diseases and may be useful as a biomarker or therapeutic target for their treatment.

Future work:

There are several directions in which this project could be extended: for the inflammasome study, it would be interesting to measure pro-IL-1β levels from the cell lysates after TLR2 and TLR4 stimulation to show that pro-IL-1β release was not affected by the inhibitors used, and hence that pannexin-1 was more associated with inflammasome assembly than pro-IL-1β cleavage. As a control, I could also measure IL-18 secretion, as IL-18 synthesis does not depend on TLR signalling. Measuring ATP levels in cell supernatants and lysates would indicate that endogenous levels of ATP are sufficient for inflammasome activation and caspase-1 cleavage. It would also be interesting to elucidate the role of TRIF in inflammasome assembly after TLR4 activation using a gene knock-down approach. This would verify what was observed with the signalling inhibitors used in my thesis. I would also characterise specific pathways in human cells that lead to pro-IL-1β cleavage after TLR4 stimulation. It is known that caspase 11 in mice is responsible for inflammasome activation, however caspase 11 was not found in human monocytes. I could verify the role of potential alternative caspases in human cells, for example caspase 4 and 5 to investigate their role in inflammasome assembly and IL-1β secretion. Finally, it would be useful to measure pannexin-1 and P2XR protein expression by western blot after siRNA knock-down.

One of the major limitations of the COPD study was the number of subjects we used. This would be greatly improved by performing a bigger study using at least n=20 for each group and also to include age-matched smokers as an additional control group. This would allow me to determine whether any changes in responses to PAMPs are due to the smoking status or as a consequence of the disease process. Finally, I would measure the expression of TLRs on blood cells to see whether any differences in the sensitivity to PAMPs were due to differences in levels of TLR expression.

To investigate further the role of TLRs activation on cell proliferation and apoptosis I would use other TLR8 agonists to investigate whether cell death observed in my study was TLR8 mediated or due to non-specific toxicity of the drug. It would be also interesting to use another technique capturing the cell death e.g. Cellomics or confocal microscopy. I could also combine TLR8 agonists with other chemotherapeutic agents and extend this work to in vivo studies; the mouse Lewis lung model would be appropriate for this work.

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