University of Glasgow

The Role of Trained Immunity against *Streptococcus pneumoniae*

Tamadhour Alem

MBBS,MSc

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Institute of Infection and Immunity

College of Medical, Veterinary & Life Sciences

University of Glasgow

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Abstract

Background: *Streptococcus pneumoniae* is a gram-positive bacterium associated with multiple serious illnesses. According to the CDC antimicrobial resistance reports, pneumococcal bacteria are resistant to one or more antibiotics in more than 30% of cases. This high percentage necessitates the need for alternative therapeutic targets that stem from a better understanding of immune defences against the bacteria. The bacteria possess several virulence factors and can cause significant morbidity and mortality in humans. This thesis examines the innate immune responses activated in defence against *Streptococcus pneumoniae*. It investigates whether these responses involve the secretion of type I interferons and other pro-inflammatory cytokines, such as tumour necrosis factor alpha and interleukin-1 beta. Another aspect of this thesis is to investigate the phenomenon of trained immunity, in which innate immune cells produce a more robust response to a secondary infection by the same or a different pathogen. To do this, multiple cell models were used, including primary dendritic cells, macrophages, and cell lines. These cells were pre-treated with innate immune stimulants such as ß-glucans, Zymosan derived from fungi, and LPS derived from *E. coli*, a gram-negative bacterium. Subsequently, the secretion of pro-inflammatory cytokines, TNF alpha and IL-1 beta, was measured in response to the pre-treatment of these cells and their infection with *Streptococcus pneumoniae*. This was done to explore whether pretreatment impacts cytokine release following infection in innate immune cells.

In other chapters, mRNA transcription of TNF alpha gene in response to pre-treatment with LPS and infection with *Streptococcus pneumoniae* was assessed. Also, the epigenetic changes underlying trained immunity were explored, specifically DNA methylation in response to LPS pre-treatment and infection with *Streptococcus pneumoniae* to compare the methylation percentages between different conditions. Finally, cellular proteins involved in toll-like receptor 4 (TLR4) signalling were examined using protein lysates, through gel electrophoresis and western blotting. These were done to determine which proteins are responsible for the pro-inflammatory effects of LPS pre-treatment and *Streptococcus pneumoniae* infection.

Main results: None of the cell models used in the study, including primary monocyte-derived dendritic cells, primary monocyte-derived macrophages, the BEAS-2B bronchial epithelial cell line, and U937 monocyte-derived macrophages, produced type 1 interferons following infection with *Streptococcus pneumoniae*. However, both primary cells and the U937 cell line produced pro-inflammatory cytokines, such as TNF alpha and IL-1 beta, in response to *Streptococcus pneumoniae* infection.

Innate immune stimulants, including beta-glucan, zymosan, and LPS, all appeared to stimulate the release of TNF alpha and IL-1 beta 24 hours after infection with *Streptococcus pneumoniae*. Additionally, LPS pretreatment of U937 cells and subsequent infection with *Streptococcus pneumoniae* appear to boost the transcription of TNF-alpha mRNA. The mRNA expression is at similar levels in cells that were pre-treated only. However, ELISA results indicate that the pretreated infected cells produced more TNF alpha compared to the pretreated only cells.

TNF alpha gene methylation following pre-treatment was studied. The first segment examined is in the promoter region. DNA methylation percentages revealed patchy results. However, it seems there's a trend of increased methylation in LPS pre-treated and infected cells compared to pre-treated only cells, but this difference didn't reach statistical significance. Increased methylation in the promoter region of a gene is associated with gene silencing. Similar results are seen in the second segment, which is in the gene's first exon.

Lastly, cellular proteins involved in TLR4 signalling were examined. It appears that LPS pretreatment combined with infection boosts JNK phosphorylation.

Conclusion: Based on these results, it appears that *Streptococcus pneumoniae* infection doesn't lead to IFN secretion. However, it boosts the secretion of the pro-inflammatory cytokines TNF alpha and IL-1 beta. Pretreatment of innate immune cells with innate immune stimulants prior to infection increases the secretion of TNF alpha and IL-1 beta 24 hours following infection. When combining the ELISA results with qPCR, it appears that both pre-treated infected cells and pre-treated-only cells undergo increased TNF alpha mRNA

transcription; however, the infection seems to work post-transcriptionally to enhance mRNA translation through an unknown mechanism.

Regarding the TNF alpha methylation data, it appears that LPS pretreatment may cause increased methylation and gene silencing. However, when this result is added to previous findings, it could suggest that LPS pretreatment works through a different mechanism to induce TNF alpha gene transcription.

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Author's Declaration

I hereby declare that this thesis is an embodiment of original work generated by my own efforts. Appropriate acknowledgements have been made where practical support was provided. Neither has this work, nor any part of it, been submitted for or as part of another degree at the University of Glasgow or at any other institution.

Tamadhour Alem

Definitions/Abbreviations

Definitions	Abbreviations
12-O-tetradecanoylphorbol13-acetate	TPA
4E-binding protein	4EBP
Absent in melanoma	AIM2
Activating transcription factor -2	ATF-2
Activator protein-1	AP-1.
Adenosine triphosphate	ATP
Antigen presenting cell	APC
Bactericidal/permeability-increasing protein fold containing family A, member 1	BPIFA1
Brain Heart Infusion	BHI
c-Jun N-terminal kinases	JNK
C-type lectin receptor	CLR
C-X-C Motif Chemokine Ligand 1	CXCL-1
CC-chemokine ligand 2	CCL2
CC-chemokine ligand 5	CCL5
Centers for Disease control	CDC
Chronic obstructive pulmonary disease	COPD

Cleavage and polyadenylation specificity factor	CPSF
Cleavage stimulation factor	CSTF
colony forming unit	CFU
Coronavirus disease 19	COVID-19
Cross reacting material 197	CRM197
Cryopyrin-associated periodic syndrome	CAPS
Cyclic-GMP-AMP-synthase	cGAS
Cytosine phospho-guanine	CpG
Damage Associated Molecular Patterns	DAMPS
Dendritic cell	DC
Deoxyribonucleic acid	DNA
Downstream sequence elements	DSEs
Enzyme-Linked Immunosorbent Assay	ELISA
Ethylenediaminetetraacetic acid	EDTA
Eukaryotic translation initiation factor 2	eIF2
European center for disease prevention and control	ECDC
European Union / European Economic Area	EU/EEU
extracellular signal-related kinase	ERK
Formyl peptide receptor 1	Fpr1

Glyceraldehyde 3-phosphate dehydrogenase	GAPDH
glycosylphosphatidylinositol	GPI
Granulocyte-macrophage colony-stimulating factor	GM-CSF
Group B streptococci	GBS
Guanosine-5'-triphosphate	GTP
Human immunodeficiency virus	HIV
IL-1 receptor-associated kinase	IRAK
Immunoglobulin A 1	IgA1
Immunoglobulin M	IgM
Inflammatory bowel disease	IBD
Interferon beta	IFN β
interleukin 1 receptor	IL-1R
Interleukin one beta	IL-1 β
Interleukin-1 receptor-associated kinase 1 c	IRAK1c
Invasive pneumococcal diseases	IPD
IκB Kinase	IKK
Lactate dehydrogenase	LDH
lipopolysaccharide	LPS
Lipoteichoic acid	LTA

LPS binding protein	LBP
Macrophage colony-stimulating factor	M-CSF
major histocompatibility complex molecules	MHC I/II
Mammalian target of rapamycin	mTOR
Mitogen-activated protein kinase	MAPK
Myeloid differentiation primary response protein 88	MyD88
Myeloid differentiation protein 2	MD-2
Natural killers' cells	NK cells
Nod-like receptor	NLR
Nuclear factor-κB	NF-κB
nucleotide-binding domain and leucine-rich repeat (NLR) protein	NLRC4
Pathogen associated molecular patterns	PAMPs
Pattern recognition receptors	PRRs
Peptidoglycan	PG
Phorbol 12-myristate 13-acetate	PMA
Platelet-activating factor	PAF
Pneumococcal conjugate vaccine	PCV
Pneumolysin	PLY

Poly(A) polymerase	PAP
poly(A)-binding protein	PABP
Polymerase Chain Reaction	PCR
Protein kinase C	PKC
Ribonucleic acid	RNA
RIG-I-like receptor	RLR
Serum amyloid A 3	Saa3
Severe combined immunodeficiency	SCID
Small nuclear ribonucleic particles	snRNPs
stimulator of interferon genes	STING
TANK-binding kinase 1	TBK1
The Bacillus Calmette-Guérin Vaccine	BCG vaccine
The Institute for Genomic Research (TIGR), 4th pneumococcal isolate sequenced.	TIGR4
TIR Domain-Containing Adaptor Protein	TIRAP
TNF Receptor-Associated Factor 6	TRAF6
Toll-like receptor 2	TLR2
Toll-like receptor 4	TLR 4
Toll/IL-1R homology domain	TIR domain

Toll/Interleukin-1 receptor (TIR) domain-containing adaptor-inducing IFN-β	TRIF
Transcriptional start site	TSS
Transforming growth factor beta	TGF- β
Transforming Growth Factor-β-Activated Kinase 1	TAK1
TRIF-related adaptor molecule	TRAM
tris-acetate-EDTA	TAE
Tris-Borate-EDTA	TBE
Tris-buffered saline with Tween-20	TBST
Tumour necrosis factor alpha	TNF α
United states food and drug administration	US FDA
World Health Organization	WHO

1 Introduction

1.1 Streptococcus pneumoniae

Streptococcus pneumoniae, also known as pneumococcus, is a gram-positive, rounded, lancet-shaped bacterium. It is an alpha-haemolytic (produces incomplete hemolysis) member of the genus *Streptococcus*.

1.1.1 *S. pneumoniae* Virulence Factors

Streptococcus pneumoniae possesses several virulence factors that enhance its ability to cause infections in humans. The most significant virulence factor is a polysaccharide capsule, which has an anti-phagocytic effect that prevents bacterial clearance through phagocytosis. Opsonisation by antibodies or complement is required for efficient phagocytosis [1]. Nonetheless, the polysaccharide capsule also inhibits the organism's mechanical removal by mucus and can hinder autolysis and antibiotic exposure.

Pneumolysin (PLY), another virulence factor of *Streptococcus pneumoniae*, is a 53-kDa pore-forming toxin [2]. It binds to membrane cholesterol and leads to large pores formation and subsequent cell death. Moreso, it activates the classical complement pathway, and at lower concentrations, it induces a pro-inflammatory response and produces reactive oxygen mediators. These effects are potentially facilitated by the PLY's ability to interact with the lipopolysaccharide receptor, Toll-like receptor 4 (TLR4) [3]. In animal studies, it was observed that mice that were infected with pneumococcal mutants without PLY (PLY− strains) exhibited less inflammation and delayed cellular recruitment in their lung tissue compared to those infected with wild types of bacteria [4].

Notably, other pneumococcal proteins interfere with host defence at mucosal surfaces, such as IgA1 protease and hyaluronidase, interfere with host defence at mucosal surfaces. Hyaluronidase breaks down the hyaluronic acid component of connective tissue and extracellular matrix [36]. The degradation of hyaluronic acid may help bacterial spread and colonisation. While neuraminidase helps the attachment to epithelial cells. Other proteins that contribute to the virulence of *S. pneumoniae* include pneumococcal surface protein A and autolysin. Pili also

play a role, permitting the organism to adhere to cellular surfaces and induce host inflammation [5].

1.1.2 *S. pneumoniae* Serotypes

Based on its capsular polysaccharides, *S. pneumoniae* can be classified into different serotypes. Approximately 100 serotypes are known, and their importance is emphasised when designing a vaccine or studying immunity against pneumococcus. According to Public Health Scotland, In the first three quarters of 2024, the total number of IPD cases were 372 cases. Typing results were available for 274 of the 372 cases reported. The four most common serotypes are listed in Table 1-1.

Table 1-1. *Streptococcus pneumoniae* serotypes reported in Scotland in the first three quarters of 2024 [6]

Serotype	Number of cases
Serotype 8	38
Serotype 22F	28
Serotype 3	27
Serotype 9N	23

The most virulent Streptococcus pneumoniae serotypes are listed in Table 1-2.

Table 1-2 The Most Virulent *Streptococcus pneumoniae* Serotypes [7]

Serotype	Clinical Significance	Vaccine Coverage
3	Severe pneumonia, meningitis	PCV13
19A	Meningitis, bacteremia	PCV13
1	Rapid-onset pneumonia	PCV13
7F	Invasive disease in children	PCV13
4	Pneumonia, invasive disease	PCV13
8	Invasive disease in adults	PCV20

In this study, we used the TIGR4 strain, which belongs to serotype 4. TIGR4 stands for The Institute for Genomic Research (TIGR), and the number 4 indicates it was their fourth pneumococcal isolate sequenced. The strain was isolated from a pneumonia patient and is widely used today to study pneumococcal virulence and immunity against it [8].

1.1.3 Diseases caused by *S. pneumoniae*

S. pneumoniae infections are categorized into two types: invasive and non-invasive.
Invasive pneumococcal infections or diseases (IPD) affect major organs and cause conditions such as pneumonia, meningitis, bacteraemia, and septic arthritis. On the other hand, non-invasive infections exclude the major organs, with conditions such as otitis media, bronchitis and sinusitis.
Streptococcus pneumoniae colonizes the upper respiratory tract in a significant portion of the population without causing disease. In the 1990s, it was found that almost 40%- 50% of healthy children and 20%—30 % of healthy adults were carriers [9]. Following the introduction of the vaccine, carriage rates significantly decreased among children and adults [10, 11]. Risk factors for invasive pneumococcal disease (IPD) include patients who are younger than 2 years of age or older than 65, smokers, asthmatics/COPD, alcohol abusers, and (splenectomised patients) lacking a spleen.

1.1.3.1 Community-Acquired Pneumonia

Pneumonia is an infection that causes inflammation in the alveoli of one or both lungs. *S. pneumoniae* is the leading cause of community-acquired pneumonia. It colonizes the nasopharynx and spreads directly to the respiratory tract, probably through microaspiration events [12].

Symptoms include a sudden onset of fever and chills, chest pain, productive cough with rusty sputum, difficulty breathing, palpitations, and weakness.
The case-fatality rate is 5–7% and may be much higher among elderly and patients with underlying co-morbid conditions. Complications may include empyema, pericarditis, and respiratory failure [13].

1.1.3.2 Meningitis

Meningitis, also known as inflammation of the meninges, refers to the inflammation of the protective membranes surrounding the brain and spinal cord. It is a life-threatening infection. *S. pneumoniae* gains access to the meninges by spreading from other sites of infection. In one study, the primary foci of infections were examined and analyzed. It was found that in 30% of

patients, the primary focus was otitis media (middle ear), pneumonia (lungs) in 18% of patients, sinusitis in 8%, and in 42% of patients, the primary focus was not identified [14]. Symptoms include headache, lethargy, vomiting, fever, irritability, neck rigidity, seizures, and coma. The case-fatality rate of pneumococcal meningitis is approximately 8% in children and 22% among adults [13, 15]. Complications include septic shock, multi-organ failure and disseminated intravascular coagulation. Neurological sequelae, such as intellectual and behavioural disabilities, seizures, motor deficits and hearing loss, can occur in up to 50% of survivors of pneumococcal meningitis [13, 14].

1.1.3.3 Acute otitis media

Otitis media is an infection of the middle ear that causes inflammation (redness and swelling) and fluid accumulation behind the eardrum. Symptoms include ear pain, fever, hearing difficulty, ear discharge, and irritability. In young children, earache may show as irritability, changes in sleeping or eating habits, or rubbing the ear. Fever, ear drainage, and hearing loss may also be present. Complications of pneumococcal otitis media may include meningitis and mastoiditis [13].

1.1.3.4 Other Pneumococcal diseases

These include bronchitis, rhinitis, acute sinusitis, conjunctivitis, sepsis, osteomyelitis, septic arthritis, cellulitis, peritonitis, pericarditis, and endocarditis.

1.1.4 Treatment of *S. pneumoniae* Diseases

Penicillin and its derivatives are effective and inexpensive antibiotics for the treatment of pneumococcal infections caused by susceptible isolates. Penicillins can be administered orally or parenterally to eliminate the infection by inhibiting cell wall synthesis. Penicillin G is the drug of choice for the parental treatment of susceptible *S. pneumoniae* infections.

First-generation cephalosporins can be used to treat penicillin-susceptible strains as their mechanism of action and methods of resistance are similar to that of Pencillin.

Moreso, Macrolides can be used to treat Pneumococcal pneumonia but not meningitis, as they are unable to cross the blood-brain barrier. Between 1998 and 2011, macrolide resistance increased to 25%-45% in the United States [16]. Respiratory fluoroquinolones such as levofloxacin, moxifloxacin, or gatifloxacin are other options for treatment. Glycopeptide antibiotics, including vancomycin, dalbavancin, and telavancin, are also effective. Vancomycin, in combination with third-generation cephalosporin, is the drug of choice for treating penicillin-resistant pneumococcal meningitis. Clindamycin may also be used to treat non-meningeal *S. pneumoniae* infections. Approximately 5-10% of *S. pneumoniae* strains in the United States are resistant to clindamycin [17].

Carbapenems are effective but are reserved as the last antibiotics to be used due to their broad spectrum of coverage and the fear of resistance emergence [18]. According to the CDC antimicrobial resistance reports, pneumococcal bacteria are resistant to one or more antibiotics in more than 30% of cases [19]. According to ECDC, in the EU/EEA, the mean percentage of penicillin resistance in 2019 was 12.1%, while macrolide resistance was 14% [20].

1.1.5 *S. pneumoniae* Vaccines

There are two types of pneumococcal vaccines: conjugate vaccines and polysaccharide vaccines. The pneumococcal polysaccharide vaccine, which is the most commonly used recently, consists of purified polysaccharides from 23 serotypes (1, 2, 3, 4, 5, 6b, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F). Immunity is induced by the stimulation of B-cells and the release of Immunoglobulin M (IgM) without the involvement of T cells [21].

Notably, the pneumococcal conjugate vaccine is composed of capsular polysaccharides attached to the diphtheria toxoid CRM197, which is highly immunogenic but non-toxic. This combination induces a more vigorous immune response by recruiting CRM197-specific helper T cells, which allow immunoglobulin type switching (to produce non-IgM immunoglobulin) and production of memory B cells [22]. The need for the conjugate vaccine stems from the nature of the polysaccharide vaccine, as the immunity it produces is T-

cell independent. It does not produce an immune response in children under two years of age because their immune system is not fully developed. This also applies to patients with functional or anatomical asplenia [23]. Vaccination results in mucosal immunity through the production of IgA. However, conjugated vaccines only cover a subset of the serotypes covered by the polysaccharide vaccines. While the polysaccharide vaccine is 23-valent, conjugate vaccines can only be 7,10, or 13-valent [21].

The CDC recommends pneumococcal vaccination for individuals with risk factors for pneumococcal infection, including young children (aged < 5 years) and adults aged 65 years or older. It is also recommended for patients who are immunocompromised (e.g., HIV, cancer) or have functional or anatomic asplenia, cochlear implants or cerebrospinal fluid leaks. Usually, a dose is enough for adults, while two or more doses for babies because two or more doses are required to achieve sufficient immunity [24, 25].

1.1.6 The Burden of *S. pneumoniae*

According to the World Health Organization (WHO), diseases caused by *Streptococcus pneumoniae* represent a major public health problem worldwide. Young children and the elderly are the most affected age groups in the developing world. It is estimated that about one million children die from pneumococcal disease every year [26]. Since the introduction of pneumococcal vaccines in the 1980s, there has been a significant decline in invasive pneumococcal disease (IPD) amongst young children and adults. According to the Centre for Disease Control (CDC), from 1998 through 2021, IPD rates among children under 5 years of age decreased by 95% overall and 99% for diseases caused by serotypes covered by PCV13. Declines in IPD were seen as early as 2001 among adults, and these declines were due to the introduction of pneumococcal conjugate vaccines in children through community or herd immunity [15].

Despite the introduction of vaccines, the global burden of pneumococcal infections remains. A study that included Austria, Finland, Netherlands, New Zealand, and Sweden found that PCV20 serotypes caused 3000-345,000 disease cases across these countries, resulting in annual costs ranging from $1.3 million

USD to 44.9 million annually. In aggregate, PCV20 serotypes caused 1,234,000 cases and $213.5 million in annual medical costs in children under the age of 5. Despite the success of PCV10 and PCV13 in reducing pneumococcal disease, a considerable clinical and economic burden remains due to serotypes not contained in the current vaccines [27].

The burden of pneumococcal diseases, particularly pneumonia, increases in crowded settings. The Hajj, the world's largest annual gathering in Saudi Arabia, has shown that between 2004 and November 2013, 23% of total admissions to general wards in Makkah were due to pneumococcal infections, while 20% of all ICU admissions were because of pneumonia. In many centres, including in Makkah, strains of *S. pneumoniae* are showing increasing resistance rates to penicillin and several other antibiotics. Pharyngeal swab samples taken from asymptomatic Hajj pilgrims in 2011 and 2012 revealed over 50 serotypes, with the most prevalent serotypes being non-susceptible to antibiotics [28].

1.1.7 The Burden of *S. pneumoniae* in Relation to the COVID-19 Pandemic

Prior to the COVID-19 Pandemic, the prevalence of *S. pneumoniae* invasive diseases was reported in the EU/EUU to be 6.4 cases per 100,000 population in 2018, with an increase in numbers starting in 2014. Age-specific rates were highest in adults aged 65 years or older (18.7 confirmed cases per 100 000 population) and infants under one year (14.4 confirmed cases per 100 000 population), with higher rates reported in males than females. Among all cases in children under five years of age, 75% were caused by a serotype not included in any pneumococcal conjugate vaccine (PCV) [29].

After the COVID-19 Pandemic, the incidence of *S. pneumoniae* invasive diseases in children was examined in England. The COVID-19 pandemic, along with associated lockdowns, social isolation, and other measures, led to significant declines in respiratory infections, including invasive pneumococcal disease (IPD) [30].

IPD cases declined by 30% in England after the first lockdown in March 2020. Cases remained low during the following winter until February 2021, when cases

increased by 8% above the 3-year pre-pandemic mean incidence for February [31].

However, IPD cases increased slowly when the third national lockdown ended in March 2021, after the emergence of the SARS-CoV-2 Alpha variant; case numbers remained 25% lower than pre-pandemic levels by June 2021. By June 2021, case numbers remained 25% lower than pre-pandemic levels, but a proportionately higher increase in cases was observed among children <15 years of age [31].

This global burden of *S. pneumoniae* indicates the significance of more research in infectious diseases, specifically in understanding the immunological responses to infection.

1.2 Innate Immunity

The immune system has evolved to provide a host defence against infection. It can recognize a wide variety of structures found in bacteria, viruses, fungi, and non-living materials such as toxins, chemicals, and drugs.

The body's ability to detect and kill pathogens depends on two types of immune responses. One is innate immunity, the first line of defence against pathogens and relatively 'non-specific'. The other is adaptive immunity, which is more specific and requires longer initiation. Innate immune elements recognise these pathogens through Pattern Recognition Receptors (PRRs); these are molecules designed to bind to Pathogen Associated Molecular Patterns (PAMPs) or Damage Associated Molecular Patterns (DAMPS). DAMPS can be uric acid crystals or cellular proteins. PAMPS include lipopolysaccharide (LPS), bacterial or viral DNA, and RNA. PAMPS can be found in multiple pathogens, making innate immune recognition broader in the spectrum. This feature prevents microbial mutants from escaping innate immune detection and allows the innate immune cells with a limited number of receptors to detect a wide range of antigens.

1.2.1 Components of the innate immune system

The components of the innate immune system include physical barriers, such as skin and mucosal membranes, and effector cells, such as granulocytes, monocytes/macrophages, dendritic cells, natural killer (NK) cells, and innate lymphoid cells[32] (Table 1-3).

Table 1-3. Components of Innate Immune System [32]

COMPONENT	FUNCTION
Barriers	
Skin	prevents microbial entrance
Mucosa	prevents microbial entrance, secretes proteins and enzymes, absorbs metabolic substrates
Effector cells	
Granulocytes	phagocytosis, cytokine production, protein and enzyme secretion, destruction of pathogens
Monocytes/macrophages	phagocytosis, cytokine production, protein and enzyme secretion, destruction of pathogens
Dendritic cells	antigen presentation, phagocytosis, cytokine production, protein and enzyme secretion, destruction of pathogens
Natural killer (NK) cells	lysis of infected and tumoral cells, activation of macrophages through cytokine production
Innate lymphoid cells	mediate immune response and regulate tissue homeostasis and inflammation
Endothelial/epithelial cells	microbial recognition, cytokine production
Antimicrobial peptides	
Cathelicidins (LL-37)	destruction of invading pathogens, immune cells recruitment, enhances wound healing

COMPONENT	FUNCTION
Defensins (α and ß)	Anti-microbial activity, chemotaxis and helps to maintain the integrity of mucosal barriers
Soluble mediators	
Cytokines	
TNF-α, IL-1, chemokines	mediate immune response and inflammation
IFN-α	involved in resistance to viral infection
IFN-γ	involved in resistance to intracellular pathogen infection and activation of macrophages
IL-12	stimulates IFN-γ production by NK cells and T lymphocytes
IL-15	stimulates NK cell proliferation
IL-10	regulates and controls the inflammation process
TGF-ß	regulates and controls the inflammation process
Serum proteins	
Complement system (Particularly Alternative Pathway Proteins, Lectin Pathway Proteins, and Terminal Pathway Proteins (shared by all pathways) C5, C6, C7, C8, amphipathic C9 (MAC components)	opsonization, direct killing of pathogens (by pore formation, cell lysis), B cell activation, inflammation and immune cells recruitment
Granzymes (A, B, K, M, and H) and Perforin-1	pore formation, and apoptosis.
Collectins	opsonization of pathogens and complement activation

COMPONENT	FUNCTION
C reactive protein	opsonization of pathogens and complement activation
Coagulation system	localization of damage or infected tissue
Cellular receptors	
TLRs	recognize a variety of microbial components
NLRs	sense bacterial components present in the cytoplasm
CLRs	recognize sugar moieties of bacteria and fungi
RLRs	sense viral RNA

1.2.2 Pattern Recognition Receptors

These are germline-encoded receptors. They are divided into two types:

1. Membrane-bound Receptors: toll-like receptors (TLRs), C-type lectin receptors (CLRs).

2. Cytosolic Receptors: include nucleotide-binding oligomerization domain-like receptors or NOD-like receptors (NLRs), retinoic acid-inducible gene (RIG)-like receptors (RLRs), and absent in melanoma (AIM2) [33].

As mentioned previously, these receptors detect pathogens and the damage they cause. They are expressed by several cell types, including the immune cells of focus in this study: monocytes, macrophages, and dendritic cells [33]. PRRs bind to different ligands, as listed in (Table 1-3).

1.2.2.1 Toll-Like Receptors:

This study focused on TLRs, which are crucial sensors of microbial elements. Cells prominently expressing TLRs include antigen-presenting cells (APCs), such as dendritic cells and macrophages. There are many TLR types in humans, starting from TLR1 to TLR-10 [34].

- TLRs 1,2,4,5,6 and 10 are located on the cellular membrane.

- TLRs 3,7,8, and 9 are located on intracellular vesicles.

TLRs characterized by the extracellular domains contain variable numbers of leucine-rich-repeat (LRR) motifs and a cytoplasmic signalling domain homologous to that of the interleukin 1 receptor (IL-1R), named the Toll/IL-1R homology (TIR) domain (Figure 1-1) [35].

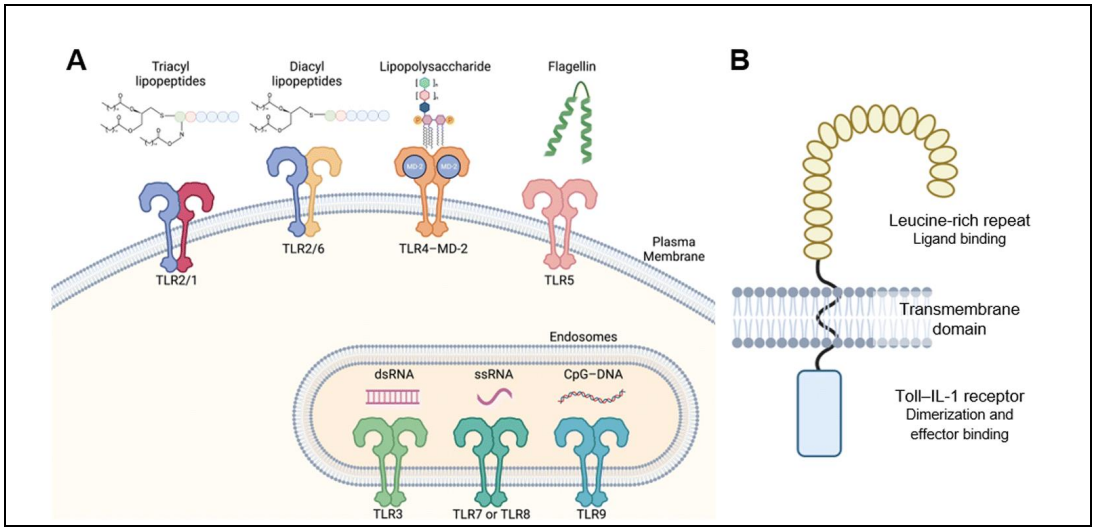

Figure 1-1 Toll-like Receptors and their structure [36].

TLRs bind to a variety of ligands. This study focuses on TLR2 and TLR4. TLR2 binds to Mycoplasma elements, such as diacyl lipopeptides, Mycobacterial elements, Triacyl lipopeptides, and Lipoarabinomannan.

Lipoteichoic acid (LTA) is found in Group B Streptococcus. Peptidoglycan (PG) is a cell wall component in Gram-positive bacteria, and Porins are found in Neisseria. In fungi, TLR2 binds to Zymosan from Saccharomyces cerevisiae, Phospholipomannan from Candida albicans, and Glucuronoxylomannan from Cryptococcus neoformans. among other ligands [33](Table 1-4).

TLR4 binds to Lipopolysaccharides (LPS), which are found in Gram-negative bacteria, Mannan, Candida albicans, and host factors such as Heat Shock proteins 60, 70, and Fibrinogen [33] (Table 1-4).

TLR signalling is facilitated through several adaptor proteins, mainly myeloid differentiation primary response 88 (MyD88), toll/Interleukin-1 receptor (TIR) domain-containing adaptor-inducing IFN-β (TRIF), and TRIF-related adaptor molecule (TRAM). The recruitment of these adaptor proteins triggers downstream signalling cascades that produce pro-inflammatory cytokines [37].

TLRs stimulants of interest in this work are LPS, Zymosan and Gram-Positive bacterial elements.

Among the bacterial cell wall elements recognized by TLRs, LPS is the most effective immunostimulant. A lipid portion of LPS termed “lipid A” is responsible for most of the pathogenic effects associated with Gram-negative bacterial infection, such as endotoxin shock. LPS is released from Gram-negative bacteria by LPS-binding protein (LBP), an acute-phase protein found in the blood, and then binds to CD14, a glycosylphosphatidylinositol (GPI) linked protein expressed on the cell surface of phagocytes. LPS is then transferred to MD-2, which links with the extracellular portion of TLR4, followed by oligomerization of TLR4, a crucial molecule of LPS signalling [38].

LPS used in this thesis is derived from Escherichia coli O111:B4-LPS (1 µg/ml) (Sigma), an enteric bacterium. However, it has been found that different bacteria produce different LPS molecules, which vary in their phosphate patterns, numbers of acyl chains, and fatty acid composition. This variation results in varied biological effects of Lipid A. LPS preparations from non-enteric bacteria, such as *Legionella pneumophila* and *Leptospira interrogans*, which were reported to act as TLR2 but not as TLR4 agonists. However, such results should be interpreted with caution because of the difficulty of removing impurities from LPS preparations [39].

TLR2 recognises gram-positive bacterial elements, such as LTA and PG. The importance of TLR2 in the host defence against Gram-positive bacteria has been exhibited using TLR2-deficient (*TLR2*−/−) mice, which were highly susceptible to infection with *Staphylococcus aureus* or *Streptococcus pneumoniae* [40] .

Table 1-4 TLR Recognition of Microbial Components[33]

Microbial Components	**Species**	**TLR Usage**
Bacteria		
Lipopolysaccharides (LPS)	Gram-negative bacteria	TLR4
Diacyl lipopeptides	Mycoplasma	TLR6/TLR2
Triacyl lipopeptides	Bacteria and mycobacteria	TLR1/TLR2
Lipoteichoic acid (LTA)	Group B Streptococcus	TLR6/TLR2
Peptidoglycan (PG)	Gram-positive bacteria	TLR2
Porins	Neisseria	TLR2
Lipoarabinomannan	Mycobacteria	TLR2
Flagellin	Flagellated bacteria	TLR5
CpG-DNA (5'C-phosphate-G3')	Bacteria and mycobacteria	TLR9
Fungus		
Zymosan	*Saccharomyces cerevisiae*	TLR6/TLR2
Phospholipomannan	*Candida albicans*	TLR2
Mannan	*Candida albicans*	TLR4
Glucuronoxylomannan	*Cryptococcus neoformans*	TLR2 and TLR4
Host		
Heat-shock protein 60, 70		TLR4
Fibrinogen		TLR4

Innate Immune Cells

The effector cells considered in this thesis are monocytes, macrophages, and dendritic cells.

1.2.2.2 Monocytes

Monocytes are an essential component of innate immunity; they are white blood cells derived from common myeloid progenitor cells in a process called haematopoiesis, which is facilitated by macrophage colony-stimulating factor (M-CSF). They later differentiate into macrophages or dendritic cells (Figure 1-2).

Monocytes are found primarily in the bloodstream and serve two main functions: circulating within the body to detect infection or inflammation and organizing the innate immune response to eliminate pathogens. They can also act as antigen-presenting cells to initiate an acquired immune response [41].

In response to a pathogen threat, monocytes are recruited from the circulation, recognizing a PAMP using their toll-like and other innate immune receptors. They can further differentiate into macrophages to enhance host defence. In the circulation, they will undergo apoptosis within a day or two.

Through opsonization, pathogens can be phagocytosed by monocytes, which means coating the pathogen with complement or antibodies that favour its phagocytosis and elimination through specific complement or antibody Fc receptors. Another method for eliminating pathogens is antibody-dependent cell-mediated cytotoxicity [42].

Monocytes have various specific cell-surface receptors, such as CD14 and CD16, which can help identify this lineage. They also possess various innate immune pathogen recognition receptors [43].

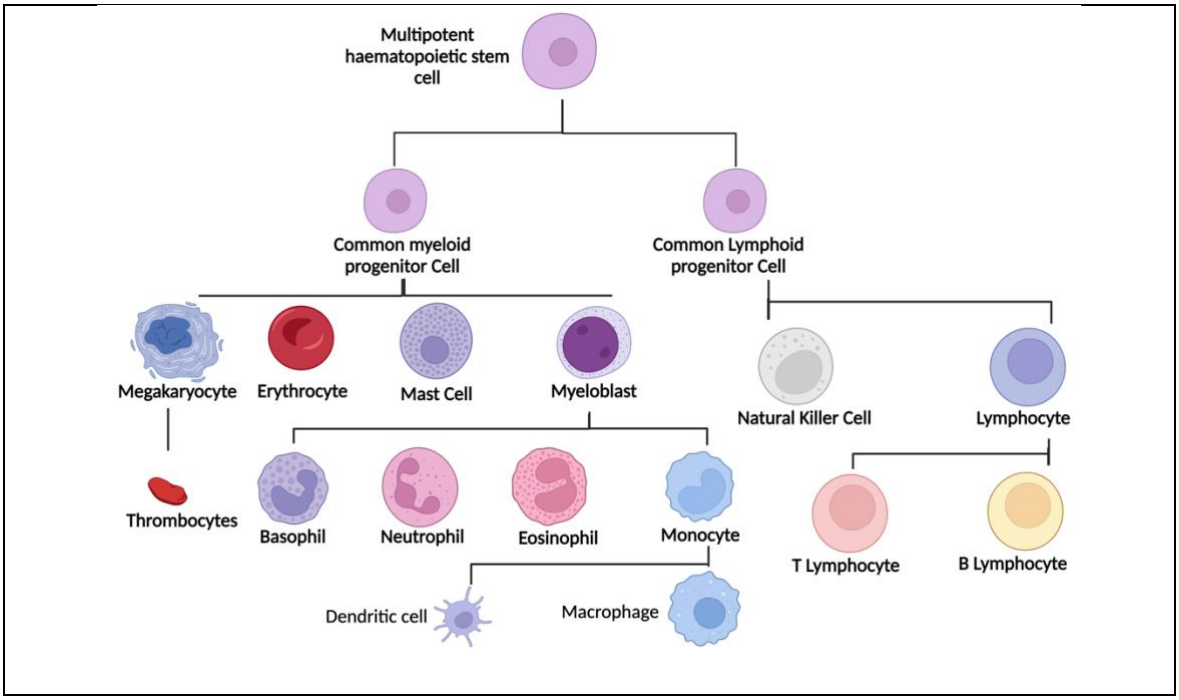

Figure 1-2 Differentiation of hematopoietic stem cells
Figure created by the author using BioRender.com

1.2.2.3 Macrophages

Macrophages are derived from monocytes, and as their name suggests, their function is phagocytosis.
They are found essentially in all tissues and screen for pathogens. Depending on their location, they are given different names, such as Kupffer cells in the liver, microglia in the brain, and alveolar macrophages in the alveoli [44].
Their primary function is phagocytosis, which involves engulfing the micro-organisms and killing them through oxygen-dependent free radicals generation or oxygen-independent mechanisms. Macrophages also possess several receptors needed for microbial elimination, such as Toll-like receptors (TLRs).
TLR1,2,4,5,6 and 10 are all found on its cell surface, while TLR7,8,9 are located within cellular compartments [45].

In addition to eliminating the pathogen, macrophages also function as antigen-presenting cells to naïve T-cells. After engulfing the pathogen, they present peptide antigens on major histocompatibility complex molecules (MHC I and II). $CD8^+$ T cells recognize peptides presented in class I MHC domains, and $CD4^+$ T cells recognize peptides in class II MHC domains through their T cell receptors. However, this binding alone is insufficient to activate T cells, so an additional

co-stimulatory signal and other cytokines produced by macrophages are required. The co-stimulatory signal is achieved by binding the CD28 receptor on T cells to two costimulatory molecules on macrophages, B7-1 (CD80) and B7-2 (CD86). If this occurs, T cells will be activated; otherwise, they will undergo apoptosis or cell cycle arrest.
Once activated, T cells differentiate into different types: CD4+ T cells differentiate into T helper 1 or 2, T helper 17, and T regulatory cells, while CD8+ T cells differentiate into effector T cells. This differentiation process into each type is dependent on cytokines produced by macrophages. Details of antigen presentation and T-cell differentiation are presented in (Figure 1-3) [46].

Another role of macrophages is to lyse tumour cells and maintain tissue re-organization [47]. They secrete several products:

1. Cell differentiation factors
2. Colony stimulation factors
3. Cytotoxic factors
4. Tumour necrosis factor-α
5. Hydrolytic enzymes: collagenase, lipase, phosphatase endogenous pyrogen
6. Interleukin 1
7. Complement components: C1 to C5, properdin, factors B, D, I, H, α-interferon
8. Plasma proteins
9. Coagulation factors
10. Oxygen metabolites: H_2O_2 and superoxide anion
11. Arachidonic acid metabolites: prostaglandins, thromboxanes, leukotrienes

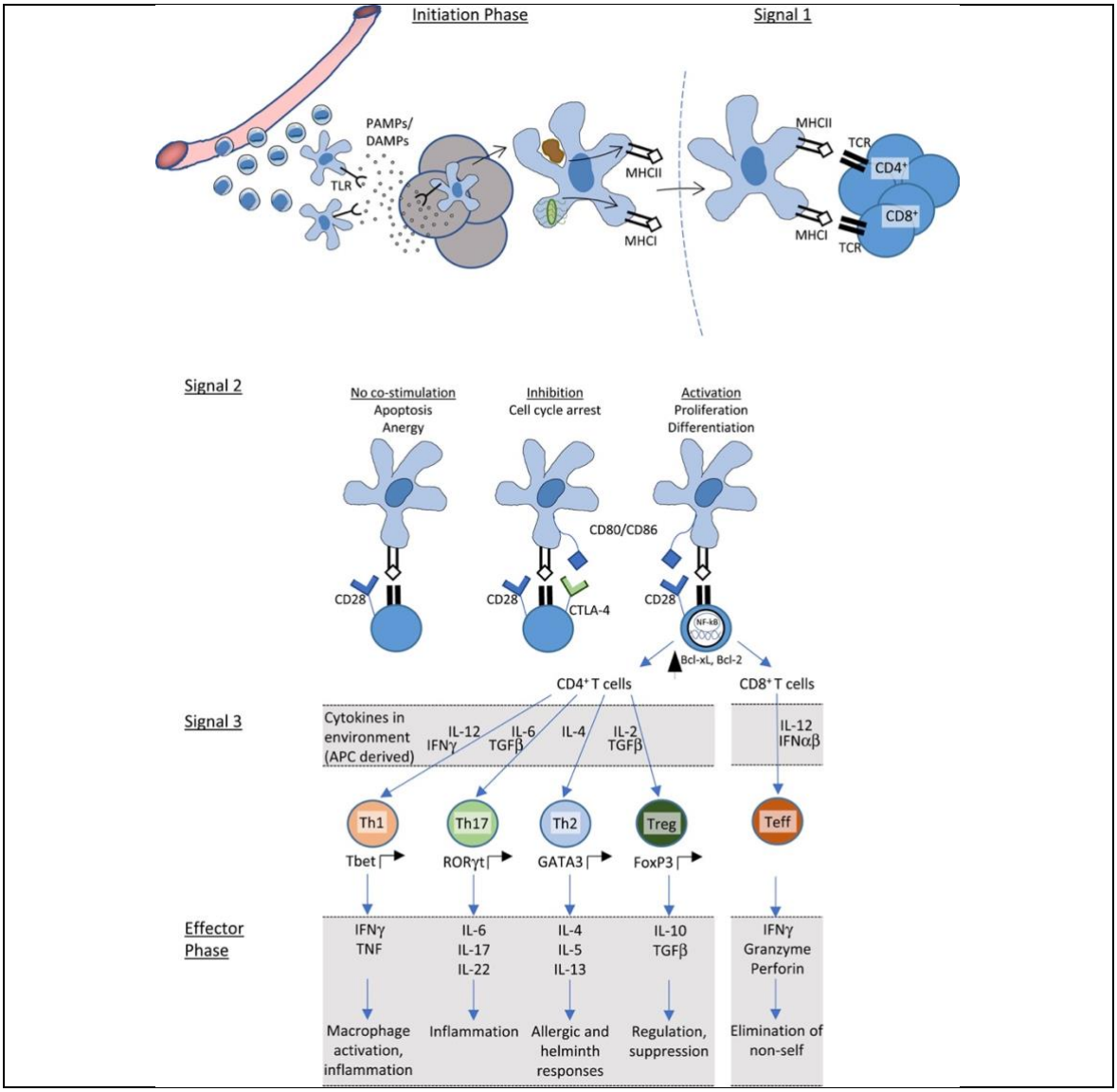

Figure 1-3 T cells Activation and differentiation. [46]

1.2.2.4 Dendritic Cells

Dendritic cells produce projections called dendrites when developed, which is reflected in their name.

They are found in all tissues and also have several types, including conventional dendritic cells (cDC) and plasmacytoid dendritic cells (pDC).

As mentioned previously, conventional dendritic cells stimulate Th1 differentiation by producing IL-12 and IL-18.

Plasmacytoid dendritic cells resemble plasma cells in morphology. They produce a large amount of type I interferons, enabling them to produce potent antiviral responses [48].

Dendritic cells are important antigen-presenting cells to naïve T cells, thus serving as an essential link between innate and adaptive immune responses. This study examined dendritic cells for IFN production following *Streptococcus pneumoniae* infection, as dendritic cells are among the most potent cells in interferon production [49].

1.2.3 Cytokines

Cytokines are small proteins that are essential in cell signalling. They serve their function through attachment to cellular cytokine receptors on target cells. Cytokines include chemokines, interferons, tumour necrosis factors, interleukins, and lymphokines. They are produced by many immune cells. This research focused on the following cytokines: Tumour necrosis factor alpha (TNF-α), Interleukin-1 beta (IL-1ß), and Interferons (IFN-α, IFN-ß, and IFN-γ).

1.2.3.1 Tumour necrosis factor-alpha

Tumour necrosis factor alpha (TNF-α), a 17 kDa protein, is a cytokine that has two forms: the first is a transmembrane form (mTNF-α), while the second is a soluble form (sTNF-α), which is produced from mTNF-α by proteolytic cleavage through the action of TNF-α converting enzyme [50]. The two isoforms bind to different receptors: mTNF-α binds to TNFR1 and TNFR2, while sTNF-α binds to TNFR1 only [51].

While Many cells produce TNF-α, its production predominantly originates from activated macrophages, T lymphocytes, and natural killer (NK) cells. Triggers for TNF-α production include viruses, LPS, Zymosan, bacteria and yeasts.

The primary role of TNF-α is to regulate the immune response. It acts as a pyrogen-inducing fever and causes apoptosis and inflammation. It can inhibit tumorigenesis and viral replication. TNF-α is one of the most critical cytokines in the induction of the clinical condition of sepsis and its associated pathophysiological changes.

The binding of tumour necrosis factor (TNF) to TNF receptor 1 (TNFR1) induces inflammation by activating the nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) signalling pathways, this activation leads to the

transcriptional upregulation of genes encoding proinflammatory mediators such as cytokines and chemokines. TNFR1 activation also indirectly promotes inflammation by triggering cell death. This occurs as a result of a series of reactions that lead to lytic cell death and the release of DAMPs into the cellular surroundings. when detected by neighbouring cells' PRRs, causes them to produce pro-inflammatory cytokines [52].

TNF-α is a growth factor that stimulates the proliferation of hematopoietic and B cells and is also needed for macrophage maturation. TNF-α signalling promotes the survival and differentiation of monocytes into dendritic cells. TNF-α also regulates lymphokine-activated killer T cells' growth, differentiation, and maturation [42].

Another vital role of TNF-α is in the infection with *Mycobacterium tuberculosis*. The bacterium responsible for TB is capable of surviving in an intracellular environment, evading the killing mechanisms of macrophages and remaining alive inside the cell [53]. The pathological hallmark of tuberculosis infection is the formation of a granuloma. Which is thought to contain the infection and prevent its further spread. The tuberculous granuloma typically comprises macrophages, multinucleated giant cells (formed by fusion of macrophages), neutrophils, lymphocytes (CD4+ and CD8+ T cells, and B cells), and occasionally fibroblasts. In human tuberculosis, the granulomas often have a necrotic centre, known as caseous necrosis.
TNF-α plays an essential role in granuloma formation along with IFN-γ. It activates macrophages and mediates apoptosis of the infected cells. Studies have shown that mice deficient in TNF are unable to control *M. tuberculosis* infection, and granulomas do not form properly in their lungs [54].

TNF production has been associated with a variety of human diseases, including Alzheimer's disease, major depression, rheumatoid arthritis, cancer, psoriasis, and inflammatory bowel disease (IBD), This makes it a critical therapeutic target to treat these diseases or controlling their symptoms [55].

1.2.3.2 Interleukin-1 beta (IL-1β)

Interleukin-1 beta (IL-1ß) is a protein with 17.5 kDa size. IL-1ß is a member of the interleukin 1 family of cytokines. Activated macrophages, monocytes, and dendritic cells produce this cytokine.

Similar to TNF-α, IL-1ß is a pro-inflammatory cytokine. It is produced as an inactive precursor called pro-IL-1ß. Pro-IL-1ß, which is then cleaved by a protease named Caspase-1. The activation of caspase-1 occurs through its recruitment to a multi-protein complex (the inflammasome). Following pro-IL-1ß cleavage, mature IL-1ß is rapidly secreted from the cell.

In human monocytes, IL-1ß requires two signals to be released in its active form. First, the gene for pro-IL-1ß needs to be induced, typically through the activation of innate immune signalling receptors or pro-inflammatory cytokines such as TNF-α. Second, the action of the inflammasome allows the processing of the pro-IL-1ß to its active form, which is then released from the cell [56].

Triggers for inflammasome activation are diverse and depend on the complex's molecular makeup. For example, flagellin and Gram-negative Type III secretion system components activate inflammasomes based on the nucleotide-binding domain and leucine-rich repeat (NLR) protein NLRC4. The release of mature IL-1ß requires the action of the pore-forming protein gasdermin-D, which also results in cell death, termed pyroptosis [57].

The IL-1 receptor family has several members. IL-1 binds to IL-1R1, which recruits the IL-1 type 3 receptor (IL-1R3) along with adaptor IL-1 receptor-associated kinase (IRAK) and myeloid differentiation primary response protein 88 (MyD88). IL-1R1 initiates inflammatory responses when binding to the ligands IL-1α and IL-1ß, which are expressed by T-lymphocytes, fibroblasts, epithelial cells, and endothelial cells [58].

IL-1R2 does not initiate signal transduction, is expressed in various hematopoietic cells, and serves as a decoy receptor, and does not initiate signal transduction [59].

IL-1ß promotes the differentiation of monocytes into conventional dendritic cells (DCs) and M1-like macrophages. It also supports the proliferation of activated B lymphocytes and their differentiation into plasma cells. IL-1 with IL-2 promoted the proliferation of NK cells and CD4+ CD8+ T-lymphocytes [60].
IL-1ß produced by activated antigen-presenting cells (APCs) induces type 1 immune responses, which produces cytotoxic T lymphocyte and causes the polarisation of CD4+ T -lymphocytes towards T-helper cell type 1 (Th1) [61].

IL-1ß plays a role in numerous diseases, including rheumatoid arthritis, septic shock, atherosclerosis, Alzheimer's disease, migraine, kidney diseases, endometriosis, and in some tumours like liver, bladder, and gastric cancer [62].

Targeting IL-1ß with neutralizing antibodies has proven effective in treating some autoinflammatory diseases. Canakinumab is a monoclonal antibody that specifically targets IL-1ß and is classified as an IL-1ß inhibitor. it has been successful in treating cryopyrin-associated periodic syndrome (CAPS), systemic onset juvenile idiopathic arthritis and gout [58].

1.2.3.3 Interferons

Interferons (IFNs) are a protein sub-class of cytokines named after their interference with viral replication. There are three main types of IFNs: Type I IFN, Type 2 IFN, and Type 3 IFN.

Type I IFNs consist of several IFNs, such as IFN-α, IFN-ß, IFN-ε, IFN-κ, and IFN-ω. They bind to a common receptor, which is IFNAR [63]. IFNs production is induced by viral PAMPs, amongst other triggers. IFNAR receptor has two subunits, IFNAR1 and IFNAR2. When Type 1 IFNs bind to it, the IFNAR1 subunit interacts with tyrosine kinase 2, and IFNAR2 will interact with Janus-activated kinase (JAK) 1. These interactions will lead to the activation of these molecules. Their activation will result in rearrangement and dimerization of the receptor's subunits, followed by autophosphorylation and activation of classical JAK-STAT (signal transducer and activator of transcription)-signalling pathways.

Type 2 IFN, IFN-γ, binds to the IFNGR, the type II IFN receptor. IFNGR has two subunits, IFNGR1 and IFNGR2. The interaction of IFN-γ with these subunits is

similar to the aforementioned cascade in Type 1 IFNs. However, in the initial step, the IFNGR1 subunit associates with JAK1, while IFNGR2 is constitutively associated with JAK2 (Figure 1-4) [63].

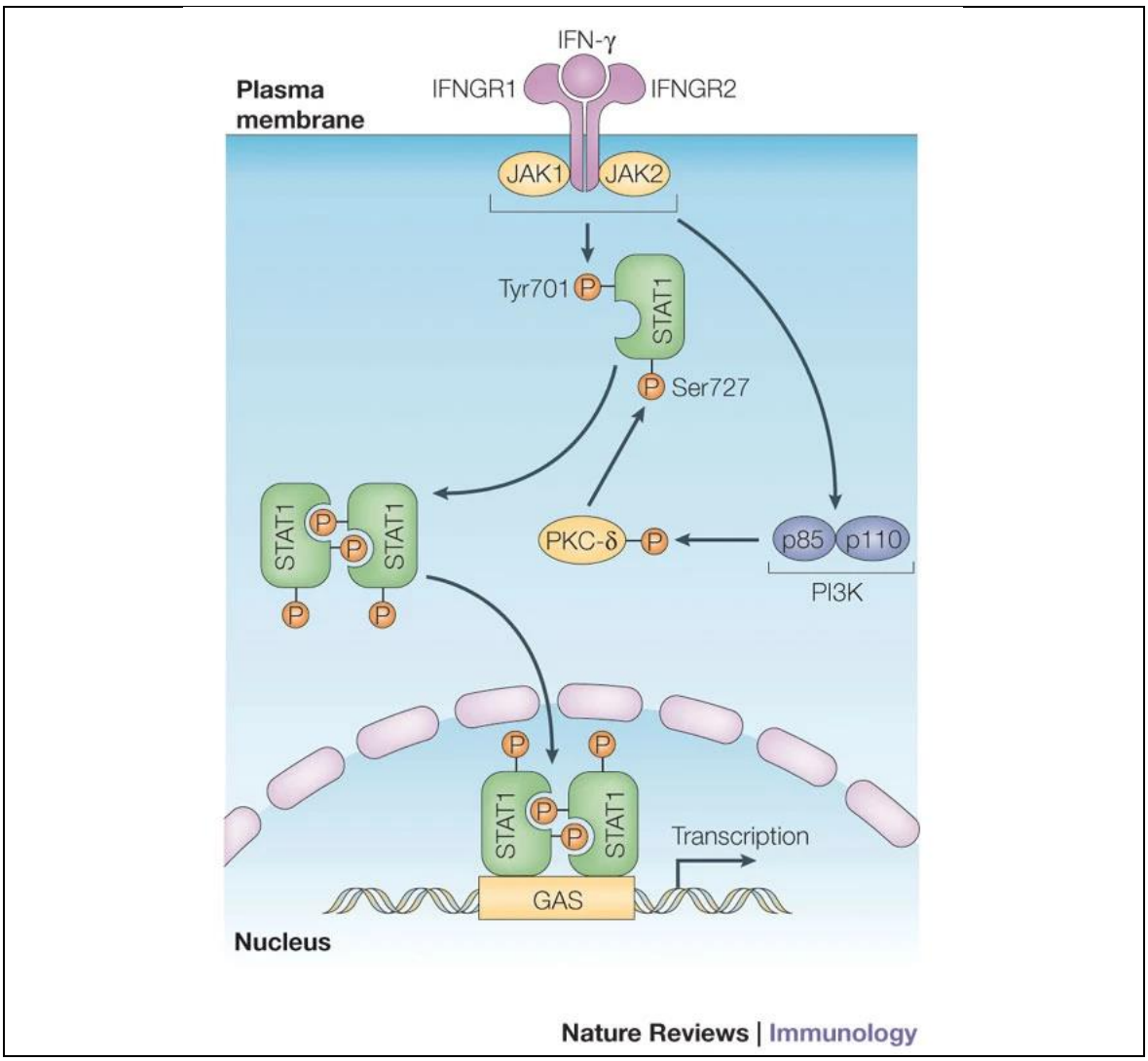

Figure 1-4 Interferon receptors and activation of classical JAK-STAT pathways by type I and II interferons [63].

These signalling pathways stimulate the transcription of IFN-stimulated gene factors, thereby activating macrophages and NK cells by increasing their antigen presentation. This is achieved by stimulating the expression of their major histocompatibility complex (MHC) antigens. Type II interferons are released by cytotoxic T cells and type-1 T helper cells. They prevent the proliferation of type-2 T helper cells, inhibiting a Th2 immune response and further inducing a Th1 immune response. Additionally, they limit viral spread by promoting apoptosis [64].

Type 1 IFNs have a prominent role in viral infections. In this thesis, their role against pneumococcal infection was examined. In pneumococcal infection, it was found that IFN-β increases immune cell recruitment and decreases the transmigration of bacteria to the blood. This is achieved by stimulating the genes that encode tight junction proteins and decreasing the expression of platelet-activating factor (PAF), a cellular receptor that is used by the bacteria to gain access to the host cell [65].

Overall, IFN-β has a protective role against *Streptococcus pneumoniae* infection. Interestingly, while prior expression of IFN-β has a detrimental role in secondary *Streptococcus pneumoniae* infection, it was found that prior IFN-β release leads to decreased release of neutrophil chemokines [66]. Moreso, it suppresses the production of IL-17 by T cells [67, 68], which diminishes neutrophil recruitment and reduces the secretion of anti-bacterial peptides Lipocalin 2 and BPIFA1[69].

The signalling pathway for *S. pneumoniae* starts with the detection of bacterial DNA using Toll-like Receptor 9 (TLR9) [33, 70], which is a membrane-bound receptor that is found on the cell membrane and the endoplasmic membrane within the cell and binds to DNA that is unmethylated at CG dinucleotides. Eukaryotic DNA is typically methylated at CG dinucleotides, making the recognition of unmethylated CG DNA selective for prokaryotic pathogens or viruses. Binding to TLR9 leads to activation of the transcription factors nuclear factor-kB (NF-KB) [71]and Interferon Regulatory Factor 7. After translocating to the nucleus, these will activate the expression of IFN genes, which leads to the production of interferon-alpha (IFNα) that contributes to the inflammatory

response. In addition, they will activate genes involved in tumour progression and cellular stress [72].

After translocating to the nucleus, a sensor that can bind cytosolic dsDNA is the Cyclic-GMP-AMP-synthase (cGAS)[73]. cGAS is an enzyme found in various subcellular compartments that detects DNA [74]. Upon binding to DNA, it will convert guanosine-5'-triphosphate (GTP) and adenosine triphosphate (ATP) to Cyclic guanosine monophosphate- adenosine monophosphate (cGAMP). cGAMP will stimulate an adaptor molecule, the stimulator of interferon genes (STING). which is a transmembrane protein in the endoplasmic reticulum membrane [75]. STING then activates TANK-binding kinase 1 (TBK1). which phosphorylates STING. Together, they recruit IRF3, which thereafter stimulates the transcription of genes encoding type I interferons, particularly IFNα and IFN-ß [76-78] (Figure 1-5). Furthermore, it was found that the cGAS/STING pathway promotes microbial clearance through autophagy [79, 80]. When the STING-TBK1 complex is activated, it leads to the pathogen becoming ubiquitinated and subsequently degraded via the proteasome. After ubiquitination, autophagy markers (P62 and NDP52) are recruited [81, 82], leading to phagophore formation surrounding the bacteria and subsequent removal [83].

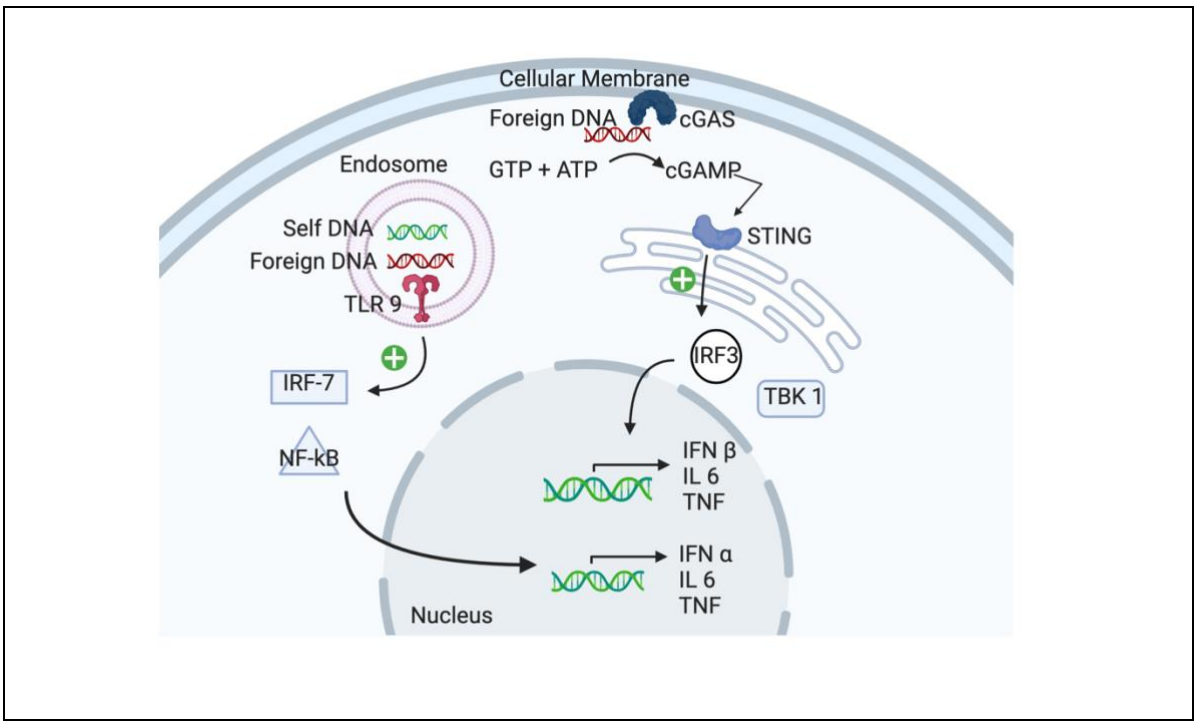

Figure 1-5 cGAS-STING Signaling Pathway.
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1.2.4 Innate immunity against *Streptococcus pneumoniae*

The innate immune response against *Streptococcus pneumoniae* is the focus of this thesis. Like many infections, this response produces both pro- and anti-inflammatory cytokines. This is done to fine-tune the response and ensure there is no overshooting or excessive inflammation. The pro-inflammatory cytokines produced by innate immune cells following infection are shown in Table 1-5.

Table 1-5 Pro-inflammatory Cytokines Secreted Post-*Streptococcus pneumonia*e Infection [84]

Cytokine	Role	Timing	Cell Source(s)
TNF-α	Initiates inflammation, promotes fever, vasodilation, and neutrophil recruitment	Early (within hours)	Macrophages, monocytes, dendritic cells
IL-1β	Fever induction, endothelial activation, promotes leukocyte infiltration	Early (within hours)	Macrophages, monocytes, epithelial cells
IL-6	Acute-phase protein production, B cell stimulation, fever	Early to Intermediate (hours to 1 day)	Macrophages, T cells, endothelial cells
IL-8 (CXCL8)	Neutrophil chemotaxis and activation	Very early (within 1–3 hours)	Epithelial cells, macrophages, fibroblasts
IFN-γ	Activates macrophages, enhances antigen presentation	Intermediate (1–3 days)	NK cells, Th1 $CD4^+$ T cells, $CD8^+$ T cells
IL-17	Recruits neutrophils and supports mucosal defence	Later (2–5 days)	Th17 cells ($CD4^+$), γδ T cells

Shortly after, anti-inflammatory cytokines emerge to lower the immune response and tame it Table 1-6. Some present early after the infection and some later, the balance between the two different groups of cytokines (pro- and anti-) creates the main outcome for the immune response.

Table 1-6 Anti-inflammatory Cytokines Secreted Post-*Streptococcus pneumoniae* Infection [84]

Cytokine	Role	Timing	Cell Source(s)
IL-10	Suppresses TNF-α, IL-1β, IL-6; inhibits Th1/Th17 responses	Intermediate to Late (1–5 days)	Regulatory T cells (Tregs), macrophages, dendritic cells
TGF-β	Immune suppression, promotes tissue repair, induces Tregs	Late (days to weeks)	Tregs, macrophages, epithelial cells
IL-1Ra	Competitively inhibits IL-1 signalling, reducing inflammation	Early to Intermediate	Monocytes, macrophages, neutrophils

1.3 Trained Immunity and Epigenetic Changes

Since the 1940s, it was believed that immunological memory is a characteristic of adaptive immune response, not innate immunity. However, this concept has changed in recent years. as several studies examined innate immune cells and found that they possess some adaptive-like characteristics and can develop memory[85, 86]. This memory provides a stronger immune response following a re-infection with the same pathogen and sometimes against other pathogens. In other cases, it gives a lower immune response following an infection [87].

The term “Trained immunity” was given to this phenomenon. Trained immunity is the functional reprogramming of innate immune cells produced by external or internal factors. This reprogramming could result in an altered immune response against a second infection after the cells have returned to their non-active state[87]. The secondary immune response can be either more or less intense than the primary response. The nature of secondary response is dependent on several factors, including the cytokines produced, which can be either pro-inflammatory or anti-inflammatory, as well as the timing of the response, as previously mentioned [84]. Figure 1-6.

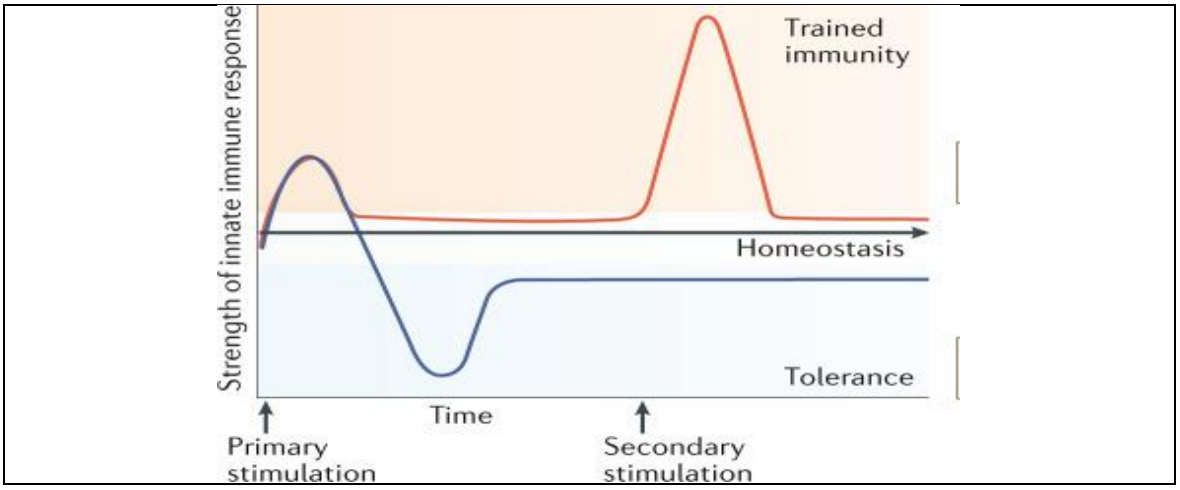


Figure 1-6 Primary and Secondary Innate Immune Responses, and Trained Immunity[87]

There are two types of trained immunity: central trained immunity which occurs in bone marrow progenitor cells, and peripheral trained immunity that occurs in monocytes and macrophages. For example, ß-glucan (fungal cell wall element) is one of the stimuli used to elicit trained immunity, it was found that it can promote the expansion of myeloid progenitors in hematopoietic stem cells [88] Another stimulus is BCG vaccine; it was found to reprogram hematopoietic stem cells toward myelopoiesis in the bone marrow [89].
These changes were found to arise from epigenetic changes rather than genetic recombinational changes, as observed in adaptive immune cells [90]. Epigenetic changes refer to heritable alterations in gene expression that do not result from a change in the gene's DNA sequence [91].

Chromatin, the mixture of DNA and histone proteins in eukaryotic cells, is highly condensed in the normal state. Following various stimuli, including infection, the chromatin structure undergoes several chemical changes, such as acetylation or methylation of its histones. As a result, the chromatin becomes loose. Once the chromatin becomes loose, the gene promoter segments of the DNA become exposed to transcription, and further translation of these genes into proteins is necessary for the immune defence to take place. Nonetheless, once the infection is cleared, the chromatin may not completely return to its initial state, with some changes persisting that affect the immune response against subsequent infection Figure 1-7 [92].
These epigenetic changes are reversible and short-lived, lasting from 3 months to a year but not for a lifetime, as seen in adaptive immune memory [93, 94].

Epigenetic changes can be achieved through several mechanisms. One is DNA methylation; where the addition of a methyl group to a DNA segment prevents proteins from attaching to it, thereby blocking that segment. Another mechanism is via histone modification, whereby chromatin becomes tightly wrapped around a histone so it cannot be read. using a non-coding RNA or "silent RNA" is another mechanism that has a complementary segment to the gene we wish to silence; by binding to the mRNA segment, it blocks its translation into that particular protein[95].

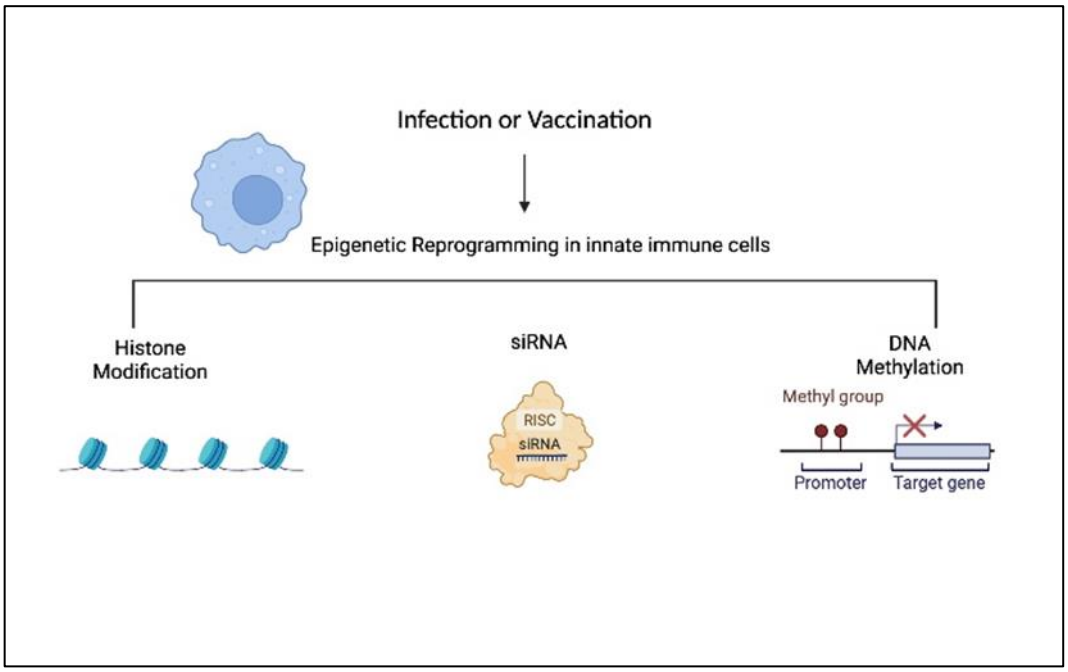


Figure 1-7 Epigenetic changes following infection.

Figure created by the author using BioRender.com

Epigenetic changes can be transgenerational. In a study by Katzmarski *et al.*, the progeny of male mice exposed to sub-lethal doses of *Candida albicans* exhibited a better immune response against subsequent *C.albicans* infection, with increased levels of pro-inflammatory cytokines INF-γ and TNF-α compared to the progeny of male mice that were not exposed to *C.albicans* [96].

1.3.1 Trained Immunity and Timing

To train innate immune cells (monocytes, macrophages, and dendritic cells), it takes time. Usually, to produce trained cells, the following phases occur [95, 97, 98]:

- Initial Priming Phase: During this phase, immune cells need to be primed. In this thesis, the cells were primed using immune stimulants such as beta-glucan, zymosan, and LPS. The priming phase generally lasts between 0 and 24 hours.

- Resting Phase: It typically lasts between 1-7 days following the initial priming. During this period, innate cells revert to their baseline cytokine levels but stay primed. They may exhibit altered chromatin accessibility and increased cytokine gene expression. Haematopoietic progenitor cells in the bone marrow might also be reprogrammed, indicating central trained immunity.

- Secondary Challenge Phase: In reality, this can occur at any time following a challenge after the resting phase between weeks and months, but in our experiments, it was conducted 24-48 hours after priming. Upon the secondary challenge, trained cells generally rapidly produce higher levels of pro-inflammatory cytokines (TNF-α, IL-6, IL-1ß). This leads to increased phagocytosis, reactive oxygen species (ROS) production, and killing efficiency. Neutrophil recruitment is amplified due to enhanced chemokine secretion. Faster bacterial clearance is usually observed 1-6 hours after exposure [95, 97, 98].

1.3.2 Stimulants of trained immunity

Examples of stimulants of trained immunity include fungal cell wall elements such as beta-glucan and zymosan, LPS from gram negative bacteria and the BCG vaccine for tuberculosis among many others[99].

1.3.2.1 Beta glucan (β-glucan)

Beta-glucan (ß-glucan) is a significant component of the fungal cell walls. In one study by Qunitin *et al.*, it was found that ß-glucan obtained from the fungus *Candida albicans* provided protection against *C. albicans* reinfection and increased cytokines secretions (TNF-α and IL-6). These effects were achieved through epigenetic reprogramming and upregulation of histone tri-methylation H3K4me3 [100, 101]. The study by Hetland et al. showed that ß-glucan was

protective against pneumococcal infection in mice. Bacterial levels in the blood were significantly lower in mice exposed to ß-glucan and they showed better survival rates [102]. Another study found that treatment with the fungal ligand ß-glucan protected mice against subsequent infection with *Staphylococcus aureus* [103].

1.3.2.2 Zymosan

Zymosan is another cell wall component derived from the fungus *Saccharomyces cerevisiae*. It activates macrophages through Toll-like Receptor 2 (TLR-2). Both ß-glucan and Zymosan are recognised by Dectin-1, a phagocytic receptor found on macrophages and dendritic cells [104, 105].

1.3.2.3 Lipopolysaccharide (LPS)

Lipopolysaccharides (LPS) are large molecules composed of a lipid and a polysaccharide. found in the outer membrane of Gram-negative bacteria. They have been shown to induce a lower inflammatory response in re-infection "immune tolerance" [106]. LPS-activated macrophages induce certain changes that upregulate the metabolite itaconate, which produces anti-inflammatory activity and counteracts induced trained immunity produced by ß-glucan [107].

1.3.2.4 Bacillus Calmette-Guérin (BCG) vaccine

The Bacillus Calmette-Guérin (BCG) vaccine is a live-attenuated vaccine against *Mycobacterium tuberculosis*. and serves as another stimulator of trained immunity. In a study by Kleinnijenhuis et al., blood samples were collected from volunteers prior to receiving the BCG vaccine, 2 weeks after vaccination, and 3 months after vaccination. Peripheral blood mononuclear cells were isolated and then infected with *M. tuberculosis*, *S. aureus* and *C. albicans* to assess the production of proinflammatory cytokines (IFN-γ, TNF-α, and IL-1ß). The results showed that BCG vaccination increased the nonspecific production of proinflammatory cytokines (IFN-γ, TNF-α, and IL-1ß) in response to the aforementioned infections after BCG vaccine administration [97].

In other studies, it was shown that the BCG vaccine provided immunity against other micro-organisms causing other infections such as yellow fever and malaria [108]. It has also reduced the incidence of respiratory tract infections (including community- and hospital-acquired pneumonia) in elderly patients who were given BCG at birth and another dose at 65 years or more [109]. BCG vaccine is approved by the US FDA to be used as treatment for bladder cancer and to treat other malignancies such as lymphoma and melanoma [108, 110].

1.3.3 Possible Benefits and Uses of Trained Immunity

Regulating trained immunity can be a potent therapeutic approach in different disease contexts. Depending on the condition, inducing trained immunity could be beneficial in assisting specific cancer therapies or in managing the immune paralysis associated with sepsis. Other therapeutic targets involve inhibiting an excessively trained innate immune state in chronic inflammatory diseases or inhibiting the detrimental trained immunity in organ transplantation [111]. In Africa, it was found that administering the BCG vaccine to low-weight African children at birth, rather than in the subsequent weeks or months, was associated with a one-third decrease in neonatal mortality [112].

In the context of cancers, the BCG vaccine has been developed into a novel immunotherapy for cancer treatment, as mentioned previously [108, 110]. ß-Glucan has been used in Asia to boost immune responses in cancer patients, such as those with breast cancer. BCG vaccine is currently in clinical trials in the USA in combination with immune checkpoint inhibitors as a treatment for cancers [113]. Another therapeutic strategy involves injecting tumours with immunostimulatory agents such as activators of NLRP3, STING, Toll-like receptors and RIG1 pathways [114].

1.3.4 Trained Immunity in *Streptococcus pneumoniae* Infection

A few studies have been conducted to understand the role of trained immunity on *Streptococcus pneumoniae* infection. In one of the studies, mice were exposed to intranasal ambient amounts of LPS. 6 days later, they were infected with *Streptococcus pneumoniae*. The alveolar macrophages were isolated and studied, and the results showed that mice exposed to LPS had an enhanced

reactivity to pneumococci. LPS-pretreated mice displayed enhanced bacterial clearance and reduced lung tissue inflammation 48 hours after infection. Additionally, LPS-exposed alveolar macrophages secreted higher amounts of multiple cytokines and chemokines, including C-X-C Motif Chemokine Ligand (CXCL)-1, interleukin (IL)-1β, IL-10, IL-12p40 and IL-6 compared to control (saline-exposed) alveolar macrophages [115]. Interestingly, the IL-6 responses of alveolar macrophages remained elevated two and six weeks after intranasal LPS treatment. This indicates long-lasting cellular reprogramming following ambient LPS exposure [115]. Another study examined the role of bacterial flagellin derived from *Salmonella enterica* serovar Typhimurium on pneumococcal infection outcomes. Mice were divided into two groups; the first received coadministration of flagellin alongside *S. pneumoniae*, while the second was infected with *S. pneumoniae* only. It was found that animals receiving both flagellin and *S. pneumoniae* intranasally had significant reductions of bacterial counts in their lungs during the first 24 hours after the challenge compared to those that received *S. pneumoniae* alone. In addition, their survival rate increased by 75%-100% in different experiments [116]. To study the role of flagellin pre-treatment, a group of mice received flagellin intranasally 12- 24 h before pneumococcal challenge. These animals all survived, while all control mice died by day 4 [116]. Collectively, these results suggest that flagellin can locally activate innate immunity and increase resistance to acute pneumonia. Flagellin mucosal treatment improved *S. pneumoniae* lung clearance and promoted survival after infection [116].

The flagellin effect observed is thought to be attributed to TLR5 signalling, as demonstrated in this study [116] [117] using a flagellin mutant that is unable to signal through Toll-like receptor 5 (TLR5) [116]. It was found that flagellin induced neutrophil infiltration into the airways and upregulated the expression of genes coding for interleukin 6 (IL-6), tumour necrosis factor-alpha (TNF-α), CXCL1, CXCL2, and CCL20. The mechanism of flagellin protection is believed to exclude T and B cells. This is because when SCID mice were studied, they cleared the pneumococcal challenge similarly to immunocompetent animals. This suggests that the mechanism is primarily reliant on innate immunity [118].

1.4 Control of DNA Transcription and Translation

Inflammation serves as the first line of defence against infection and is essential for repairing and restoring damaged tissues. However, uncontrolled or prolonged inflammation can be detrimental to the body, leading to diseases such as cancer and atherosclerosis. Therefore, coordination and organisation of this inflammatory process are crucial [119].
Acute Inflammation begins when germline-encoded pattern recognition receptors (PRRs), located in cellular compartments, bind to microbial elements. Once activated, these receptors trigger signalling cascades that lead to activating transcription factors. These factors regulate gene expression that is involved in the immune response, such as genes for phagocytosis, cell migration, and tissue repair [120] .
Several genes are regulated by transcriptional “on” and “off” controls. Multiple levels of regulation ensure an adequate and appropriate inflammatory response. These include chromatin state, histone or DNA modifications, and recruitment of transcription factors to control gene expression [121]. Although transcription is an essential first step, the accurate regulation of immune genes also involves several additional post-transcriptional checkpoints. These occur at the level of mRNA splicing, mRNA polyadenylation, mRNA stability, and protein translation [120].

In regular transcription, a gene consists of both introns and exon segments. The exons are the coding segments of the DNA transcript, while the introns are the non-coding segments. The gene then undergoes mRNA splicing, whereby the introns will be removed, leaving only the exons in the final mRNA product. These exons code for amino acids that, when combined, form a protein, which is the final translation product (Figure 1-8).

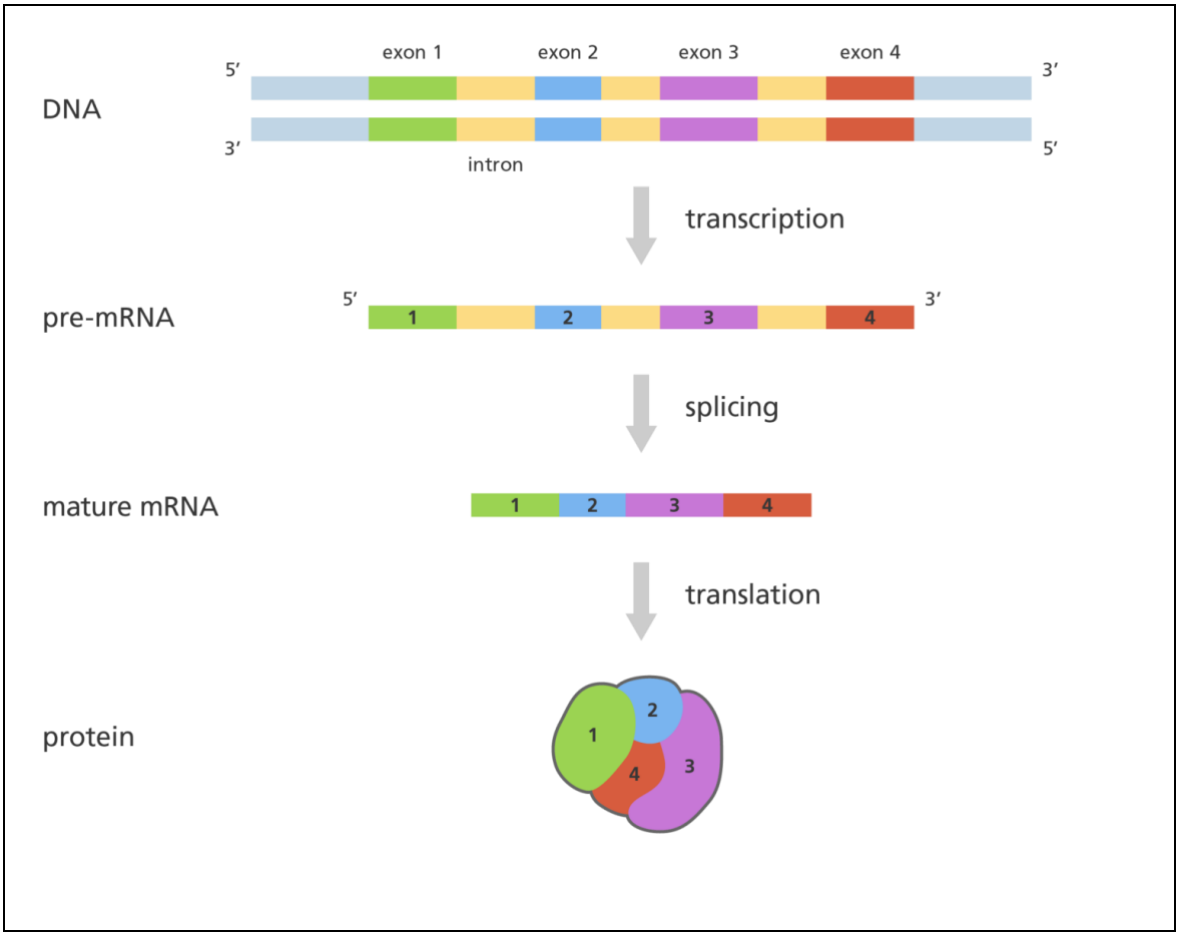


Figure 1-8 DNA Transcription and RNA Splicing.
In regular transcription, the gene consists of introns and exons segments. The exons are the coding segments of the DNA transcript, and the introns are the non-coding segments. The intron's function is to control the gene expression. The gene will then undergo mRNA splicing, in which the introns will be removed, and the final mRNA product contains exons only. These exons will code for amino acids that, when combined, will form a protein, which is the final translation product. Figure Adapted from [122]

1.4.1 Control of DNA Transcription and Pre-mRNA Processing

1.4.1.1 Alternative mRNA Splicing

Polyadenylation is defined as the process of adding a poly(A) tail to an RNA transcript, typically mRNA. The Poly(A) tail consists of multiple adenosines. A method of post-transcriptional control is alternative mRNA splicing. Usually, in transcription, the introns' splice sites are recognized by small nuclear ribonucleic particles (snRNPs) U1 and U2. More so, a poly(A) tail is added to the 3' end of transcripts. These poly(A) tail signals and nearby U-rich or GU-rich downstream sequence elements (DSEs) are detected by two multiprotein complexes: cleavage and polyadenylation specificity factor (CPSF) and cleavage stimulation factor (CSTF). These protein complexes promote cleavage of the

pre-mRNAs. Poly(A) polymerase (PAP) consequently catalyzes the addition of a stretch of adenosines from the cleavage site [123]. One study has shown that more than 94% of human genes are subject to alternative splicing and/or alternative polyadenylation [124]. Such mechanisms are extensively involved in TLR signalling. The TLR signalling pathway is subject to broad post-transcriptional regulation, in which more than 256 alternatively processed transcripts encode variants of receptors, adaptors, and signalling molecules. Every TLR gene has numerous alternatively spliced variants[125], and TLR1 to TLR7 all have between two and four predicted alternative polyadenylation sites [126].

LPS stimulation enhances the expression of soluble TLR4 (smTLR4) by macrophages, and overexpression of smTLR4 inhibits LPS-mediated activation of nuclear factor-κB (NF-κB) and the production of tumour necrosis factor (TNF) [127].

An analogous TLR4 mRNA isoform, which contains a premature stop codon is upregulated following LPS stimulation of human monocytes. Induction of this isoform is significantly lower in monocytes from cystic fibrosis patients who, compared with healthy controls, produce more TNF in response to LPS. These findings suggest that the production of a truncated form of TLR4 generates negative feedback that limits excessive inflammation [128].

Another component of this negative feedback mechanism is the TLR4 co-factor MD2. Shortened MD2 isoforms have been described in human monocytic cell lines. The mRNA encoding the human MD2s variant lacks all of exon 2. MD2s expression is upregulated by LPS, as well as by IFNγ and interleukin-6 (IL-6). MD2s proteins bind TLR4 as competently as full-length MD2. But they fail to facilitate signalling. MD2s inhibits the binding of full-length MD2 to TLR4. Thus, these shortened forms of MD2 inhibit macrophage stimulation by LPS. Together, these results suggest that the production of altered forms of either TLR4 or MD2 modulate macrophage responses to LPS and bacterial pathogens [129].

Another spliced variant that affects TNF-α production in response to LPS stimulation is Interleukin-1 receptor-associated kinase 1 c (IRAK1c). IRAK-1 is an enzyme that plays a role in TNF production in response to LPS. IRAK1c lacks exon

11 and has no kinase activity. It suppresses both NF-κB activation and TNF production in response to LPS [130].

1.4.1.2 mRNA Stability

Cellular mRNA levels are established by both mRNA production and degradation. Analysis of gene transcription and RNA decay rates showed that increases in RNA levels are provoked by pro-inflammatory stimuli that produce changes in transcription rates, while the duration of these responses is controlled by the rate of RNA decay [131]. In macrophages, LPS stimulation of TNF showed a negative correlation between the rate of transcript induction and intrinsic mRNA stability [132].

LPS treatment of DCs modifies the stability of 6% of the expressed mRNAs. Interestingly, the affected transcripts are enriched for inflammatory and immune signalling genes, as well as NF-κB targets. These results indicate that the regulation of mRNA degradation plays a vital role in influencing innate immune responses [131].

1.4.2 Control of mRNA Translation

Another method to shape the inflammatory response is by inducing changes at the translational levels. which has to do with the modification of the translation of pre-existing mRNAs. This provides faster responses, e.g. LPS stimulation of DCs induces an immediate and massive increase in new protein synthesis within the first 60 minutes [133].

1.4.2.1 Regulation of translation initiation factor activity

Eukaryotic translation initiation factor 2 (eIF2) is a regulator of innate immunity. eIF2 forms a complex with the initiator methionyl-tRNA and a molecule of GTP, which binds to the 40S ribosomal subunit, this complex is essential for start codon recognition and recruitment of the 60S ribosomal subunit [134]. Upon positioning the 40S subunit at the start codon, eIF2 hydrolyses the bound GTP, which causes the release of eIF2 from the ribosome. The resulting eIF2–GDP is then recycled to form a new ternary complex that is ready for a new round of

translation. Phosphorylation of eIF2 is triggered by double-stranded RNA, among other causes. The phosphorylation of eIF2 causes global translational repression of most cellular and viral mRNAs. Suppression of translation mediated by eIF2 phosphorylation is beneficial during viral infections. As it inhibits the production of new viral proteins and limits viral spread [135].

1.4.2.2 Regulation by mTOR and 4EBPs

Mammalian target of rapamycin (mTOR) is a serine/threonine kinase that responds to many cellular stimuli, including TLR ligands. LPS activation of macrophages leads to mTOR-dependent 4EBP phosphorylation, which induces the translation of TNF, IL-6 and CXC-chemokine ligand 1 (CXCL1) [136]. In unstimulated cells, 4EBPs act as negative regulators of innate immunity. They regulate the expression of IFN-regulatory genes and help prevent an excessive innate immune response against pathogens. inactivation of mTOR by the *Leishmania* spp. Protein Leishmanolysin leads to translational repression of macrophage transcripts, which is significant for pathogen survival [137]. Other studies have shown that activation of mTOR by bacterial infections was found to stimulate the expression of pro-inflammatory genes. Infection of macrophages with a virulent strain of *Legionella pneumophilia* leads to mTOR ubiquitylation and degradation, thereby suppressing its function [138].

1.4.2.3 Regulation of poly(A) length.

The poly(A) tail located at mRNA 3‘ ends has an essential role in translation by serving as a binding site for poly(A)-binding protein (PABP).
In unstimulated macrophages, TNF mRNA is expressed, but it lacks a poly(A) tail and fails to participate in the translation machinery and cannot produce TNF protein [139]. However, following LPS stimulation, TNF transcripts acquire poly(A) tails, which activate their translation, enabling the fast and abundant expression of TNF protein. Similarly, in resting memory CD8+ T cells, expressed mRNA that encodes CC-chemokine ligand 5 (CCL5) lacks a poly(A) tail and is translationally repressed until the T cell receptor is re-engaged. This re-engagement triggers poly-adenylation of the pre-existing pool of CCL5 mRNA, Facilitating rapid translation and CCL5 protein secretion [140].

Other controls of translation include alternative translation initiation pathways, gene-specific regulation, regulation by GAIT complex, and regulation of translation elongation. The abundance of these control measures highlights the importance of fine-tuning the immune response to avoid over or under-stimulation[120].

1.5 Hypothesis

The hypothesis underlying this project is that treatment of innate immune cells by inflammatory modulators will result in functional reprogramming of the cellular response to infection, leading to altered responses on infection with *Streptococcus pneumoniae*, consistent with induction of trained immunity and epigenetic modification of the immune cell genome. We hypothesise that innate immune priming (trained immunity) enhances pro-inflammatory cytokine responses to *Streptococcus pneumoniae* through epigenetic and post-transcriptional mechanisms.

1.6 Aim

This project aims to test this hypothesis by measuring the effect of pre-treatment of innate immune cells with inflammatory mediators on subsequent innate immune cell response to infection with Streptococcus pneumoniae. I will determine whether altered responses to infection by such treatments are accompanied by changed expression of key innate immune response genes, together with altered gene CG methylation status, as well as any changes in signal transduction pathways.

1.7 . Objectives

This study aims to investigate the innate immune response to *Streptococcus pneumoniae*, with a particular focus on cytokine production and the potential for trained immunity. The specific objectives are:

1. To determine whether infection with *Streptococcus pneumoniae* (TIGR4 strain) induces the production of cytokines, including interferons (IFNs),

TNF-α, and IL-1β, in bronchial epithelial cells, primary monocyte-derived dendritic cells, and macrophages.

2. To assess whether pre-treatment with immune stimulants, including fungal cell wall components (β-glucan and zymosan) or lipopolysaccharide (LPS), alters cytokine production upon subsequent exposure to *Streptococcus pneumoniae*, consistent with the concept of trained immunity.

3. To investigate the mechanisms underlying altered responses, by determining whether the effects of pre-treatment occur at the transcriptional or post-transcriptional level and if they affect the epigenetic CG methylation status of key innate immune cytokine genes.

4. To identify key cellular proteins and signalling pathways involved in the innate immune response to *Streptococcus pneumoniae* infection and whether these are altered by pre-treatment with inflammatory mediators.

Results of these investigations will allow a better understanding of the innate immune response to infection with *Streptococcus pneumoniae,* and how this can be shaped by previous inflammatory stimuli. Such stimuli could then be utilised in enhancing immune responses to vaccination against *Streptococcus pneumoniae*.

2 Materials and Methods

2.1 Cell Culture

2.1.1 Human Bronchial Airway Epithelial Cell Line, normal lung, University of Iowa (Nu-Li1)

The research in this thesis was conducted using the Bronchial Airway Epithelial Cell Line, normal lung, University of Iowa (Nu-Li1) for the first objective. The objective aimed to determine whether bronchial epithelial cells infected with *Streptococcus pneumoniae* TIGR4 lead to the production of cytokines such as IFNs, TNF α, or IL-1β.

These immortalized cells, which exhibit epithelial morphology, were isolated from the normal lungs of a 36-year-old male. This cell line may serve as a valuable model for studying Chloride (Cl^-) ion transport physiology, therapeutic interventions for cystic fibrosis, and innate immunity. NuLi-1 cells were selected for the *Streptococcus pneumoniae* infection model due to their origin as human airway epithelial cells, which closely mimic the natural site of respiratory infection. Their well-differentiated epithelial characteristics provide a relevant and physiologically meaningful in vitro system to study host-pathogen interactions specific to the respiratory tract. The cells were gifted to us by Dr. Andrew Bowie from Trinity College, Dublin. Cells were shipped on dry ice and thawed immediately upon arrival. The cell culture flasks were coated with placental collagen type 4 (60 µg/ml) (Sigma) to enhance cellular attachment, following the Nu-Li1 ATCC protocol.

After thawing the cells, Airway Epithelial Cell Basal Medium (ATCC® PCS-300-030) was added, supplemented with the Bronchial Epithelial Cell Growth Kit (ATCC® PCS-300-040). The cells were then centrifuged at 200 G for 6 minutes at room temperature. The supernatant was discarded, and the pellet was resuspended in fresh media before being transferred into a cell culture flask. The tissue culture was placed in the incubator overnight at 37°C in a 5% CO_2 and 95% air atmosphere. Cell handling, subculturing, and cryopreservation were performed following the Nu-Li1 ATCC protocol. Mycoplasma testing was not conducted following receipt.

2.1.2 Human Bronchial Epithelial Cell Line (BEAS-2B)

This normal human bronchial epithelium is derived from autopsies of non-cancerous individuals and has been used to study pneumococcal infection. These cells, like NuLi-1 cells were chosen for the *Streptococcus pneumoniae* infection model due to their origin as human airway epithelial cells, which closely mimic the natural site of respiratory infection. This cell line was cultured in BEGM™ (Bronchial Epithelial Cell Growth Medium BulletKit)™ (Lonza) at 37 °C and 5% CO_2. The cells were gifted from the University of the West of Scotland, transported in dry ice, and thawed immediately upon arrival. Handling was done according to a protocol provided by ATCC®. Mycoplasma testing was not conducted following receipt. After thawing and passaging, the cells were inoculated with the selected strains of *Streptococcus pneumoniae*.

2.1.3 Human U937 Cell Line:

U937 is a cell line (CRL-1593.2 -ATCC) which exhibits a monocyte morphology that was derived in 1974 from malignant cells obtained from the pleural effusion of a 37-year-old white male patient with histiocytic lymphoma.

The U937 cell line was used in this study because primary cells exhibited variability and inconsistent results, which limited reproducibility. U937 cells provide a standardized, well-characterized model that reliably mimics key aspects of monocyte/macrophage behaviour during bacterial infection, making them a practical choice for controlled infection experiments. The cell line was tested for *Mycoplasma* for quality control testing before its purchase and delivery, according to the supplier.

Upon receiving the frozen cell vial, the cells were thawed in a 37°C water bath, washed with complete tissue culture media [(RPMI 1640 with L-glutamine and sodium bicarbonate (R8758-500ml, Sigma), 10% heat-inactivated foetal bovine serum (Invitrogen), and 1% Penicillin-streptomycin (Gibco)], spun at 100 × g for 7 minutes, and resuspended in complete tissue culture media again. The cells were incubated subsequently at 37°C and 5% CO_2. The cells were maintained at a density of 1×10^6 viable cells/ml. Fresh media were added to the cell culture every 3 days or less depending on cell density.

More so, Cryopreservation was carried out using a freezing media consisting of 90% FBS, 10% DMSO at a cell density of 1×10^7 cells/ml. Cells were initially frozen at -80°C for 24-48 hours and then moved into liquid nitrogen for long-term storage. All cell handling was conducted under aseptic conditions in a Biosafety level 2 containment hood.

2.1.4 PMA Induction:

Phorbol 12-myristate 13-acetate (PMA) (InvivoGen) also known as 12-O-tetradecanoylphorbol13-acetate (TPA), is a specific activator of protein kinase C(PKC), which activates nuclear factor-kappa B(NF-κB). NF-κB is a transcription factor that regulates numerous physiological functions and plays a vital role in the pathogenesis of various diseases. It has been identified as a potential therapeutic target in inflammatory processes, cancer, and autoimmune diseases[141]. PMA activates U937 monocytes leading to their differentiation into macrophages. Activation was achieved by incubating the cell culture with phorbol myristate acetate (PMA) for 24 h at 37°C. PMA working concentration was 100 ng/ml.

2.1.5 Human Primary Monocyte-derived Dendritic Cells and Macrophages:

Primary cells were used in this study due to their closer physiological relevance and more accurate reflection of *in vivo* cellular responses during infection, thereby providing a more faithful model of host-pathogen interactions. These were primary cells isolated using leukocyte cones (sourced from NHS blood bank, Newcastle, UK). The blood was layered on Ficoll-Paque PLUS (GE Healthcare) and then spun at 600 G for 40 minutes at 18°C, acceleration 9, deceleration 0 to obtain the buffy coat. After obtaining the buffy coat, the cells were washed three times with ice-cold PBS (Sigma). After each wash, they were spun at 500 G for 7 minutes, at 4°C, with both acceleration and deceleration set to 9. After washing, they were enumerated using a hemocytometer (Sigma).

Following enumeration, CD14+ monocytes were magnetically isolated using a negative selection kit (Miltenyi Biotech) according to the manufacturer's instructions. After isolation, the cells were washed with PBS and spun at 500 G

for 7 minutes at 4°C, with both acceleration and deceleration set to 9, and were subsequently counted.

The CD14+ monocytes were suspended in culture media consisting of IMDM+glutamax (Thermo Fisher), 5% heat-inactivated foetal calf or bovine serum (Sigma),1.25 µg/ml fungizone (Gibco), 100 µg/ml streptomycin, and 100U/ml penicillin (Thermo Fisher). Monocytes were differentiated into dendritic cells by the addition of GM-CSF (50ng/ml) and IL-4 (500U/ml: Peptrotech) at a cell density of 5×10^5 cells/ml. The cells were incubated at 37°C. On day 3, an additional 1 ml of media containing IL-4 (500U/ml) * and GM-CSF (50ng/ml) was added to each well.

On day 5, monocyte derived-DCs were harvested by placing the wells containing DCs on ice for 20-30 minutes, to reduce cell adherence to the plate prior to harvesting. Cells were released by tapping the sides of the plate gently and then pipetting the cells up and down. Afterwards, the plates were washed with PBS to collect any remaining cells.

Macrophages were harvested using Accutase cell detachment solution (Thermofisher; 10 mL per 75- cm^2 surface area) to detach the cells. The cells were incubated with Accutase for 10 minutes at 37°C and then aspirated. Following the harvesting, Dendritic Cells/Macrophages were washed in ice-cold HBSS (Sigma) with 10% FBS twice. After each wash, the cells were spun at 600 G for 7 minutes, at 4°C, to remove residual antibiotics, IL-4 and GM-CSF.

Resultant DCs/Macrophages were suspended at 5×10^5 cells per well in 1 ml antibiotic-free culture media (IMDM+glutamax with 5% heat-inactivated FBS) with 50 ng/ml GM-CSF only (no IL-4). IL-4 was only added to differentiate monocytes into dendritic cells but was not used for the differentiation of Macrophages. The cells were not tested for *Mycoplasma*.

2.2 Cellular Cytotoxicity Analysis

Cell viability following infection was measured by assessing LDH release (Lactate dehydrogenase) using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, UK), according to the kit's manual. Results were obtained using a

plate reader to measure the absorbance at 492 nm. For the results calculations, the no-cell control reading was subtracted from all experimental condition wells. The maximum LDH release control was then used to calculate the percentage cytotoxicity of each experimental condition well. The percentage was calculated using the following formula:

$$\text{Cytotoxicity percentage} = 100 \times \frac{\text{Experimental LDH release}}{\text{Maximum LDH release}}$$

2.3 *Streptococcus pneumoniae* Growth and Culture

2.3.1 *Streptococcus pneumoniae* Culture

Streptococcus pneumoniae TIGR4 strain [142] was provided by Prof. Evans. The bacteria were grown on blood agar plates, prepared by mixing Blood agar base with 5% de-fibrinated Sheep blood (TCS Biosciences Ltd) or 5% de-fibrinated Horse blood (EO Labs). A 20-25 ml volume of the blood agar mixture was used per petri dish plate (MVLS Stores), and plates were left to air dry in the culture hood.

An Optochin disc (Sigma) was used to ensure that the alpha-haemolytic colonies were *Streptococcus pneumoniae* and not another alpha haemolytic *Streptococci*. *Streptococcus pneumoniae* is optochin- sensitive whereas other-alpha haemolytic Streptococci are resistant. Bacterial Liquid Culture was prepared using Brain Heart infusion (BHI) media and Todd Hewitt broth media (Sigma).

2.3.2 *Streptococcus pneumoniae* Growth

Bacterial growth was measured using optical density at 600 nm using a spectrophotometer at certain time points (zero → 6 hours, and again at 14 → 25 hours). In addition, bacteria were cultured on blood agar plates after serial dilution to count the number of colonies at those time points and their corresponding optical densities.

2.3.3 Preparing Frozen stock of *Streptococcus pneumoniae*

To ensure an accurate number of *S. pneumoniae*, bacterial colonies were transferred from blood agar into liquid culture, and grown in liquid culture at 37°C, 5% CO_2, for 4-6 hours. Samples of liquid culture were taken for enumeration. Serial dilutions of the bacterial cultures were prepared and plated on blood agar and then incubated overnight.

Bacteria were harvested at 6000 G for 10 minutes at room temperature. The supernatant was then discarded, and bacteria were resuspended in freezing media consisting of 90% BHI and 10% Glycerol. Bacteria were frozen in vials at -20°C for short-term storage.

The following day, bacterial colonies on the blood agar plates were counted to determine the colony-forming unit (CFU) number per ml of frozen media.

2.4 Cytokines of interest:

This research initially focused on studying type I IFNs and their role in bacterial infections, potentially paving the way for investigating the involvement of the cGAS-STING pathway in the induction of type I IFNs following such bacterial infections. However, after multiple experiments using different cell lines and primary cells, it appeared that the *Streptococcus pneumoniae* TIGR4 strain does not induce type I IFNs. As a result, the focus of the study was switched to investigate the pro-inflammatory cytokines, TNF-α and IL-1β, as well as examining the effect of innate immune stimulants and trained immunity on their levels post-infection with *Streptococcus pneumoniae* TIGR4.

2.5 Infection Protocol

Cells were grown in tissue plates. The infections were performed using various bacterial growth time points. Most experiments utilised mid-log phase bacteria (2-4 hours post-culture) for infection. However, some experiments used different time points, such as 16 hours post-culture, to obtain more bacteria. Bacterial load was determined using a growth curve and frozen stock to achieve a multiplicity of infection (MOI) of 0.1-10. MOI indicates the ratio of bacteria to host cells. Choosing the correct MOI is vital for balancing infection efficiency and cell viability. Different MOIs were tested to study their impact on cell survival and cytokine production. Typically, an MOI of 1 was used to standardise host-pathogen interactions and avoid overwhelming the cells. The infection protocol steps were consistent in all experiments. However, some modifications were done in some experiments, and these were mentioned in their specific sections. E.g., the incubation period after infection depends on the specific experiment. In ELISA and cytokine secretion measurements, incubation times of 2, 4, 6, and 24 hours were used, as cytokine secretion generally occurs within this window [143, 144]. For RT-qPCR experiments measuring mRNA expression, shorter incubation times of 0 and 6 hours were employed. The 6-hour time point allows sufficient time for bacterial recognition, intracellular signalling, and transcriptional activation of the genes without excessive cell death [145]. For Protein quantification and Western blotting, cells were incubated for 10, 20, and 30 minutes before processing. Time points at 10, 20, and 30 minutes were chosen to capture the rapid activation and peak phosphorylation of proteins

IκBα, JNK, and p38, which typically occur within this window during bacterial infection. This timing is consistent with established kinetics of key inflammatory signalling pathways in immune cells[146].

2.5.1 Cellular Pre-treatment with Stimulants of Innate Immunity

Pre-treatments were added to primary cell culture on day 5, for 24 hours prior to infection, and to cell lines one day before infection. All pre-treatments were prepared in antibiotic-free tissue culture media. The pre-treatments used were:

- Beta glucan (100µg/ml) (InvivoGen).
- Zymosan cell wall preparation of *S. cerevisiae* (10 µg/ml) (InvivoGen).
- Lipopolysaccharides from Escherichia coli O111:B4-LPS (1 µg/ml) (Sigma).

On the day of infection, the media was changed, and cells were infected with TIGR4 (MOI 0.1-10). Positive controls for cytokine secretions included Poly I:C (10µg/ml) (Sigma), LPS (1 µg/ml)+ATP (5 mM-Sigma), and LPS (1 µg/ml -Sigma). The negative control consisted of cells only. Cell culture supernatants were collected at 2, 4, 6, and 24 hours after infection and were frozen for further testing.

The rationale for selecting these stimulants is due to their ability to enhance the secondary innate immune response upon re-infection, consistent with the induction of trained immunity. In previous papers, ß-glucan was shown to improve infection outcomes and increase pro-inflammatory cytokines including TNF and IL-6 production. Most importantly and relevant to this thesis is the work by Hetland et al, the paper found that mice pre-treated with ß-glucan showed enhanced resistance upon re-infection with *Streptococcus pneumoniae*. This was demonstrated by reduced mortality, lower bacterial loads, and elevation of cytokines (e.g., TNF-α, IL-6), compared to controls. However, the work in this study was conducted in mice, in this thesis we aimed to examine it in human cells for greater biological resemblance [102].

In Stothers et al., similar results are observed in mice pre-treated with β-glucan prior to infection with *Pseudomonas aeruginosa* at various times post-β-glucan treatment 1, 3, 7, or 14 days before challenge. These mice showed increased recruitment of innate leukocytes to the infection site. The number of monocytes in the mice peritoneal cavities was significantly higher in mice treated with β-glucan at 1 and 3 days before infection, with the numbers returning to control levels at day 7. They also demonstrated improved local clearance of bacteria, an effect that persisted for more than 7 days [147].

Another study showed similar effect of β-glucan in *Mycobacterium tuberculosis* infection. Moorlag et al. (2020) showed that β-glucan induces trained immunity that increases protection against *Mycobacterium tuberculosis* in mice and human monocytes. This effect was dependent on IL-1 signalling and involved epigenetic reprogramming, as well as increased cytokine production. Trained macrophages showed improved bacterial control and broad, non-specific innate memory [148].

With regard to Zymosan, In Ciarlo et al, Mice pre-treated with zymosan showed higher survival and reduced bacterial load after *E. coli* peritonitis. Zymosan induced long-term activation of innate immunity, including increased phagocyte recruitment and cytokine production. E.g. TNF-α, IL-6. Re-stimulation assays showed elevated inflammatory responses, suggesting functional reprogramming of innate cells. The effect was independent of adaptive immunity, confirming a trained immunity mechanism [149].

Lastly, regarding LPS, studies show a more complex picture of its pre-treatment effects. LPS produces a more combined effect of tolerance and stimulation to innate immunity. In Kang et al, LPS pre-treatment in mice led to a protection against challenge with *Streptococcus pneumoniae,* pre-treated mice had lower bacterial loads in the lungs, reduced lung inflammation, and improved survival rates following *S. pneumoniae* infection. These effects seem to be achieved through enhanced phagocytosis and increased cytokine production (TNF-α, IL-6, and IL-1β). The same experiment was conducted using a different secondary challenge, SARS-CoV-2 (COVID-19), and LPS pre-treatment led to minimal protection. This may suggest that its innate immune effects are pathogen-specific [150]. However, since *S.pneumoniae* is the pathogen of choice in this

thesis infection model, LPS was considered as one of the immune stimulants to be used.

Later in the thesis, beta glucan was dropped because it didn’t show significant effect on cytokines production.

2.6 Enzyme-Linked Immunosorbent Assay (ELISA)

This was performed using an ELISA duo set kit (enzyme-linked immunosorbent assay) (R&D system ®) protocol, along with the Duo Set ELISA Ancillary Reagent Kit. ELISA was conducted to measure the secretion of the following cytokines: IFNα, IFN-ß, IFNγ, TNF-α, and IL-1ß, according to the manufacturer's protocol [151-153]. Later IFNs assays were dropped, due to repeated negative results in different cell culture models, and the main focus shifted to TNF-α, and IL-1ß.

2.7 DNA Methylation Assay in TNF-α Promoter and First Exon regions:

The experimental controls and conditions for this experiment were:

1- Negative control: U937 macrophages that are neither pre-treated nor infected.

2- Positive control: U937 macrophages stimulated with LPS (1 µg/ml) for 2 hours before processing.

3- Infected Cells: U937 macrophages that are infected with TIGR4 (MOI=1) for 2 hours, before processing. Without pre-treatment with LPS.

4- Pre-treated Cells: U937 macrophages that are pre-treated with LPS (0.1 µg/ml), for 4 hours.

5- Pretreated Infected Cells: U937 macrophages that are pre-treated with LPS (0.1 µg/ml) for 4 hours and infected on the next day with TIGR4 (MOI=1) for 2 hours, prior to processing.

U937 cells were pre-treated with LPS (0.1 µg/ml) and later infected with *Streptococcus pneumoniae*. The cells were then lysed, and DNA is obtained to study the TNF-α gene, particularly the promoter and 1st exon regions.

2.7.1 DNA Extraction:

DNA extraction was performed using the DNeasy Blood and Tissue Kit (Qiagen), following the manufacturer's instructions. The desired DNA segment was amplified using Polymerase Chain Reaction (PCR).

PCR was carried out to amplify elements of the TNF-α promoter and 1st exon regions, using TNF-α promoter set 10 primers for the complete promoter region, TNFp3 primers were used to amplify a smaller segment of the promoter region. TNF-α E1U/TNF-α E1D primers were used for the first exon region. Primers were purchased from Integrated DNA Technologies (IDT, Belgium). Primer sequences are listed in Table 2-4. PCR was done under the following cycling conditions, using Platinum Taq DNA polymerase (Invitrogen) Table 2-1.

Table 2-1 PCR Cycling Conditions

Step	Temperature	Time
1-Initial Denaturation	94°C	2 minutes
2-Denaturation	94°C	30 seconds
3-Annealing	55°C	30 seconds
4-Extension	72°C	2 minutes, repeat step 2 for 35X Cycles.
Hold	4°C	∞

2.7.2 Agarose Gel Electrophoresis:

To verify that the PCR products were of the right size, agarose gel electrophoresis was carried out. A 1% Agarose gel was prepared by mixing 300 mg of agarose powder, 30 ml of 1X TBE buffer, and 3 µl of SYBR safe DNA gel stain (Thermo Fisher).

5X TBE (Tris-Borate-EDTA) buffer was prepared by mixing 54 g of Tris base, 27.5 g of boric acid, and 20 ml of 0.5 M EDTA (PH-8) in distilled water. The final solution's pH was adjusted to 8.3, and the final volume was adjusted using distilled water.

After mixing, the solution was heated up, poured into the gel frame, and left to cool down for 10 minutes. The electrophoresis solution is a TBE buffer. 1 KB+ DNA Ladder (Invitrogen) was used, or 100 bp DNA Ladder (Promega), depending on the segment target size. Samples (1-2 µl per sample) were mixed with gel dye (5 µl per sample—New England Biolabs).

Gel running conditions: 100-volt running power, 300 mAmp running speed, for 30 minutes. In later experiments, TBE buffer was replaced with TAE (tris-acetate-EDTA) buffer (Sigma-Aldrich).

2.7.3 Bisulfite DNA Conversion

Conversion of unmethylated cytosines to uracil was performed using bisulfite. this was performed using the EpiJET Bisulfite Conversion Kit (ThermoFisher), according to the manuals' instructions [154]. To verify if the products were of the right size, PCR was done using Phusion U Hot Start DNA Polymerase (ThermoFisher). Tnfp3 Primers were used to amplify the first amplicon located in the TNF promoter regions and TNF-α E1U/TNF-α E1D primer for the first exon region DNA target (Table 2-4), the thermal cycler was set to the following (Table 2-2):

Table 2-2 PCR Cycling Conditions

Step	Temperature	Time
1- Initial Denaturation	98°C	30 Seconds
2- Denaturation	98°C	10 Seconds
3- Annealing	55°C	30 Seconds
4- Extension	72 °C	1 minute, repeat step 2 35X Cycles.
5- Final Extension	72 °C	10 minutes
6- Hold	4°C	∞

2.7.4 Cloning the PCR products:

Cloning of PCR products was performed using StrataClone PCR Cloning Kit (Agilent), following the manual's instructions [155]. After cloning, 10 colonies were picked for each condition and were grown overnight on LB-Ampicillin plates at an ampicillin concentration of 50 µg/ml.

2.7.5 Plasmid Purification:

Plasmid DNA was extracted using QIAprep Spin Miniprep Kit (Qiagen), according to the manual's instructions [156]. The plasmids were then sent for sequencing.

2.7.6 Plasmid Sequencing:

DNA sequencing was performed by sending the plasmids to DNA Sequencing and Services Medical Sciences Institute, School of Life Sciences, University of Dundee. The sequences were then analyzed using Seaview software.

2.8 Western Blotting

In these experiments, U937 cells were cultured in antibiotic-free media and primed with PMA (100 ng/ml). Some of the cells were pre-treated with LPS (0.1 µg/ml) for 4 hours, after which the media was changed to wash out the pre-treatment with antibiotic-free media and PMA. The next day, a subset of the cells was infected with *Streptococcus pneumoniae* TIGR4 (MOI=1), the negative control is cells without pre-treatment of infection, and the positive control is LPS (1 µg/ml) stimulated cells. LPS stimulation (0.1–10 µg/ml) causes IκBα protein phosphorylation [157], P38 phosphorylation [158], ERK-1 phosphorylation [159], and JNK phosphorylation [160].

Cells were incubated for 10, 20, and 30 minutes. At each time point, protein lysates were prepared, and protein concentration was measured. Afterwards, gel electrophoresis was conducted, and the gel was transferred to a membrane by western blotting. The membrane was exposed to several antibodies to check for cellular proteins in the samples.

2.8.1 Protein Lysates Preparation

Lysates were prepared according to the following steps. The media was removed from the cell culture plate, and then the cells were washed with 500 µl of ice-cold PBS. The cells were harvested, and the washing step was repeated by centrifugation at 100 G for 5 minutes at 4°C. The supernatant was discarded, and the tubes were placed on ice.

An additional 500 µl of ice-cold PBS was added to the wells, and the cells were scraped from the bottom of the wells using a scraper or pipette tip. The cells-PBS mixture was then added to their corresponding tubes, spun again, and the supernatant was discarded. This step was performed to ensure maximal retrieval of cells from the culture.

The cell pellet was resuspended in cell lysis buffer, which consists of 5 ml RIPA buffer, 0.005M NaF, 0.005M EDTA, 0.001M NaH_4PO_4, 0.01M NaP, and a pill of protease/phosphatase inhibitor (Pierce Protease and Phosphatase Inhibitor Mini Tablets- Thermo Fisher). The lysis buffer was vortexed, after which 150 µl of cell

lysis buffer was added to each tube. The amount of cell lysis buffer can be reduced depending on the size of the cell pellet. The tubes were left on ice for 30 minutes and then spun at 21,000 G for 15 minutes at 4°c. The supernatant was then carefully isolated into new tubes and stored for protein concentration assay.

2.8.2 Protein Concentration Assay

Protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Scientific™) according to the kit's manual [161]. After measuring the concentrations, some samples were diluted with RIPA buffer to standardize the protein concentration across all samples before further analysis. For western blotting, only samples with a total protein mass of ≥ 10 µg were used.

2.8.3 Gel Electrophoresis

Samples for gel electrophoresis were prepared. The protein lysates for western blotting were thawed on (in) ice. The samples for loading were then prepared by adding 1X Nupage Sample reducing agent (ThermoFisher Scientific, UK), 1X Nupage LDS Sample Buffer (ThermoFisher Scientific, UK), and a maximum of 19.5 µl sample ± RIPA buffer. This gave 30 µl of sample for loading. Sample tubes were then put in a hot block at 95°C for 5 minutes to denature the proteins.

After denaturing, 25 µl of each sample was loaded along with 10 µl of Novex™ Sharp Pre-stained Protein Standard (ThermoFisher) on NuPAGE® Novex® Bis-Tris Gel with MES SDS Running buffer (Sigma). A smaller sample volume was loaded to prevent the formation of air bubbles in the gel.

Gel running conditions were 200 V constant voltage and 125 mA current for 35 minutes, or until the dye front was close to the bottom of the gel. Once gel electrophoresis was complete, the pre-cast gel cassette was opened, and the gel was used for blotting as described below.

Samples were loaded onto the gel in following order:
Lane 1; Negative control; untreated U937 cells + PMA at time zero.
Lane 2; U937 cells pre-treated with LPS (0.1 µg/ml), at time zero.

Lane 3; U937 cells infected with *Streptococcus pneumoniae* TIGR4 (MOI=1), 10 minutes.
Lane 4; U937 cells pre-treated with LPS (0.1 µg/ml) + infected with *Streptococcus pneumoniae* TIGR4 (MOI=1), 10 minutes.
Lane 5; U937 cells infected with *Streptococcus pneumoniae* TIGR4 (MOI=1), 20 minutes.
Lane 6; U937 cells pre-treated with LPS (0.1 µg/ml) + infected with *Streptococcus pneumoniae* TIGR4 (MOI=1), 20 minutes.
Lane 7; U937 cells infected with *Streptococcus pneumoniae* TIGR4 (MOI=1), 30 minutes.
Lane 8; U937 cells pre-treated with LPS (0.1 µg/ml) + infected with *Streptococcus pneumoniae* TIGR4 (MOI=1), 30 minutes.
Lane 9; The positive control; U937 cells stimulated with LPS (1 µg/ml) + PMA at 30 minutes.

2.8.4 Membrane Transfer Protocol

Western blotting was performed by assembling the blot sandwich in the following order: a sponge, 2 sheets of paper, gel from the previous step, nitrocellulose membrane, 2 sheets of paper and a sponge. The sandwich was then enclosed with the apparatus frames and inserted into the transfer chamber. Transfer buffer was then added (14.4 g Glycine, 3 g Tris, 800 ml distilled water (dH2O), and 200 ml methanol).

Transfer conditions were 70 V constant voltage for one and a half hours (90 minutes).

2.8.5 Antibody detection

After blotting, the membrane was removed, and Ponceau stain was applied to assess the transfer quality. If the ponceau stain was used, the membrane was washed several times with Tris-Buffered Saline with Tween-20 (TBST) wash buffer to remove any residual stain.

10X TBST is made of 121 g TRIS, 400 g NaCl, and 50 ml Tween 20. The solution pH was 7.6. Afterwards, the membrane was blocked using a solution of 5 grams of skimmed milk powder dissolved in 100 ml of TBST. Blocking was performed at

room temperature for 1 hour on the shaker. After blocking, if the primary antibody was diluted in milk, then there was no need to wash the membrane. However, if the primary antibody was diluted in Bovine Serum Albumin (BSA), then the membrane must be washed a couple of times with TBST.

After washing, the primary antibody solution was added. The Primary antibody was diluted 1:1000, either in 5% milk solution as above or 5% BSA (5% BSA is diluted in TBST). The membrane was then placed in a cold room on a shaker overnight.

The next day, the membrane was washed four times with TBST, each wash lasting five minutes. After washing, the secondary antibody solution was added. The Secondary antibody solution was diluted 1:2000 in 5% BSA (5% BSA was diluted in TBST). The membrane was then placed on a shaker at room temperature for one hour.

Afterwards, the membrane was washed 4 times with TBST, with each wash lasting 5 minutes.

2.8.6 Developing the membrane

After washing, the protein ladder on the membrane was marked using a chemiluminescent pen (WesternBright ChemiPen—Advansta). Then, substrate solutions were added, either WesternBright ECL HRP substrate or, for a stronger signal, WesternBright Sirius HRP substrate, which is more sensitive. The membrane was incubated with the substrate solution for 2 minutes before detection.

The membrane acquisition was performed using C-DiGit® Blot Scanner (Li-cor) and analysed using the iStudio software.

2.8.7 Membrane Stripping

If the membrane is to be used to detect another antibody after development, it will be stripped using a stripping buffer (500 ml). The stripping buffer consists of 3.75 gm of 0.1M Glycine and 4.35 gm of 0.15M NaCl at pH 2-2.6.

The stripping buffer was added to the membrane twice, each time for 10 minutes, on the shaker at room temperature. After stripping, the membrane was washed twice with TBST. It was then blocked with 5% milk, as described in the previous protocol, and antibody detection and development were carried out as outlined above.

2.8.8 List of Antibodies

The following is the list of antibodies used in western blotting to detect cellular proteins in the final chapter.

- Phospho-IKKß (D30C6) Rabbit mAb, Cell Signalling Technology.
- IKKß Antibody, Cell Signalling Technology.
- Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP® Rabbit mAb, Cell Signalling Technology.
- p44/42 MAPK (Erk1/2) Antibody, Cell Signalling Technology.
- Phospho-IκBα (Ser32) (14D4) Rabbit mAb , Cell Signalling Technology.
- IκBα Antibody, Cell Signalling Technology, Cell Signalling Technology.
- Phospho-SAPK/JNK (Thr183/Tyr185) (81E11) Rabbit mAb, Cell Signalling Technology.
- SAPK/JNK Antibody, Cell Signalling Technology.
- Phospho-p38 MAPK (Thr180/Tyr182) (D3F9) XP® Rabbit mAb, Cell Signalling Technology.
- p38 MAPK Antibody, Cell Signalling Technology.
- Phospho-IRAK1, St John's Laboratory.
- IRAK-1 Antibody, St John's Laboratory.
- Goat anti-Rabbit IgG (H+L) Secondary Antibody, HRP, abcam.

2.8.9 Protein Levels Normalization

Band intensities were quantified using iStudio software. For phosphorylated proteins, signal intensity of the phospho-specific band (e.g., p-JNK) was normalized to the corresponding total protein band (e.g., JNK) to account for protein loading and expression levels. All values were then expressed relative to the untreated control condition.

2.9 Quantitative Polymerase Chain Reaction (qPCR)

2.9.1 Cell Lysis

qPCR was carried out following the steps outlined below. Cell lysis was achieved using the Cell Lysis Buffer from the Purelink® RNA Mini Kit (ThermoFisher Scientific, UK) [10]. For every 3 mL of lysis buffer, 120 µL of 1 M Dithiothreitol was added. Fresh Dithiothreitol was added prior to cell lysis. Notably, the supernatant was removed from the tissue culture plate, and cell lysis buffer was added 0.3 ml per well in a 24-well plate. Cells were then scraped from the bottom of the well using a cell scraper or pipette tip. The Lysates were aspirated into 2 ml tubes and stored at -20°C until further testing.

2.9.2 RNA Extraction

RNA extraction was performed using the Purelink® RNA Mini Kit (ThermoFisher Scientific, UK), following the manufacturer's instructions [162].

2.9.3 RNA Quantification

The mRNA concentrations were measured using a Nanodrop machine. The concentrations from the RNA quantification were inputted into a spreadsheet to calculate the total amount of RNA present, the volume of each sample, and the volume of nuclease-free water required to give 100 ng of RNA. The maximum amount of RNA sample ± water required for reverse transcription is 14.2 µl.

2.9.4 Reverse Transcription

Reverse transcription was performed using a High-Capacity RNA-to-cDNA™ Kit (Applied Biosystems™), according to the kit manual [163], with cycling conditions as listed in (Table 2-3). The resulting cDNA was then stored either in a fridge for short-term or a -20°C freezer for long-term storage.

All qPCR reactions included the following controls: no-template controls (NTCs) to detect contamination, no-reverse transcriptase controls (No-RT) to verify the absence of genomic DNA contamination, and positive controls to confirm assay performance. Housekeeping gene (GAPDH) was used for normalization, and standard curves were generated to assess PCR efficiency.

Table 2-3 PCR Cycling Conditions

Step	Temperature	Time
1	37°C	60 minutes
2	95°C	3 minutes
3	4°C	∞

2.9.5 Quantitative Polymerase Chain Reaction (QPCR)

The qPCR primers used included forward and reverse primers for human IL-1ß and TNF-α. For qPCR, GAPDH was used as the housekeeping gene. Sequences are shown in (Table 2-4). Primers were purchased from Integrated DNA Technologies (IDT, Belgium).

Upon their delivery, primers were centrifuged for 30 seconds before adding an amount of nuclease-free water equal to 10X the nmole amount from the optical density measurement stated on the primer container. This gives a primer stock of 100 mM, which then allows 0.1 µl of each primer to be used for the qPCR process. cDNA was used for qPCR using PowerUp SYBR Green Master Mix (ThermoFisher Scientific, UK) according to the manufacturer's manual [164]. The PCR plate was then inserted into the real-time PCR machine (QuantStudio 7 Flex or StepOnePlus Real-Time PCR System; Applied Biosystems, UK), and the thermal cycling conditions were set to standard using SYBR Green reagents. A melt curve was also included in the real-time PCR instrument settings to check for non-specific DNA amplification. To calculate the relative gene expression, several formulae were used. The formulae used for quantification are as described in Livak et al. [165]:

ΔCT = Test gene average – housekeeping gene average

ΔΔCT = ΔCT of experimental condition – ΔCT of negative control "Cells Only"

mRNA fold increase = 2(-ΔΔCT)

Table 2-4 Primer Sequences

Primer Name	Forward Primer Sequence (5'→3')	Reverse Primer Sequence (5'→3')
- GAPDH	GATCATCAGCAATGCCTCCT	GAGTCCTTCCACGATACCAAAG
- IL-1β	CCTAAACAGATGAAGTGCTCCT	TGAAGCCCTTGCTGTAGTG
- TNF-α	GTAGCCCATGTTGTAGCAAAC	CTGGTTATCTCTCAGCTCCAC
- Primetime® qPCR Primer TNF	TGCACTTTGGAGTGATCGG	TCAGCTTGAGGGTTTGCTAC
- Primetime® qPCR Primer HPRT1	TTGTTGTAGGATATGCCCTTGA	GCGATGTCAATAGGACTCCAG
- TNF-α Promoter Set 10	TGGAAGCCAAGACTGAAACC	CATCTTTCACCCATCCCATCTC
- Tnfp3	AGTGGYTTAGAAGATTTTTTYG GAAT	AATCTATAATTRCTTCTCTCCCT CTTAR
- TNF-α E1U/E1D	TTTTTTGGAAAGGATATTATG AGTATTG	AAAATAAATAAAAACTAACCA AACACTC

2.10 Statistical Analysis

Experiments were conducted in duplicates or triplicates, with graphs and statistics performed using GraphPad Prism. Statistical analysis was carried out using an independent samples (unpaired) t-test where the variables were equal to or less than two. In cases where the variables were more than two, a one-way ANOVA test was used to analyse the data. Multiple comparisons were done using follow up Dunnett's test to compare the samples to a specific control. Results of statistical analysis were interpreted based on the P value obtained, to be significant or non-significant. Non-significant when a P value > 0.05. Significant when P value ≤ 0.05. While cytokine secretion (ELISA readings) was measured in

picograms per ml (pg/ml), cytokine/mRNA expression (qPCR readings) was measured in fold increase or induction caused by infection. Cytotoxicity readings are presented as percentage cytotoxicity vs the condition studied. All readings denote means ± standard error of the mean.

For percentages in chapter 5 of this thesis, statistical significance was determined and compared to each other using the significance level (P-Value) obtained using the Chi-squared test as recommended by Campbell (2007) and Richardson (2011) to determine whether the percentage change is significant or not[166, 167].

3 Model to Study Cytokines Production Following *Streptococcus pneumoniae* Infection

3.1 Introduction

Models for studying cytokine production following *Streptococcus* infection have been attempted by various authors with multiple cytokines including interferons, TNF- α and IL-1ß studied and identified[168, 169].

In the present study we hypothesized that *Streptococcus pneumoniae* infection leads to interferon production. This was observed in Nakamura et al.; however, unlike that study, the present work uses a pure bacterial infection model without co-infection with influenza virus [170].

Streptococcus pneumoniae, a gram-positive, rounded, lancet-shaped bacterium is an alpha-haemolytic (produces incomplete hemolysis) member of the genus *Streptococcus*. It is responsible for multiple diseases, which can be divided into invasive and non-invasive categories. Invasive infections include pneumonia, meningitis, and septic arthritis, while non-invasive infections include otitis media, sinusitis, and bronchitis [13].

Streptococcus pneumoniae has multiple virulence factors, including the polysaccharide capsule [1] and pneumolysin [2]. Additionally, other pneumococcal proteins hinder host defence at mucosal surfaces. For example, IgA1 protease is a proteolytic enzyme that cleaves certain peptide bonds in the human immunoglobulin A1 (IgA1) hinge region sequence. Another pneumococcal protein is hyaluronidase, an enzyme that breaks down the hyaluronic acid in connective tissue and extracellular matrix. Additional virulence factors of *S. pneumoniae* include pneumococcal surface protein A, autolysin, and pili, which allow the bacterium's adherence to mucosal surfaces [5].

Interferons are a group of cytokines produced by most cells in the human body. and are classified into two types: type 1 and type 2. The type I IFNs in humans include IFN-α, IFN-ß, IFN-ε, IFN-κ and IFN-ω, while type II IFNs are IFNγ in humans [171]. Type I IFNs are crucial for defence against viruses; they induce

the antiviral effector molecules encoded by IFN-stimulated genes. These IFNs contribute to the immunopathology that occurs in acute viral infections. Conversely, they also contribute to immunosuppression and loss of virus control during chronic viral infections [172, 173].

Type I IFNs and type II IFNs orchestrate the immune response. They have antagonising actions toward each other. Type I IFNs, for instance, can antagonise the action of IFNγ by reducing the responsiveness of macrophage activation induced by IFNγ [63, 173].

In the context of bacterial infections, which is the scope of this thesis, it was found that IFNs can have both beneficial and detrimental effects on bacterial infection outcomes. Most of IFNs' effects were observed to be protective in bacterial infections. However, detrimental effects were observed in chronic intracellular bacterial infections such as *Listeria monocytogenes* and *Mycobacterium tuberculosis*. This is due to high concentrations of type I IFNs, which may block B cell responses or lead to the production of immunosuppressive molecules and decrease the responsiveness of macrophages to activation by IFNγ, as demonstrated in the aforementioned intracellular bacterial infections [174, 175].

The protective effects of IFNs were observed in many bacterial models, most notably in *Streptococcus pneumoniae* infection. *Ifnar1*$^{-/-}$ mice had reduced survival compared with wild-type controls and/or increased bacterial growth [65]. This effect was also observed in other bacterial models, including infections caused by group B Streptococcus (GBS), *E. coli*, *H. pylori* and *S. pyogenes* infections [176] [177-180].

In the case of the immune response to group B streptococci (GBS), *E. coli* and *S. pneumoniae*, IFNα/β signalling was found to support optimal macrophage activation, particularly to enhance their production of TNF and nitric oxide (NO)[177]. *Streptococcus pneumoniae* is known to be a prevalent cause of pneumonia following viral infections and is associated with significant morbidity and mortality. Studies have investigated the role of IFNs in bacterial pneumonia following influenza virus infection. In mice, Influenza virus-infected Ifnar1-/- mice survived secondary infection with *Streptococcus pneumoniae* better than

wild-type controls, with augmented bacterial clearance [66, 170, 181]. This improved outcome was associated with increased production of the neutrophil chemo attractants CXC-chemokine ligand 1 (CXCL1) and CXCL2 [66], enhanced production of the macrophage chemoattractant CC-chemokine ligand 2 (CCL2)[170], and the enhancement of the γδ T cell response [181].

This chapter aims to optimize a model to study cytokine production following *Streptococcus pneumoniae* infection in innate immune cells. The specific objectives which were to employ experiments to determine the ability of these innate immune cells to produce type I interferons, TNF-α, or IL-1β, in response to *Streptococcus pneumoniae* infection.

3.2 Results and Discussion:

3.2.1 *Streptococcus pneumoniae* TIGR4 Growth:

First, the optimal conditions for bacterial growth were determined to obtain reproducible numbers of bacteria for the infection experiments. The growth and enumeration of bacterial colony-forming units were done as follows. *Streptococcus pneumoniae* TIGR4 bacterial strain were grown on blood agar plates. Bacterial Liquid Culture was prepared using Brain Heart Infusion (BHI) media and Todd-Hewitt broth media as previously described in section 2.3.1. The bacterial counts were determined from the bacterial liquid culture using serial dilution (Figure 3-1). A few drops were placed on a blood agar plate to count the number of colonies at certain time points (Figure 3-2) or at certain optical densities. Density was measured using a spectrophotometer OD_{600}.

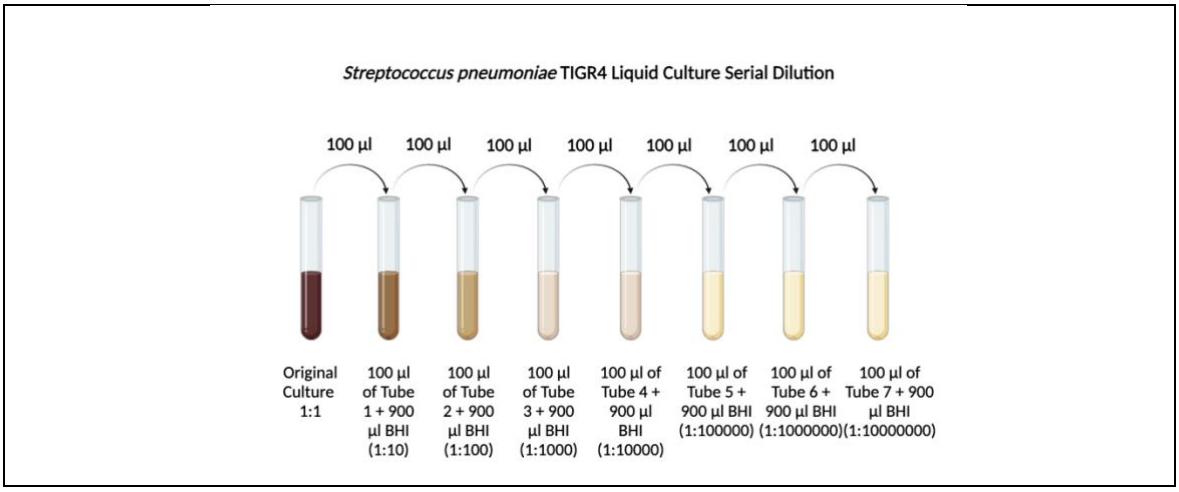


Figure 3-1 *Streptococcus pneumoniae* TIGR4 liquid culture serial dilution.

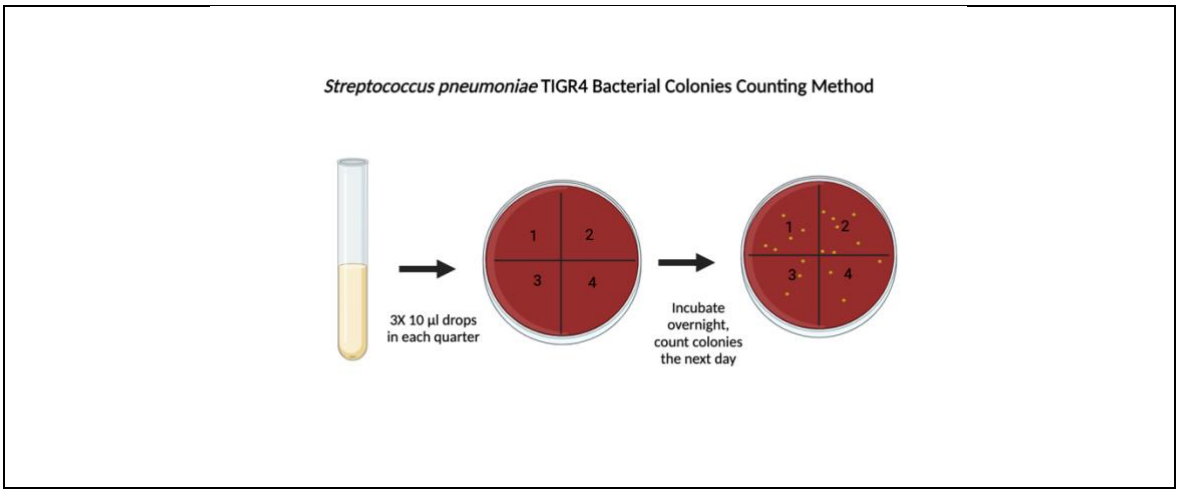


Figure 3-2 *Streptococcus pneumoniae* TIGR4 Colonies Counting Method.

The results indicate an increase in bacterial growth over time. The lag phase of growth seems to start at time zero hours and lasts until approximately 3 hours after inoculation. The log phase occurs 3 -5 hours after inoculation and is characterised by rapid growth. Additionally, around 16 hours after inoculation, the bacterial count begins to decline, indicating the onset of the death phase. This decline persists until about 19 hours after inoculation, at which point the bacterial count reaches zero CFU/ml (Figure 3-3).

For the experiments in this study, a time point between 4-6 hours after inoculation was selected for either the use or freezing of TIGR4, due to the highly viable bacterial count at this time. The time vs optical density (OD) curve shows that optical density rises with time; however, the OD seems to plateau at 14 - 25 hours at 0.23 (Figure 3-3). The comparison between viable counts and OD shows that the OD lags behind the incubation times and hence does not provide an accurate indication of the viability of the bacteria. The OD vs bacterial count curve shows that there seems to be a direct relationship between the two (Figure 3-3). However, some variability in bacterial count was observed at the same OD; for future testing, ensuring the accurate number of TIGR4 is needed to achieve the desired multiplicity of infection (MOI). Therefore, it was decided to grow bacteria to a mid-log phase, determine their concentration through serial dilution and plating, and then freeze aliquots for future experiments.

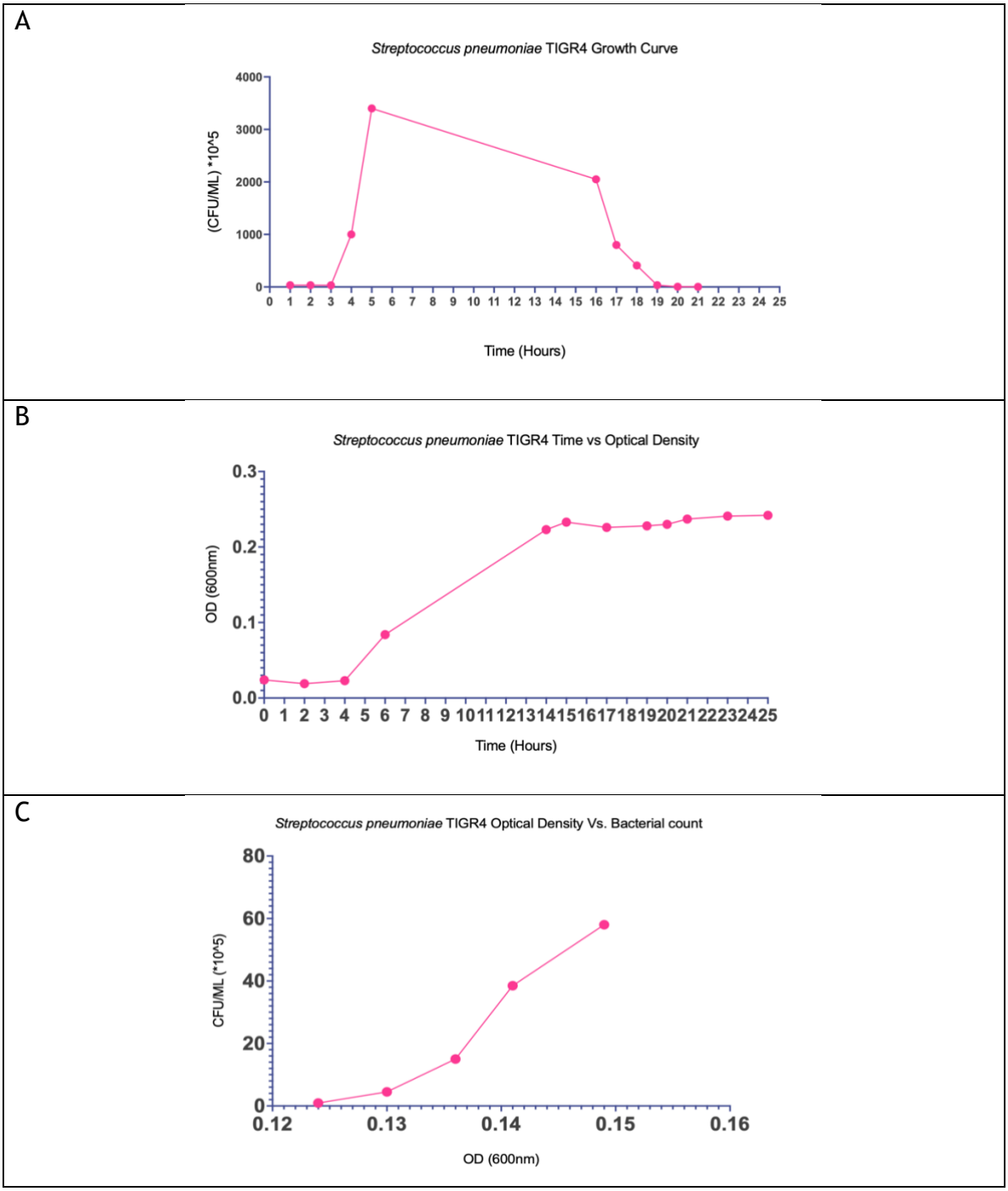


Figure 3-3 Graphs showing Streptococcus pneumoniae TIGR4 growth curves including (A) TIGR4 Time vs. Count. (B) Time vs. Optical Density (C) Optical Density vs. Count.

3.2.2 Interferon Production in NuLi-1 Cells Post Pneumococcal Infection

The NuLi-1 cells did not exhibit any growth or attachment, even after transfer into a new collagen-coated flask. One possible reason for their lack of growth is the passage number, as it was observed in these cells that they start to change after the tenth passage. However, the passage number in these cells is unknown, as they were gifted to us from another laboratory.

3.2.3 Interferons Production in BEAS-2B Cells Post Pneumococcal Infection:

The cells exhibited good growth in culture. After passaging, infection with pneumococcus was carried out. Cell culture supernatants were collected, and ELISA was done to determine the interferon levels produced by the BEAS-2B cells following infection. Assays were conducted to check for IFNα (Data not shown), IFN-β and IFNγ(Figure 3-4).

The cells did not produce significant levels of either interferon. Subsequent modifications to the culturing conditions, including the use of Collagen Type 4 coating of the tissue culture wells, did not lead to any noticeable change in interferon production(Figure 3-5) . Overall, there was no significant production of IFN-β or IFN-α.

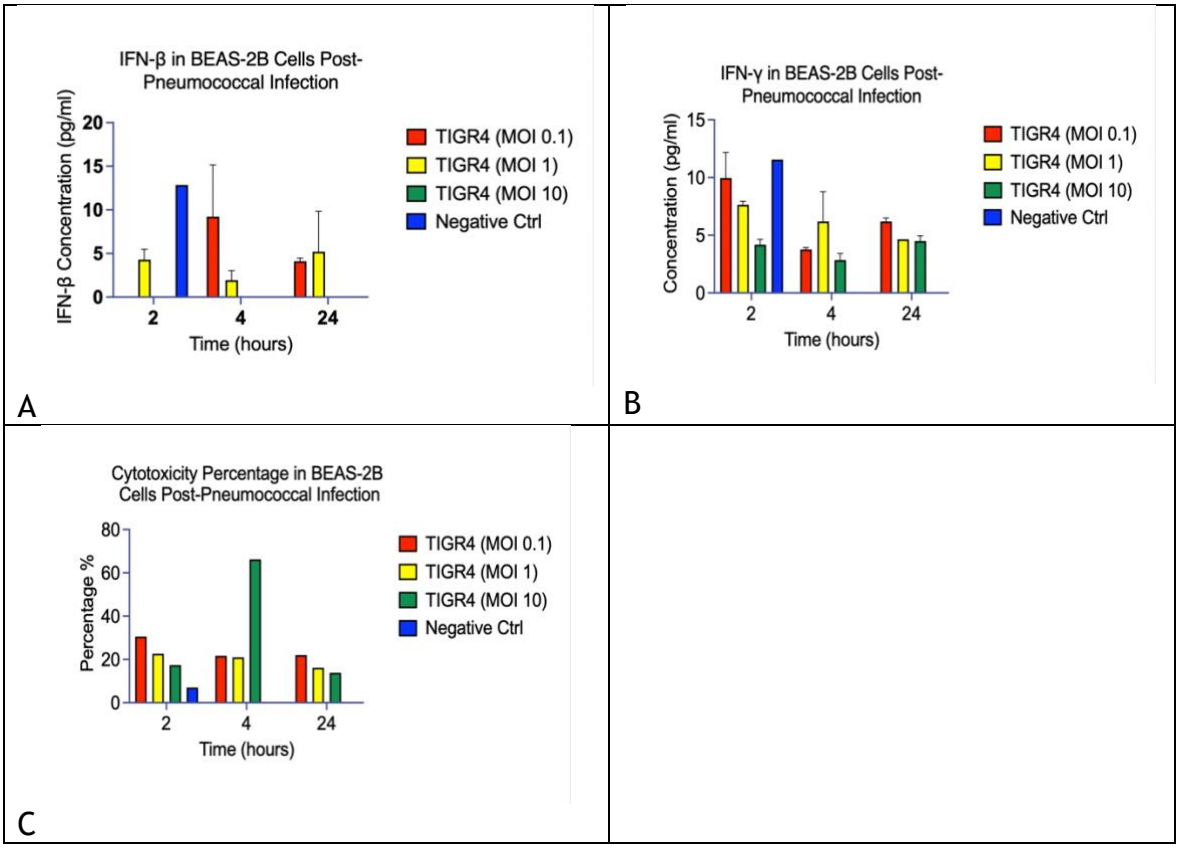


Figure 3-4 Interferons β and γ Concentrations in BEAS-2B cells post pneumococcal infection
(A) IFN-β in BEAS-2B Cells Post Pneumococcal Infection (B) IFN-γ in BEAS-2B Cells Post Pneumococcal Infection BEAS-2B cells were infected with Streptococcus pneumoniae TIGR4 (MOIs = 0.1,1,10) for 2,4, and 24 hours. The Negative control is untreated cells. The kit's detection limit = (7.81- 500) pg/ml for IFNβ, and (9.38- 600) pg/ml for IFN γ. The data are representative of one experiment with biological replicates (n=2), columns show the mean results; error bars are the SEM. (C) Cytotoxicity percentage % in BEAS-2B cells post Pneumococcal infection. Percentage cytotoxicity was calculated relative to a maximum lysis control. Data represent the average of 2 biological replicates (independent wells).

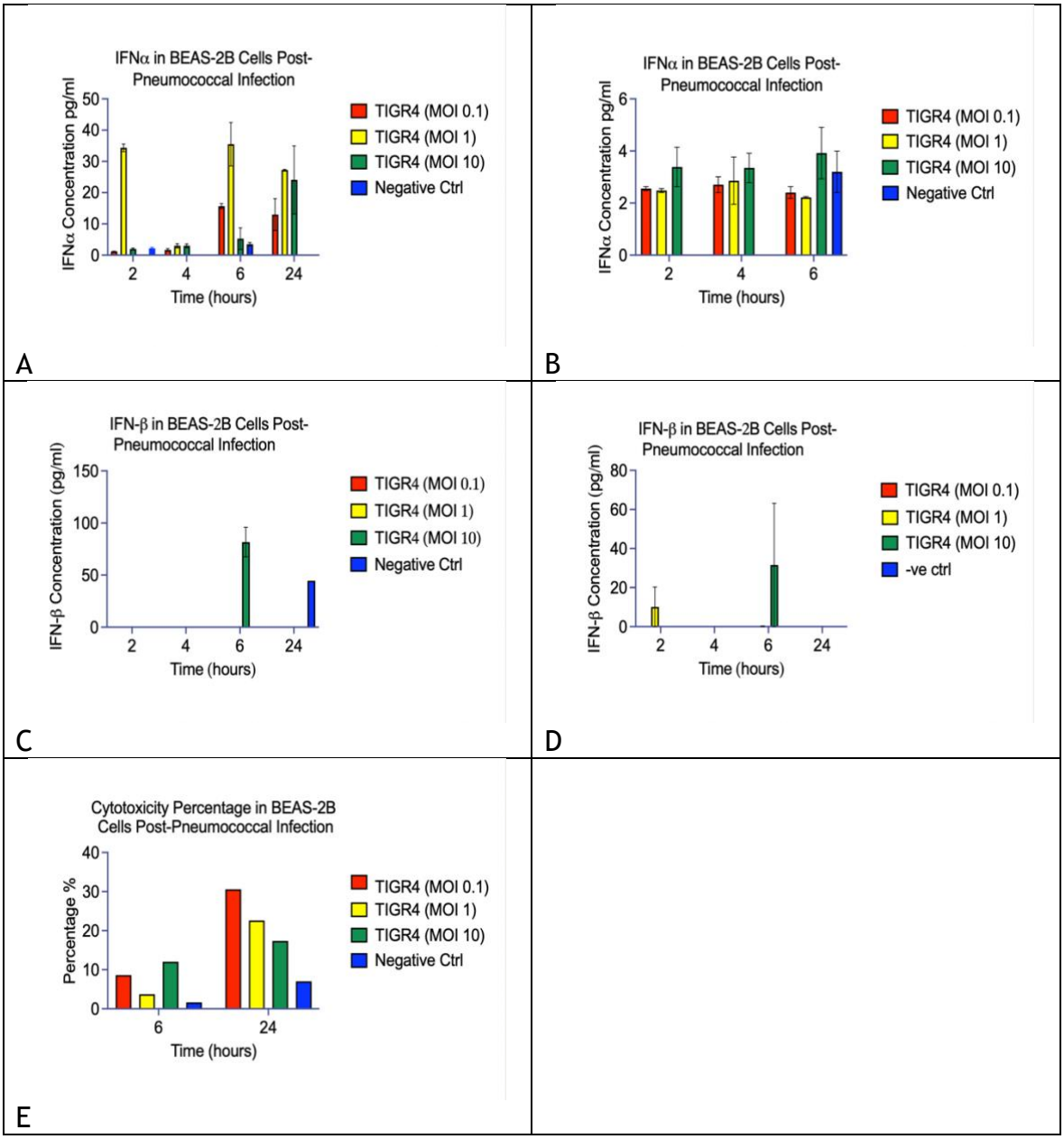


Figure 3-5 Interferon α and β Concentrations in BEAS-2B cells post pneumococcal infection IFN-α in BEAS-2B Cells Post Pneumococcal Infection, cultured in flasks coated with Placental Collagen type 4 for (A) 3 hours prior to the experiment (B) 24 hours prior to the experiment (C) IFN-β in BEAS-2B Cells Post Pneumococcal Infection, cultured in flasks coated with Placental Collagen type 4 for (C)3 hours prior to the experiment (D) 24 hours prior to the experiment Cells were infected with *Streptococcus pneumoniae* TIGR4 (MOIs = 0.1,1,10) for 2,4,6, and 24 hours. The Negative control is untreated cells. The kit's detection limit = (7.81- 500) pg/ml for IFNβ, and (3.12 – 200) pg/ml for IFNα. The data are representative of one experiment with biological replicates(n=2), columns show the mean values; error bars show the SEM. (E) Cytotoxicity percentage in BEAS-2B cells. Cell membrane integrity was assessed by measuring lactate dehydrogenase (LDH) release into the supernatant using a colorimetric LDH cytotoxicity assay. Percentage cytotoxicity was calculated relative to a maximum lysis control. Data represent the average of 2 biological replicates (independent wells).

3.2.4 TNF-α Production in BEAS-2B Cells Post Pneumococcal Infection:

To investigate whether TIGR4 infection stimulated a pro-inflammatory response in BEAS-2B cells, the cells were infected with TIGR4 and assessed for TNF-α using ELISA. The results showed no production of TNF-α, suggesting that the cells may have lost their biological characteristics, it is essential to note that there was no detectable levels of TNF-α produced by positive control cells (treated with Poly I:C) this suggests there is a technical error, defective signalling pathway, or low expression of poly I:C receptors (TLR3, MDA5 and RIG-I). This loss of function could be due to prolonged passaging (

Figure 3-6).

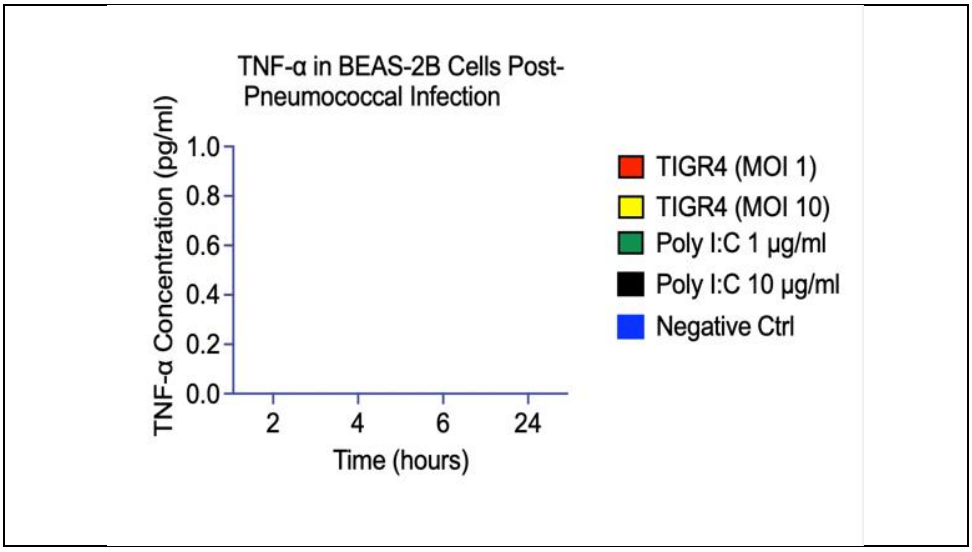


Figure 3-6 TNF-α in BEAS-2B Cells Post Pneumococcal Infection.
BEAS-2B cells were infected with *Streptococcus pneumoniae* TIGR4 (MOIs = 1,10) for 2,4,6, and 24 hours. The data are representative of one experiment with biological replicates(n=2), Positive control is Poly I:C (1,10 µg/ml), Negative control is untreated cells. The kit's detection limit = (15.6-1000) pg/ml.

3.2.5 Pro-inflammatory Cytokines TNF-α and IL-1β in U937 Cells Post Pneumococcal Infection:

At first, the cells were used in cell culture without priming with Phorbol myristate acetate (PMA), an activator of the NF-kB. However, following pneumococcal infection, the cells did not produce any TNF-α or IL-1β (Data not shown). This suggested that PMA priming would be necessary in future experiments. In an additional pilot experiment, the effect of two reagents, granulocyte-macrophage colony-stimulating factor (GM-CSF) and Phorbol myristate acetate (PMA) on the differentiation of U937 cells into macrophages was examined.

The cells were incubated in antibiotic-free media, with some cells primed with (GM-CSF ,50 ng/ml) and others with (PMA – 200 ng/ml) for 24 hours. On the following day, the cells were infected with *Streptococcus pneumoniae* TIGR4 at MOI of 2 and 6. Cell culture supernatants were collected to test for IL-1β secretion levels using ELISA. The results showed no significant difference in IL-1β levels produced by U937 cells primed with GM-CSF compared to U937 cells primed with PMA (Figure 3-7).

Statistical analysis with an unpaired t-test was done to compare the IL-1β levels produced by U937 cells primed with GM-CSF vs IL-1β levels produced by U937 cells primed with PMA. 4 hours after infection, a significant difference was observed between cells primed with PMA and infected (MOI=2) and cells primed with GM-CSF and infected (MOI=2).

The cells that were primed with GM-CSF produced higher levels of IL-1β than the cells primed with PMA at 4 hours. The test shows a similar result at 6 hours after infection (

Figure 3-7). While there was a significant difference between the two priming agents at two time points, the rest of the time points and conditions did not show a significant difference in IL-1β levels between the two. This suggests that these reagents have a similar effect on U937 cells regarding IL-1β production. Hence, in future experiments, only one was used, which is PMA, to differentiate the cell line into macrophages.

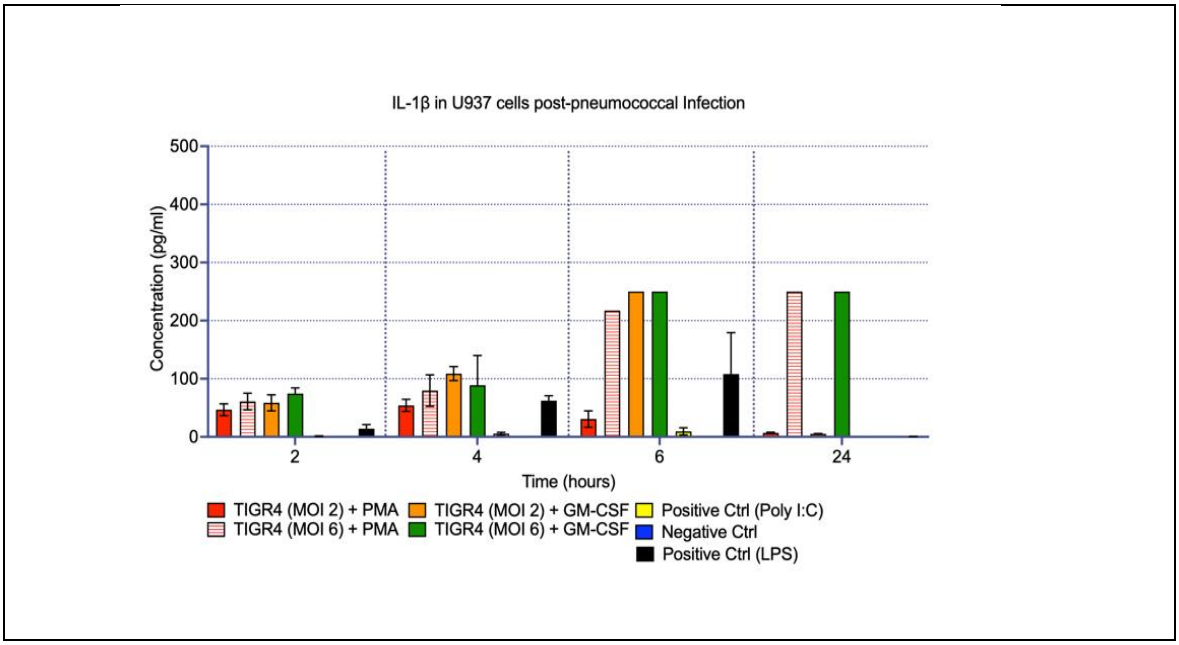


Figure 3-7 IL-1ß Concentrations produced by U937 cells post-pneumococcal Infection. U937 cells were infected with *Streptococcus pneumoniae* TIGR4 (MOIs = 2,6) for 2,4,6, and 24 hours. The data are representative of one experiment with biological replicates (n=3), Columns show the mean, Error bars show SEM. Positive control is LPS + PMA (1 µg/ml); negative control is untreated cells + PMA. The kit's detection limit is (3.91-250) pg/ml. Statistical analysis in the form of an unpaired T-test was done to compare the IL-1ß concentrations produced by U937 cells primed with GM-CSF vs IL1ß concentrations produced by U937 cells primed with PMA. The results revealed no overall significant difference, P value > 0.05.

3.2.6 Interferon-β Production in Monocyte-Derived Dendritic Cells Post-Pneumococcal Infection:

Monocyte-derived dendritic cells (MDDCs) were infected with *Streptococcus pneumoniae* TIGR4, at different multiplicities of infection. However, the levels of IFN-β produced by MDDCs were undetectable post-infection, although stimulation with poly I:C, a TLR3 agonist, as a positive control resulted in good levels of IFN-β. The experiment was repeated twice (Figure 3-8).

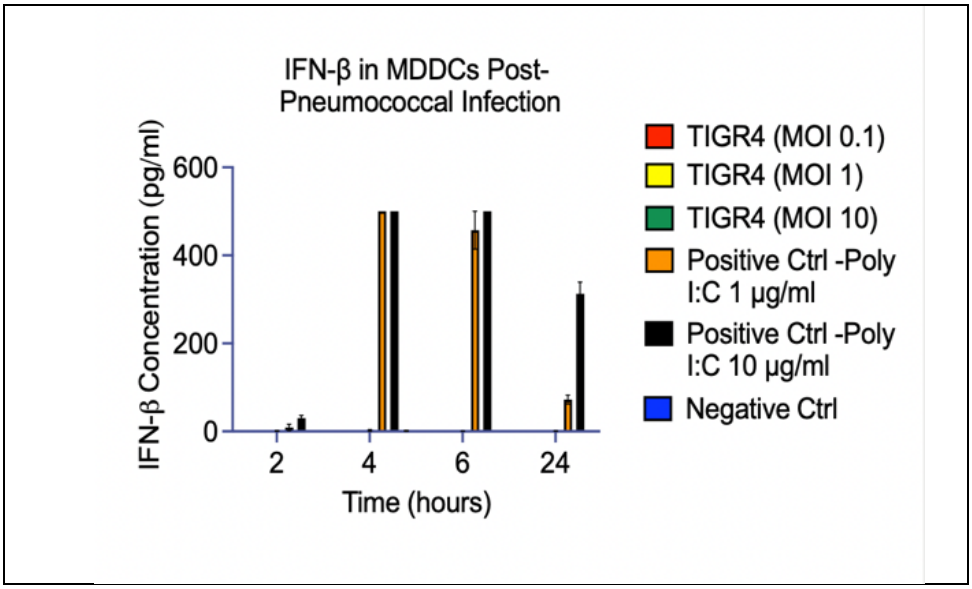


Figure 3-8 IFN-β in MDDCs Post Pneumococcal Infection.
MDDCs were infected with *Streptococcus pneumoniae* TIGR4 (MOIs = 0.1,1,10) for 2,4,6, and 24 hours. The data are representative of one experiment with biological replicates(n=2), Columns show the mean, Error bars show SEM. Positive control is Poly I:C (1,10 µg/ml), Negative control is untreated cells. The kit's detection limit = (7.81-500) pg/ml.

3.2.7 TNF-α Production in Monocyte-Derived Dendritic Cells Post-Pneumococcal Infection:

MDDCs produced significant levels of TNF-α following infection with *Streptococcus pneumoniae* TIGR4 at MOI=10 at 2, 4, and 6 hours after infection. These results may suggest that *Streptococcus pneumoniae* infection has a pro-inflammatory effect on MDDCs, while having little to no effect on interferon production. Interestingly, infection at MOI of 1 did not result in TNF-α secretion, in contrast to the responses observed at MOIs of 0.1 and 10. This discrepancy may be due to an error in bacterial loading (Figure 3-9).

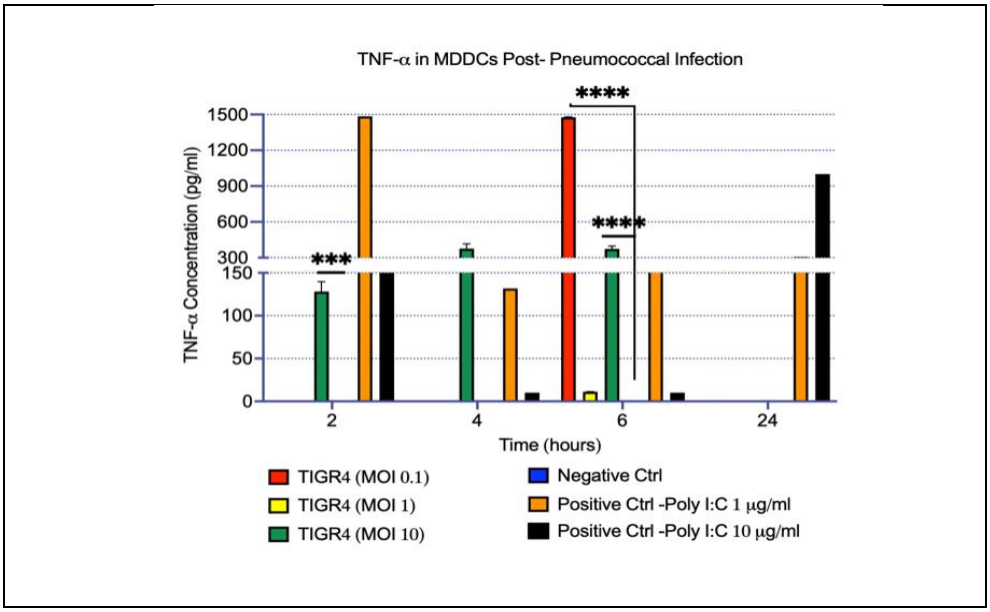


Figure 3-9 TNF-α in MDDCs Post Pneumococcal Infection.
MDDCs were infected with Streptococcus pneumoniae TIGR4 (MOIs = 0.1,1, and 10) for 2,4,6, and 24 hours. The data are representative of one experiment with biological replicates (n=2), columns show the mean, Error bards show SEM. Positive control is Poly I:C (1,10 µg/ml); negative control is untreated cells. The kit's detection limit = (15.6-1000) pg/ml. Statistical analysis was performed using a One-way ANOVA test with a follow-up Dunnett test to compare the results to the negative control.
Symbols: ns means P value > 0.05. * Means P value ≤ 0.05. ** means P value ≤ 0.01. * means P value ≤ 0.001. **** means P value ≤ 0.0001.**

3.3 Conclusion

A key marker of the altered immune responsiveness which characterises trained immunity is the increased production of cytokines in addition to surface receptors[99]. The aim of this chapter was to establish an optimal experimental model for the study of cytokine production in response to pneumococcal infection. The initial model involved growing cells in culture for 24 hours prior to infection, and culturing them in antibiotic-free media. The following day, the cells were infected with *Streptococcus pneumoniae* TIGR4 (at varying MOIs ranging from 0.1 to 10). Cell culture supernatants were then collected at 2-, 4-, 6-, and 24-hours post-infection for cytokine analysis. These time points were selected based on previous studies, which noted that the maximum concentration of TNF-α released from monocyte-derived macrophages following LPS stimulation occurs within 8 hours [143].

Regarding IL-1β, in one study, it was found that in human adherent monocytes, IL-1β mRNA accumulated rapidly after LPS stimulation and was associated with the later appearance of intracellular IL-1β protein, which was then released from the cells (60% at 9 hours) [144]. In another study, it was seen that LPS stimulation of whole blood led to high IL-1β levels after 8 hours [182]. According to Grahames et al., it was found that the THP-1 monocyte cell line, when primed with PMA and stimulated with LPS and ATP, produced high levels of IL-1β between 4-6 hours, with good cytotoxicity percentages among these cells [183]. Based on the findings of the previous two studies, 8 hours would be an optimal time point to measure pro-inflammatory cytokine production. However, due to technical reasons, a 6-hour time point was selected instead.

Regarding the cells used in the infection model, the two cell lines used initially did not produce promising results. Nuli-1 cells did not grow, and BEAS-2B cells did not produce any IFNs (α, β, or γ), even when tissue culture flasks were coated with collagen type 4 for 3 hours prior to culture. BEAS-2B cells were used to assess for TNF-α secretion post *Streptococcus pneumoniae* TIGR4 infection, but no TNF-α production was observed. As a result, both cell lines were deemed unsuitable for use in this study. It is crucial to point out that BEAS-2B cells showed good cell viability percentages at 24 hours after infection (appro. 60%),

which suggests that their lack of cytokine production is not because of cell death.

Upon using U937 cells, which are a monocytic cell line, a more suitable infection model was established. At first, the cells were used as a model to examine TNF-α and IL-1β levels without the use of PMA to prime the cells to differentiate them into macrophages. Under these conditions, the cells did not produce any of the mentioned cytokines. This finding aligns with previous studies, which reported that PMA induced differentiation of THP-1 monocytes into macrophages, elicited a substantial expression of TNF-α [143, 184], suggesting the importance of PMA priming in such experimental setups. Next, these cells were used, with two different priming conditions: PMA and GM-CSF. Both reagents are commonly used to differentiate monocytes into macrophages. The aim was to examine whether there was a difference between the use of these two reagents on IL-1β secretion. The results show no overall significant difference between the two priming agents when it comes to IL-1β secretion post *Streptococcus pneumoniae* infection.

When primary cells were utilised, MDDCs did not produce any IFN-β following *Streptococcus pneumoniae* infection. However, the cells did produce TNF-α after infection, suggesting that *Streptococcus pneumoniae* induces a pro-inflammatory response but does not affect type I IFN levels. It is critical to point that MDDCs had good cell viability percentages at 24 hours after infection (appro. 80%), which suggests that their lack of IFNs production is not because of cell death. Some papers in the literature report IFN secretion after pneumococcal infections; however, it is important to note that these studies involved models with co-infection or sequential infection of *Streptococcus pneumoniae* and Influenza virus, rather than infection with pneumococcus alone [170, 185]. Viral infection plays a crucial role in analysing post-infection IFNs levels.

In Zangari et al. [185], mice were infected with the same strain of *Streptococcus pneumoniae* used in this thesis, TIGR4 (or T4), along with Influenza A virus. Infected mice showed elevated levels of interferon-stimulated genes (ISGs), such as Ifit2, Mx1, and Oasl2, between 2 and 21 days post-infection. However, it is important to highlight that there was no increase in the expression of IFNA or IFNB genes 2 days after TIGR4 infection, indicating that a longer time window

might be necessary for affecting IFN-1 secretion. Nakamura et al. reported that nasopharyngeal colonisation of mice with Pneumococcus induced type I IFNs[170]. However, it is important to note that the strain employed in this study was P1121, a type 23F clinical isolate that does not cause invasive infection in mice. Strain differences are important to consider; in this thesis, TIGR4, a highly virulent strain of *S. pneumoniae* belonging to serotype 4, was used. This might explain the differing results, as P1121 and P1547 are various strains, both belonging to serotype 6A.

Based on the several negative results of IFNs secretion, it seems that *Streptococcus pneumoniae* TIGR4 infection does not induce type I IFNs secretion but has a pro-inflammatory effect in producing TNF-α and IL-1β [184].

The initial focus of the thesis was placed on investigating interferon production following *Streptococcus pneumoniae* infection. However, consistent negative results were obtained with regard to the interferons, indicating that this pathway might not be significantly involved or detectable under the experimental conditions used.

However apart from interferons, several other cytokines are known to play important roles in mediating inflammatory responses and innate immune cell function[186]. These cytokines that have been previously identified to drive the induction and maintenance of trained immunity includes IL-1β, TNF-α, IL-6, and IL-18. As a result, the focus was shifted to the study of trained immunity against *Streptococcus pneumoniae*, using alternative cytokines as markers. This ultimately also allowed for the exploration of a broader and emerging aspect of the innate immune response, providing more promising and insightful data for the thesis.

4 The Role of Trained Innate Immunity against Bacterial Infection

4.1 Introduction

Trained immunity is the functional reprogramming of innate immune cells by external or internal factors. This reprogramming could lead to a modified immune response against a second infection after the cells have returned to their non-active state [87].

The changes to the immune response might amplify or reduce the subsequent response (Figure 4-1) [187]. An example of reducing the subsequent inflammatory response is endotoxin tolerance, in which an initial response to a stimulus triggers molecular events that prevent the induction of a subset of genes upon subsequent exposure to a similar stimulus [188].

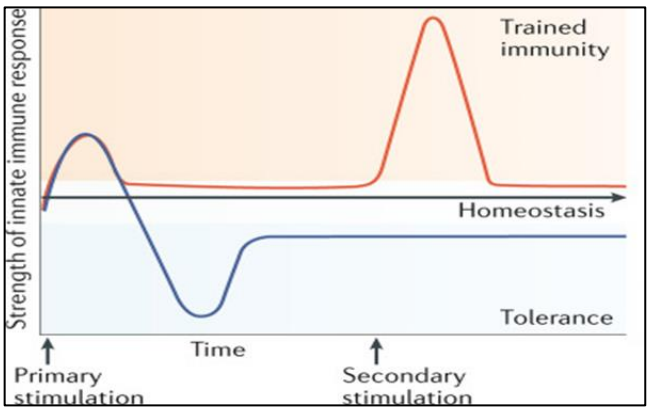


Figure 4-1 Primary and Secondary Innate Immune Responses, and Trained Immunity[87].

Endotoxin tolerance refers to an event that occurs after a cell is exposed to LPS (bacterial endotoxin), resulting in the cell becoming unresponsive or less responsive to a second prolonged exposure to the same initial stimulus [188]. It has been found that a group of LPS-induced genes assembles into a repressive chromatin configuration following this initial stimulus. This initial stimulus produces a memory that inhibits the transcription of these genes following exposure to the second stimulus [189].
More so, trained immunity could be an amplified secondary response. An example of that is seen when initial LPS stimulation leads to the induction of transcription of specific genes following a second stimulus, such as Fpr1 and

Saa3 genes [165]. FPR-1 is an important regulator of neutrophil recruitment and a tissue-specific driver of pulmonary fibrosis [190]. Saa3 gene serves as a biomarker for infection and inflammation and plays a protective role against *P. aeruginosa* infection-induced lung injury [191].
These changes were seen due to epigenetic changes rather than genetic recombinational changes, which occur in adaptive immune cells [90] . Epigenetic changes are heritable changes to gene expression that do not result in a change in the DNA sequence of the gene itself [91].

There are a few epigenetic changes, such as histone modifications or DNA methylation. Examples of histone modification are histone acetylation, in which an acetyl group ($-COCH_3$) is added to a histone tail, resulting in active chromatin. The second example is histone methylation, in which a methyl group (CH_3) is added to the histone tail, resulting in repressed chromatin. The third example is histone phosphorylation, which is adding a phosphate group (PO_4) to a histone tail, and this leads to loose chromatin [92].
Another epigenetic change is DNA methylation, in which a methyl group (CH_3) is added to a cytosine residue that exists in a CpG sequence (Figure 4-2),(Table 4-1).

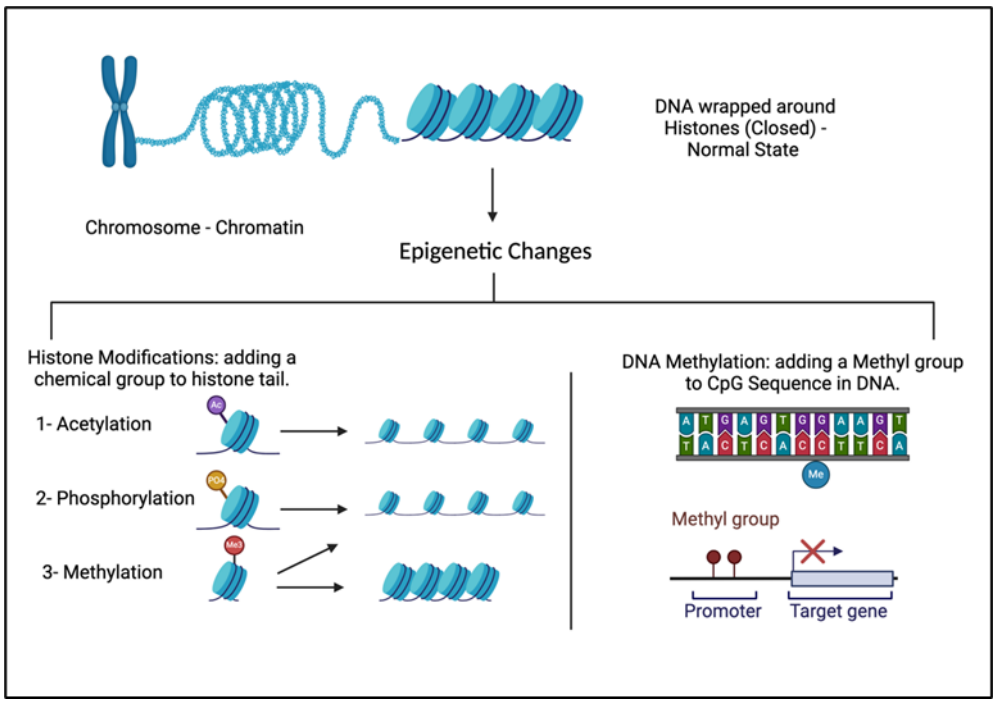


Figure 4-2 Types of Epigenetic Changes.
Figure created by the author using BioRender.com

Table 4-1 Epigenetic Changes and Their Effects on Gene Expression, Adapted from[90, 192, 193]

Epigenetic Change	Effect on Gene Expression	Stability	Function
DNA methylation (promoter)	Silencing	Stable / long-term	Gene repression, genomic stability
Histone acetylation	Activation	Transient / dynamic	Active transcription
Histone deacetylation	Repression	Dynamic	Chromatin condensation
Histone Methylation H3K4me3	Activation	Relatively stable	Active promoters
Histone Methylation H3K27me3	Repression	Stable	Developmental gene silencing
X-inactivation	Silencing	Very stable / permanent	Gene dosage equalization
Histone phosphorylation	Increased (context-dependent)	Transient	Stress response, signaling

These epigenetic changes are reversible and temporary, lasting from 3 months to a year but not for a lifetime, as seen in adaptive immune memory [93, 94]. Also, these epigenetic changes are cell-selective; for instance, only cells that express TLR4 will elicit a robust response to lipopolysaccharide (LPS), which is a stimulant used in this study. LPS stimulation of various macrophages and dendritic cells leads to a strong induction of the Il12b gene, which encodes the p40 subunit of the IL-12 and IL-23 cytokines, both of which play a crucial role in the polarization of T helper cell responses. However, this gene remains transcriptionally inactive in LPS-stimulated fibroblasts, even though many other genes are commonly induced in both macrophages and fibroblasts [194].

The transcriptional response to a stimulus can be divided into primary and secondary responses [195]. Primary response gene activation requires transcription factors, whose activities are induced by post-translational mechanisms in response to the initial stimulus. Examples of post-translational induction mechanisms include the nuclear translocation of NF-κB following the phosphorylation and degradation of the cytoplasmic IκB-family inhibitors [196]. Furthermore, transcription factors induced by post-translational mechanisms often assist in the activation of secondary response genes. However, these genes require the activity of at least one protein that is newly synthesized during the primary response [92].

In this chapter, the roles of three innate immune stimulators- ß-glucan, Zymosan, and LPS are examined in primary cells, which include monocyte-derived dendritic cells and macrophages. A separate section uses the U937 monocytic cell line as a cell model.

To clarify the results' discussion, the following labels were used to compare the cell culture supernatant results:

1- Pretreated Infected cells: refers to cells pre-treated with either (ß-glucan, Zymosan, and LPS) on the first day and infected with *Streptococcus pneumoniae* TIGR4 the following day.

2- Pre-treated cells: refer to cells pre-treated with either (ß-glucan, Zymosan, and LPS) only without infection.

3- Infected cells: refers to cells infected with *Streptococcus pneumoniae* TIGR4, without pre-treatment.

4.2 Results and Discussion

4.2.1 Effect of Pre-treatment with innate immune activators on Monocyte derived dendritic cells:

We tested if pre-treatment with innate immune activators could alter the subsequent cytokine release following pneumococcal infection. Three reagents were introduced for the pre-treatment process: ß-glucan, Zymosan, and Lipopolysaccharide (LPS). The initial set of experiments served as a pilot study to determine the ideal conditions for establishing the best combinations of (pre-treatment and infection) to generate definitive results.

Monocyte-derived dendritic cells (MDDCs) were exposed to these reagents for 24 hours and then infected with TIGR4 at various multiplicities of infection (MOIs). Cell culture supernatants were collected at different time points after the infection, and ELISA assays were performed to assess the levels of the following cytokines: TNF-α, IL-1ß, and IFN-ß.

For IFN-ß, MDDCs did not seem to produce any after pneumococcal infection. The experiment was conducted in duplicates and repeated twice to confirm the same results. These findings suggest that *Streptococcus pneumoniae* TIGR4 infection has no apparent effect on IFN-ß levels. Therefore, interferons were removed from the list of cytokines to be included in this study (Figure 4-3).

For TNF-α, it was found that the levels were higher in samples pre-treated with ß-glucan, zymosan, and LPS compared to the negative control samples (untreated cells). Similar results were observed, although with lower levels of IL-1ß production (data not shown).

These results could suggest that ß-glucan and zymosan have an immune-boosting effect. However, to investigate this effect further, conditions with MDDCs treated with these pre-treatment reagents alone, without *Streptococcus pneumoniae,* should be included. This should be in addition to conditions with MDDCs infected with *Streptococcus pneumoniae* without pre-treatment. Unfortunately, these conditions were not included in these pilot experiments due to limited cell numbers.

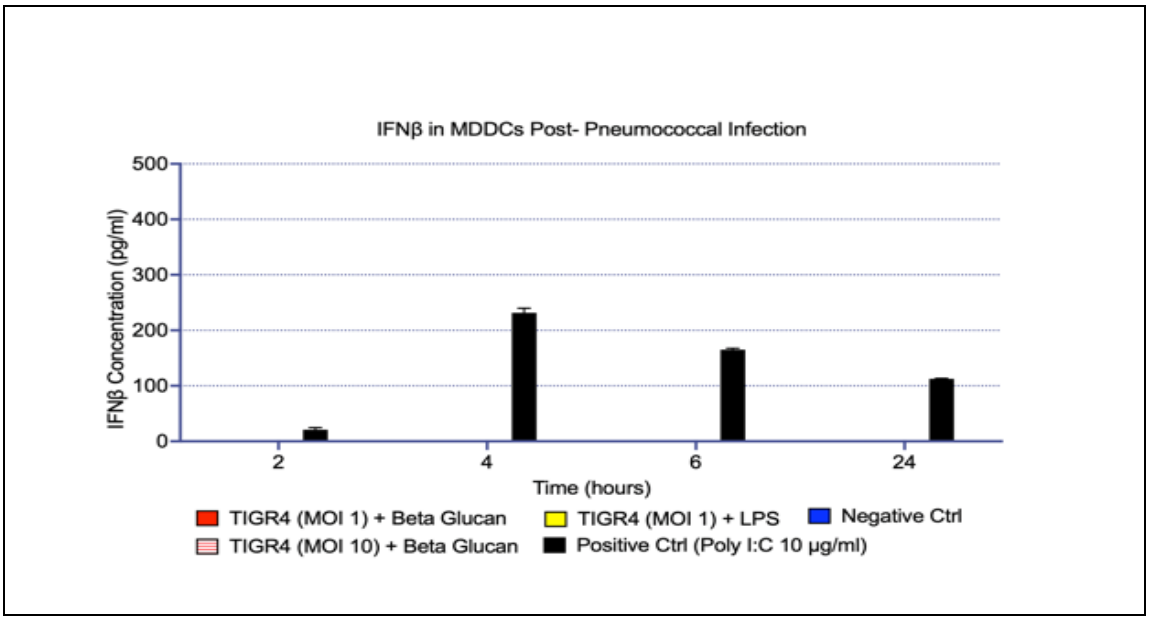


Figure 4-3 Concentrations of IFN-β produced by Monocyte-derived dendritic cells Post-Pneumococcal Infection.
MDDCs were infected with *Streptococcus pneumoniae* TIGR4 (MOIs =1, and 10) for 2,4,6, and 24 hours. Some MDDCs were pre-treated with β-glucan and some with LPS. The data are representative of two experiments with biological replicates (n=2). Columns show the mean, Erorr bars show SEM. Positive control is Poly I:C (10 µg/ml); negative control is untreated cells. The kit's detection limit = (7.81- 500) pg/ml.

The experiment was then repeated with pre-treatment-only controls to further examine the role of these reagents. In this experiment, MDDCs were cultured as usual; the cultures were exposed to ß-glucan, zymosan, and LPS for 24 hours. Some were then infected the next day with *Streptococcus pneumoniae*, while others remained uninfected. ELISA assays were performed to determine the levels of TNF-α, IL-1ß, and IFN-ß.

MDDCs did not produce significant IFN-ß levels (data not shown). As for TNF-α, ß-glucan pre-treatment seems to induce high levels of TNF-α production. However, this effect appears to be due to the ß-glucan itself rather than a trained immunity effect, as the levels of TNF-α in pre-treated infected cells (cells pre-treated with ß-glucan and infected with *Streptococcus pneumoniae* TIGR4 the following day), didn't vary much from the levels of TNF-α from pre-treated cells (ß-glucan only), except at 6 and 24 hours, when the pre-treated cells secreted more TNF-α than the pre-treated infected cells. To investigate this further, another condition must be added, which is infected cells (cells infected with *Streptococcus pneumoniae* TIGR4 without pre-treatment). Unfortunately, this was not included in the current experiment due to limited cell numbers (Figure 4-4).

Similar results were observed with zymosan, as it seems to induce TNF-α production by MDDCs. This effect seems to be due to the zymosan pre-treatment rather than a trained immunity effect. This is because levels of TNF-α in pre-treated infected cells did not vary significantly from the levels produced by pre-treated cells, except at 6 hours, when pre-treated infected cells produced significantly higher levels of TNF-α than pre-treated cells (Figure 4-4). To explore this further, another condition must be added, which is infected cells (cells infected with *Streptococcus pneumoniae* TIGR4 without pre-treatment). Unfortunately, that wasn't included in this experiment for the reason mentioned previously.

TNF-α levels with LPS pre-treatment results were omitted because the samples did not yield readable amounts due to an unknown error.

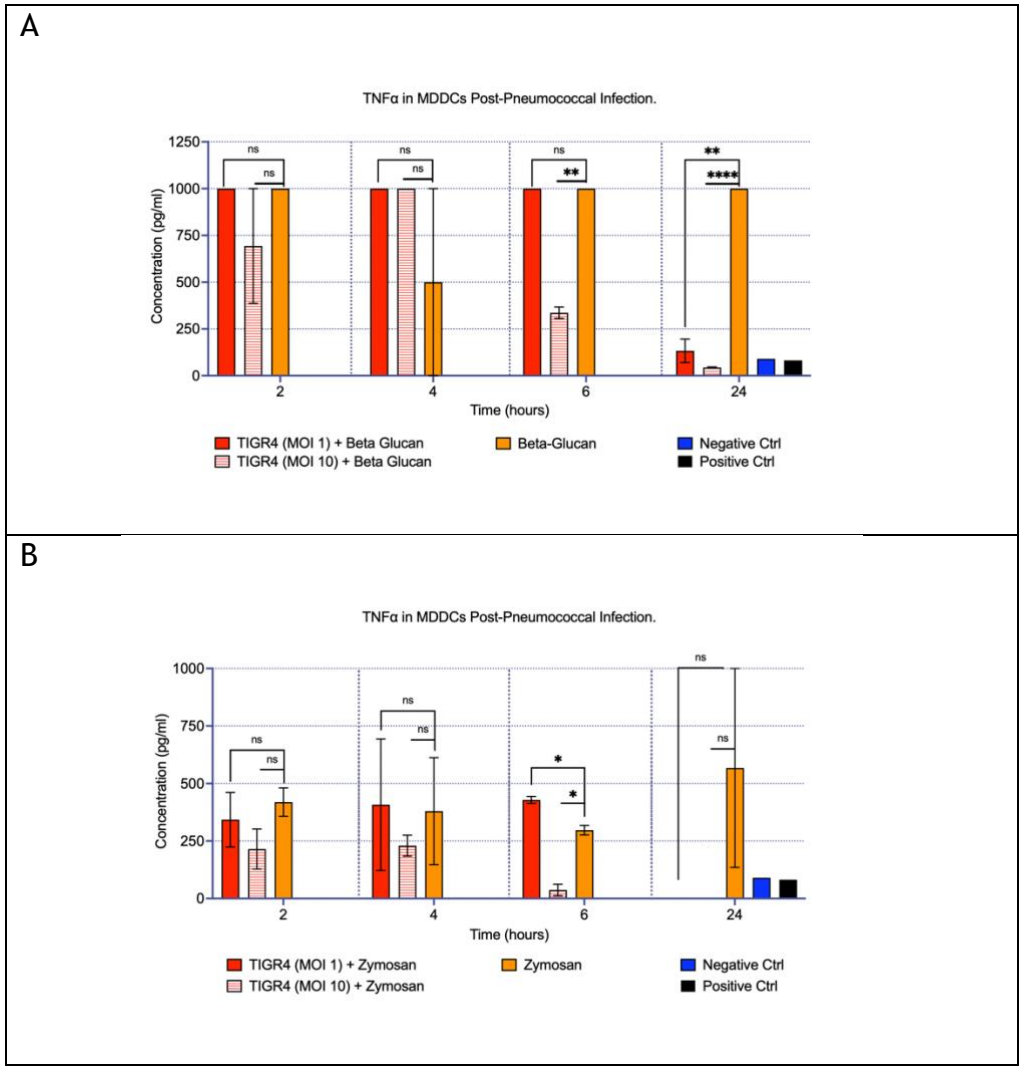


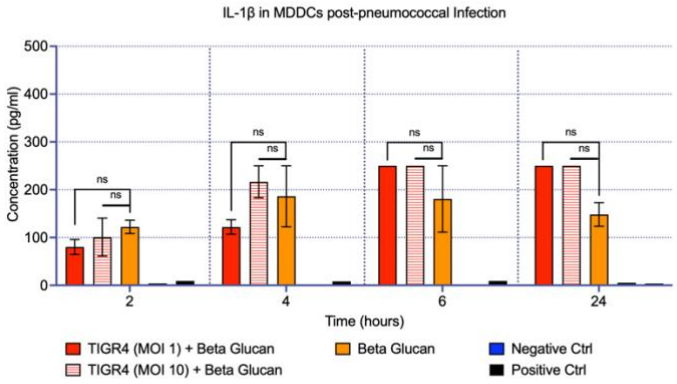
Figure 4-4 Concentrations of TNF-α produced by Monocyte-derived dendritic cells Post-Pneumococcal Infection
(A)pretreated with β-Glucan and (B)pretreated with Zymosan. MDDCs were infected with *Streptococcus pneumoniae* TIGR4 (MOIs = 1, and 10) for 2,4,6, and 24 hours. The data are representative of one experiment with biological replicates (n=2), Columns show the mean, Error bars show SEM. Positive control is Poly I:C (1,10 µg/ml), No measurable concentrations of TNF-α were observed with the positive control, possibly due to a technical error. Negative control is untreated cells. The kit's detection limit = (15.6-1000) pg/ml. Statistical analysis was performed using an unpaired t-test.
Symbols: ns means P value > 0.05. * Means P value ≤ 0.05. ** means P value ≤ 0.01. * means P value ≤ 0.001. **** means P value ≤ 0.0001.**

Another cytokine that was assessed is IL-1ß. MDDCs did not show a significant difference in the amount of IL-1ß secreted between pre-treated infected cells and pre-treated cells (Figure 4-5) .

For zymosan pre-treatment, pre-treated infected cells secreted higher levels of IL-1ß than those pre-treated only at all time points. Further testing is required to establish conclusive results. pre-treated infected cells produced higher IL-1ß levels than pretreated cells at 2, 4, and 6 hours. This could indicate that the elevated IL-1ß levels are not due to zymosan pre-treatment alone but rather to the combined effects of both pre-treatment and infection. However, this needs to be investigated further (Figure 4-5).

No significant difference in IL-1ß levels in LPS-pretreated infected cells compared to pre-treated cells at the majority of timepoints (Figure 4-5).

A



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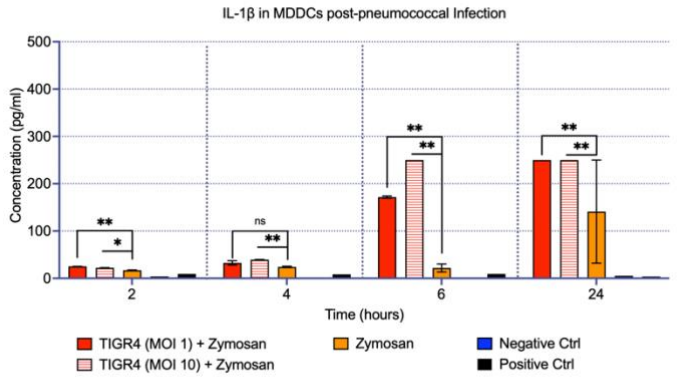

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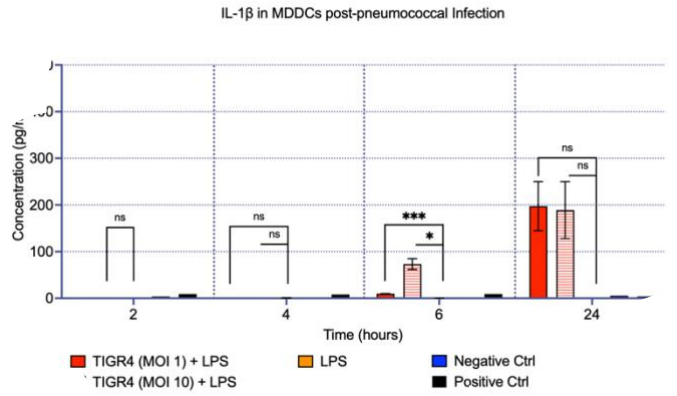


Figure 4-5 Concentrations of IL-1β produced by monocyte-derived dendritic cells post-pneumococcal infection.
(A) pretreated with β-Glucan (B) pretreated with Zymosan, (C) pretreated with LPS. MDDCs were infected with *Streptococcus pneumoniae* TIGR4 (MOIs =1, and 10) for 2,4,6, and 24 hours. The data are representative of one experiment with biological replicates (n=2), Columns show the mean, Error bars show SEM. No positive control: negative control is untreated cells. The kit's detection limit = (3.91-250) pg/ml. Statistical analysis was performed using an unpaired T-test. Symbols: ns indicates P value > 0.05; * indicates P value ≤ 0.05; ** indicates P value ≤ 0.01; * indicates P value ≤ 0.001; **** indicates P value ≤ 0.0001**

Next, to compare the effects of pre-treatments on *Streptococcus pneumoniae* TIGR4 infection, the experiment was repeated with a TIGR4-only condition. Levels of TNF-α, IL-1ß, and IFN ß levels were measured using ELISA.

TNF-α results:

- For ß-glucan: Infected cells secreted more TNF-α compared to ß-glucan pre-treated infected cells at all time points. Statistical analysis indicates a significant difference between the two. However, there is no significant difference in TNF-α levels between pretreated infected cells and the pre-treated cells (Figure 4-6).

- For zymosan: Infected cells secreted more TNF-α compared to zymosan pre-treated infected cells. Pre-treated infected cells secreted higher TNF-α at 24 hours compared to pre-treated cells (Figure 4-6).

- For LPS: Infected cells secreted more TNF-α compared to the pre-treated cells at 2 hours. At 6 and 24 hours after infection, both pre-treated and infected cells secreted similarly high levels of TNF-α. Pretreated infected cells secreted higher levels of TNF-α compared to pre-treated cells at 6 and 24 hours (Figure 4-6).

- Data collection at 4 hours was not possible due to the absence of measurable concentrations, possibly because of an error.

It is challenging to draw conclusions from these results due to their variability, which can be attributed to donor differences.

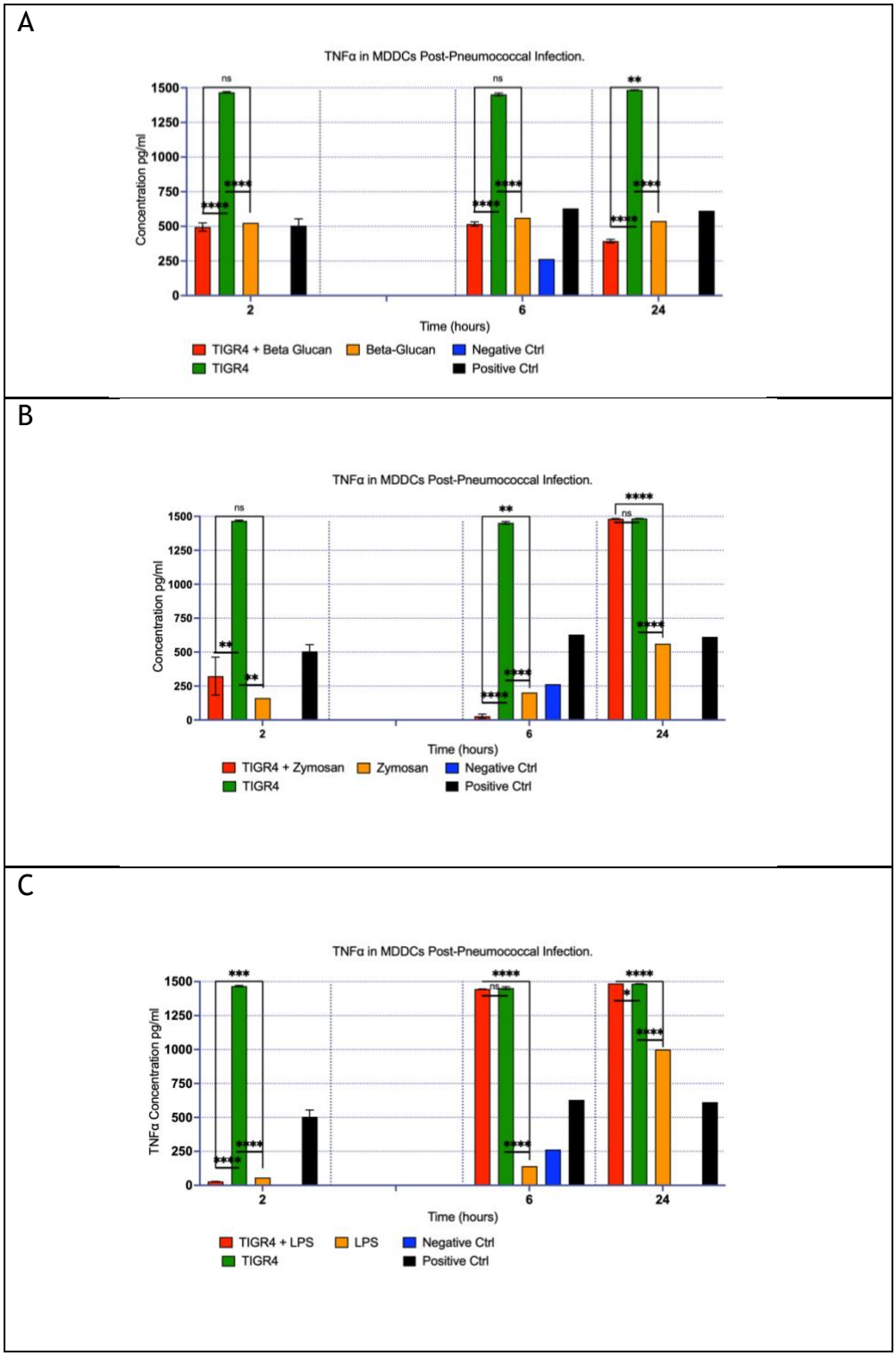


Figure 4-6 Concentrations of TNF-α produced by Monocyte-derived dendritic cells Post-pneumococcal Infection
(A) pre-treated with β-glucan (B) pre-treated with zymosan, post-pneumococcal infection (c) pre-treated with LPS. Figure shows data for MDDCs infected with *Streptococcus pneumoniae* TIGR4 (MOI =1) for 2, 6, and 24 hours. The data for 4 hours were omitted due to the absence of measurable concentrations. The data are representative of one experiment with biological replicates (n=2), Columns show the mean, Error bars show SEM. The positive control is Poly I:C (10 µg/ml); the negative control is untreated cells. The kit's detection limit = (15.6 – 1000) pg/ml. Statistical analysis was performed using a One-way ANOVA test followed by a Dunnett test to compare results to the TIGR4 control in parts A, B, and C, as well as an unpaired T-test. Symbols: ns indicates P value > 0.05; * indicates P value ≤ 0.05; ** indicates P value ≤ 0.01; * indicates P value ≤ 0.001; **** indicates P value ≤ 0.0001.**

IL-1β results:

- For β-glucan, pre-treated infected cells secreted higher levels of IL-1β at 2 and 4 hours compared to infected cells. At 6 and 24 hours post-infection, there does not appear to be a significant difference in IL-1β levels between the two. There is no significant difference in IL-1β levels between pre-treated infected cells and pre-treated cells (Figure 4-7).

- For zymosan, there was no significant difference in IL-1β levels between pretreated infected cells and infected cells. However, pretreated infected cells produced significantly higher IL-1β levels compared to pre-treated cells at 6 and 24 hours. (Figure 4-7).

- For LPS: Like the zymosan results, there is no significant difference in IL-1β levels between LPS-pretreated infected cells and infected cells. also, pretreated infected cells produced significantly higher IL-1β levels compared to pre-treated cells at 6 and 24 hours (Figure 4-7).

IFN-β results: Levels were exceedingly low in all samples (data not shown).

These varying results could be attributed to donor variability, as these experiments were conducted in primary cells sourced from different donors for each experiment. Additionally, there was an issue with establishing a consistent TIGR4 number each time using the growth curve, despite adhering to the same culture conditions.

These issues were resolved in later experiments by using cell lines and frozen TIGR4 stock to ensure the appropriate amount was used in the experiments.

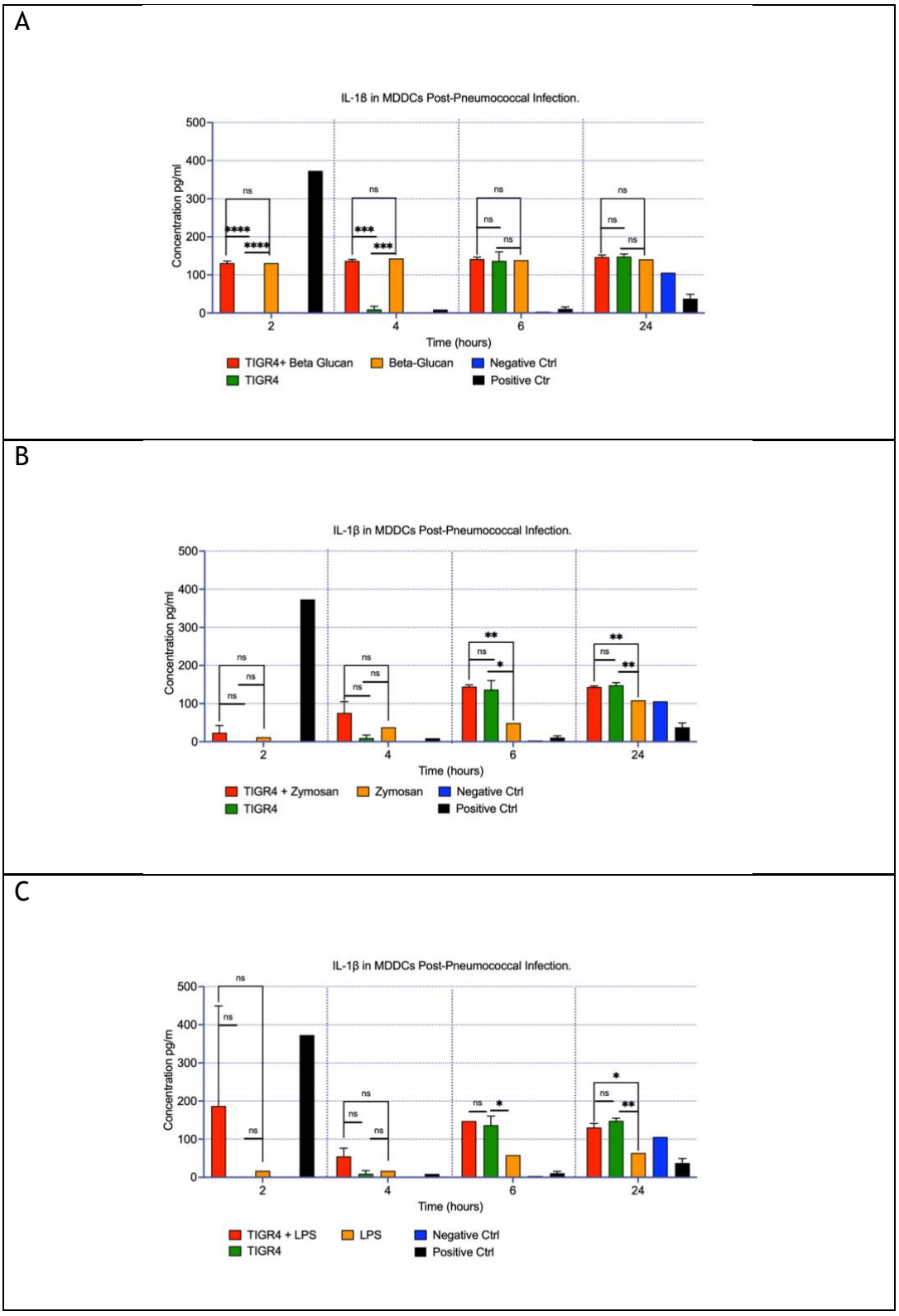


Figure 4-7 Concentrations of IL-1 β produced by monocyte-derived dendritic cells post-pneumococcal infection
(A) pre-treated with β-glucan (B) pre-treated with zymosan (C) pre-treated with LPS. MDDCs were infected with *Streptococcus pneumoniae* TIGR4 (MOI =1) for 2,4,6, and 24 hours. IL-1β concentrations produced by the negative control at 4 hours were omitted, as the values were high due to possible contamination. The data are representative of one experiment with biological replicates (n=2), Columns show the mean, Error bars show SEM. The positive control is Poly I:C (10 µg/ml); the negative control is untreated cells. The kit's detection limit = (3.91-250) pg/ml. Statistical analysis of the results was performed using a one-way ANOVA test followed by a Dunnett test to compare results to the TIGR4 control in A, B, and C, and an unpaired T-test. Symbols: ns indicates P value > 0.05; * indicates P

value ≤ 0.05; ** indicates P value ≤ 0.01; * indicates P value ≤ 0.001; **** indicates P value ≤ 0.0001.**

4.2.2 Effect of Pre-treatment with innate immune activators on primary Macrophages:

The macrophages were grown in culture and pre-treated with ß-glucan (100 µg/ml), zymosan (10 µg/ml), and LPS (1 µg/ml) for 24 hours. After 24 hours, the cells were infected with *Streptococcus pneumoniae* TIGR4 at MOI=1. Cell culture supernatants were collected at 2, 4, 6, and 24 hours for TNF-α level testing.

TNF-α results:

- For ß-glucan, pre-treated infected cells produced significantly higher levels of TNF-α at all time points compared to infected cells. These findings suggest that ß-glucan has an immune-stimulatory effect. Pre-treated infected cells produced more TNF-α than pretreated cells at all time points, suggesting that this effect could be due to trained immunity (Figure 4-8).

- For zymosan: Similar results are seen, pre-treated infected cells produced significantly higher levels of TNF-α at 2, 4, and 6 hours compared to infected cells. Pre-treated infected cells produced more TNF-α than pretreated cells at the 2, 4, and 6-hour time points, suggesting that this effect could be due to trained immunity (Figure 4-8).

- For LPS: Pretreated infected cells produced significantly higher TNF-α than infected cells at 2, 4, and 6 hours post-infection. Additionally, pretreated infected cells produced higher TNF-α levels than pretreated cells at the same time points, suggesting that this effect could be due to trained immunity (Figure 4-8).

These results suggest that pre-treatment with the above reagents has an immune-stimulatory effect on TNF-α production by macrophages following pneumococcal infection. Because no rest period was provided in this experiment, this effect could be due to trained immunity or to an amplified acute immune response to *Streptococcus pneumoniae following* pre-treatment.

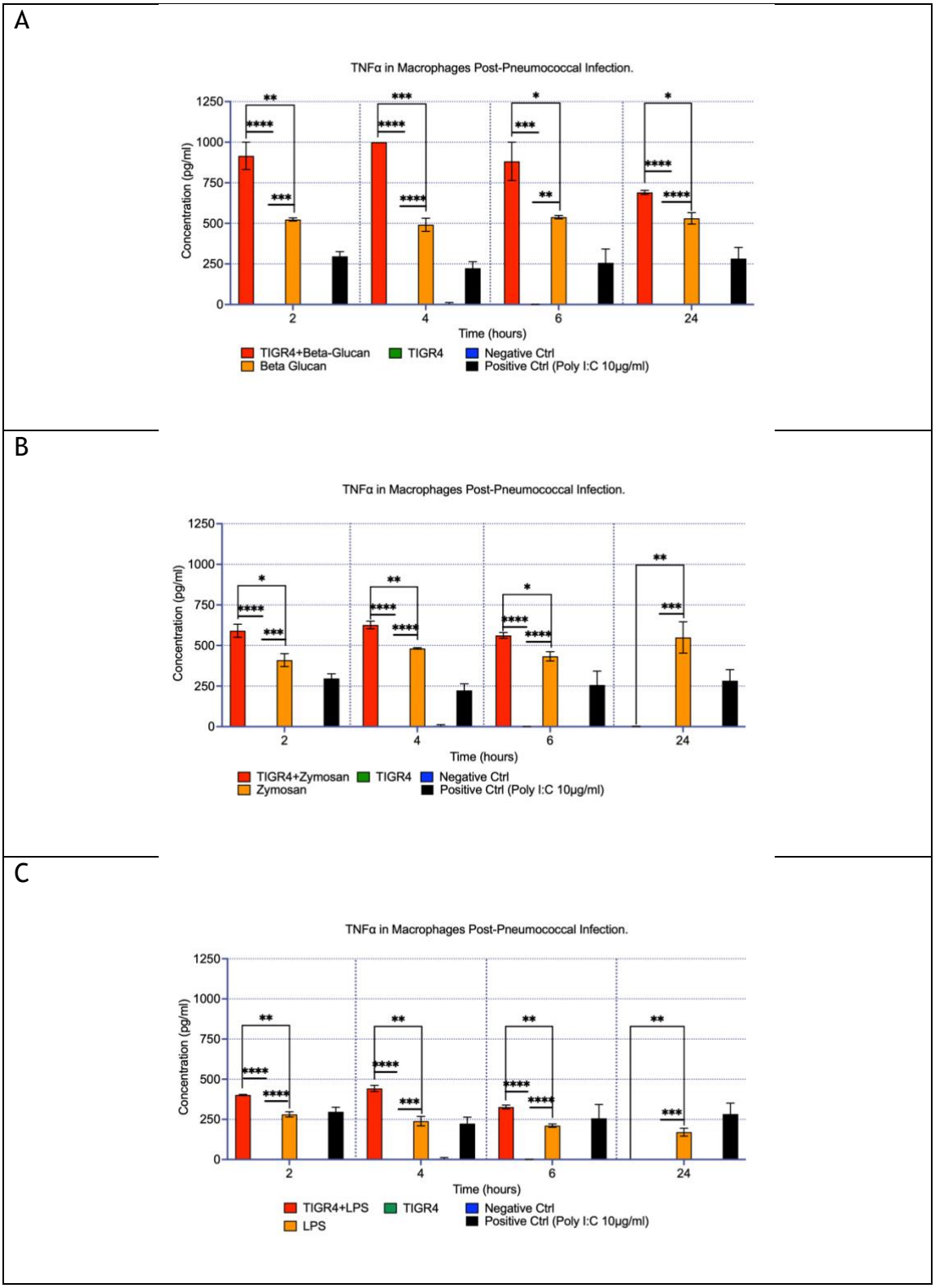


Figure 4-8 Concentrations of TNF α Produced by Macrophages Post-Pneumococcal Infection

(A) pre-treated with β-Glucan (B) pre-treated with zymosan (C) pre-treated with LPS. Macrophages were infected with Streptococcus pneumoniae TIGR4 (MOI =1) for 2,4,6, and 24 hours. Some cells were pre-treated with beta-glucan, some with zymosan, and some with LPS. The data are representative of one experiment with biological replicates (n=3). Columns show the mean, Error bars show SEM. The positive control is Poly I:C (10 µg/ml); the negative control is untreated cells. The kit's detection limit = (15.6-1000) pg/ml. Statistical analysis of the results was performed using a One-way ANOVA test with a follow-up Dunnett test to compare the results to the TIGR4 control in A, B, and C, as well as an

unpaired T-test. Symbols: ns indicates a P value > 0.05. * Indicates a P value ≤ 0.05. ** indicates a P value ≤ 0.01. * indicates a P value ≤ 0.001. **** indicates a P value ≤ 0.0001.**

The experiment was repeated to consolidate the results. Macrophages were grown in culture and pre-treated with ß-glucan (100µg/ml), zymosan (10 µg/ml), and LPS (1 µg/ml) for 24 hours. After this period, cells were infected with *Streptococcus pneumoniae* TIGR4 at (MOI=0.1). This time (LPS+ATP) was added as a positive control for IL-1ß testing [197]. LPS (1 µg/ml) was added on the day prior to infection, and ATP (5 mM-Sigma) was added one hour prior to each time point for cell culture supernatant collection.

Cell culture supernatants were collected at 2, 4, 6, and 24 hours for testing of TNF-α and IL-1ß levels.

TNF-α results

- For ß-glucan: Infected cells produced low levels of TNF-α, which could be attributed to the low MOI. Pre-treated infected cells produced significantly higher levels of TNF-α compared to the levels produced by infected cells, except at 24 hours. This indicates that pre-treatment has a stimulatory effect on TNF-α production. There was no significant difference in TNF-α levels between pre-treated infected cells and pre-treated cells , except at 24 hours. (Figure 4-9).

- For zymosan: Pre-treated infected cells produced significantly higher levels of TNF-α compared to the levels produced by infected cells at all time points. However, when comparing TNF-α production in pre-treated cells versus those pre-treated and infected, there was a significant difference between the two only at 24 hours. When the zymosan pre-treated cells produced higher levels of TNF-α compared to pre-treated infected cells (Figure 4-9).

- For LPS: Similar results were seen. Pretreated infected cells produced significantly higher levels of TNF-α than infected cells. This suggests that LPS has an immune-stimulatory effect on TNF-α production. In addition, pretreated cells exhibited a trend of higher TNF-α production at all time points than pretreated infected cells; however, the difference did not reach statistical significance (Figure 4-9).

TNF-α results suggest that in this particular experiment, ß-glucan, zymosan, and LPS pre-treatment have an immune-stimulatory effect on TNF-α production (Figure 4-9) regardless of infection status. The difference from the previous results is likely due to variations among the donors of these primary cells.

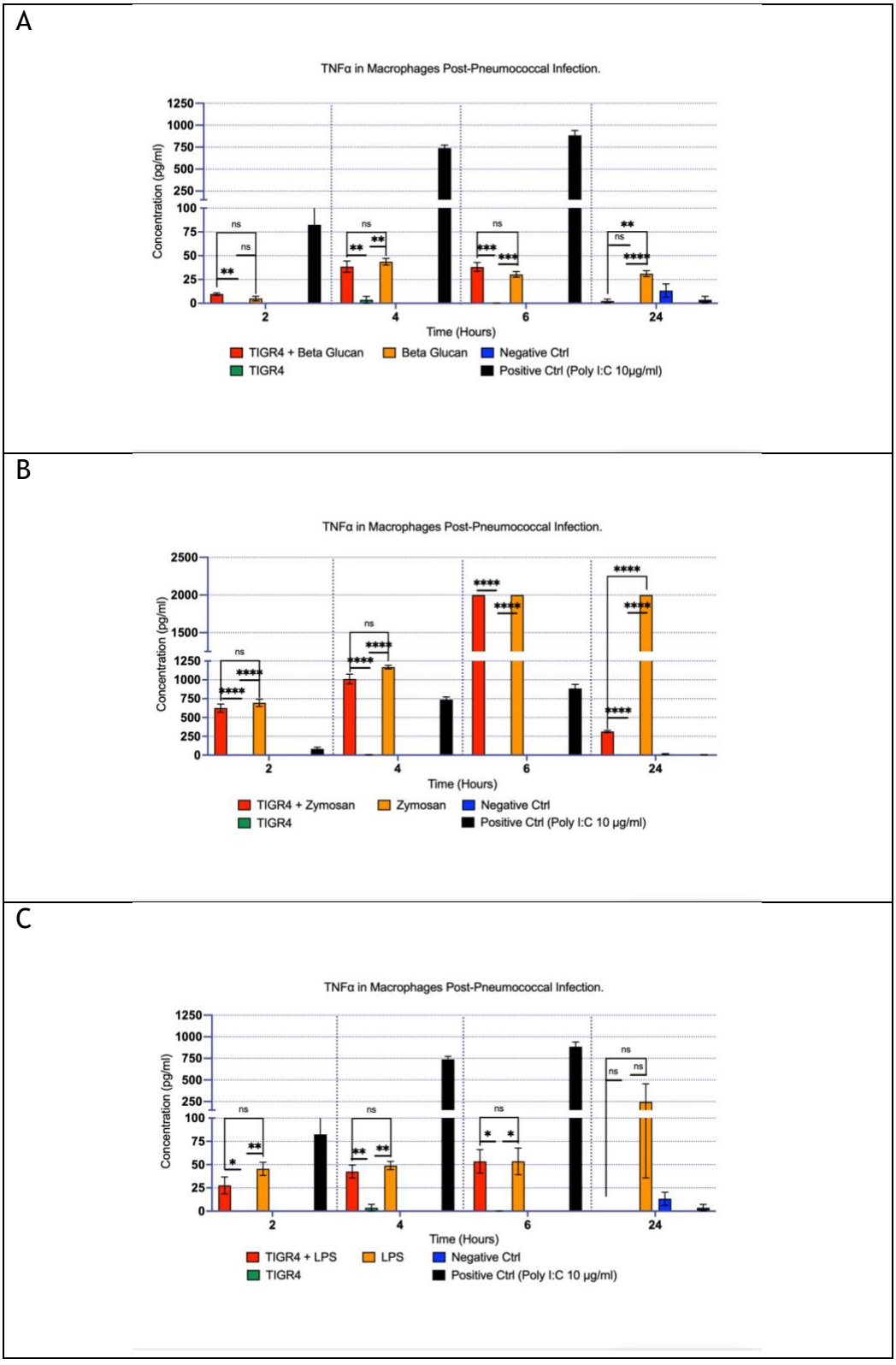


Figure 4-9 Concentrations of TNF-α Produced by Macrophages Post-Pneumococcal Infection

(A) pre-treated with β-glucan (B) pre-treated with zymosan (C) pre-treated with LPS. Macrophages were infected with *Streptococcus pneumoniae* TIGR4 (MOI =0.1) for 2,4,6, and 24 hours. Some cells were pre-treated with beta-glucan, some with zymosan, and some with LPS. The data are representative of one experiment with biological replicates (n=3). Columns show the mean, Error bars show SEM. The positive control is Poly I:C (10 µg/ml); the negative control is untreated cells. The kit's detection limit = (15.6-1000) pg/ml. Statistical analysis of the results was performed using a One-way ANOVA test with a follow-up Dunnett test to compare the results to the TIGR4 control in A, B, and C, as well as an unpaired T-test.

Symbols: ns indicates a P value > 0.05. * indicates a P value ≤ 0.05. ** indicates a P value ≤ 0.01. * indicates a P value ≤ 0.001. **** indicates a P value ≤ 0.0001.**

IL-1β results:

- For β-glucan: Infected cells produced low levels of IL-1β, which could be attributed to the low MOI. There was no significant difference in IL-1β levels between pretreated infected cells and pretreated cells, except at 24 hours when pre-treated infected cells secreted higher IL-1β than the pretreated cells (Figure 4-10).

- For zymosan, pretreated cells showed a trend of higher IL-1β production at most of the time points compared to pretreated infected cells, and such difference reached statistical significance at 2 hours (Figure 4-10).

- For LPS: it appears that pre-treated infected cells produced higher IL-1β levels compared to pretreated cells at 4 and 24 hours (Figure 4-10).

IL-1β results indicate that LPS pre-treatment has an immune-stimulatory effect on IL-1β production, but not β-glucan nor zymosan (Figure 4-10).In these experiments also, differences from previous results might be attributed to differences between donors in each experiment.

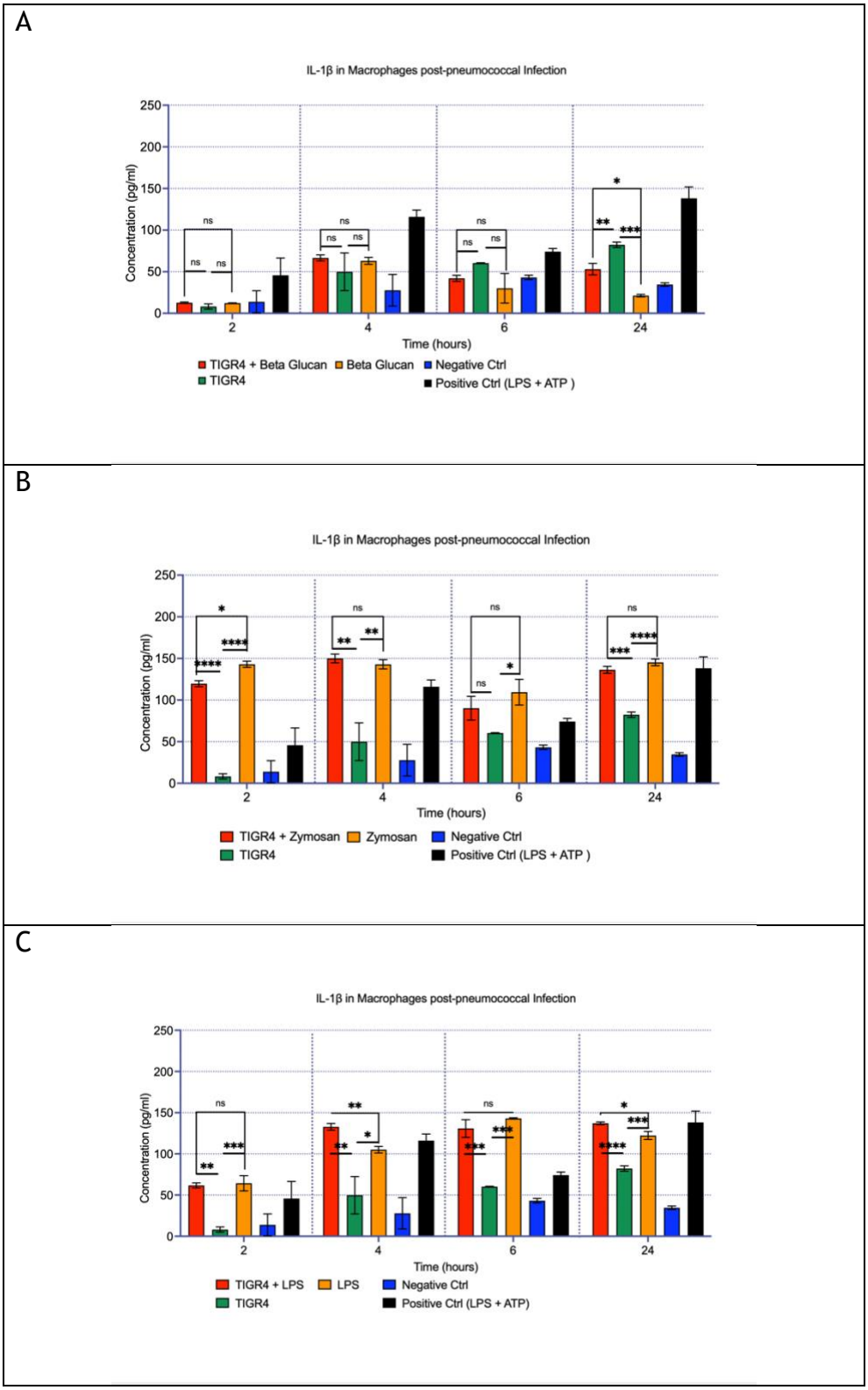


Figure 4-10 Concentrations of IL-1 β Produced by Macrophages Post-Pneumococcal Infection
(A) pre-treated with β-glucan (B) pre-treated with zymosan (C) pre-treated with LPS. Macrophages were infected with *Streptococcus pneumoniae* TIGR4 (MOI =0.1) for 2,4,6, and 24 hours. The data are representative of one experiment with biological replicates (n=3). Columns show the mean, Error bars show SEM. The positive control is Poly I:C (10 µg/ml); the negative control is untreated cells. The kit's detection limit = (3.91-250) pg/ml. Statistical analysis of the results was performed using a One-way ANOVA test with a follow-up Dunnett test to compare the results to the TIGR4 control in A, B, and C, as well as an unpaired T-test.
Symbols: ns indicates a P value > 0.05. * indicates a P value ≤ 0.05. ** indicates a P value ≤ 0.01. * indicates a P value ≤ 0.001. **** indicates a P value ≤ 0.0001.**

4.2.3 Effect of Pre-treatment with Innate Immune Activators on U937 Monocytic Cell Line:

4.2.3.1 The role of PMA Priming on Cytokine Release by U937 Monocyte-derived Macrophages Cell Line Following *Streptococcus pneumoniae* TIGR4 infection

Due to the variability in the results obtained from primary cells, such as monocytes differentiated into dendritic cells or macrophages, we conducted additional experiments using the U937 monocytic human cell line. First, the cells were cultured without priming with Phorbol myristate acetate (PMA), an NF-kB activator. However, in the absence of priming, the cells did not produce any TNF-α or IL-1ß following pneumococcal infection (Data not shown).

4.2.3.2 The role of PMA Priming vs. GM-CSF Priming on Cytokine Release by U937 Monocyte-derived Macrophages Cell Line Following *Streptococcus pneumoniae* TIGR4 infection

Next, in another pilot experiment, we wanted to examine the effect of two reagents that can be used to differentiate U937 monocytes into macrophages: granulocyte-macrophage colony-stimulating factor (GM-CSF) and Phorbol myristate acetate (PMA). The cells were incubated in antibiotic-free media; some were primed with GM-CSF (50 ng/ml) and others with PMA (200 ng/ml) for 24 hours. The following day, the cells were infected with *Streptococcus pneumoniae* TIGR4 at a multiplicity of infection (MOI) of 2 and 6.

Cell culture supernatants were collected to measure IL-1ß secretion levels using ELISA. The results have shown no significant difference in IL-1ß levels produced by U937 cells primed with GM-CSF compared to those primed with PMA.

Unpaired T-test was done to compare the IL-1ß levels produced by U937 cells primed with GM-CSF vs those produced by U937 cells primed with PMA. The results have shown no significant difference between the two conditions, except at 4 and 6 hours. Cells that were primed with GM-CSF and infected (MOI=2) produced higher levels of IL-1β than those primed with PMA and infected (MOI=2) (Figure 4-11).

While a significant difference was observed between the two priming agents at two time points, the remaining time points and conditions did not show a

significant difference in IL-1ß levels. This suggests that these reagents have a similar effect on U937 cells in terms of IL-1ß production; hence, in future experiments, only one can be used, and that was PMA, to differentiate the cell line into macrophages.

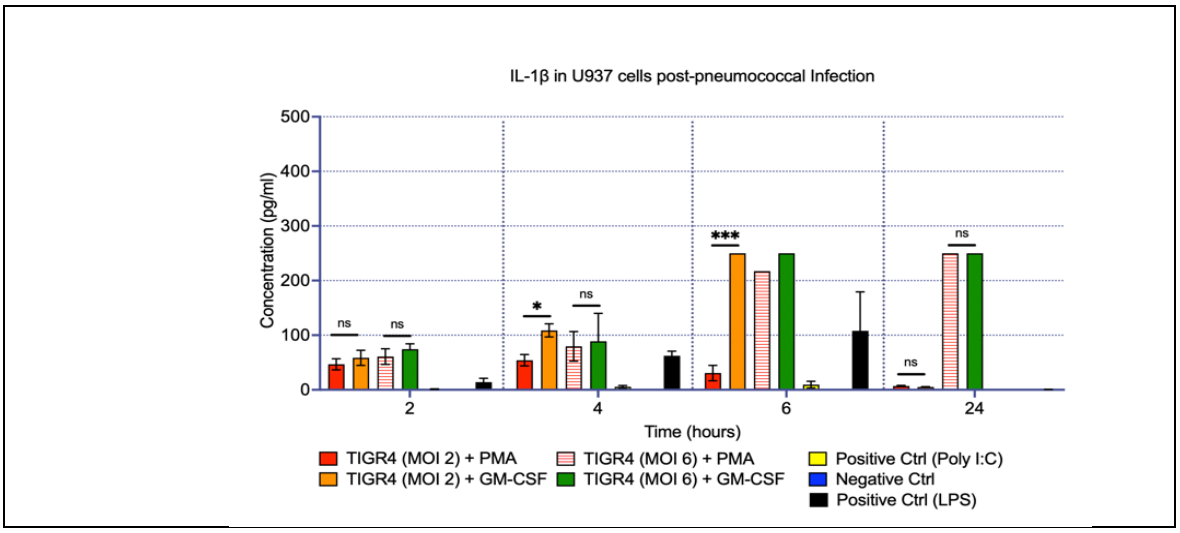


Figure 4-11 Concentrations Produced by U937 cells Post-Pneumococcal Infection. U937 cells were infected with *Streptococcus pneumoniae* TIGR4 (MOIs = 2,6) for 2,4,6, and 24 hours. The data are representative of one experiment with biological replicates (n=3). Columns show the mean, Error bars show SEM. Positive control is LPS + PMA (1 µg/ml); negative control is untreated cells + PMA. The kit's detection limit is (3.91-250) pg/ml. Statistical analysis in the form of an unpaired T-test was done to compare the IL-1β concentrations produced by U937 cells primed with GM-CSF vs IL1β concentrations produced by U937 cells primed with PMA. The results revealed no overall significant difference, P value > 0.05.

4.2.3.3 Pre-treatment Effect on Cytokine levels produced by U937 Monocyte-derived Macrophages Cell Line Following *Streptococcus pneumoniae* TIGR4 infection

To test the effect of pre-treatment with innate immune stimulants on U937 cells, the cells were cultured in antibiotic-free media for 24 hours, and PMA (100 ng/mL) was added to prime the cells and facilitate their differentiation into macrophages. After 24 hours, the cells were pre-treated with the following reagents and concentrations for 24 hours: ß-glucan (100 µg/ml), zymosan (10 µg/ml), and LPS (1 µg/ml). After 24 hours, cells were infected with *Streptococcus pneumoniae* TIGR4 at MOI=1. Results are presented in (Figure 4-12).

TNF-α results:

- For ß-glucan, pretreated infected cells showed a trend toward higher TNF-α production compared to infected cells. Such observation reached statistical significance at the 4-hour mark. To assess the effect of pre-treatment, pre-treated cells were compared to pre-treated infected cells in terms of TNF-α production. Pretreated infected cells produced higher TNF-α levels at 2 and 4 hours, although no significant differences were noted at other time points (Figure 4-12).

- For zymosan: Pretreated infected cells produced significantly higher levels of TNF-α compared to infected cells. This effect was observed at all time points following infection. However, there was no significant difference in TNF-α levels between pretreated infected cells and pretreated cells, except at 6 hours, when pretreated infected cells produced a higher level of TNF-α compared to pretreated cells. (Figure 4-12).

- For LPS: Pretreated infected cells produced significantly higher TNF-α levels than infected cells. This effect was observed at all time points following infection. When assessing the pre-treatment effect, there was no significant difference in TNF-α level between pretreated infected cells except at 4 hours, when pretreated infected cells produced a higher level of TNF-α compared to pretreated cells. At 6 and 24 hours, both conditions

yielded the highest detectable levels of TNF-α, making it difficult to comment on the difference in levels (Figure 4-12).

These results suggest that pre-treating U937 cells with the innate immune stimulant ß-glucan led to high levels of TNF-α secreted post-infection at early time points. However, there was no significant difference observed with zymosan and LPS pretreatment.

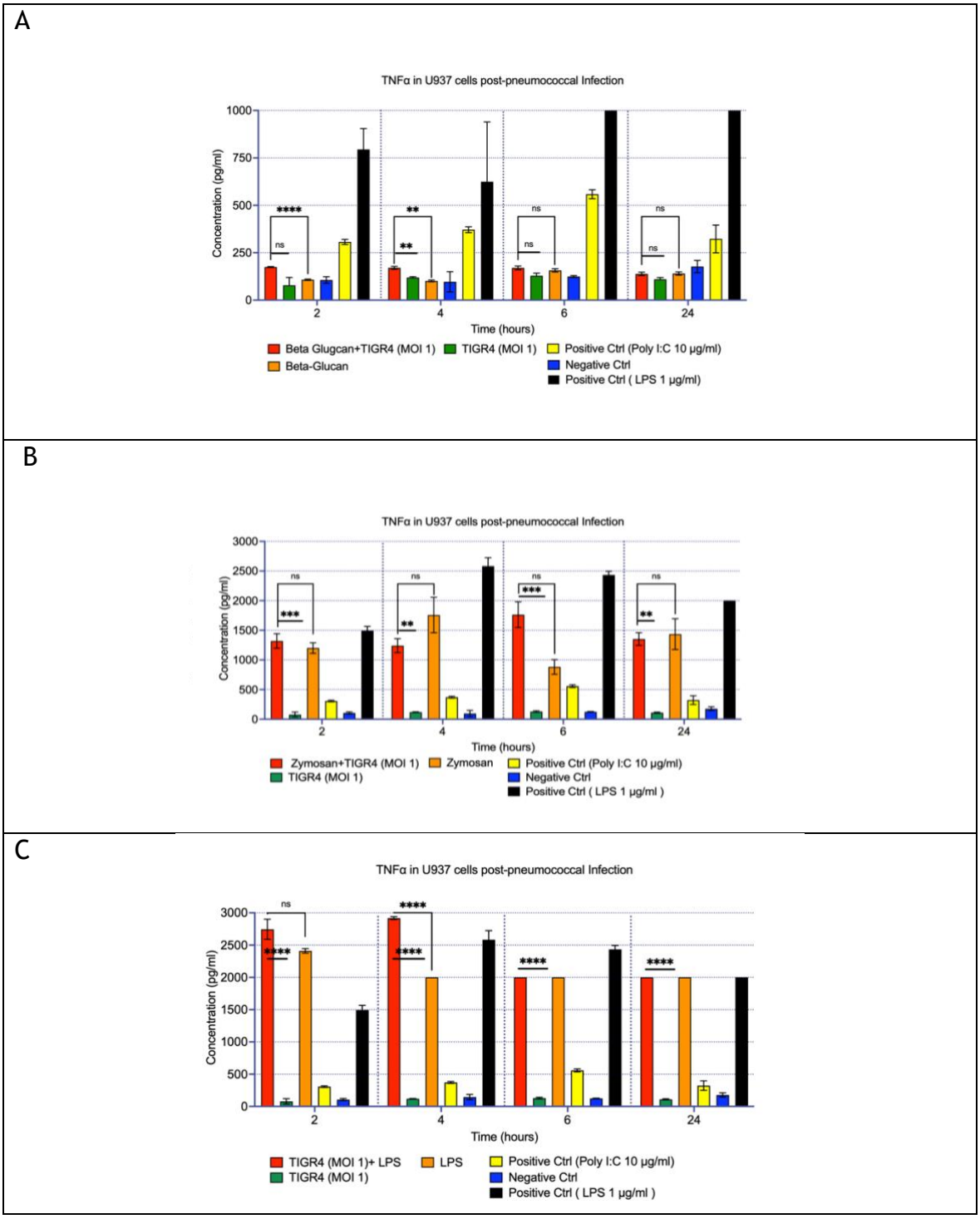


Figure 4-12 TNF-α Concentrations Produced by U937 cells Post-Pneumococcal Infection (A) Cells pre-treated with β-glucan (B) Cells pre-treated with zymosan (C) Cells pre-treated with LPS. U937 cells were infected with *Streptococcus pneumoniae* TIGR4 (MOI =1) for 2,4,6, and 24 hours. Some cells were pre-treated with beta-glucan, some with zymosan, and some with LPS. The data are representative of one experiment with biological replicates (n=3). Columns show the mean, Error bars show SEM. Two positive controls: one is Poly I:C (10 µg/ml)+ PMA; the other is LPS (1 µg/ml) + PMA; the negative control is untreated cells + PMA. The kit's detection limit = (15.6-1000) pg/ml. Statistical analysis of the results was performed using a One-way ANOVA test with a follow-up Dunnett test to compare the results to the TIGR4 control in A, B, and C, as well as an unpaired T-test. Symbols: ns indicates a P value > 0.05. * indicates a P value ≤ 0.05. ** indicates a P value ≤ 0.01. * indicates a P value ≤ 0.001. **** indicates a P value ≤ 0.0001.**

IL-1β results:

- For β-glucan: No difference in IL-1β levels between pretreated infected cells and infected cells, except at the 24-hour time point. When comparing TNF-α levels between pre-treated infected cells and pre-treated cells, there was a small yet significant difference observed at 24 hours, with the former cells produced higher IL-1β compared to the latter (Figure 4-13).

- For zymosan: Pre-treated infected cells produced significantly higher levels of IL-1β than infected cells. This effect was consistent across all time points following infection. However, there was not a significant difference in IL-1β level between pre-treated infected cells and pre-treated cells, except at 4 hours, when the former produced a higher level of IL-1β compared to the latter (Figure 4-13).

- For LPS: Pre-treated infected cells produced significantly higher levels of IL-1β than infected cells; this effect was observed at all time points following infection. Pre-treated infected cells showed a trend toward higher IL-1β production than pre-treated cells at 4, 6, and 24 hours. However, none reached statistical significance (Figure 4-13).

These results resemble those of TNF-α in the sense that β-glucan had a minor effect, while zymosan and LPS did not show a significant effect on IL-1β production.

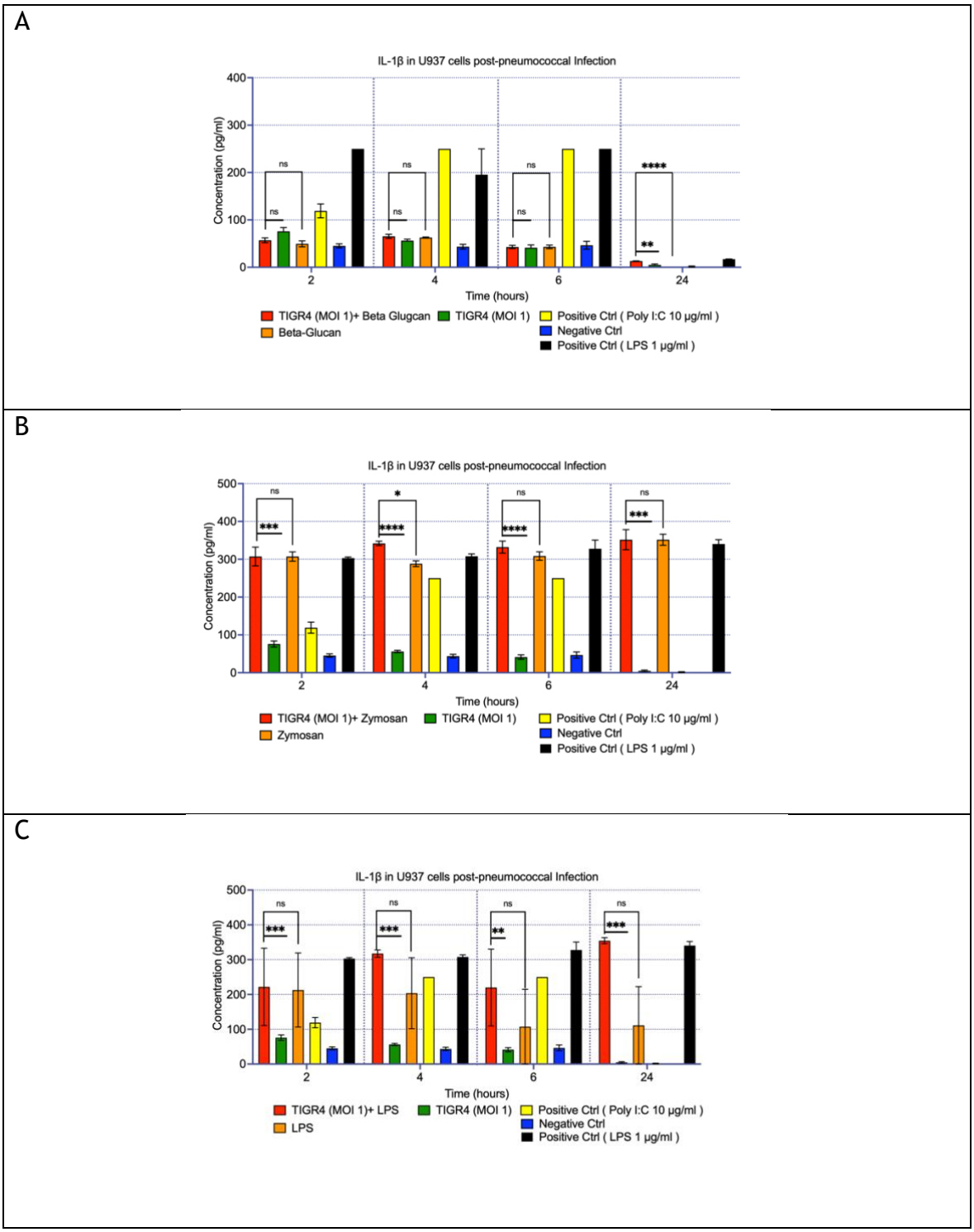


Figure 4-13 IL-1β Concentrations Produced by U937 cells Post-Pneumococcal Infection (A) pre-treated with β-glucan (B) cells pre-treated with zymosan (C), cells pre-treated with LPS. U937 cells were infected with *Streptococcus pneumoniae* TIGR4 (MOI =1) for 2,4,6, and 24 hours. Some cells were pre-treated with beta-glucan, some with zymosan, and some with LPS. The data are representative of one experiment with biological replicates (n=3). Columns show the mean, Error bars show SEM. Positive control is LPS (1 µg/ml) + PMA; the negative control is untreated cells + PMA. The kit's detection limit = (3.91-250) pg/ml. Statistical analysis of the results was performed using a One-way ANOVA test with a follow-up Dunnett test to compare the results to the TIGR4 control in A, B, and C, as well as an unpaired T-test.
Symbols: ns indicates a P value > 0.05. * indicates a P value ≤ 0.05. ** indicates a P value ≤ 0.01. * indicates a P value ≤ 0.001. **** indicates a P value ≤ 0.0001.**

4.2.3.4 The Effects of Different Priming and Pretreatment Orders on Cytokine Levels Produced by U937 Monocyte-derived Macrophages Cell Line Following *Streptococcus pneumoniae* TIGR4 Infection

Next, we examined whether the order of PMA priming and cellular pre-treatment might contribute to differences in our results. Therefore, the experiment was repeated this time as follows:

- Some U937 cells were primed with PMA (100 ng/ml) and simultaneously pre-treated with innate immune stimulants: ß-glucan (100µg/ml), zymosan (10 µg/ml), and LPS (1 µg/ml) for 4 hours. Afterwards, the cells were washed, spun, and resuspended in fresh antibiotic-free media with PMA only.

- The other group was pre-treated with the same innate immune stimulants: ß-glucan (100µg/ml), zymosan (10 µg/ml), and LPS (1 µg/ml) for 4 hours. They were then washed, spun, and resuspended in fresh antibiotic-free media containing PMA. i.e. the priming with PMA occurred after pre-treatment.

- On the next day, both groups were infected with *Streptococcus pneumoniae* TIGR4 at MOI=1. Cell culture supernatants were collected at time points 2, 4, 6, and 24 hours for further cytokine analysis.

TNF-α results:

- For ß glucan: Comparing TNF-α production in pre-treated later primed cells versus pre-treated and primed cells, there was no significant difference between the two conditions at 2 and 4 hours post-infection. At 6 hours, the pre-treated later primed cells secreted significantly higher TNF-α post-infection compared to the pre-treated and primed cells. However, there was a tendency for the opposite at 24 hours, which reached statistical significance, i.e. higher levels in pre-treated and primed cells than in the other group (Figure 4-14).

- For zymosan: There was no significant difference between pretreated later-primed cells and pre-treated primed cells at 2, 4, and 6 hours. However, at 24 hours, pre-treated and primed cells secreted significantly higher TNF-α than pre-treated later-primed ones (Figure 4-14).

- For LPS: Pre-treated later primed cells secreted less TNF-α than pre-treated primed cells across all time points. The differences were statistically significant (Figure 4-14).

Based on these results, it appears that the order of priming with PMA and pre-treatment with innate immune stimulants didn't produce a significant effect, except in the case of LPS, where it appears that simultaneous pre-treatment with LPS and priming with PMA led to higher levels of TNF-α. Therefore, in later experiments, priming with PMA and pre-treatments were introduced simultaneously.

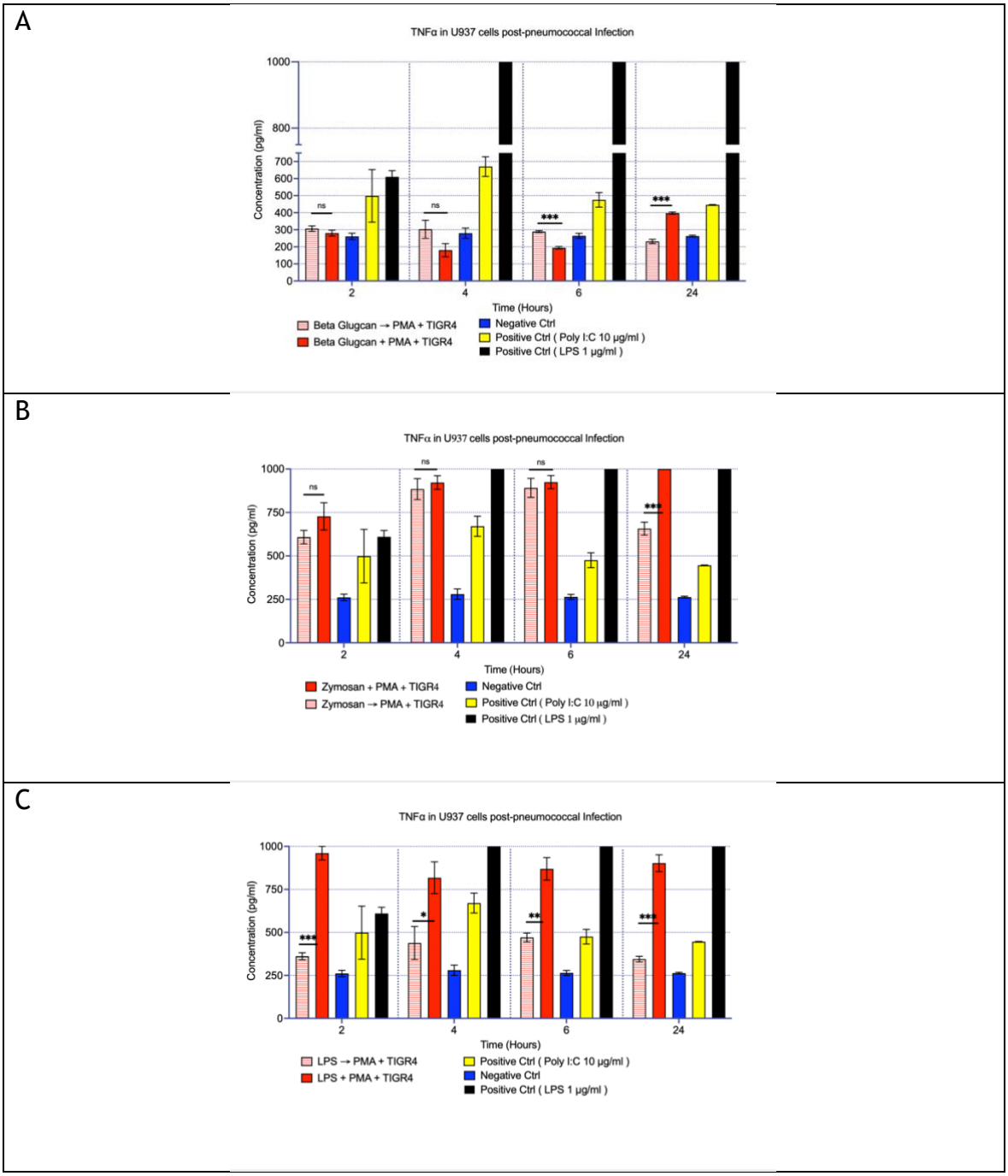


Figure 4-14 TNF-α Concentrations Produced by U937 cells Post-Pneumococcal Infection (A) cells pre-treated with β-glucan (B) cells pre-treated with zymosan (C) cells were pre-treated with LPS. For each stimulant, "+ TIGR4" denotes cells that received simultaneous stimulant treatment and PMA priming for 4 hours, whereas "→ PMA + TIGR4" denotes cells pre-treated with the stimulant for 4 hours, washed, and subsequently primed with PMA. In all cases, cells were infected with TIGR4 the following day. U937 cells were infected with *Streptococcus pneumoniae* TIGR4 (MOI =1) for 2,4,6, and 24 hours. The data are representative of one experiment with biological replicates (n=3). Columns show the mean, Error bars show SEM. Positive control is LPS (1 µg/ml) + PMA; the negative control is untreated cells + PMA. The kit's detection limit = (15.6-1000) pg/ml. Statistical analysis of the results was performed using a One-way ANOVA test with a follow-up Dunnett test to compare the results to the TIGR4 control in A, B, and C, as well as an unpaired T-test. Symbols: ns indicates a P value > 0.05. * Indicates a P value ≤ 0.05. ** indicates a P value ≤ 0.01. * indicates a P value ≤ 0.001. **** indicates a P value ≤ 0.0001.**

Using the same experiment settings, IL-1β levels were determined.

IL-1β results:

- For β-glucan: the order of PMA priming and β-glucan pre-treatment made a difference only at 24 hours post-infection. Pretreated and primed cells secreted significantly higher levels of IL-1β compared to those that were pre-treated and later primed (Figure 4-15).

- For zymosan: It appears there is no significant difference between the levels of IL-1β secreted by pre-treated later primed cells vs pretreated and primed cells. However, particularly at 4 and 6 hours, both conditions secreted the highest readable levels of IL-1β. Therefore, it is not feasible to interpret the difference (Figure 4-15).

- For LPS: There was a trend of higher IL-1β production by pretreated and primed cells compared to those pre-treated later primed. However, this difference only reached statistical significance at 2 hours (Figure 4-15).

Based on these results, it seems that there is neither a consistent nor significant difference in IL-1β production between the two groups. Therefore, in future experiments, cells will be pre-treated with innate immunity stimulants and primed with PMA simultaneously.

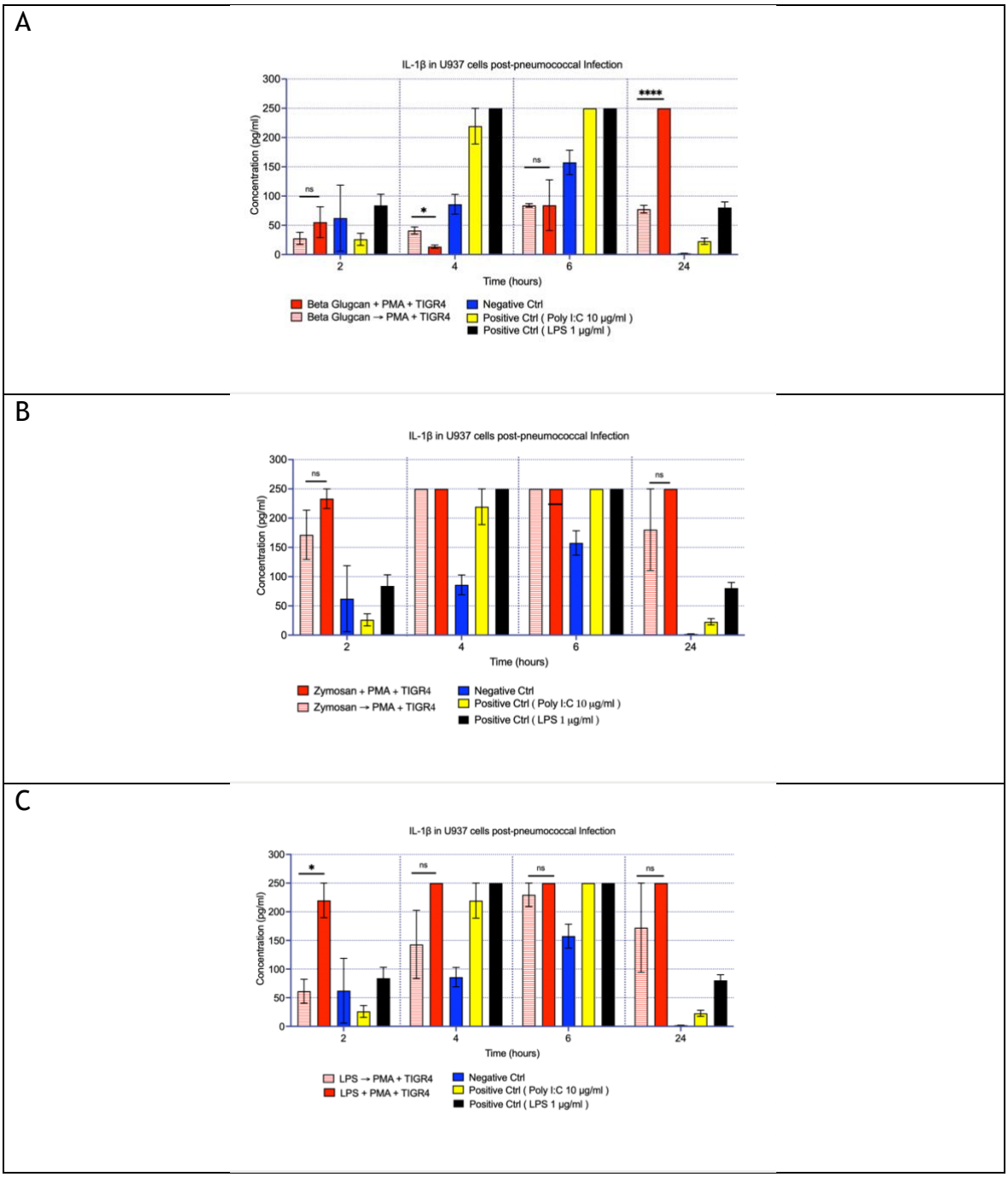


Figure 4-15 IL-1β Concentrations Produced by U937 cells Post-Pneumococcal Infection (A) cells pre-treated with β-glucan (B) cells pre-treated with zymosan (C) cells pre-treated with LPS. For each stimulant, "+ TIGR4" denotes cells that received simultaneous stimulant treatment and PMA priming for 4 hours, whereas "→ PMA + TIGR4" denotes cells pre-treated with the stimulant for 4 hours, washed, and subsequently primed with PMA. In all cases, cells were infected with TIGR4 the following day. U937 cells were infected with *Streptococcus pneumoniae* TIGR4 (MOI =1) for 2,4,6, and 24 hours. The data are representative of one experiment with biological replicates (n=3). Columns show the mean, Error bars show SEM. Positive control is LPS (1 µg/ml) + PMA; the negative control is untreated cells + PMA. The kit's detection limit = (3.91-250) pg/ml. Statistical analysis of the results was performed using a One-way ANOVA test with a follow-up Dunnett test to compare the results to the TIGR4 control in A, B, and C, as well as an unpaired T-test. Symbols: ns indicates a P value > 0.05. * indicates a P value ≤ 0.05. ** indicates a P value ≤ 0.01. * indicates a P value ≤ 0.001. **** indicates a P value ≤ 0.0001.**

The same set of experiments was conducted to examine the role of pre-treatments on TNF-α levels following pneumococcal infection, with PMA added simultaneously with the various pre-treatment reagents.

TNF-α results:

- For ß-glucan, there was no significant difference in TNF-α levels between pre-treated infected cells and infected cells at 2 and 4 hours. However, at 6 hours, infected cells secreted significantly higher levels of TNF-α than pre-treated infected cells. Conversely, at 24 hours, they secreted significantly lower levels of TNF-α.

  Another important comparison is between the pretreated infected cells and pre-treated cells. It appears that there were significant differences in TNF-α levels at most time points, particularly at 24 hours, when the pretreated infected cells produced significantly higher TNF-α levels than the pretreated cells (Figure 4-16).

- For zymosan: Pretreated infected cells consistently and significantly secreted higher levels of TNF-α compared to infected cells at all time points. This suggests that zymosan pretreatment has a pro-inflammatory effect.

  More so, there was a trend for secreting higher TNF-α by pretreated infected cells when compared to pretreated cells at multiple time points. This observation reached statistical significance at 24 hours (Figure 4-16).

- For LPS: Pretreated infected cells consistently and significantly secreted higher TNF-α compared to infected cells across all time points. There was a trend showing higher TNF-α secretion by the pretreated infected cells compared to pre-treated cells across all time points. Such a difference reached statistical significance at 24 hours (Figure 4-16).

Based on these results, it appears that innate immune stimulants evoke a delayed pro-inflammatory response. This is supported by the increase in TNF-α levels at 24 hours post-infection.

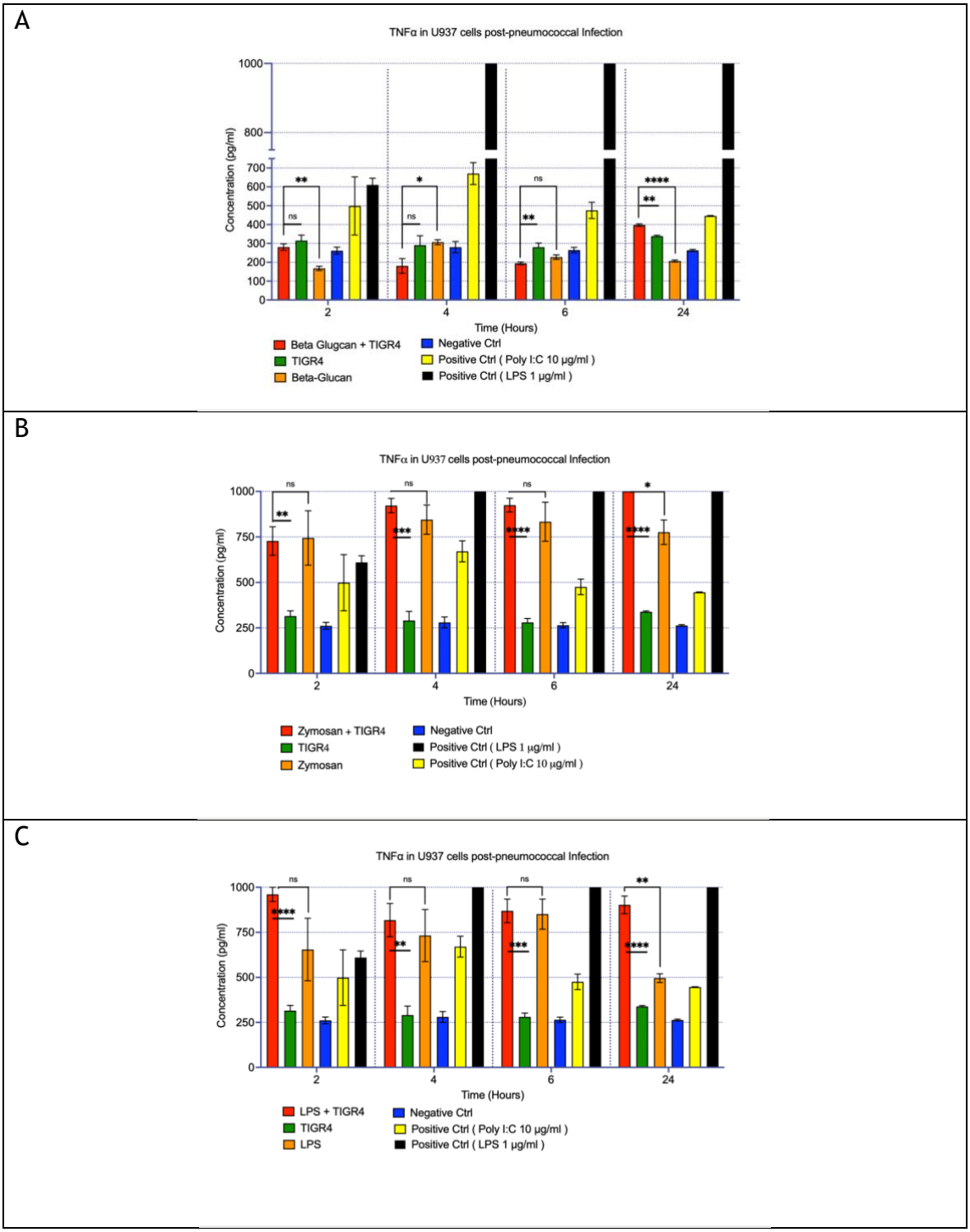


Figure 4-16 TNF-α Concentrations Produced by U937 Cells Post-Pneumococcal Infection (A) cells pre-treated with β-glucan (B) cells pre-treated with zymosan (C) pre-treated with LPS. U937 cells were infected with *Streptococcus pneumoniae* TIGR4 (MOI =1) for 2,4,6, and 24 hours. Some cells were pre-treated with beta-glucan, some with zymosan, and some with LPS. The data are representative of one experiment with biological replicates (n=3). Columns show the mean, Error bars show SEM. Positive controls are Poly I:C (10 µg/ml)+ PMA; the other is LPS (1 µg/ml) + PMA; the negative control is untreated cells + PMA. The kit's detection limit = (15.6-1000) pg/ml. Statistical analysis of the results was performed using a One-way ANOVA test with a follow-up Dunnett test to compare the results to the TIGR4 control in A, B, and C, as well as an unpaired T-test. Symbols: ns indicates a P value > 0.05. * indicates a P value ≤ 0.05. ** indicates a P value ≤ 0.01. * indicates a P value ≤ 0.001. **** indicates a P value ≤ 0.0001.**

IL-1β Results:

- For β glucan: pre-treated infected cells produced less IL-1β than infected cells at most times, except at 24 hours. There was no significant difference in TNF-α levels between pretreated infected cells and pretreated cells at most time points, except at 24 hours. when the pre-treated infected cells produced more IL-1β than pre-treated cells (Figure 4-17).

- For zymosan: pretreated infected cells secreted significantly higher levels of IL-1β than infected cells at most time points. It appears that there was no significant difference between pretreated infected cells and pre-treated cells in terms of IL-1β levels, except at 24 hours when pretreated infected cells produced more IL-1β (Figure 4-17).

- For LPS: pretreated infected cells secreted significantly higher levels of IL-1β than infected cells at most time points. Although there was no significant difference in IL-1β levels between pre-treated infected cells and pre-treated cells, except at 24 hours, pre-treated infected cells produced more IL-1β. However, at 4 and 6 hours, these results reached the highest readable concentration of 250 pg/ml, necessitating further dilution to comment more accurately on the difference in levels (Figure 4-17).

- Based on these results, there is not a major difference in IL-1β levels between pre-treated cells and pretreated infected cells, except at 24 hours post-infection, which suggests that the pretreatments (β glucan, zymosan, and LPS) have a delayed effect in boosting cytokine secretion.

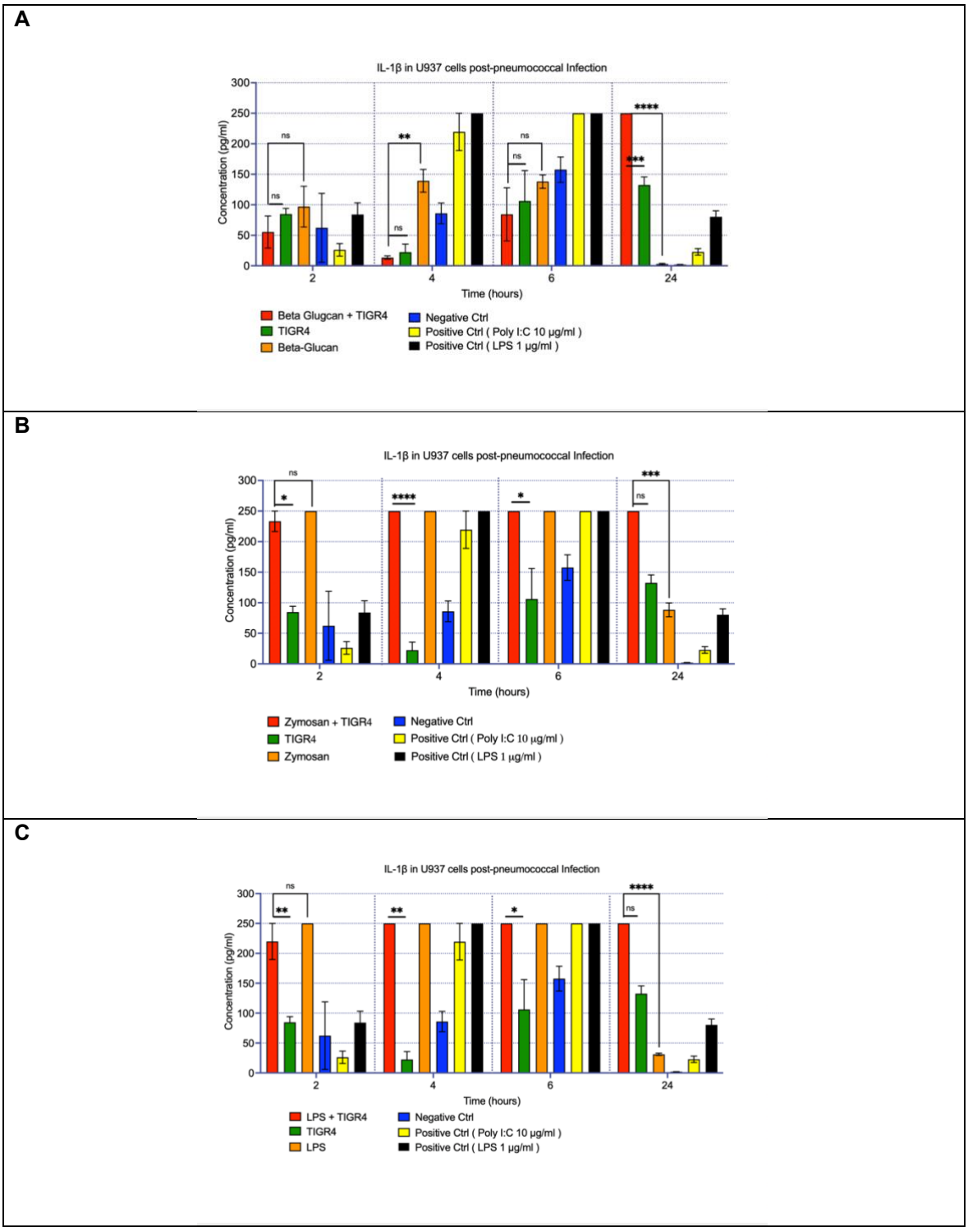


Figure 4-17 IL-1β Concentrations Produced by U937 Cells Post-Pneumococcal Infection (A) Cells pre-treated with β-glucan (B) cells pre-treated with zymosan (C) cells pre-treated with LPS. U937 cells were infected with *Streptococcus pneumoniae* TIGR4 (MOI =1) for 2,4,6, and 24 hours. Some cells were pre-treated with beta-glucan, some with zymosan, and some with LPS. The data are representative of one experiment with biological replicates (n=3). Columns show the mean, Error bars show SEM. Positive control is LPS (1 µg/ml) + PMA; the negative control is untreated cells + PMA. The kit's detection limit = (3.91-250) pg/ml. Statistical analysis of the results was performed using a One-way ANOVA test with a follow-up Dunnett test to compare the results to the TIGR4 control in A, B, and C, as well as an unpaired T-test. Symbols: ns indicates a P value > 0.05. * indicates a P value ≤ 0.05. ** indicates a P value ≤ 0.01. * indicates a P value ≤ 0.001. **** indicates a P value ≤ 0.0001.**

4.2.3.5 The Effect of Different Pre-treatment Concentrations on Cytokine Levels Produced by U937 Monocyte-derived Macrophages Cell Line Following *Streptococcus pneumoniae* TIGR4 Infection

Next, we examined whether changing the pre-treatment concentrations produced different effects. U937 cells were cultured with different concentrations of pre-treatments, along with PMA (100 ng/ml), for 8 hours. Cell culture supernatants were collected and tested for TNF-α and IL-1ß levels using ELISA. An 8-hour time point was chosen because previous studies showed that the time for maximum TNF-α concentration released from monocyte-derived macrophages following LPS stimulation is 8 hours [143].

The concentrations used for pre-treatment were:

ß glucan: 100 µg/ml, 10 µg/ml, and 1 µg/ml.

zymosan: 10 µg/ml, and 1 µg/ml, and 0.1 µg/ml.

LPS: 1 µg/ml,0.1 µg/ml, and 0.01 µg/ml.

The TNF-α results indicated that ß-glucan, when used at different concentrations, did not produce a change in TNF-α levels. However, both Zymosan and LPS resulted in lower TNF-α levels when used at lower concentrations (Figure 4-18).

These different concentrations had no effect on IL-1ß levels (Figure 4-18).

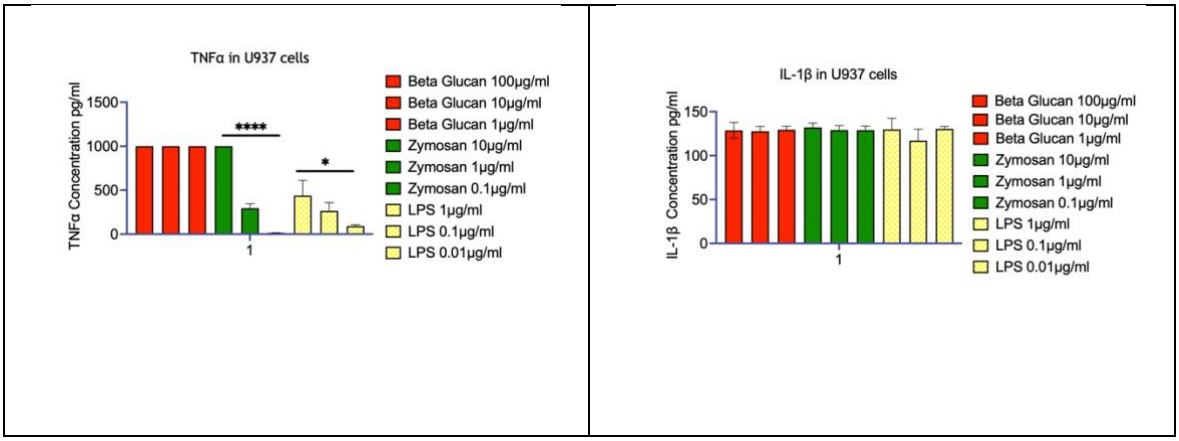


Figure 4-18 TNF-α and IL-1β concentrations produced by U937 Cells Following Pre-treatments
with β-glucan: 100 µg/ml, 10 µg/ml, and 1 µg/ml; zymosan: 10 µg/ml, 1 µg/ml, and 0.1 µg/ml and LPS: 1 µg/ml,0.1 µg/ml, and 0.01 µg/ml. U937 cells were cultured with different concentrations of pre-treatments, along with PMA (100 ng/ml) for 8 hours. Cell culture supernatants were collected and tested for TNF-α and IL-1β concentrations using ELISA. The experiment was conducted in triplicate (n=3). Columns show the mean, Error bars show SEM. The kit's detection limit TNF-α (15.6-1000) pg/ml, and IL-1β (3.91-250) pg/ml. A statistical analysis of the results was performed using a one-way ANOVA test. Symbols: ns indicates P value > 0.05. * indicates P value ≤ 0.05. ** indicates P value ≤ 0.01. * indicates P value ≤ 0.001. **** indicates P value ≤ 0.0001.**

Following this trial experiment, ß-glucan was excluded as a pre-treatment since it did not produce any significant differences in cytokine levels at various concentrations. Next, U937 cells were cultured in antibiotic-free media and primed with PMA (100 ng/ml) along with three different concentrations of zymosan and LPS for 24 hours. The concentrations used for Zymosan were 10 µg/ml, 1 µg/ml, and 0.1 µg/ml; for LPS, they were 1 µg/ml,0.1 µg/ml, and 0.01 µg/ml.

The following day, the cells were infected with *Streptococcus pneumoniae* TIGR4. Cell culture supernatants were collected at 2,4,6, and 24 hours for TNF-α testing.

TNF-α Results:

- For Zymosan (10 µg/ml): Pretreated infected cells produced more TNF-α than infected cells at most time points. Additionally, pretreated infected cells secreted more TNF-α compared to pretreated cells; however, this reached statistical significance at 6 hours (Figure 4-19).

- For zymosan (1 µg/ml): pretreated infected cells secreted higher levels of TNF-α compared to infected cells, except at 24 hours. There appears to be a trend where pretreated infected cells produce higher TNF-α levels compared to pre-treated cells. This reached statistical significance at 6 and 24 hours (Figure 4-19).

- For zymosan (0.1 µg/ml): there appears to be no significant difference in TNF-α levels produced by pretreated infected cells compared to infected cells at any time point. Pre-treated infected cells secreted higher levels of TNF-α than pre-treated cells; however, this only reached statistical significance at 4 and 6 hours (Figure 4-19).

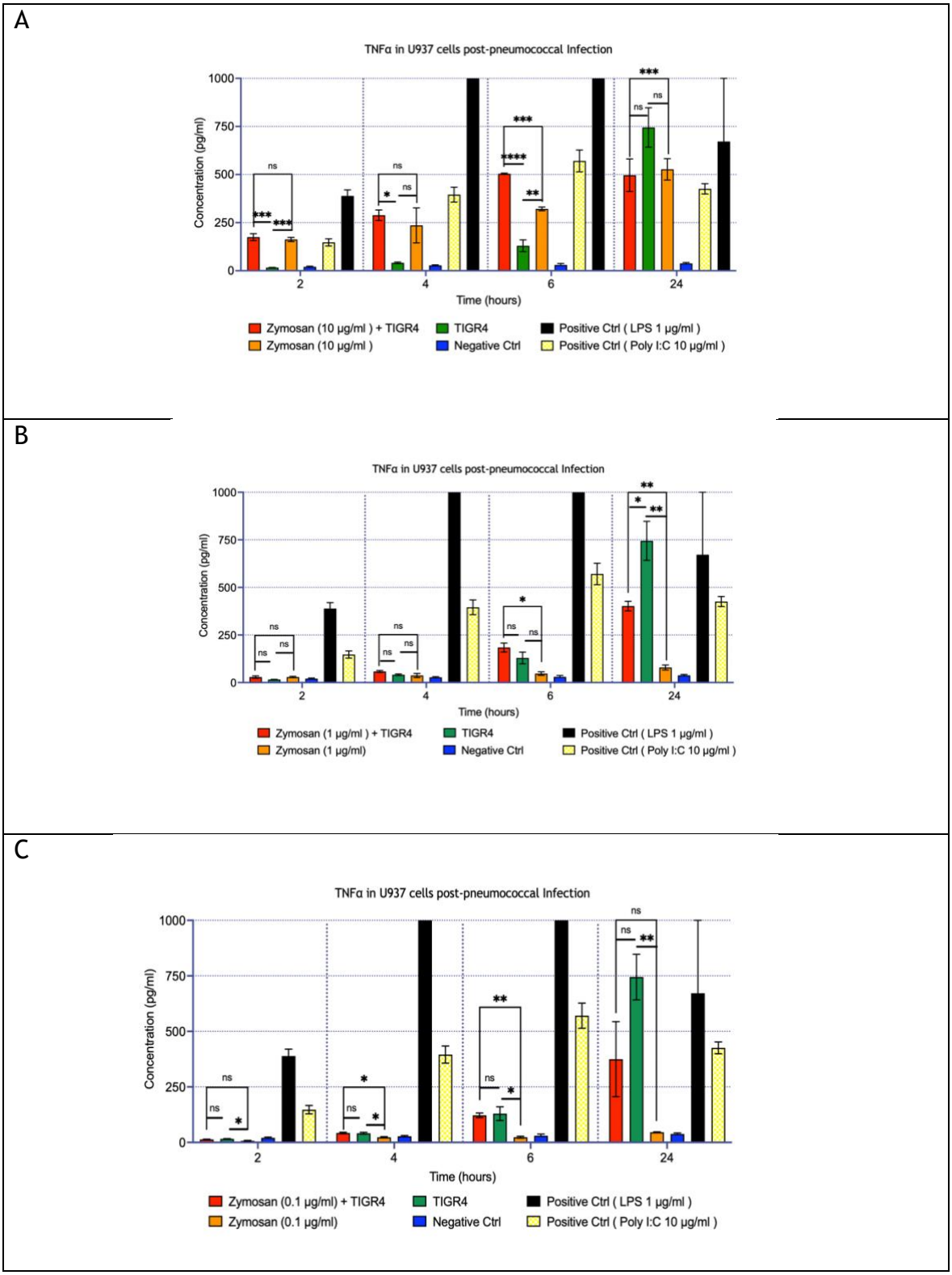


Figure 4-19 TNF-α Concentrations Produced by U937 Cells, Pretreated with Zymosan (A)10 µg/ml (B)1 µg/ml AND (C) 0.1 µg/ml. U937 cells were primed with PMA (100 ng/ml). the next day, cells were infected with *Streptococcus pneumoniae* TIGR4 (MOI =1) for 2,4,6, and 24 hours. Some cells were pre-treated with zymosan (0.1,1, and 10 µg/ml). The data are representative of one experiment with biological replicates (n=3). Columns show the mean, Error bars show SEM. Positive controls are Poly I:C (10 µg/ml)+ PMA; the other is LPS (1 µg/ml) + PMA; the negative control is untreated cells + PMA. The kit's detection limit = (15.6-1000) pg/ml. Statistical analysis of the results was performed using a One-way ANOVA test with a follow-up Dunnett test to compare the results to the TIGR4 control in A, B, and C, as well as an unpaired T-test. Symbols: ns indicates a P value > 0.05. * indicates a P value ≤ 0.05. ** indicates a P value ≤ 0.01. * indicates a P value ≤ 0.001. **** indicates a P value ≤ 0.0001**

TNF-α Results continued:

- For LPS (1 μg/ml): Pre-treated infected cells produced higher levels of TNF-α compared to infected cells, except at 24 hours. This could suggest that the effect of pre-treatment starts to wear off after the first day. However, there was no significant difference in TNF-α levels between pre-treated infected cells and pre-treated cells (Figure 4-20).

- For LPS (0.1 μg/ml): Pre-treated infected cells secreted higher TNF-α levels than infected cells at 2 and 4 hours. Conversely, at 24 hours, infected cells produced more TNF-α compared to pre-treated infected cells. This suggests that the effect of pre-treatment begins to fade after the first day. Additionally, pre-treated infected cells produced higher levels of TNF-α compared to pre-treated cells at 4,6 and 24 hours (Figure 4-20).

- For LPS (0.01 μg/ml): There is no significant difference in TNF-α levels between pre-treated infected cells and infected cells at any time point. When comparing pre-treated infected cells to pre-treated cells, it appears there is a significant difference in TNF-α; the pre-treated infected cells secrete more TNF-α at 4, 6, and 24 hours post-infection (Figure 4-20).

Based on the results from zymosan and LPS, the concentration chosen for later experiments will be 0.1 μg/ml for both pre-treatments, as this concentration demonstrated the most significant effect on TNF-α levels.

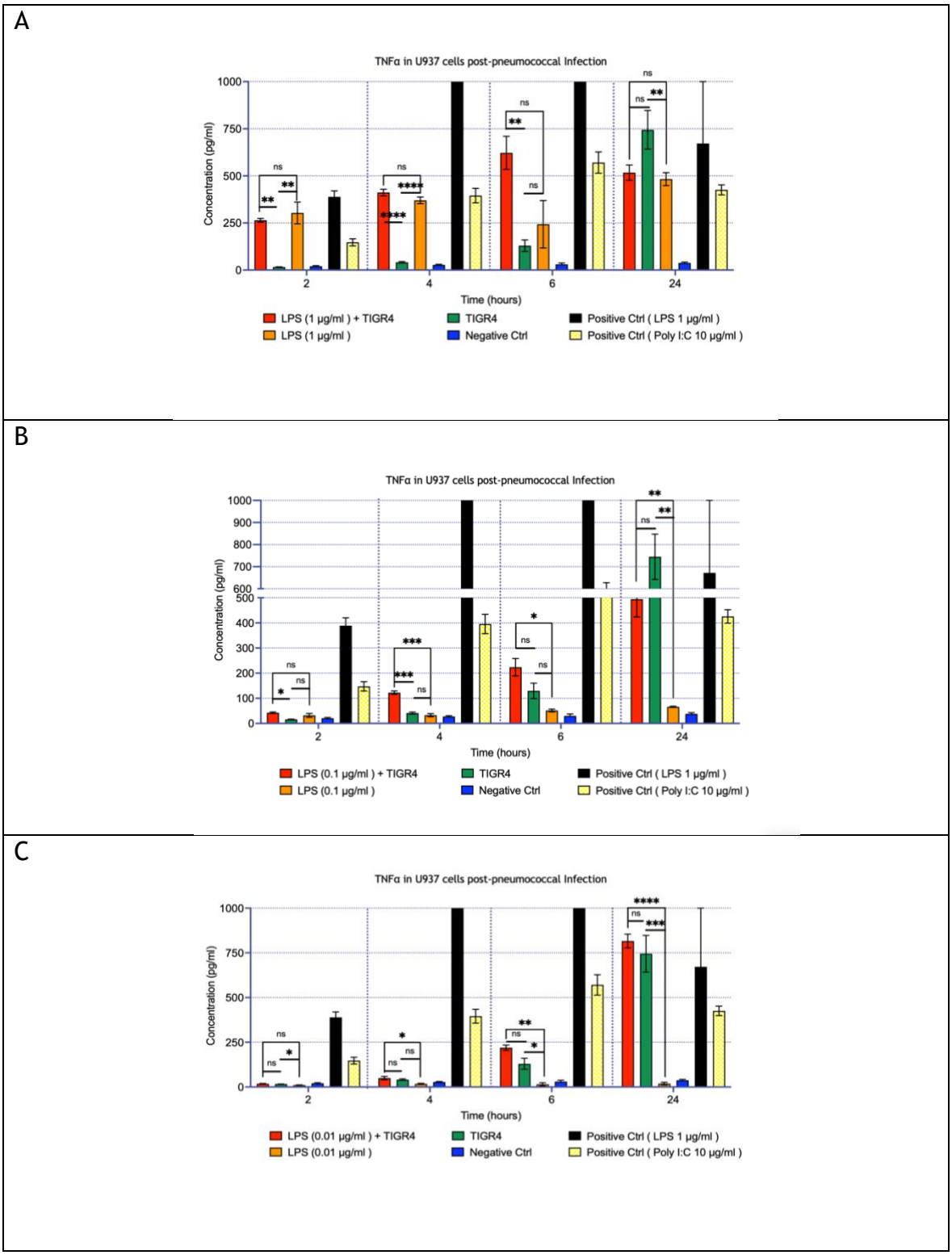


Figure 4-20 TNF-α Concentrations Produced by U937 Cells, pre-treated with LPS (A) LPS.1 µg/ml (B)0.1µg/ml (C) 0.01 µg/ml. U937 cells were primed with PMA (100 ng/ml). the next day, cells were infected with *Streptococcus pneumoniae* TIGR4 (MOI =1) for 2,4,6, and 24 hours. Some cells were pre-treated with LPS (0.01, 0.1, and 1 µg/ml). The data are representative of one experiment with biological replicates (n=3). Columns show the mean, Error bars show SEM. Positive controls are Poly I:C (10 µg/ml)+ PMA; the other is LPS (1 µg/ml) + PMA; the negative control is untreated cells + PMA. The kit's detection limit = (15.6-1000) pg/ml. Statistical analysis of the results was performed using a One-way ANOVA test with a follow-up Dunnett test to compare the results to the TIGR4 control in A, B, and C, as well as an unpaired T-test. Symbols: ns indicates a P value > 0.05. * indicates a P value ≤ 0.05. ** indicates a P value ≤ 0.01. * indicates a P value ≤ 0.001. **** indicates a P value ≤ 0.0001.**

Next, to examine whether the effects of zymosan and LPS are due to trained immunity or amplified acute immune response, the experiments were repeated with the introduction of rest period.

The U937 cells were cultured in antibiotic-free media with PMA, zymosan and LPS (0.1µg/ml) were added to a subset of cells, which were then incubated for 4 hours. Following this, the cell culture media was replaced with fresh antibiotic-free media and PMA to introduce a rest period. The following day, the cells were infected with *Streptococcus pneumoniae* TIGR4 MOI=1. Cell culture supernatants were collected at 2, 4, 6, and 24 hours for TNFα levels testing.

TNFα Results:

- For zymosan: there was no significant difference in TNFα levels between pretreated infected cells and infected cells at any time point. It may be that the (0.1µg/ml) concentration is below what is needed to stimulate the immune response. There is significant difference in levels between pretreated infected cells and pretreated cells at 24 hours when pretreated infected cells secreted more TNFα than pretreated cells (Figure 4-21).

- For LPS: pre-treated infected cells secreted significantly higher TNFα levels than infected cells at all time points. This confirms that LPS has a stimulatory effect on TNFα levels following pneumococcal infection. There is a significant difference in TNFα levels between pre-treated infected cells and pre-treated cells at 24 hours, when the pre-treated infected cells produced significantly higher TNFα than pre-treated cells (Figure 4-21).

These results suggest that zymosan and LPS has a immune stimulatory effect that can be attributed to trained immunity.

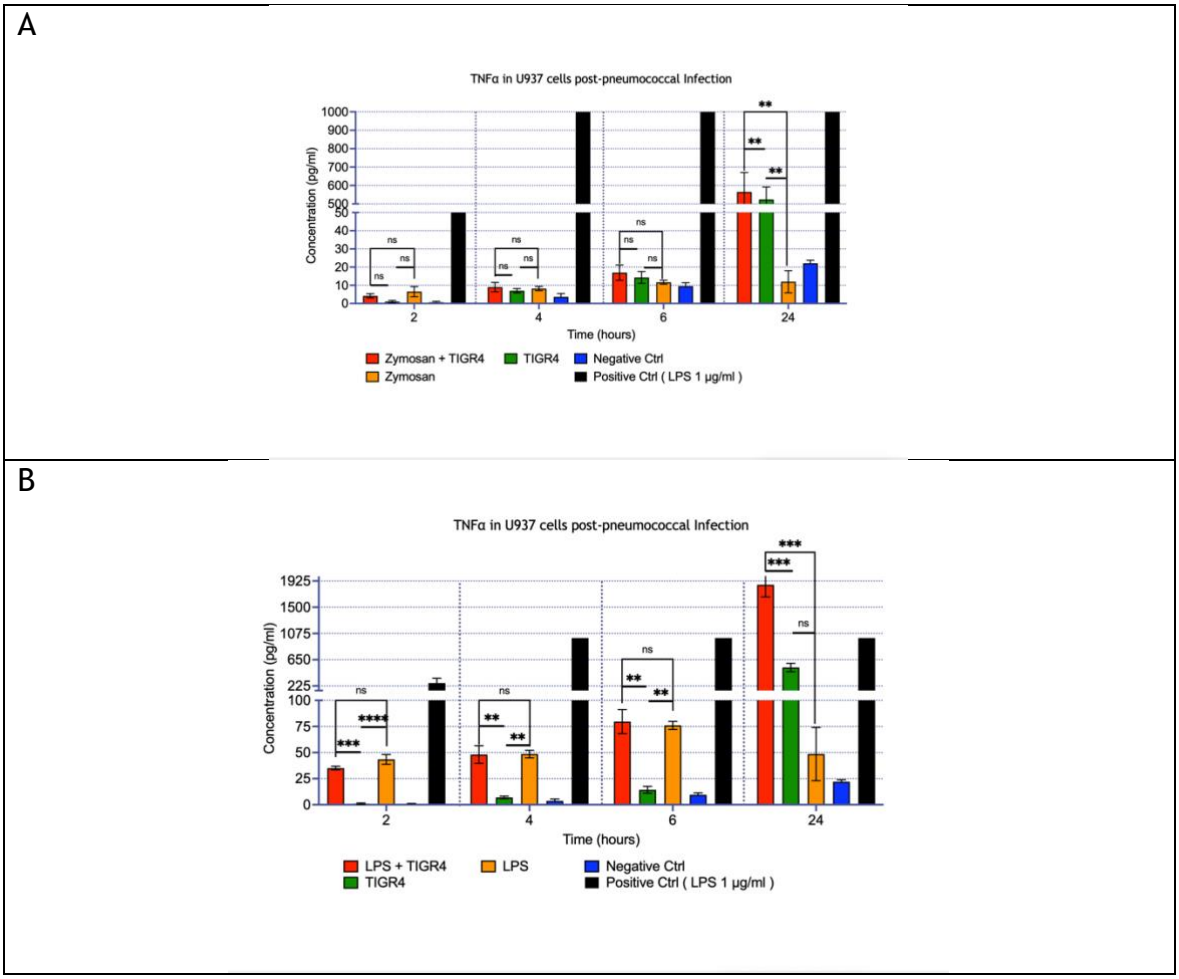


Figure 4-21 TNFα Levels Produced by U937 Cells Post-Pneumococcal Infection (A)Pre-treated with Zymosan. (B)Pre-treated with LPS. U937 cells were cultured in antibiotic-free media with PMA, zymosan and LPS (0.1µg/ml) were added to a subset of cells, for 4 hours. Following this, the cells were washed, and fresh antibiotic-free media and PMA were added to introduce a rest period. The following day, the cells were infected with *Streptococcus pneumoniae* TIGR4 MOI=1 for 2, 4, 6, and 24 hours. The data are representative of one experiment with biological replicates (n=3). Columns show the mean, Error bars show SEM. Positive control is LPS (1 µg/ml) + PMA; the negative control is untreated cells + PMA. The kit's detection limit = (15.6-1000) pg/ml. Statistical analysis of the results was performed using a One-way ANOVA test with a follow-up Dunnett test to compare the results to the TIGR4 control, as well as an unpaired T-test. Symbols: ns indicates a P value > 0.05. * indicates a P value ≤ 0.05. ** indicates a P value ≤ 0.01. * indicates a P value ≤ 0.001. **** indicates a P value ≤ 0.0001.**

To consolidate the results, the previous experiment was repeated.

The U937 cells were cultured in antibiotic-free media containing PMA. Following this, Zymosan and LPS (0.1µg/ml) were added to a subset of cells, and they were incubated for four hours. After four hours, the cell culture media was replaced with fresh antibiotic-free media and PMA, to introduce a rest period. The following day, the cells were infected with *Streptococcus pneumoniae* TIGR4 at MOI=1. Cell culture supernatants were collected at 2, 4, 6, and 24 hours for TNFα level testing.

TNFα Results:

- For zymosan: there isn't a significant difference in TNFα levels between pre-treated infected cells and infected cells at all time points, except at 6 hours. When pre-treated infected cells produced more TNFα than infected cells. This goes with previous results. It could be that (0.1µg/ml) concentration is below what is required to produce immune stimulation. Pretreated infected cells produced significantly higher TNFα than pretreated cells 24 hours after infection. This also goes with previous results (Figure 4-22).

- For LPS: pre-treated infected cells secreted significantly higher TNFα levels than infected cells at all time points, except for 24 hours. This confirms that LPS has a stimulatory effect on TNFα levels following pneumococcal infection. Additionally, pre-treated infected cells produced higher levels of TNFα than pre-treated cells at 24 hours, which aligns with previous results (Figure 4-22).

- These results align with the previous one, and they suggest that zymosan and LPS have an immune stimulatory effect that can be attributed to trained immunity.

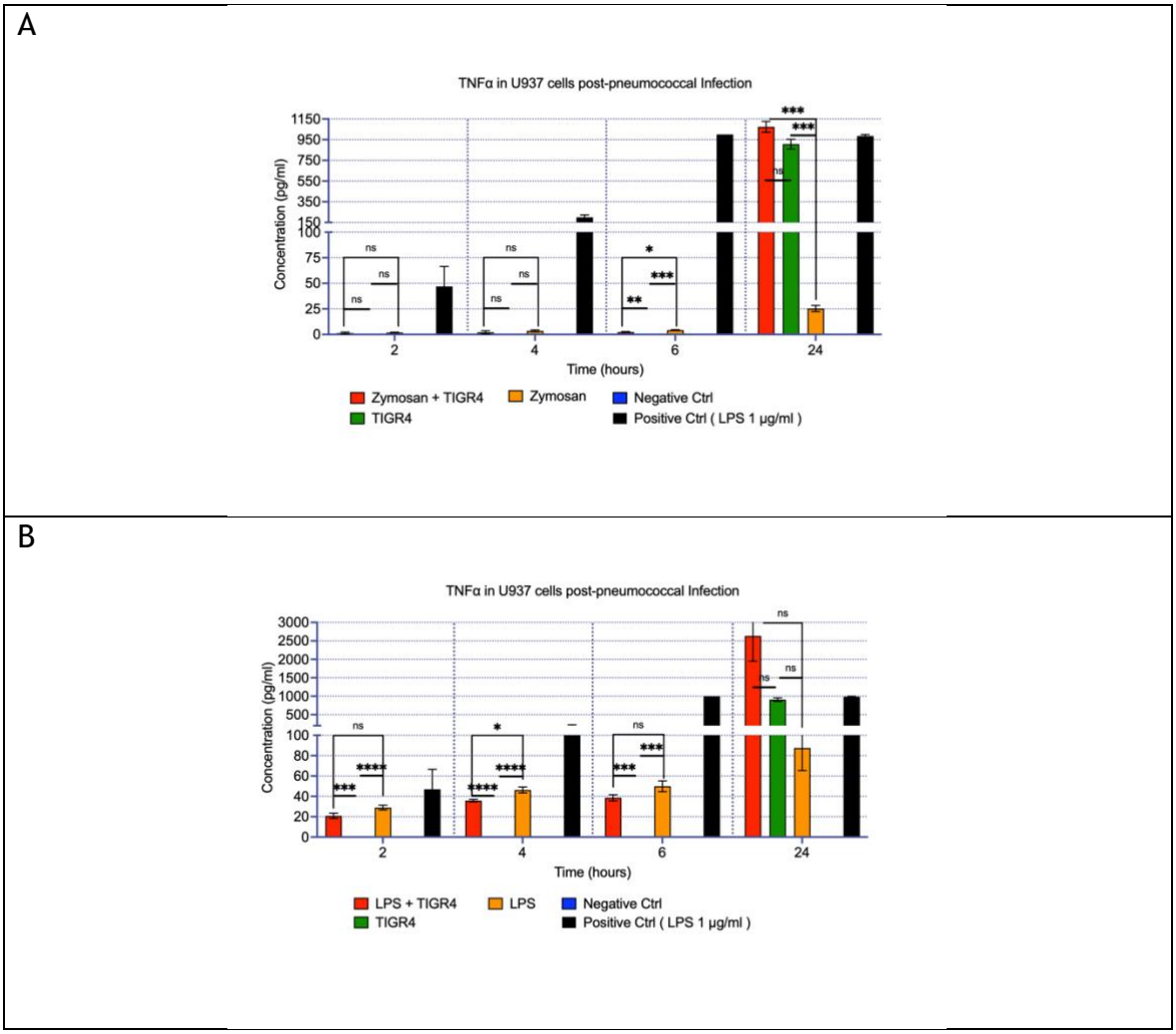


Figure 4-22 TNFα Levels Produced by U937 Cells Post-Pneumococcal Infection (A)Pre-treated with Zymosan. (B)Pre-treated with LPS. U937 cells were cultured in antibiotic-free media with PMA, zymosan and LPS (0.1µg/ml) were added to a subset of cells, for 4 hours. Following this, the cells were washed, and fresh antibiotic-free media and PMA were added to introduce a rest period. The following day, the cells were infected with *Streptococcus pneumoniae* TIGR4 MOI=1 for 2, 4, 6, and 24 hours. The data are representative of one experiment with biological replicates (n=3). Columns show the mean, Error bars show SEM. Positive control is LPS (1 µg/ml) + PMA; the negative control is untreated cells + PMA, The TNFα levels produced by the negative control at 24 hours were omitted because the values were high due to potential contamination. The kit's detection limit = (15.6-1000) pg/ml. Statistical analysis of the results was performed using a One-way ANOVA test with a follow-up Dunnett test to compare the results to the TIGR4 control, as well as an unpaired T-test. Symbols: ns indicates a P value > 0.05. * indicates a P value ≤ 0.05. ** indicates a P value ≤ 0.01. * indicates a P value ≤ 0.001. **** indicates a P value ≤ 0.0001.**

4.2.4 TNF-α mRNA Expression following LPS Pre-treatment

We then examined the effects of LPS (0.1µg/ml) pre-treatment on TNF-α mRNA expression, both with and without infection. The U937 cells were cultured in antibiotic-free media with PMA; some were pre-treated with LPS (0.1µg/ml) for 4 hours, while others received no treatment. The following day, the cells were infected with *Streptococcus pneumoniae* TIGR4 at MOI=1. At time zero and 6 hours after infection, the cells were lysed, RNA was extracted, and after reverse transcription, Quantitative Polymerase Chain Reaction (QPCR) was performed. TNF-α gene expression was calculated in comparison to the housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

The results show that LPS pre-treatment seems to boost TNF-α mRNA expression in pre-treated non-infected cells (pre-treated only), with an approximately 30-fold increase compared to non-treated controls (negative control). However, previous ELISA results indicate that the secretion of TNF-α by these cells is very low, which does not match the mRNA expression. This may imply a post-transcriptional inhibitory signal that prevents or interferes with the translation of the mRNA into TNF-α protein.

However, in the case of pre-treated infected cells U937 cells that were pre-treated with LPS and later infected with *Streptococcus pneumoniae* TIGR4, there was a similar increase in TNF-α mRNA expression, with an approximately 30-fold rise compared to the non-treated control (negative control). Based on previous TNF-α readings using ELISA, TNF-α is secreted significantly more by these cells (LPS+TIGR4) compared to the pre-treated cells (pre-treatment only). This suggests that the infection exerts a post-transcriptional effect on TNF-α, allowing the mRNA to be translated (Figure 4-23). Further studies are needed to confirm these results and understand the involved pathways; unfortunately, this couldn’t be repeated and verified due to limited timing. However, some studies have observed such effects in different infections. In Willeaume et al., mice monocytes-macrophages were stimulated with either Sendai virus or LPS, which resulted in increased TNF-α mRNA transcription and translation. Moreover, in the case of viral stimulation, most of the TNF-α produced—whether from existing or induced mRNA—was due to enhanced translation rather than increased transcription [173]. In Mogensen et al, they studied TNF-α production in mice

macrophages following *Streptococcus pneumoniae* infection. They found that pneumococcal infection induces TNF-α production, as seen in this thesis, but also that it stabilizes TNF-α mRNA through AREs in the 3' UTR [198].which goes with the previous results that pneumococcal infection exerts a transcriptional and post-transcriptional effect to induce TNF-α.In the absence of *S. pneumoniae,* TNF-α mRNA half-life is approximately 30 minutes, but with live *S. pneumoniae*, the half-life is increased up to 6 hours. allowing more protein synthesis [198].

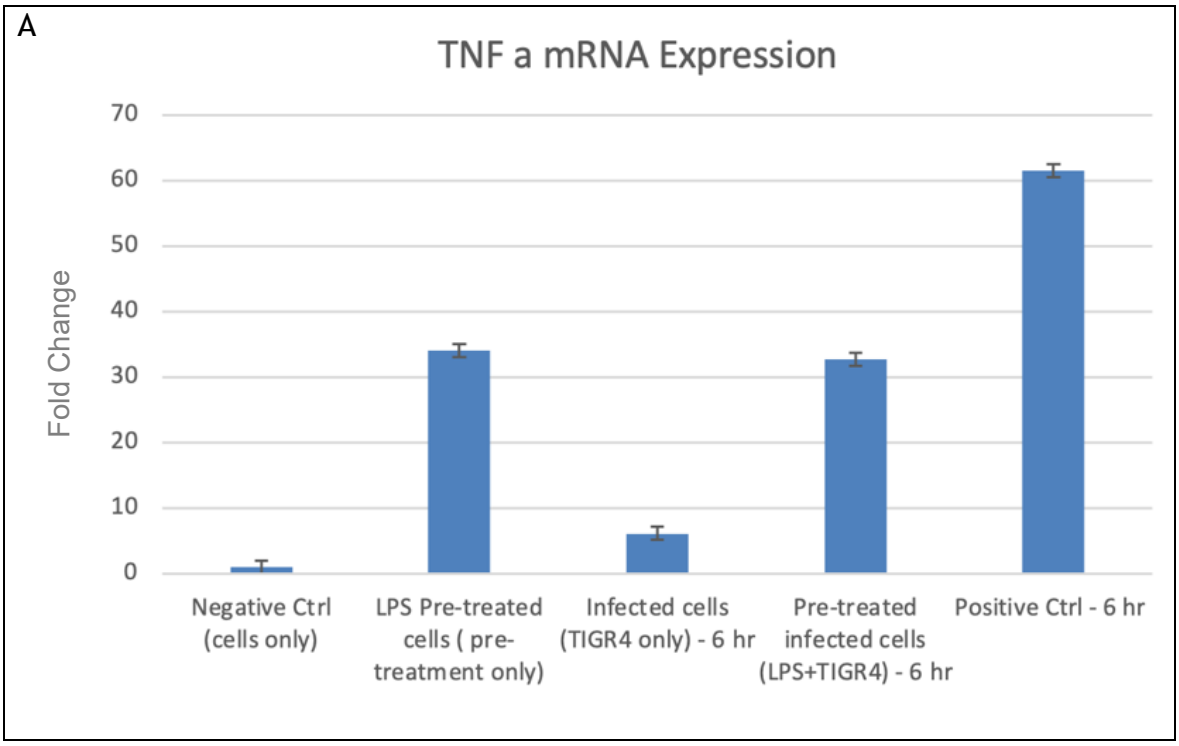


Figure 4-23 TNF-α mRNA Expression Concentrations in U937 cells Post-pneumococcal Infection.
The U937 cells were cultured in antibiotic-free media supplemented with PMA; some were pre-treated with LPS (0.1 µg/ml) for 4 hours, while others received no treatment. The following day, the cells were infected with Streptococcus pneumoniae TIGR4 at (MOI=1). The positive control is LPS (1 µg/ml) + PMA; the negative control is untreated cells + PMA. Samples were processed at time zero and 6 hours after infection. The data are representative of one experiment with biological replicates (n=3). Columns show the average of data points ±SEM.

4.3 Conclusion

This chapter explores the effect of innate immune stimulants pre-treatment on various cell types following *Streptococcus pneumoniae* TIGR4 infection. In summary, the results of this chapter present a complex picture. With findings that show trained immunity effect following the cellular pre-treatment with immune stimulants, and some results challenging that. The variability in these findings is mostly attributed to two main variables: donor variability (In primary cells) and variable TIGR4 bacterial numbers.

Although this chapter's experiments faced these two important limitations to draw conclusions from them. They still provide an insight into the need to investigate the role of trained immunity against bacterial infections further. Suggesting that simple explanations may not fully capture the complexity of such phenomena. Also, this will hopefully enable us to better understand trained immunity to use it as a therapeutic mechanism against infections in the future.

Regarding type 1 Interferons, none of the cells used in this thesis produced significant concentrations of IFN-β following *Streptococcus pneumoniae* TIGR4 infection. The experiment was repeated three times; each time, the MDDCs were obtained from a different donor, but the same negative results were observed, suggesting that TIGR4 does not have a stimulatory effect on IFN-β secretion. Cells showed good viability percentage 24 hours following infection (approx.80%), suggesting that cell death is not the reason for the lack of IFNs production.

Regarding other pro-inflammatory cytokines, such as TNF-α. In monocytes-derived dendritic cells, the experiment to measure its levels following pre-treatment and infection was repeated three times. Both ß-glucan and Zymosan, appear to induce TNF-α, mostly as a combined aggravated effect of immune stimulant and infection rather than trained immunity.LPS was found to induce TNF-α and possibly produce a trained immunity effect at 24 hours following infection.

In macrophages, the experiment was repeated twice; ß-glucan, Zymosan, and LPS all seem to induce TNF-α. However, the results are inconsistent when it comes to the trained immunity effect.

Regarding IL-1ß, similar results are seen in monocyte-derived dendritic cells. Therefore, it is difficult to conclude if IL-1ß induction was due to combined effect of infection and immune stimulants, or trained immunity effect. afterwards, a cell line was used to substitute for primary cells and donor variabilities.

The inconsistency in pro-inflammatory cytokine levels is most likely due to variable bacterial growth and donor variability. The variable bacterial growth, however, didn't seem to affect cell viability to the same level as it did with cytokine production. The majority of MDDCs and Macrophages infected remained viable 24 hours after infection, in 6 different experiments, regardless of the different actual MOI each time.

In the third part of the chapter, the U937 cell line was chosen to examine the role of innate immune stimulants on the trained immunity of these cells. The cells were differentiated into macrophages using PMA for either 24 or 48 hours prior to infection, and they were pre-treated with the stimulants for 4 or 24 hours before infection.

The same experiment was carried out to examine the effect of ß-glucan, zymosan, and LPS pretreatments. They all led to significantly higher TNF-α and IL-1ß levels 24 hours post-infection compared to pre-treated only cells. This indicates that these pre-treatments have a delayed effect on cytokine release.

A trial experiment was conducted to examine the effect of different concentrations of innate immune stimulants on cytokine levels.

Regarding TNF-α it was found that varying concentrations of ß-glucan (100,10 and 1 µg/ml) did not result in different concentrations of TNF-α. However, Zymosan (10, 1, and 0.1 µg/ml) and LPS (1, 0.1, and 0.01 µg/ml) their different concentrations led to variable TNF-α levels, which were directly proportional to

the pre-treatment concentration. The lower the pre-treatment concentration, the lower the concentration of TNF-α secreted.

Regarding IL-1ß, none of the pre-treatments with different concentrations produced variable levels of IL-1ß.

To understand the role of these different concentrations on trained immunity better, cells were primed with PMA and pre-treated with varying concentrations for 24 hours, and the next day, they were infected with *Streptococcus pneumoniae* (MOI=1).

When used in different concentrations (10, 1, 0.1 µg/ml), zymosan pre-treatment appears to induce TNF-α secretion at various times following infection. The zymosan concentration that produced the highest increase in TNF-α levels was 0.1 µg/ml; therefore, this concentration was selected as the working concentration for subsequent experiments. Similar results are seen with LPS pre-treatment at a concentration of 0.1 µg/ml. Based on these results, a working concentration of 0.1 µg/ml for both zymosan and LPS was chosen for future experiments.

It is crucial to make a distinction between two significant effects that may play a role in these experiments' outcomes: Trained immunity vs adjuvant immune effect. Trained immunity, as described earlier, is A long-lasting, memory-like enhanced response of innate immune cells to secondary stimuli, mediated by epigenetic and metabolic reprogramming. Adjuvant immune effect is an acute and short-lived, transient enhancement of immune response due to co-stimulation. It doesn't involve lasting reprogramming. The Effect relies on co-exposure or very recent exposure to immune stimulants.

In our experiments, the cells were usually pre-treated with a specific stimulant (ß-glucan, zymosan, or LPS) for 4-24 hours and then infected with TIGR4 the following day, which means in some of these experiments, the cells had a resting period of 24 hours. Epigenetic changes responsible for trained immunity, such as DNA methylation, usually start within hours after the initial stimulus and are complete within 24-72 hours [199, 200]. Meanwhile, histone modifications, another type of epigenetic change, occur faster—beginning minutes to hours

after the initial stimulus and peaking at 24 hours [201]. To classify the observed effects in the primary cells and U937 cells as trained immune effects, a longer resting period is required to indicate that it is indeed a trained immune response rather than an adjuvant stimulatory effect. In most trained immunity models, the recommended resting period for more reliable results is 3 to 7 days after the initial stimulus, allowing enough time for epigenetic changes to establish. Unfortunately, in our experiments, due to concerns about cell viability (especially when using primary cells), we used the minimum resting period of 24 hours. Had we had more time, it might have been worthwhile to repeat the experiments with longer resting periods to compare the effects of bacterial infection and pre-treatment on cytokine production. So based on the experimental design used, the observed increase in TNF-α in pre-treated, infected cells suggests innate immune priming, but due to the short interval between priming and challenge, this is more consistent with a short-term adjuvant or priming effect rather than established trained immunity.

To definitively establish "trained immunity", a longer resting period and potentially further epigenetic or metabolic profiling are necessary. In the following chapter, epigenetic modifications, particularly DNA methylation, is examined in U937 cells after these experimental conditions and analyzed to determine the occurrence of epigenetic changes after pre-treatment. Another approach to differentiate between trained immunity effects and adjuvant stimulatory effects is to use inhibitors of epigenetic changes, such as methyltransferase inhibitors or histone acetylation blockers [101]. This will assist in confirming the effect as trained immunity in response to epigenetic modification.

Finally, regarding the mRNA levels of the TNF-α gene following LPS pre-treatment and infection *with Streptococcus pneumoniae*. It was found that cells pre-treated with LPS and infected 6 hours post-infection boosted TNF-α gene expression to 30 times higher than the negative control at time zero. Additionally, LPS pre-treatment alone at time zero also appears to elevate gene expression to 30 times higher compared to the negative control at time zero. These results suggest that LPS pre-treatment induces the gene expression of TNF-α to the same level as LPS pretreatment plus infection at 6 hours. However,

when these results are combined with the previous ELISA results, TNF-α is secreted significantly higher by pretreated infected cells compared to the pre-treated only cells. This suggests that the infection seems to exert a post-transcriptional effect on TNF-α, as seen previously in the literature [198, 202], allowing the mRNA to be translated. Further studies are needed to consolidate these results and understand the pathways involved.

Based on the results seen here, it is necessary to explore the epigenetic changes that may be responsible for the trained immunity effect in our experiments. This will be explored in the next chapter.

5 Epigenetic Changes in Tumour Necrosis Factor α Gene Following *Streptococcus pneumoniae* Infection and LPS Pre-treatment

5.1 Introduction

Trained Immunity was discussed in the previous chapter. It is a phenomenon observed in innate immune cells when exposed to a stimulant or pathogen. It enables them to generate a more robust immune response following re-infection with the same or a different pathogen.

Trained Immunity is facilitated by epigenetic changes instead of genetic modification, as seen in adaptive immunity. This chapter will explore epigenetic changes to enhance our understanding of them in the context of *Streptococcus pneumoniae* infection. The epigenome comprises all epigenetic information. It differs from the genome because it varies between cells and over time. This variation occurs due to different conditions [203].

There are two prominent types of epigenetic changes: histone modification and DNA methylation. However, this chapter focuses on the DNA methylation of the TNF-α gene following *Streptococcus pneumoniae* infection. DNA methylation is an important epigenetic mechanism involved in both normal development and disease. During normal development, it plays a role in genetic imprinting and X-chromosome inactivation [204, 205]. In female chromosomes, one X chromosome is inactivated through the hypermethylation of CpG islands in the promoter regions of its genes, which leads to gene silencing [206, 207]. However, Hellman et al. found that Xi (inactivated chromosome) had less methylation compared to Xa (activated chromosome). Significantly, this added methylation occurred in gene bodies rather than promoters [208].

DNA methylation is involved in carcinogenesis, and aberrant DNA methylation is a common method of inactivating tumour suppressor genes during cancer progression. However, unlike the genetic mechanisms involved in cancer, such as gene deletion and mutation [209], epigenetic DNA methylation is potentially reversible.

DNA methylation can happen at various parts of the genome, including gene promoters and intragenic segments, such as exons and introns [210]. The relationship between DNA methylation and gene transcription and expression has been extensively studied. One study found that promoter methylation is associated with decreased transcription and expression, thus gene silencing [211]. Another study argued that intragenic methylation, particularly 1st exon methylation, is a leading indicator of gene silencing rather than promoter methylation [210].

Brenet et al. examined DNA methylation in different regions: the gene promoter, the first exon, introns, internal exons, and the last exon. They found no correlation between the methylation of introns and downstream exons and the methylation of the first exon or the transcriptional start site of the gene (TSS). Additionally, they found that methylation of the 5' end of the gene is linked to gene silencing, unlike methylation of the 3' end [210].

Another observation is that gene expression is more variable when the promoter is methylated. The methylation of the promoter TSS and the first exon are related, which could be due to overlapping elements. Intron methylation was found to be coupled with the methylation of 3' prime elements but not with methylation at the 5' end. In summary, they found that 5' methylation and 3' methylation have different functions and distinct controls. Genes with the lowest expression levels had the highest levels of 5' methylation [210].

While most DNA methylation occurs outside CpG islands (CpG = cytosine phospho-guanine dinucleotide), the methylation of CpG islands is particularly interesting for studying epigenetic changes. The vertebrate genome has fewer cytosine and guanine nucleotides, and the overall CG dinucleotide content observed/expected (O/E) CG ratio is 0.24 for the human genome [212].

Still, when present, they are often found in clusters or islands [213]. Gardiner-Garden and Frommer define CpG islands as areas of DNA that are ≥200 bp in size, CG percentage ≥ 50%, and (CG)observed/(CG)expected >0.6 [214].

Observed /Expected CpG = Number of CpG * N / (Number of C * Number of G)

* N = length of sequence [214].

However, this definition seems too lenient. In Takai et al., new and stricter criteria were adopted. CpG islands were defined as DNA areas ≥500 bp in size, with a CG percentage of≥ 55% and (CG)observed/(CG)expected >0.65 [215]. CpG islands are found in 40% of mammalian genes [216], most of which are housekeeping genes and fewer tissue-specific genes [216].CpG islands can be found in both coding and non-coding regions of genes. In non-coding regions, they typically have a mean CG percentage of 65% and lengths ranging between 200 to 400 bp, playing a primary role in gene silencing when methylated [217]. In contrast, CpG islands located in coding regions exhibit a lower CG percentage, consist of shorter segments, and have functions that remain unknown [215].

Many studies have found methylation in various regions of the genome, including gene bodies, across different species such as the *A. thaliana* plant, sea squirts, honeybees, and *Myzus persicae* insects. In all these species, gene body methylation does not affect gene expression. It may be that the impact of DNA methylation relies on the specific methylation site [203].

Brenet et al. found that DMEs (densely methylated elements) are primarily located outside the CpG islands region. Their median length is approximately 600 bp, and their O/E CG ratio is 0.49, which is why most DMEs do not fall under the CpG islands classification [218, 219] and do not overlap with CpG islands [210].

In this chapter, we amplified two segments of the TNF-α gene: the first amplicon is in the gene promoter region, and the second amplicon is found in the gene's first exon. Both are crucial regions for gene transcription control. Approximately 40-50% of gene promoters contain CpG islands, and the presence of these islands was used to predict the promoter region of genes [220].

Genes can have CpG islands in the upstream region of the translation site (5' end) or downstream of the translation site (3' end). However, the 5' CpG islands are in exons and introns, while the 3' CpG islands are found in or within exons [214]. The majority of CpG islands are located at the 5' end [214]. The TNF gene

promoter region at -150 and a region in the first exon at +450 contain CpG-rich sequences classified as CpG islands [221],the mentioned base pair positions are relative to the transcriptional start site (+1).

5.1.1 Histone Modification:

The chromosome consists of two chromatid structures formed by chromatin. Chromatin, a highly ordered structure of DNA wrapped around histone proteins, dynamically switches between open and closed states that impact gene accessibility and transcription [222]. In its normal state, chromatin is highly condensed; however, during infection, it loosens to facilitate the transcription of immune defence genes. Nevertheless, chromatin does not return to its original state after the infection is cleared. This exemplifies epigenetic change (Figure 4-2), (Table 4-1).

Examples of Histone Modifications[92]:

- **Acetylation**: an acetyl group ($COCH_3$) attached to the histone tails; this usually results in active (open) chromatin.

- **Phosphorylation**: phosphate groups (PO_4) attached to the histone tails usually result in loose chromatin, permissive of gene transcription.

- **Methylation**: A methyl group (CH_3) is attached to the histone tails, usually resulting in repressed (Closed) chromatin.

Histone modifications were studied in the TNF-α gene, the gene of interest in this thesis. It was shown that Histone H3 lysine four dimethylation and trimethylation happen at the transcription initiation site, with trimethylation significantly correlating with active transcription and dimethylation signalling a state of competence (Table 4-1)[223]. Competence is an epigenetic state of chromatin that enables a gene or region to be activated, even if it is not currently transcribed. It is often marked by specific histone modifications (e.g., H3K4me2 or H3K4me3) that prime a region for future activation [223].

Histone H3 lysine four methylation is typically associated with TNF-α active transcription. When MTA (histone methyltransferase inhibitor 5-deoxy-5-methylthioadenosine) was used, THP-1 cells' ability to secrete TNF-α was markedly reduced [221]. It was also found that histone acetylation at the TNF-α locus plays a vital role in increasing the cell's ability to produce TNF-α. Inhibition of histone deacetylases led to increased histone acetylation and more TNF-α production [224].

LPS stimulation (1 μg/ml) increased the H4 acetylation of the TNF locus at two specific regions, the distal upstream promoter (TNF4) and the enhancer region (+1417). H3 acetylation was increased only in the enhancer region (+1417). Acetylated H3 and H4 and dimethylated H3 lysine 4 appear to spot both active promoters and enhancers [225].

5.1.2 DNA Methylation

DNA methylation is adding a methyl group (CH_3) to a cytosine residue in a CpG sequence. Adding the methyl group (CH_3) to a gene promoter reduces gene transcription or gene silencing, while adding the methyl group (CH_3) to the gene body promotes transcription.

DNA methylation can cause gene silencing by impeding the binding of transcription regulation factors to the gene (Figure 4-2)[92].

In one study, TNF-α gene methylation was examined in three cell lines: the THP-1 cell line, which are monocytes isolated from peripheral blood from an acute monocytic leukaemia patient; the HL-60 cell line, which are promyeoloblasts isolated from the peripheral blood by leukapheresis from a 36-year-old, White, female with acute promyelocytic leukaemia; and the K562 cell line, which are lymphoblast cells isolated from the bone marrow of a 53-year-old chronic myelogenous leukaemia patient [221].

It was found that cell lines (THP-1 and HL60) that had TNF-α gene demethylation produced more TNF-α in response to LPS or PMA stimulation compared to K562 cells, which don't produce TNF-α; these were heavily methylated [221].

In this study, we focus on DNA methylation. The DNA methylation of the TNF-α gene promoter and first exon regions was studied using bisulfite DNA conversion sequencing.

The technique relies on converting unmethylated cytosine into uracil using sodium bisulfite. Methylated cytosines are not converted into uracil. After the conversion, PCR is performed to amplify the DNA target. Subsequently, sequencing is carried out. The sequence data is then analysed to detect the changes of cytosine into thymine. Cytosine residues that remain as cytosine will be labelled as methylated cytosine (Figure 5-1).

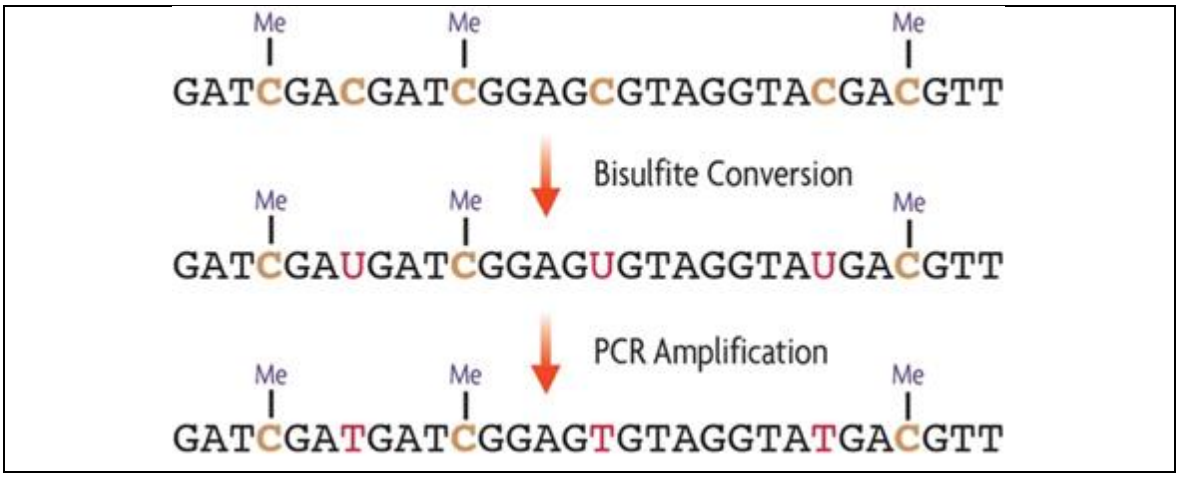


Figure 5-1 DNA Sodium Bisulfite Conversion Method. [226]

The experimental controls and conditions for this experiment were prepared as mentioned in 2.7 DNA Methylation Assay in TNF-α Promoter and First Exon regions.

My previous results showed that LPS pre-treatment induces TNF-α mRNA transcription to a similar level as pre-treated infected cells. However, when combined with previous ELISA assays, it seems that LPS pretreatment with *Streptococcus pneumoniae* infection leads to higher TNF-α secretion, which suggests that *Streptococcus pneumoniae* infection plays a post-transcriptional role in increasing the TNF-α translation and secretion.

This chapter examines whether LPS pretreatment (0.1 μg/ml) causes changes in DNA methylation levels at the promoter and 1st exon regions of the TNF-α gene.

Initially, we attempted to examine the whole CG island in the TNF-α gene, but the amplification didn't work, so we resorted to using shorter gene segments.

TNF-α gene map is outlined in (Figure 5-2). The first amplicon is seen in the promoter region, ranging from (-268 to +64) bp (see appendix for sequences—amplicon 1). The second amplicon is in the first exon, ranging from (+161 to +394) bp (see appendix for sequences—amplicon 2).

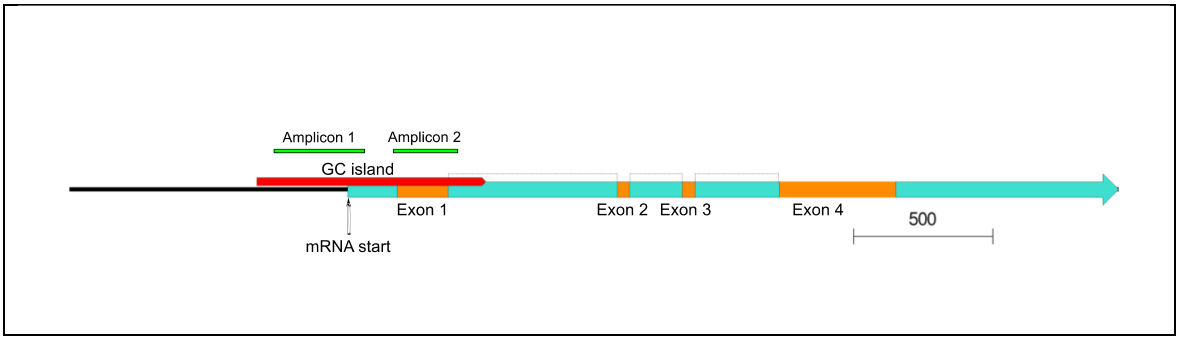


Figure 5-2 TNF-α gene map.
Gene ID -Genbank 7124, (mRNA NM_000594.4). The scale bar represents 500 bp. The TNF-α promoter region is within -1000 bp to +1 bp relative to TSS [227].

5.2 Results

5.2.1 DNA Methylation in the TNF-α Promoter Region

In these experiments, U937 cells were grown in culture. The day prior to the experiment, the cells were primed with PMA (100 ng/ml) to differentiate them into macrophages, and some were pre-treated with LPS (0.1 µg/ml) for 4 hours.

The following day, some cells were infected with *Streptococcus pneumoniae* TIGR4 (MOI=1). Cells were incubated at 37°C for 2 hours, then lysed, and DNA was extracted, as 2 hours is the most likely time for effects to be seen. This is based on a study conducted by Medzhitov et al, which demonstrated that TNF-α mRNA is rapidly induced within 1–2 hours of LPS stimulation in macrophages [228].

PCR was done using the TNFp3 primer to amplify the promoter region. Agarose gel was run to verify that the desired gene segment exists. Afterwards, sodium bisulfite DNA conversion was carried out. The converted DNA segment was then amplified using PCR; the 1st amplicon is in the promoter region, at position -268 to +64 bp relative to the mRNA start site.

The PCR product was diluted and cloned using the StrataClone kit into bacterial plasmids. After overnight bacterial growth, the plasmids were purified using the miniprep kit and sent for sequencing.

Sequencing data was analysed for methylation percentages in the TNF-α promoter segment. The data showed 100% methylation of C residues at CG sites in all samples, which is evidence of good conversion. The results were obtained to determine the level of methylation between LPS-pretreated cells and non-treated cells.

To answer the question of whether LPS pre-treatment (0.1 µg/ml) affects the TNF-α promoter methylation percentage following infection with *Streptococcus pneumoniae*, CpG sites methylation percentages were analysed (Table 5-1).and compared to each other using the significance level (P-Value) obtained using the Chi-squared test as recommended by Campbell (2007) and Richardson

(2011)[166, 167] to determine whether the percentage change is significant or not. TNF-α promoter methylation data is presented in (Table 5-1)(Figure 5-3).

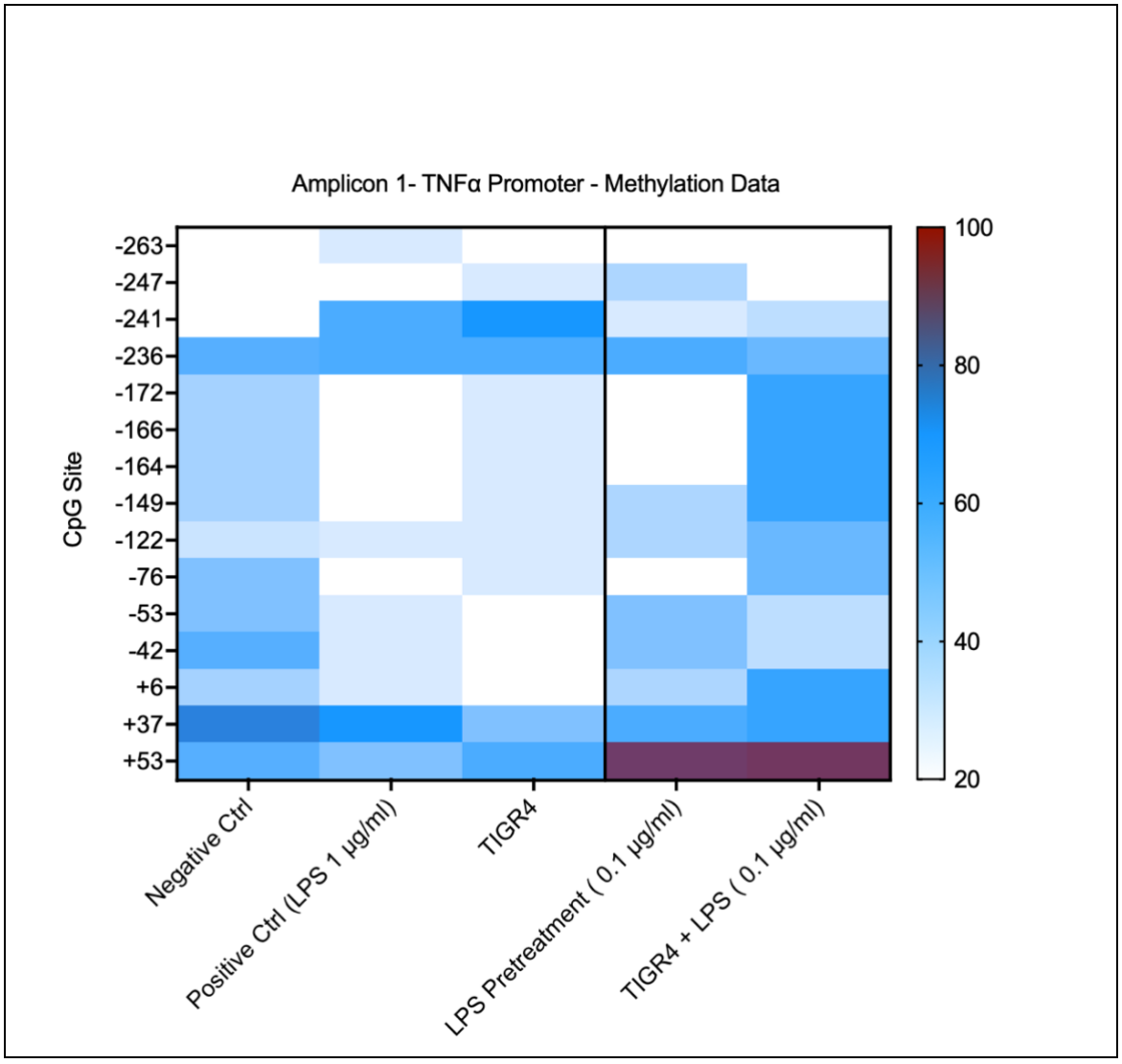


Figure 5-3 Amplicon 1 - TNF-α Promoter Segment Methylation Percentages
U937 cells were primed with PMA (100 ng/ml), and some were pre-treated with LPS (0.1 µg/ml). The next day, some of the cells were infected with *Streptococcus pneumoniae* (MOI=1). The positive control is LPS (1 µg/ml) + PMA; the negative control is untreated cells + PMA. TIGR4: refers to U937 cells infected with *Streptococcus pneumoniae* TIGR4 (MOI=1) without prior LPS pretreatment. LPS pretreatment (0.1 µg/ml): refers to U937 cells pretreated with LPS (0.1 µg/ml) without infection. TIGR4 + LPS pretreatment (0.1 µg/ml): U937 cells pretreated with LPS (0.1 µg/ml) and infected with *Streptococcus pneumoniae* TIGR4 (MOI=1). The number of sequences used for every condition to calculate methylation percentages varies between 10-12 sequences per condition.

Table 5-1 Amplicon 1 - TNF-α Promoter Methylation Percentages.

Columns represent the number of methylated cytosines at each site and the methylation percentage. Statistical analysis to calculate the significance level (P-value) obtained using the Chi-squared test.

Site	Negative Control	Positive Control LPS (1 µg/ml)	Infected Cells; TIGR4	Pre-treated Cells; LPS Pre-treatment (0.1 µg/ml)	Pre-treated Infected Cells; TIGR4+ LPS Pretreatment (0.1 µg/ml)
-263	1 / 12 (8.33%)	3 / 10 (30%)	1 / 10 (10%)	2 / 10 (20%)	0 / 11 (0%)
-247	1 / 12 (8.33%)	2 / 10 (20%)	3 / 10 (30%)	4 / 10 (40%)	2 / 11 (18.18%)
-241	2 / 12 (16.66%)	6 / 10 (60%)	7 / 10 (70%)	3 / 10 (30%)	4 / 11 (36.36%)
-236	7 / 12 (58.33%)	6 / 10 (60%)	6 / 10 (60%)	6 / 10 (60%)	6 / 11 (54.54%)
-172	5 / 12 (41.66%)	2 / 10 (20%)	3 / 10 (30%)	1 / 10 (10%)	7/11(63.63%)
-166	5 / 12 (41.66%)	2 / 10 (20%)	3 / 10 (30%)	2 / 10 (20%)	7/11(63.63%)
-164	5 / 12 (41.66%)	1 / 10 (10%)	3 / 10 (30%)	2 / 10 (20%)	7 / 11 (63.63%)
-149	5 / 12 (41.66%)	2 / 10 (20%)	3 / 10 (30%)	4 / 10 (40%)	7 / 11 (63.63%)
-122	4 / 12 (33.33%)	3 / 10 (30%)	3 / 10 (30%)	4 / 10 (40%)	6 / 11 (54.54%)
-76	6 / 12 (50%)	2 / 10 (20%)	3 / 10 (30%)	2 / 10 (20%)	6 / 11 (54.54%)
-53	6 / 12 (50%)	3 / 10 (30%)	1 / 10 (10%)	5 / 10 (50%)	4 / 11 (36.36%)
-42	7 / 12 (58.33%)	3 / 10 (30%)	1 / 10 (10%)	5 / 10 (50%)	4 / 11 (36.36%)
+6	5 / 12 (41.66%)	3 / 10 (30%)	1 / 10 (10%)	4 / 10 (40%)	7 / 11 (63.63%)
+37	9 / 12 (75%)	7 / 10 (70%)	5 / 10 (50%)	6 / 10 (60%)	7 / 11 (63.63%)
+53	7 / 12 (58.33%)	5 / 10 (50%)	6 / 10 (60%)	9 / 10 (90%)	10 / 11 (90.90%)

In the TNF-α Promoter region, at CpG site -263, it appears that TIGR4-infected samples had a methylation percentage of 10% compared to 0% percentage of TIGR4+LPS pre-treatment, while the pre-treatment led to a decrease in methylation, the difference is non-significant (P=0.29).methylation percentage of LPS pre-treated samples is 20%, which is higher than TIGR4+LPS pre-treatment, the difference is non-significant (P=0.12).

CpG site -247: It appears that TIGR4-infected samples had a methylation percentage of 30% compared to 18.18% for TIGR4+LPS pre-treatment. While the pre-treatment led to a decrease in methylation, the difference is non-significant (P = 0.53). The methylation percentage of LPS pretreated samples is 40%, higher than that of TIGR4+LPS pre-treatment. The difference is non-significant (P = 0.28).

CpG site -241: It appears that TIGR4-infected samples had a methylation percentage of 70% compared to 36.36% of TIGR4+LPS pre-treatment. While the pre-treatment led to a decrease in methylation, the difference is non-significant (P = 0.13). The methylation percentage of LPS pretreated samples is 30%, higher than TIGR4+LPS pre-treatment. The difference is non-significant (P = 0.76).

CpG site -236: It appears that TIGR4-infected samples had a methylation percentage of 60% compared to 54.54% for TIGR4+LPS pre-treatment. While the pre-treatment led to a decrease in methylation, the difference is non-significant (P = 0.80). The methylation percentage of LPS pretreated samples is 60%, higher than that of TIGR4+LPS pre-treatment. The difference is non-significant (P = 0.80).

CpG site -172: It appears that TIGR4-infected samples had a methylation percentage of 30% compared to 63.63% for TIGR4+LPS pre-treatment. While the pre-treatment led to an increase in methylation, the difference is non-significant (P = 0.13). The methylation percentage of LPS pretreated samples is 10%, which is lower than that of TIGR4+LPS pre-treatment. The difference is significant (P = 0.01).

CpG site -166: It appears that TIGR4-infected samples had a methylation percentage of 30% compared to 63.63% for TIGR4+LPS pre-treatment. While the pre-treatment led to an increase in methylation, the difference is non-significant (P = 0.13). The methylation percentage of LPS pretreated samples is 20%, which is lower than TIGR4+LPS pre-treatment. The difference is significant (P = 0.04).

CpG site -164: It appears that TIGR4-infected samples had a methylation percentage of 30% compared to 63.63% for TIGR4+LPS pre-treatment. While the pre-treatment led to an increase in methylation, the difference is non-significant (P = 0.13). The methylation percentage of LPS pretreated samples is 20%, which is lower than that of TIGR4+LPS pre-treatment. The difference is significant (P = 0.04).

CpG site -149: It appears that TIGR4-infected samples had a methylation percentage of 30% compared to 63.63% for TIGR4+LPS pre-treatment. While the pre-treatment led to an increase in methylation, the difference is non-significant (P = 0.13). The methylation percentage of LPS pretreated samples is 40%, which is lower than that of TIGR4+LPS pre-treatment. The difference is non-significant (P = 0.29).

CpG site -122: It appears that TIGR4-infected samples had a methylation percentage of 30% compared to 54.54% of TIGR4+LPS pre-treatment. While the pre-treatment led to an increase in methylation, the difference is non-significant (P = 0.26). The methylation percentage of LPS pretreated samples is 40%, which is lower than TIGR4+LPS pre-treatment. The difference is non-significant (P = 0.51).

CpG site -76: It appears that TIGR4-infected samples had a methylation percentage of 30% compared to 54.54% for TIGR4+LPS pre-treatment. While the pre-treatment led to an increase in methylation, the difference is non-significant (P = 0.26). The methylation percentage of LPS pretreated samples is 20%, which is lower than that of TIGR4+LPS pre-treatment. The difference is non-significant (P = 0.11).

CpG site -53: It appears that TIGR4-infected samples had a methylation percentage of 10% compared to 36.36% of TIGR4+LPS pre-treatment. While the pre-treatment led to an increase in methylation, the difference is non-significant (P = 0.16). The methylation percentage of LPS pretreated samples is 50%, which is higher than TIGR4+LPS pre-treatment. The difference is non-significant (P = 0.53).

CpG site -42: It appears that TIGR4-infected samples had a methylation percentage of 10% compared to 36.36% of TIGR4+LPS pre-treatment. While the pre-treatment led to an increase in methylation, the difference is non-significant (P = 0.16). The methylation percentage of LPS pretreated samples is 50%, which is higher than TIGR4+LPS pre-treatment. The difference is non-significant (P = 0.53).

CpG site 6: It appears that TIGR4-infected samples had a methylation percentage of 10% compared to the 63.63% percentage of TIGR4+LPS pre-treatment. While the pre-treatment led to an increase in methylation, the difference is significant (P = 0.01). The methylation percentage of LPS pretreated samples is 40%, which is lower than the TIGR4+LPS pre-treatment. The difference is non-significant (P = 0.29).

CpG site 37: It appears that TIGR4-infected samples had a methylation percentage of 50% compared to 63.63% for TIGR4+LPS pre-treatment. While the pre-treatment led to an increase in methylation, the difference is non-significant (P = 0.53). The methylation percentage of LPS pretreated samples is 60%, which is lower than that of TIGR4+LPS pre-treatment. The difference is non-significant (P = 0.86).

CpG site 53: It appears that TIGR4-infected samples had a methylation percentage of 60% compared to 90.9% of TIGR4+LPS pre-treatment. While the pre-treatment led to an increase in methylation, the difference is non-significant (P = 0.10). The methylation percentage of LPS pretreated samples is 90%, the same as TIGR4+LPS pre-treatment; hence, the difference is non-significant (P = 0.94).

The DNA methylation results show 15 CpG sites; 11/15 of these sites showed increased methylation in cells pre-treated with LPS prior to infection with TIGR4, compared to cells infected without pre-treatment. This is observed in loci -172 to 53 of the TNF-α promoter. Mostly, that increase is double the amount of methylation. Increased methylation in a gene promoter is associated with a reduction of transcription of that particular gene. This suggests that LPS pre-treatment may have a role in reducing TNF-α transcription (Table 5-1)(Figure 5-3) When methylation percentages of LPS pretreatment were compared to TIGR4+LPS pre-treatment samples, it showed the majority of CpG sites (9/15) showed increased methylation in TIGR4+LPS pre-treatment samples compared to samples pre-treated with LPS only (Table 5-1)(Figure 5-3).

This suggests that LPS pre-treatment may cause an increase in DNA methylation following *Streptococcus pneumoniae* infection at these sites. In previous experiments, it was shown that LPS pre-treatment caused an increase in TNF-α mRNA transcription, based on PCR results, and increased TNF-α secretion following *Streptococcus pneumoniae* TIGR4 infection, based on ELISA assays of cell culture supernatants. When these results are combined with DNA methylation studies, it appears that *Streptococcus pneumoniae* infection raises TNF-α levels in a post-transcriptional manner. The changes in methylation observed here are difficult to reconcile with the ELISA and mRNA data if methylation is reducing transcription factor binding and hence reducing mRNA transcription. However, several studies have shown that 5mC may actually enhance binding of certain transcription factors, increasing mRNA transcription [229]. This is considered further in the Discussion.

5.2.2 DNA Methylation in TNF-α 1st Exon Region

The experiment was repeated, this time to examine the methylation percentages in the TNF-α 1st exon region, which is presented in(Table 5-2)(Figure 5-4) The data showed 100% methylation of C residues at CG sites in all samples, which is evidence of good conversion. The 2nd amplicon is in the first exon, at position 161 until 394 bp relative to the mRNA start site.

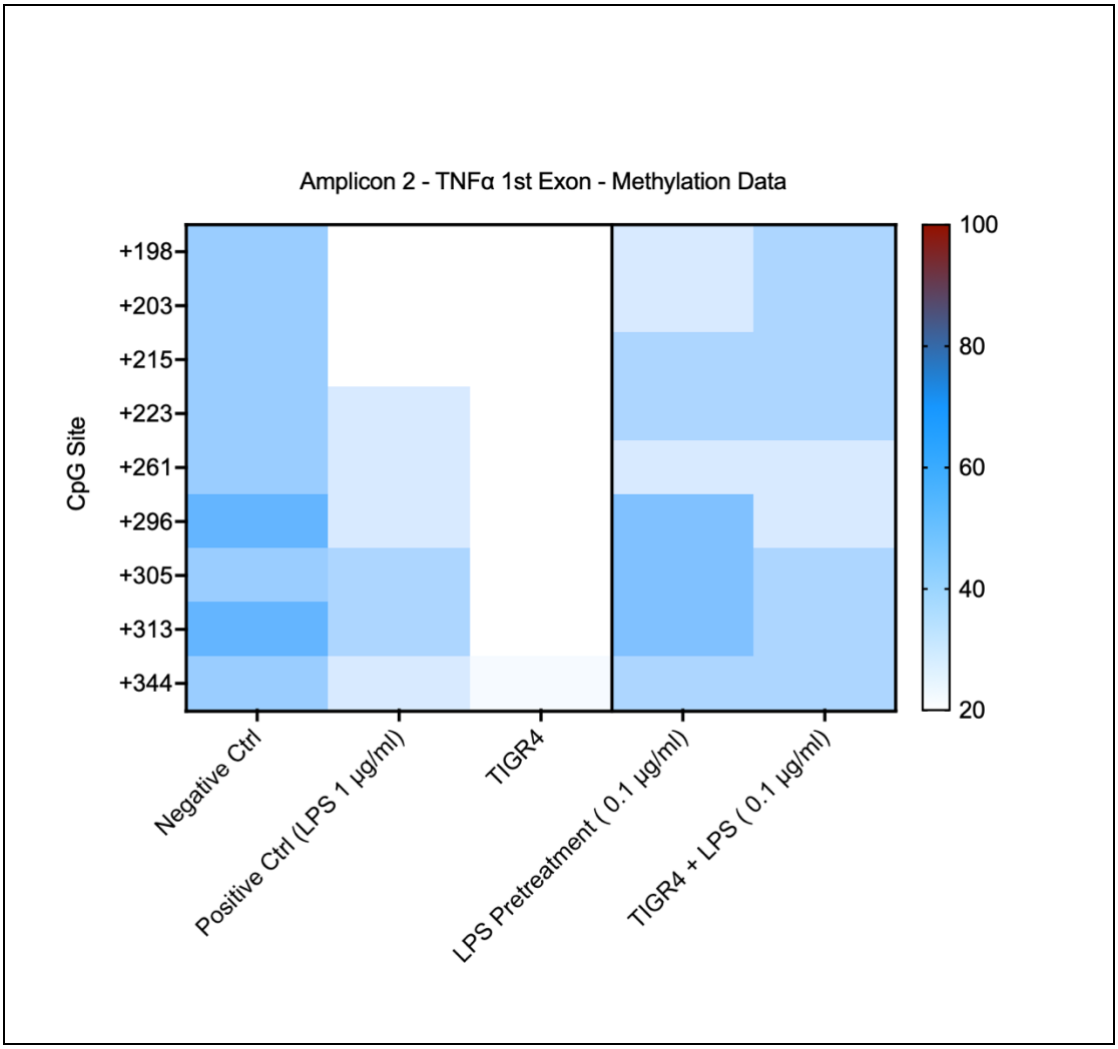


Figure 5-4 Amplicon 2 - TNF-α 1st Exon Segment Methylation Percentages.
U937 cells were primed with PMA (100 ng/ml), and some were pre-treated with LPS (0.1 µg/ml). The next day, some of the cells were infected with *Streptococcus pneumoniae* (MOI=1). The positive control is LPS (1 µg/ml) + PMA; the negative control is untreated cells + PMA. TIGR4: refers to U937 cells infected with *Streptococcus pneumoniae* TIGR4 (MOI=1) without prior LPS pretreatment. LPS pretreatment (0.1 µg/ml): refers to U937 cells pretreated with LPS (0.1 µg/ml) without infection. TIGR4 + LPS pretreatment (0.1 µg/ml): U937 cells pretreated with LPS (0.1 µg/ml) and infected with *Streptococcus pneumoniae* TIGR4 (MOI=1). The number of sequences used for every condition to calculate methylation percentages varies between 9-10 sequences per condition.

Table 5-2 Amplicon 2 - TNF-α 1st Exon Methylation Percentages.
Columns represent the number of methylated cytosines at each site and the methylation percentage. Statistical analysis to calculate the significance level (P-value) obtained using the Chi-squared test.

Site	Negative Control	Positive Control LPS (1 µg/ml)	Infected Cells; TIGR4	Pre-treated Cells; LPS Pre-treatment (0.1 µg/ml)	Pre-treated Infected Cells; TIGR4+ LPS Pretreatment (0.1 µg/ml)
+198	4 / 9 (44.44%)	2 / 10 (20%)	1 / 9 (11.11%)	3 / 10 (30%)	4 / 10 (40%)
+203	4 / 9 (44.44%)	2 / 10 (20%)	1 / 9 (11.11%)	3 / 10 (30%)	4 / 10 (40%)
+215	4 / 9 (44.44%)	2 / 10 (20%)	1 / 9 (11.11%)	4 / 10 (40%)	4 / 10 (40%)
+223	4 / 9 (44.44%)	3 / 10 (30%)	1 / 9 (11.11%)	4 / 10 (40%)	4 / 10 (40%)
+261	4 / 9 (44.44%)	3 / 10 (30%)	1 / 9 (11.11%)	3 / 10 (30%)	3 / 10 (30%)
+296	5 / 9 (55.55%)	3 / 10 (30%)	1 / 9 (11.11%)	5 / 10 (50%)	3 / 10 (30%)
+305	4 / 9 (44.44%)	4 / 10 (40%)	1 / 9 (11.11%)	5 / 10 (50%)	4 / 10 (40%)
+313	5 / 9 (55.55%)	4 / 10 (40%)	1 / 9 (11.11%)	5 / 10 (50%)	4 / 10 (40%)
+344	4 / 9 (44.44%)	3 / 10 (30%)	2 / 9 (22.22%)	4 / 10 (40%)	4 / 10 (40%)

Starting with CpG site +198, it appears that TIGR4-infected samples had a methylation percentage of 11.11% compared to a 40% methylation percentage of TIGR4+LPS pre-treatment. While the pre-treatment led to an increase in methylation, the difference is non-significant (P = 0.16). The methylation percentage of LPS pretreated samples is 30%, which is lower than that of TIGR4+LPS pre-treatment, and the difference is non-significant (P = 0.64).

CpG site +203: It appears that TIGR4-infected samples had a methylation percentage of 11.11% compared to 40% of TIGR4+LPS pre-treatment. While the pre-treatment led to an increase in methylation, the difference is non-significant (P = 0.16). The methylation percentage of LPS pretreated samples is 30%, which is lower than that of TIGR4+LPS pretreatment. The difference is non-significant (P = 0.64).

CpG site +215: It appears that TIGR4-infected samples had a methylation percentage of 11.11% compared to the 40% percentage of TIGR4+LPS pre-treatment. While the pre-treatment led to an increase in methylation, the difference is non-significant (P = 0.16). The methylation percentage of LPS pretreated samples is 40%, the same as TIGR4+LPS pre-treatment; hence, the difference is non-significant (P = 1).

CpG site +223: It appears that TIGR4-infected samples had a methylation percentage of 11.11% compared to the 40% percentage of TIGR4+LPS pre-treatment. While the pre-treatment led to an increase in methylation, the difference is non-significant (P = 0.16). The methylation percentage of LPS pretreated samples is 40%, the same as TIGR4+LPS pre-treatment; hence, the difference is non-significant (P = 1).

CpG site +261: It appears that TIGR4-infected samples had a methylation percentage of 11.11% compared to 30% for TIGR4+LPS pre-treatment. While the pre-treatment led to an increase in methylation, the difference is non-significant (P = 0.32). The methylation percentage of LPS pretreated samples is 30%, the same as TIGR4+LPS pre-treatment; hence, the difference is non-significant (P = 1).

CpG site +296: It appears that TIGR4-infected samples had a methylation percentage of 11.11% compared to 30% for TIGR4+LPS pre-treatment. While the pre-treatment led to an increase in methylation, the difference is non-significant (P = 0.32). The methylation percentage of LPS pretreated samples is 50%, higher than that of TIGR4+LPS pre-treatment. The difference is non-significant (P = 0.37).

CpG site +305: It appears that TIGR4-infected samples had a methylation percentage of 11.11% compared to 40% of TIGR4+LPS pre-treatment. While the pre-treatment led to an increase in methylation, the difference is non-significant (P = 0.16). The methylation percentage of LPS pretreated samples is 50%, which is higher than TIGR4+LPS pre-treatment. The difference is non-significant (P = 0.66).

CpG site +313: It appears that TIGR4-infected samples had a methylation percentage of 11.11% compared to 40% of TIGR4+LPS pre-treatment. While the pre-treatment led to an increase in methylation, the difference is non-significant (P = 0.16). The methylation percentage of LPS pretreated samples is 50%, which is higher than TIGR4+LPS pre-treatment. The difference is non-significant (P = 0.66).

Finally, at CpG site +344, TIGR4-infected samples had a methylation percentage of 22.22% compared to a 40% percentage of TIGR4+LPS pre-treatment. While the pre-treatment led to an increase in methylation, the difference is non-significant (P = 0.41). The methylation percentage of LPS pretreated samples is 40%, the same as TIGR4+LPS pre-treatment. Hence, the difference is non-significant (P = 1).

The results show a trend of increased methylation percentage in cells pre-treated with LPS prior to infection with TIGR4, compared to cells infected with TIGR4 without pre-treatment. This increase was observed in all CpG sites +198 to +344 (9/9 sites), and the increase is more than double the percentage of methylation (Table 5-2)(Figure 5-4). When comparing the methylation percentages of LPS pretreatment alone to those of TIGR4+LPS pre-treatment samples, the majority of CpG sites exhibited increased or equal methylation in

the TIGR4+LPS pre-treatment samples compared to those pre-treated with LPS alone. 3/9 CpG sites showed increased methylation in pretreated infected samples compared to pretreated samples, and 4/9 CpG sites displayed equal methylation in pretreated infected samples compared to pretreated samples (Table 5-2)(Figure 5-4). This suggests that LPS pre-treatment causes an increase in DNA methylation following *Streptococcus pneumoniae* infection.

In the previous chapter, PCR results indicated that LPS pre-treatment increased TNF-α mRNA transcription. ELISA assays of cell culture supernatants also revealed increased TNF-α secretion following *Streptococcus pneumoniae* TIGR4 infection. These findings are considered further in the discussion.

5.3 Conclusion

In this chapter, two regions of the TNF-α gene were examined for DNA methylation: the TNF-α promoter and 1st exon regions. The aim was to determine if LPS pre-treatment led to a different methylation percentage, affecting gene transcription, followed by an infection with *Streptococcus pneumoniae*.

The DNA methylation results in the TNF-α promoter region showed patchy results, with variable methylation data across different CpG sites. However, in most of these sites, there is increased methylation in cells pre-treated with LPS prior to infection with TIGR4 compared to cells infected without pre-treatment. This is observed in loci -172 to 53 of the TNF-α promoter, where the increase is often double the amount of methylation. Increased methylation in a gene promoter is typically associated with decreased transcription of that gene. When comparing the methylation percentages of LPS pre-treatment alone to those of TIGR4+LPS pre-treatment samples, the majority of CpG sites show increased methylation in TIGR4+LPS pre-treatment samples compared to those pre-treated with LPS only (Table 5-1)(Figure 5-3).

Similar results were observed in the TNF-α 1st exon region. The DNA methylation data indicate a variation in levels depending on the CpG site. However, at all these sites, there is increased methylation in cells pre-treated with LPS prior to infection with TIGR4 compared to cells infected without pre-treatment. This increase was noted in all CpG sites from +198 to +344, and the increase is more than double the percentage of methylation (Table 5-2)(Figure 5-4). When comparing the methylation percentages of LPS pretreatment to TIGR4+LPS pre-treatment samples, the majority of CpG sites showed increased or equal methylation in TIGR4+LPS pre-treated samples relative to LPS pretreated samples (Table 5-2)(Figure 5-4). This suggests that LPS pre-treatment leads to an increase in DNA methylation following *Streptococcus pneumoniae* infection.

Increased methylation in the gene promoter or the first exon typically results in suppression of gene expression, usually due to histone densification. However, a number of studies have shown that certain transcription factors show increased

binding at mCpG sites, actually leading to increased mRNA transcription. Further work will be required to identify which transcription factors are binding in the areas of increased methylated C residues [229, 230].

In contradistinction to the LPS pre-treatment results, TIGR4 infection alone appears to reduce methylation at CpG sites in amplicon 2 and in some of the sites in amplicon 1. TIGR4 infection alone produces a slight effect on mRNA transcription under the conditions studied here. This suggests that a different set of transcription factors may be binding at these sites under these conditions that are different from those binding with LPS pretreatment.

Post-transcriptional controls of gene expression encompass multiple mechanisms. Within the nucleus, DNA is transcribed into pre-mRNA, which includes exons (the coding regions) and introns (non-coding regions). Pre-mRNA undergoes intron splicing to produce an mRNA structure that can migrate into the cytoplasm and be translated into a protein [231]. This splicing can be carried out in alternative ways, referred to as alternative RNA splicing, which is one of the mechanisms of post-transcriptional control of gene expression. In alternative splicing, different proteins are produced from the same gene, using different intron removals and sometimes the removal of some exons as well [123, 231].

Another aspect of post-transcriptional control of gene expression is RNA stability. RNA stability is defined as the degree to which an RNA molecule maintains its structural integrity and resists degradation by exonucleases. This is done through 2 protective caps added to mRNA before leaving the nucleus. On the 5’ end, methylated GTP is added as a protective cap, and on the 3’ end, a poly-A tail is added as a protective cap. These caps include multiple repeats of the adenosine base.

Two factors control RNA stability in the cytoplasm. One is RNA-binding proteins (RBP). When these proteins bind to the RNA, that region will not be translated, hence the name untranslated region. There are two untranslated regions: 5’ UTR and 3’ UTR. External stimuli can modify the RBP through protein phosphorylation. Therefore, these RBPs can either increase or decrease RNA stability. Another factor affecting RNA stability is microRNAs. These are short,

single-stranded, non-coding RNA molecules, comprising 21 to 23 nucleotides, that function in post-transcriptional regulation. They associate with ribonucleoprotein complexes to form the RNA-induced silencing complex (RISC). RISC and miRNAs bind to RNA to degrade it, thereby decreasing its stability [132, 231].

Based on results from the current and previous chapters, we hypothesise that LPS pre-treatment before *Streptococcus pneumoniae* infection leads to increased TNF-α levels, mediated by both transcriptional and post-transcriptional changes in TNF-α expression that favour its production.

Previous studies showed that LPS stimulates TNF-α production in macrophages both transcriptionally and post-transcriptionally [232]. Translational control of TNF-α mRNA is achieved through an AU-rich sequence found in the 3' untranslated region of TNF-α mRNA, known as the AU-rich mRNA coding gene (ARE-gene). TNF-α is an example of an ARE-gene. In chronic inflammatory diseases, such as chronic inflammatory arthritis and Crohn's-like inflammatory bowel disease, mice with ZFP46 deletion, which codes for the ARE-mRNA destabilising protein TTP, exhibited severe inflammatory syndrome. This is mainly due to the increased stability of the mRNAs for TNF-α and GM-CSF, resulting in increased secretion of these cytokines [233].

In other studies, researchers observed that the translational regulation of TNF-α mRNA depends on the p38 protein [234] and c-Jun N-terminal kinases (JNK), which will be examined in the next chapter [235].

6 Cellular Proteins Involved in Innate Immune Response against *Streptococcus pneumoniae*

6.1 Introduction

In the previous chapters, it was shown that Zymosan and LPS pre-treatments have a stimulatory effect on TNF-α and IL-1β secretion following *Streptococcus pneumoniae* infection. The next step was to study the cellular proteins involved in this signalling process to understand the pathway by which these two stimulants produce this particular effect. This was performed using western blotting.

Toll-like receptor 4 (TLR4) is a transmembrane protein expressed by various cells, primarily monocytes, macrophages, and dendritic cells [236]. A major ligand for this receptor is bacterial LPS; upon their binding, a pro-inflammatory response is initiated [237].

LPS recognition occurs when LPS binds to a lipopolysaccharide-binding protein (LBP). The LPS-LBP complex moves the LPS to CD14, which is a membrane protein that binds to the LPS-LBP complex and facilitates the transfer of LPS to the MD-2 protein, which is associated with the extracellular domain of TLR4. LPS binding triggers the dimerization of the TLR4/MD-2 complex. Conformational changes in TLR4 lead to the recruitment of intracellular adaptor proteins to activate the downstream signalling pathway [238].

There are two major signalling pathways for TLR4 (Figure 6-1). One is TRIF-dependent. TRIF stands for TIR-domain-containing adapter-inducing interferon-β (TRIF). This pathway produces type I IFNs, which is not the pathway of interest in this study [239].

However, the second pathway is MyD88-dependent. Myeloid Differentiation Primary Response Gene 88 (MyD88) and TIR Domain-Containing Adaptor Protein (TIRAP) are two adaptor proteins that initiate the signalling pathway. This pathway also involves the activation of IL-1 Receptor-Associated Kinases (IRAKs) and the adaptor molecules TNF Receptor-Associated Factor 6 (TRAF6). TRAF6 activates TAK1 (Transforming Growth Factor-β-Activated Kinase 1), resulting in

the activation of MAPK cascades (Mitogen-Activated Protein Kinase) and the IκB Kinases (IKK), known as IKKα and IKKß [240]. IKKs' signalling leads to the induction of the transcription factor NF-κB, while the activation of MAPK cascades leads to the activation of another transcription factor, AP-1. These two transcription factors stimulate the expression of genes encoding pro-inflammatory cytokines, such as tumour necrosis factor α (TNF-α), interleukin (IL)-6, and type III interferons [240].

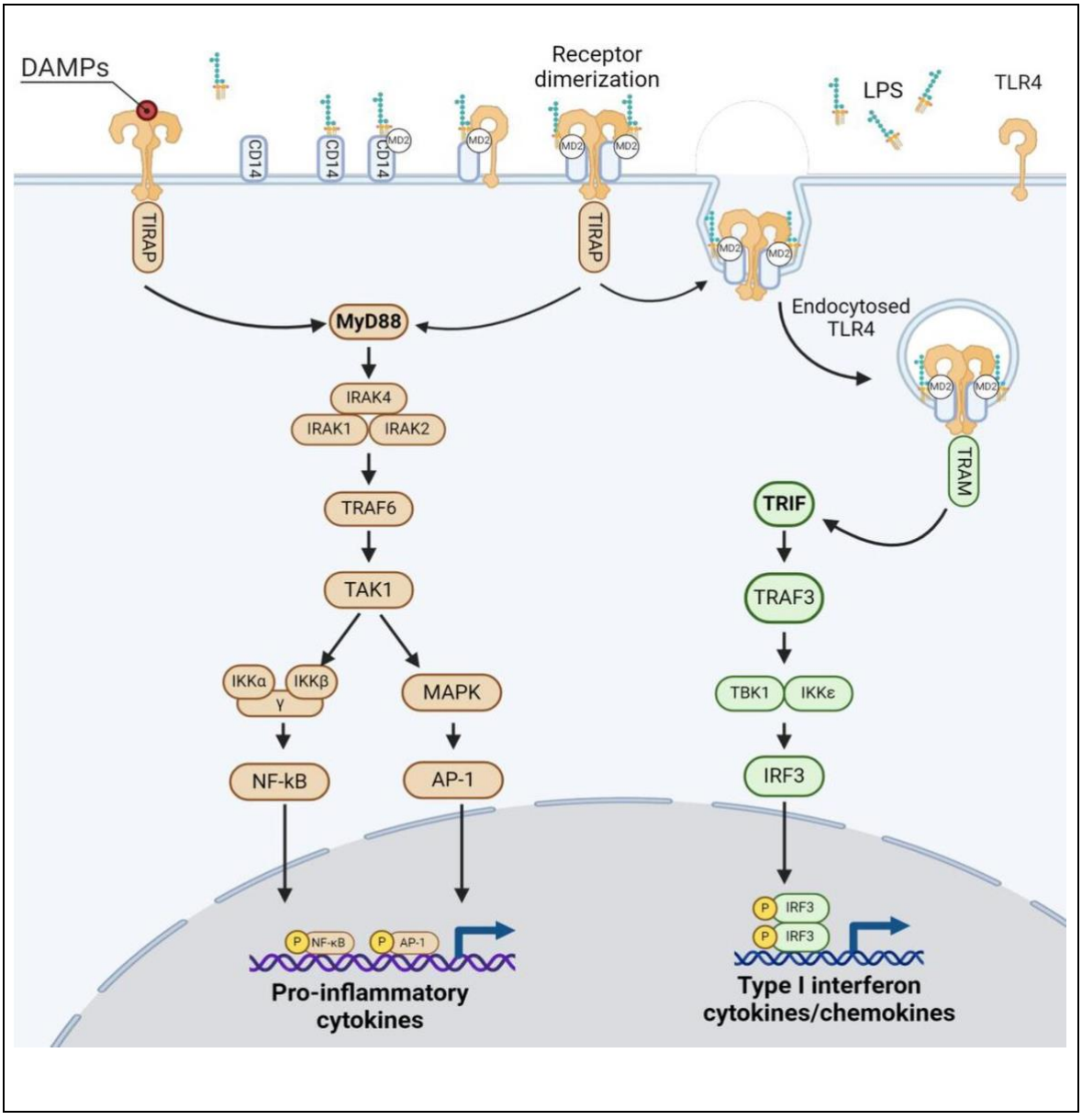


Figure 6-1 TLR4 Signalling Pathways [241].

Previous studies investigated the TNF gene in response to LPS stimulation. TNF-α is a crucial mediator of septic shock [242]. It appears that LPS stimulation in monocytes and macrophages activates the mitogen-activated protein kinase (MAPK) pathway, including the extracellular signal-related kinase (ERK), c-Jun N-

terminal kinases (JNK), and p38 cascades [243]. The transcriptional activity of c-jun depends on phosphorylation by JNK, while that of ATF-2 relies on either JNK or p38 [244]. However, another study showed that TNF-α gene transcription induction following LPS stimulation fully depends on ERK and not on P38 [245].

Tsai et al. showed that TNF-α mRNA levels were selectively inhibited (approximately 50%) by the ERK inhibitor PD98059 and were unaffected by the p38 inhibitor SB203580. Thus, ERK, but not p38, activity is required for the full induction of TNF-α transcription by LPS in J774 cells [245]. To confirm that ERK was stimulated by LPS, they performed a Western blot analysis using nuclear extracts from J774 cells and a specific antibody for the phosphorylated forms of ERK1 and ERK2. LPS stimulated the levels of phosphorylated ERK, while total ERK levels remained unaffected. The LPS-induced levels of phosphorylated ERK were, in turn, inhibited by the ERK inhibitor PD98059. Thus, in J774 cells, LPS stimulation leads to the activation of ERK1/2 through the phosphorylation of specific tyrosine residues, with PD98059 acting as an inhibitor of LPS-induced ERK phosphorylation [245].

The aim of this chapter is to examine cellular proteins involved in TLR4 signalling in an attempt to understand the pathways by which zymosan and LPS stimulants produce their pro-inflammatory effect.

6.2 Results

In these experiments, U937 cells samples were prepared as mentioned in 2.8 Western blotting section, and the following results were obtained.

6.2.1 P -IκBα:

Blots were first used to detect the respective experimental antibodies before being stripped and re-probed for the control antibody (GAPDH- glyceraldehyde-3-phosphate dehydrogenase).

The blots show an even loading of proteins, as the GAPDH levels for all conditions are similar. There does not seem to be an issue with loading, which implies that these results are valid.

IκBα (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha) is a cellular protein that is found in cytosol, with a molecular weight of 36 kDa. IκBα inhibits the NF-κB transcription factor and inhibits apoptosis [246].

To activate NF-κB, IκBα is phosphorylated, ubiquitinated and degraded by the proteasome. The phosphorylated form of this protein P- IκBα has a molecular weight of 41 kDa[39].

The blots were examined for the presence of P- IκBα protein. No significant differences in IκBα phosphorylation were observed. This indicates that LPS pretreatment does not have a significant effect on IκBα protein phosphorylation (Figure 6-2).

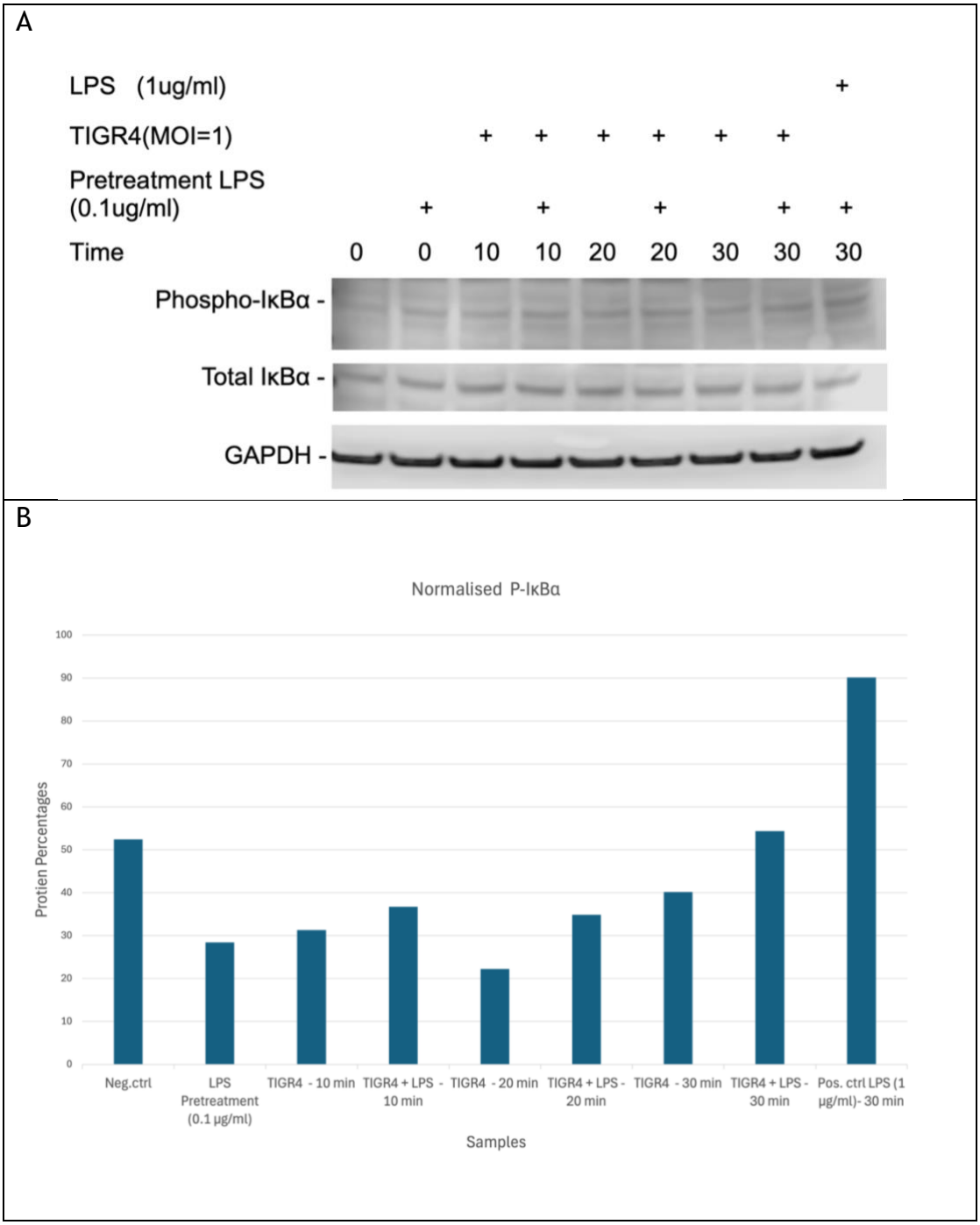


Figure 6-2 Western blot detection of P -IκBα in U937 cells.
Panel A: Blot images of P -IκBα in U937 cells .Proteins were extracted from U937 cells. The cells were cultured in antibiotic-free media and primed with PMA (100 ng/ml). Some of the cells were pre-treated with LPS (0.1 µg/ml) for 4 hours. The next day, some of the cells were infected with *Streptococcus pneumoniae* TIGR4 (MOI=1). Membranes were probed with [Phospho-IκBα (Ser32) (14D4) Rabbit mAb] to detect P-IκBα, and [IκBα Antibody] to detect IκBα at different time points of 0, 10, 20 and 30 minutes. GAPDH was used as a loading control. Blot image developed by iStudio software. Panel B: Normalised phosphorylated IκBα (p- IκBα) protein levels under the same conditions.

6.2.2 P-P38:

Mitogen-activated protein (MAP) kinases are a family of cellular proteins involved in signal transduction pathways that control intracellular events, such as acute responses to hormones and key developmental changes in organisms [247]. This family consists of three main groups: Extracellular signal-regulated kinases (ERKs),c-Jun N-terminal kinases (JNKs), and p38 mitogen-activated protein kinases (p38s) [248].

p38 mitogen-activated protein kinase is a protein involved in cell differentiation, apoptosis and autophagy [249]. It has a molecular weight of 38 kDa. Its phosphorylated form has the same molecular weight.

The blot shows bands of corresponding molecular size for P-p38 in all lanes, including the negative control, which consists of only cells. The same observation is noted in the lane for pre-treated cells, indicating increased phosphorylation of the p38 protein even at a basal level in the negative control. This could be attributed to the use of a cell line rather than primary cells, which sometimes lose some original biological properties. There doesn't appear to be a significant difference between the bands of pre-treated infected conditions and pre-treated conditions regarding the P-p38 and p38 proteins. This suggests that LPS pretreatment does not affect p38 protein phosphorylation (Figure 6-3).

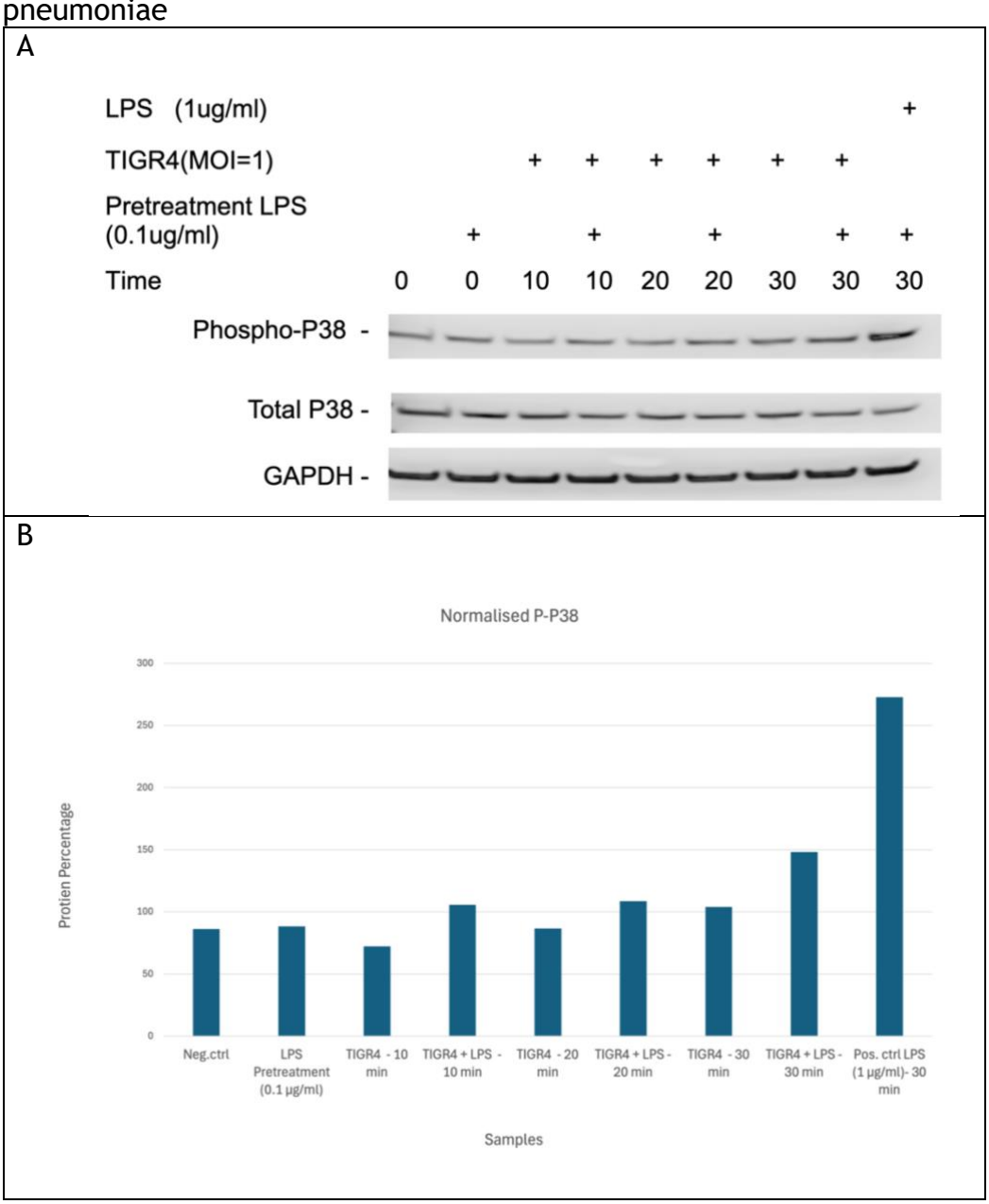


Figure 6-3 Western blot detection of P-P38 in U937 cells.
Panel A: Blot images of the P-P38 in U937 cells. Proteins were extracted from U937 cells. The cells were cultured in antibiotic-free media and primed with PMA (100 ng/ml). Some of the cells were pre-treated with LPS (0.1 µg/ml) for 4 hours. The next day, some of the cells were infected with *Streptococcus pneumoniae* TIGR4 (MOI=1). Membranes were probed with [Phospho-p38 MAPK (Thr180/Tyr182) (D3F9) XP® Rabbit mAb] to detect P-P38 and [p38 MAPK Antibody] to detect P38 at different time points of 0, 10, 20 and 30 minutes. GAPDH was used as a loading control. Blot image developed by iStudio software. Panel B: Normalised phosphorylated p38 (p-p38) protein levels under the same conditions.

6.2.3 P-ERK 1:

Another protein of the Mitogen-activated protein (MAP) kinases family of cellular proteins [247]. Extracellular signal-regulated kinases (ERK) have 7 isoforms in humans, which are ERK1, 2, 3, 5, 7, 8 and ERK3 -related [250]. ERK1, the protein in focus, has a molecular weight of 44 kDa, while its phosphorylated form has a molecular weight of 42-44 kDa.

In the blots, there are equally sized thick bands in the lanes corresponding to the experimental conditions, indicating phosphorylation of ERK1 protein. additionally, there are no significant differences in band thickness across the experimental conditions regarding active ERK1 (P-ERK1) and inactive ERK1. This suggests that LPS pretreatment does not affect the phosphorylation of ERK (Figure 6-4).

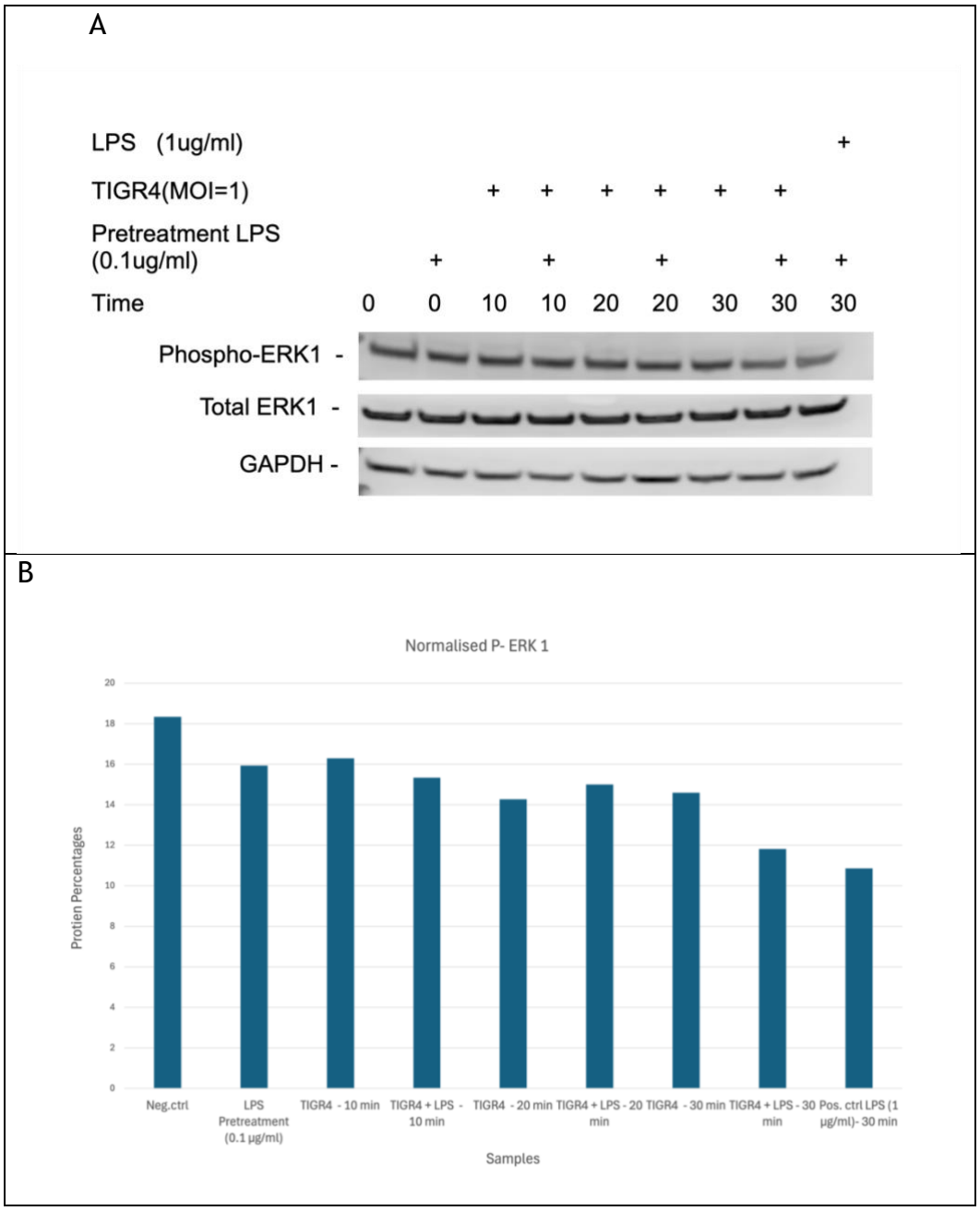


Figure 6-4 Western blot detection of P-ERK-1 in U937 cells.
Panel A: Blot images of P-ERK-1 in U937 cells. Proteins were extracted from U937 cells. the cells were cultured in antibiotic-free media and primed with PMA (100 ng/ml). Some of the cells were pre-treated with LPS (0.1 µg/ml) for 4 hours. The next day, some of the cells were infected with *Streptococcus pneumoniae* TIGR4 (MOI=1). Membranes were probed with [Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP® Rabbit mAb] to detect P-ERK1 and [p44/42 MAPK (Erk1/2) Antibody] to detect ERK-1 at different time points of 0, 10, 20 and 30 minutes. GAPDH was used as a loading control. Blot image developed by iStudio software. Panel B: Normalised phosphorylated ERK-1 (p- ERK-1) protein levels under the same conditions.

6.2.4 P-JNK:

JNK is another member of the Mitogen-activated protein (MAP) kinases family of cellular proteins [247]. c-Jun N-terminal kinase (JNK) It has a molecular weight of 48 kDa, while its phosphorylated active form, P-JNK, is 46 kDa.

Both JNK and p38 signalling pathways are reactive to stress, cytokines, ultraviolet radiation, osmotic shock, and heat shock. These MAPKs are involved in apoptosis or cell differentiation [212].

The blots show cleaved bands (double bands) in lanes corresponding to experimental conditions, with thicker bands and lanes of pretreated infected cells compared to infected cells without pre-treatment. This suggests that LPS pretreatment has a role in JNK phosphorylation and activation.

Regarding inactive JNK protein, it seems there are double bands in lanes corresponding to experimental conditions, these bands keep getting darker up until 20 minutes time point, and then it starts to fade. This may indicate that pre-treatment and infection have a role in stimulating inactive JNK for the first 20 minutes following infection, and then that effect subsides(Figure 6-5).

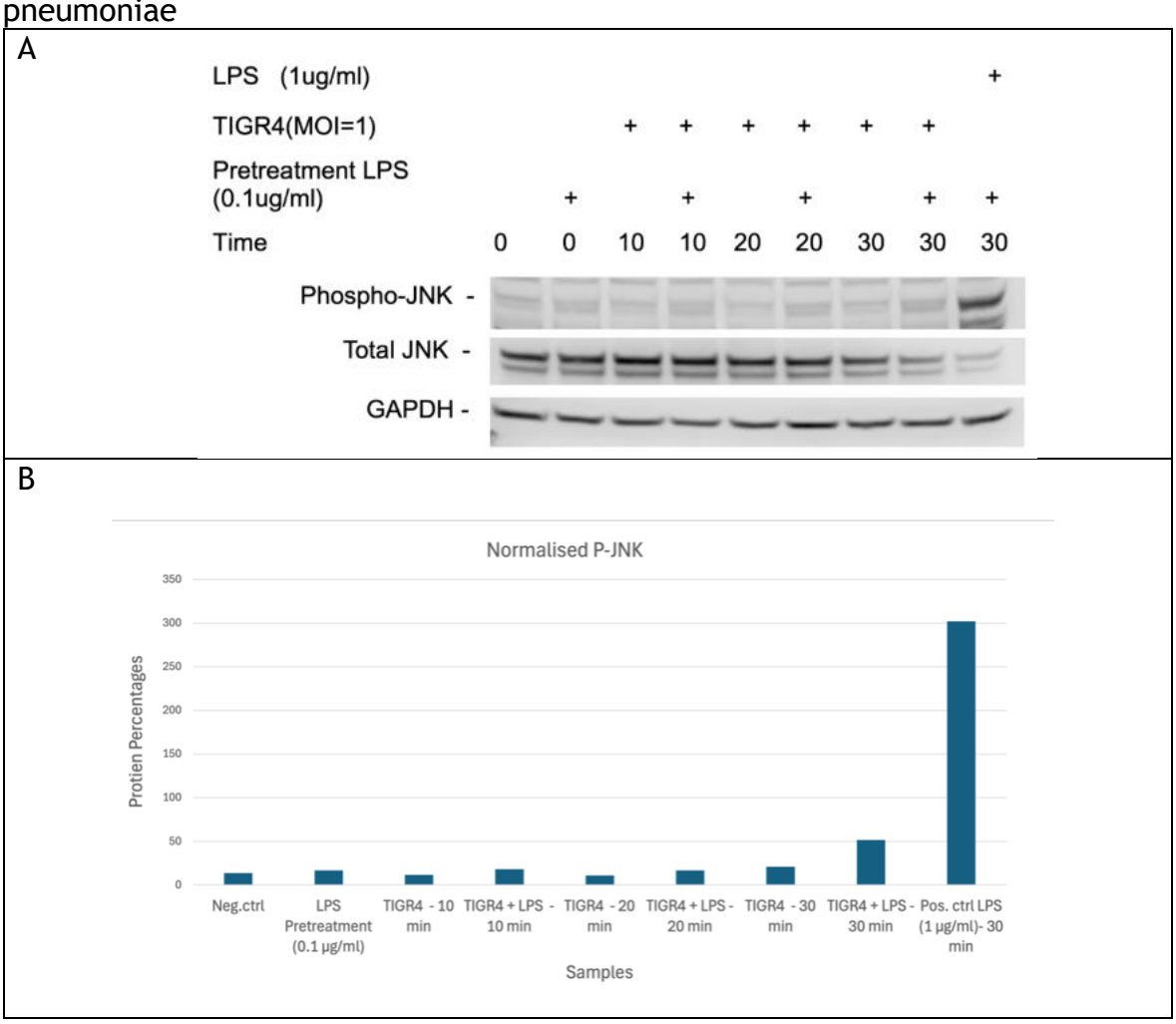


Figure 6-5 Western blot detection of P-JNK in U937 cells.
Panel A: Blot images of P-JNK in U937 cells. Proteins were extracted from U937 cells. The cells were cultured in antibiotic-free media and primed with PMA (100 ng/ml). Some of the cells were pre-treated with LPS (0.1 µg/ml) for 4 hours. The next day, some of the cells were infected with *Streptococcus pneumoniae* TIGR4 (MOI=1). Membranes were probed with [Phospho-SAPK/JNK (Thr183/Tyr185) (81E11) Rabbit mAb] to detect P-JNK and [SAPK/JNK Antibody] to detect JNK at different time points of 0, 10, 20 and 30 minutes. Blot image developed by iStudio software. Panel B: Normalised phosphorylated JNK (p- JNK) protein levels under the same conditions.

In an attempt to prevent any baseline activation of these proteins, the cells were cultured in serum-free medium before infection.

The blot doesn't show a band in the negative control lane, which could be due to the above reason. Also, the blot shows slightly darker bands in lanes corresponding to pre-treated infected cells compared to bands in lanes of infected non-treated cells and the lane of pre-treated non-infected cells. this effect is seen at 20- and 30-minutes following infection. This could indicate that LPS pretreatment increases the phosphorylation of JNK protein (Figure 6-6).

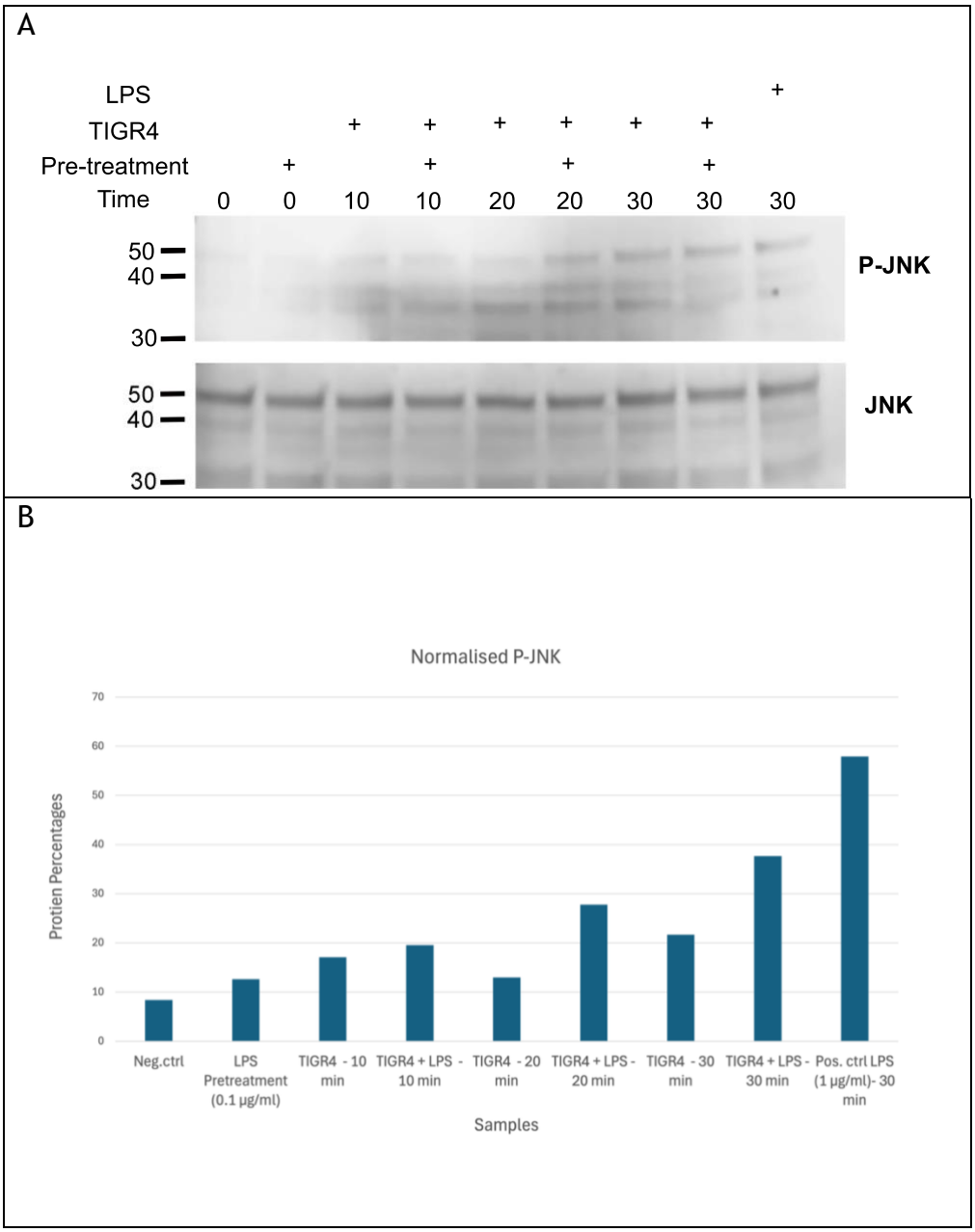


Figure 6-6 Western blot detection of P-JNK in U937 cells.
Panel A: Blot images of P-JNK in U937 cells. Proteins were extracted from U937 cells. the cells were cultured in antibiotic-free media and primed with PMA (100 ng/ml). Some of the cells were pre-treated with LPS (0.1 µg/ml) for 4 hours. The next day, some of the cells were infected with *Streptococcus pneumoniae* TIGR4 (MOI=1). Membranes were probed with [Phospho-SAPK/JNK (Thr183/Tyr185) (81E11) Rabbit mAb] to detect P-JNK and [SAPK/JNK Antibody] to detect JNK at different time points of 0, 10, 20 and 30 minutes.Blot image developed by iStudio software. Panel B: Normalised phosphorylated JNK (p- JNK) protein levels under the same conditions.

The experiment was repeated. The blot shows slightly darker bands in the lanes corresponding to pre-treated infected cells compared to the bands in the lanes of infected non-treated cells and the lanes of pre-treated non-infected cells. This effect is seen 10- and 20 minutes following infection. This could indicate that LPS pretreatment increases the phosphorylation of the JNK protein (Figure 6-7). Regarding the inactive JNK protein, there appear to be double bands in the experimental lanes (Figure 6-7). In Qin et al, LPS stimulation (10 µg/ml) of hCMEC/D3 cells led to significantly increased phosphorylation of JNK at 15, and 30 minutes following treatment [160]. This goes with these results, keeping in mind the differences in LPS concentrations. However, unlike the previous experiment, there is an increase in phosphorylation at 10 minutes. It is not clear why this increase wasn't observed in the previous experiment. Despite identical experimental conditions, minor variability in phosphorylation levels between replicate experiments was observed. These differences likely stem from the inherent biological and technical variability associated with Western blotting and the transient nature of phosphorylation signalling events.

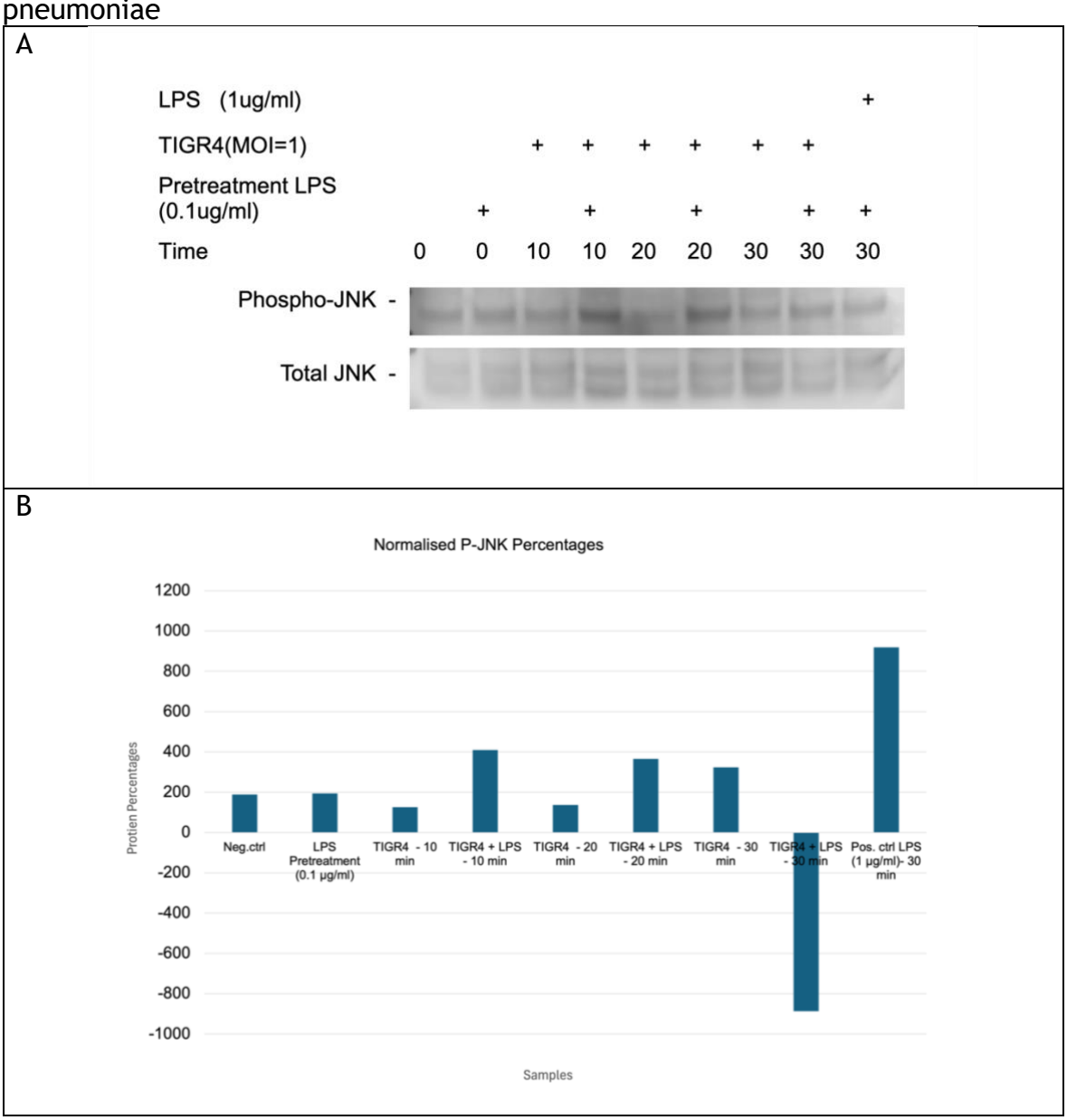


Figure 6-7 Western blot detection of P-JNK in U937 cells.
Panel A: Blot images of P-JNK in U937 cells.Proteins were extracted from U937 cells. the cells were cultured in antibiotic-free media and primed with PMA (100 ng/ml). Some of the cells were pre-treated with LPS (0.1 µg/ml) for 4 hours. The next day, some of the cells were infected with *Streptococcus pneumoniae* TIGR4 (MOI=1). Membranes were probed with [Phospho-SAPK/JNK (Thr183/Tyr185) (81E11) Rabbit mAb] to detect P-JNK, and [SAPK/JNK Antibody] to detect JNK at different time points of 0, 10, 20 and 30 minutes. Blot image developed by iStudio software. Panel B: Normalised phosphorylated JNK (p- JNK) protein levels under the same conditions.

6.3 Conclusion

In this chapter, we analysed the cellular proteins involved in the LPS pretreatment effect that causes U937 macrophages to produce more TNF-α following streptococcal infection. Pilot experiments (not shown) have suggested that this role is mediated by Toll-like receptor 4. Therefore, the cellular proteins in the TLR4 signalling pathway were chosen for examination regarding their role using gel electrophoresis and Western blotting. The blots were probed with the antibody for the protein of choice and examined using a scanner. Unfortunately, many proteins examined did not show a difference in phosphorylation between pre-treated infected cells and infected-only cells, such as P-IκBα, P-IRAK1, P-P38, P-IKKα/ß, and P-ERK, and some did not show bands at all. The results showing no difference in bands can be attributed to the use of the cell line in these experiments. Results may differ if the same experiments were conducted using primary cells.

However, blots examined for P-JNK protein showed darker bands in the pre-treated infected cell lanes compared to the lanes of infected cells without pre-treatment. This was seen at times 10, 20 and 30 minutes throughout three separate experiments with the same conditions. This suggests that LPS pre-treatment plays a role in stimulating TNF-α production through phosphorylation and activation of MAP kinase (JNK). This may indicate that the increase in TNF-α production is mediated by the AP-1 transcription factor, which is induced by JNK MAP kinase; however, further testing is required to verify this effect. Unfortunately, due to time constraints, this could not be done.

7 Conclusion

This thesis aimed to examine the innate immune response elements against *Streptococcus pneumoniae* bacteria. Initially, the focus was on designing a model to study the release of cytokines following infection.

Based on the several negative results of IFNs secretion, it seems that *Streptococcus pneumoniae* TIGR4 infection does not induce type I IFNs secretion but has a pro-inflammatory effect in producing TNF-α and IL-1β [184].

The initial focus of the thesis was placed on investigating interferon production following *Streptococcus pneumoniae* infection. However, consistent negative results were obtained with regard to the interferons, indicating that this pathway might not be significantly involved or detectable under the experimental conditions used.

At first, several experiments were done to assess interferon levels following pneumococcal infection. The experiment results came back negative, regardless of the cell type used: monocyte-derived dendritic cells, macrophages, the BEAS-2B bronchial epithelial cell line, or the U937 monocyte-derived macrophage cell line. Therefore, we conclude that *Streptococcus pneumoniae* infection doesn't lead to the production of type 1 Interferons in the infection model that was studied.

There are some papers in the literature that showed some positive results regarding streptococcus infection and type 1 IFNs. However, it is essential to highlight some key differences in these studies and this thesis.

As mentioned in chapter three, one of these are Nakamura et al. they reported that nasopharyngeal colonisation of mice with Pneumococcus induced type I IFNs[170]. However, there are major differences in the infection models between the mentioned study and this thesis. First, their infection model consisted of pneumococcus along with Influenza A virus, which may be the main reason behind the IFNs induction, since IFNs are highly produced in viral infections. Also, their research was done using in vivo mice infection model rather than in vitro human cells, which may have caused the differences in

results. As in vivo models carry out complexity of multicellular interaction. Finally, it is important to note that the strain employed in this study was P1121,a strain that belongs to serotype 6A. that is different from TIGR4, a highly virulent strain of *S. pneumoniae* belonging to serotype 4, that was used in this thesis.

This is a similar infection model to the one used in Zangari et al. [185], which states that mice that were infected with *Streptococcus pneumoniae* TIGR4 (or T4), along with Influenza A virus, showed elevated levels of interferon-stimulated genes (ISGs), such as Ifit2, Mx1, and Oasl2, between 2 and 21 days post-infection. Similar to Nakamura, the infection model used is in vivo mice model, and the infection wasn't purely bacterial but rather a co-infection of streptococcus and flu virus. Another important point to mind is that this research showed increased expression of IFN stimulated genes 2 days after infection. Not the secretion of type I IFNs.

While Zangari et al. demonstrated activation of type I interferon signaling during pneumococcal colonization, this was primarily evidenced by induction of interferon-stimulated genes and dependence on IFNAR signaling in vivo rather than by direct quantification of secreted IFN-α/ß protein levels. Therefore, the presence of IFN signaling at the tissue level does not necessarily imply robust extracellular IFN protein accumulation detectable by ELISA in isolated in vitro systems. On another note, isolated in vitro systems as used in this thesis lack the complex tissue context and often fail to show detectable IFN-I protein secretion despite engagement of innate sensing pathways.

In another paper, parker et al[178], showed that *streptococcus pneumoniae* infection in mice lung tissue lead to induced IFN beta expression. Also, that IFN beta expression was dependant on pneumolysin toxin presence and on cytosolic DNA sensors STING-TBK1-IRF3.however it is important to note that type I IFN activation in response to *Streptococcus pneumoniae* was assessed primarily by measuring IFN-ß mRNA induction and downstream STAT signalling in mouse lung tissue, rather than quantifying secreted IFN-α/ß protein levels by ELISA. This difference in methodology may explain why robust transcriptional signatures are reported in the literature even when secreted IFN proteins remain below detection in in vitro ELISA assays.

The paper showed its positive results in in vivo models, while when they used in vitro monocellular model that consisted of A549 epithelial cells, they didn't show induced IFN beta expression, which may explain why the use of the BEAS-2B bronchial epithelial cell line didn't produce any IFNs post-infection. Phagocytic activity is essential for IFN response, and capsule-deficient strains may lead to better expression of IFNs than capsule-thick strains such as TIGR4. The strain that was used in their study is D39, a strain with a less thick capsule compared to TIGR4.

Similar results were seen in Koppe et al [78]. it demonstrated STING-dependent IFNB transcription in macrophages following infection with S. pneumoniae D39, primarily assessed at the mRNA level at early timepoints. In contrast, my work measured secreted IFN-α/β protein by ELISA across multiple human cell types following infection with TIGR4. While Koppe et al. observed transcriptional induction in murine macrophages, epithelial cells did not produce type I IFN independently, and protein secretion was not the primary endpoint. The absence of detectable IFN protein in our system may therefore reflect species differences, strain-specific uptake characteristics, and the low magnitude and transient nature of pneumococcal type I IFN responses.

Second, the aim was to examine if these different cells produced pro-inflammatory cytokines such as TNF-α and IL-1β. With MDDCs, the cells produced TNF-α following infection.

Next, the study focused on the phenomenon of trained immunity, which involved using innate immune stimulants β-glucan, zymosan, and LPS on different cell types infected with *Streptococcus pneumoniae* to test whether pre-treatments affected pro-inflammatory cytokine levels following infection.

Overall, the primary cells' results were not consistent. This could be attributed to donor variability, as the cells were sourced from different donors in each experiment. Also, we experienced some problems with standardizing the TIGR4 count across different experiments despite following the same culture protocol. So, these two factors must be taken into consideration when reviewing these results.

With MDDCs, it appears that ß glucan boosts TNF-α and IL-1ß secretion. However, this effect seems to be mainly due to the pre-treatment rather than a combined effect of pre-treatment and infection. When MDDCs were pre-treated with zymosan, it seemed to boost TNF-α and IL-1ß levels. However, it's difficult to conclude if this effect is due to trained immunity because of mixed results. Furthermore, LPS seems to stimulate TNF-α and IL-1ß secretion in MDDCs possibly due to the trained immunity effect.

Next, another cell model was used: primary monocyte-derived macrophages. Results are difficult to analyze due to their lack of consistency and reproducibility. However, at MOI=1, pre-treatments seemed to induce TNF-α levels because of a combined effect of infection and pre-treatment, this is more likely to be due to adjuvant amplified immune response rather than trained immunity.

Afterwards, U937 cells were used. Several pilot experiments were done. Initially, the PMA role was assessed, and it was found that priming the cells with PMA was essential for them to produce cytokines following infection. Another pilot experiment was done to assess priming with GM-CSF versus priming with PMA, in which it was found that there was no significant overall difference between these two priming reagents and IL-1ß secretion following infection. Next, the order of priming and pre-treatment was examined to test if the order affects pro-inflammatory cytokine release following *Streptococcus* infection. In the case of ß glucan and zymosan pre-treatments, the order of priming and pretreatment did not produce a significant difference in TNF-α and IL-1ß levels. However, with LPS, it appears that priming cells with PMA and pre-treating them with LPS simultaneously produces the most TNF-α. But not IL-1ß. Eventually, it was decided to pre-treat with the above stimulants and prime the cells with PMA at the same time.

All pre-treatments appeared to stimulate TNF-α and IL-1ß due to a combined effect of pre-treatment and infection 24 hours post-infection, which indicates a delayed effect on cytokine release. This is expected, since priming the cells with either ß-glucan, zymosan, and LPS leads to NF-κB signalling, that induce the transcription of TNFα + pro IL1ß. And with the use of the strain TIGR4 that has pneumolysin, the pneumolysin can provide a second signal to promote mature IL-

1β secretion, so this combination provides higher TNF α and IL-1β at 24 h[251-253].

Furthermore, another trial experiment was conducted in which different concentrations of pre-treatments were used to check if varying concentrations produced different effects on cytokine release. The different concentrations did not show a difference in IL-1β levels. However, for TNF-α levels, there appears to be a variation; zymosan and LPS concentrations of 0.1µg/ml seem to induce the highest TNF-α levels. Therefore, these concentrations were chosen for pre-treatment. The ß-glucan stimulation resulted in similar TNF-α production across concentrations, suggesting early receptor saturation of Dectin-1 signaling[254]. In contrast, zymosan and LPS exhibited classical dose-dependent TNF-α responses consistent with TLR-mediated NF-κB activation[255, 256]. IL-1β levels were not affected by concentration changes, likely reflecting the requirement for inflammasome activation (Signal 2) rather than transcriptional priming alone, which was not done as this trail experiment didn't include the infection with TIGR4 that has pneumolysin, a potent activator of signal two[253], nor did I use ATP an activator of the second signal, so understandably there wasn't a significant difference in Il-1b concentrations between the different samples.

After establishing the increased TNF-α production following pre-treatment with LPS (0.1µg/ml) for 4 hours, resting the cells for 24 hours, and later infecting them with *Streptococcus pneumoniae*. This observed effect can be attributed to trained immunity or adjuvant stimulatory effect. Because of the short rest period, it is hard to make a distinction between the two. While epigenetic effect can occur in one day, usually to establish trained immunity, the rest period has to be within 3-7 days before the secondary challenge. This wasn't done in this thesis due to concerns regarding cell viability. So, based on the experimental conditions used, the detected increase in TNF-α in pre-treated, infected cells suggests innate immune priming. Because of the short rest period between priming and challenge, this is more consistent with a short-term adjuvant or priming effect rather than established trained immunity. In papers that studied trained immunity, Netea et al showed that trained immunity involves H3K4me3 and H3K27ac chromatin modifications, which are typically demonstrated after 3–7 days of rest, which was not done in this thesis[95]. Also, in Quintin et al, a similar rest period was employed, 6 days[100]

After observing the effect of zymosan and LPS pre-treatments on inducing innate immunity, we wanted to examine whether LPS pre-treatment affects TNF-α mRNA production. The results suggest that LPS-pretreated non-infected cells produced 30 times more TNF-α mRNA than the negative control. However, this amount of tumour necrosis factor mRNA is similar to the TNF-α mRNA produced by the pre-treated infected cells 6 hours after infection. Although previous ELISA results indicated that pre-treated infected cells produced more TNF-α than pre-treated non-infected cells, this could suggest that *Streptococcus* works to enhance TNF-α production in a post-transcriptional manner, allowing the TNF-α mRNA to be translated. This can be done through a variety of methods, including alternative mRNA splicing and RNA stability control.

Afterwards, we wanted to investigate whether LPS pre-treatment leads to an epigenetic change in the TNF-α gene. Numerous articles indicated that epigenetic changes, not genetic ones, cause trained immunity. Two regions of the TNF-α gene were investigated for DNA methylation following LPS pre-treatment and *Streptococcus* infection: the promoter region and the first exon region. In the promoter region, the overall methylation data appear to vary between different CpG sites. Still, there is an overall increase in methylation in the promoter region of the pretreated infected samples compared to the infected non-treated cells and also increased methylation in pretreated infected samples compared to pretreated samples. Increased methylation at the gene promoter region is associated with gene silencing but can also result in increased binding of certain transcription factors. This may account for the increased levels of TNF mRNA seen with pretreatment, and the increased TNF-α protein production detected by ELISA at 6 and 24 hours after infection. Further experiments will be required to investigate this further.

Another possible explanation for these results is that LPS induces the TNF-α mRNA transcription, and at the same time, it causes promoter methylation as a method of control. Infection increases the promoter methylation further, and this helps to regulate the TNF-α expression. It is also important to note that increased promoter methylation won't lead to lower protein levels immediately. That's because DNA methylation is slower in effect than acute transcriptional activation[257]. Acute TNF-α production is driven by transcription factor activation [258], and pre-existing chromatin accessibility [259]. In pacis et al

they found that gene activation occurred before DNA methylation[260]. So, this suggests that DNA methylation represents a delayed regulatory mechanism or immune tolerance.

Analysis of methylation results in the second amplicon studied, which is the TNF-α first exon region, showed that LPS pretreatment exhibited a higher degree of methylation in the first exon region compared to the TNF-α promoter. This goes with LPS pretreatment, causing transcriptional repression of TNF-α.

The methylation data of pretreated infected cells showed slightly less methylation compared to pretreated cells. It also showed less methylation in the first exon compared to the promoter; this may suggest a regulatory mechanism or delayed effect.

In the last results chapter, an attempt was made to determine the cellular proteins by which LPS pre-treatment affects TNF-α secretion following infection. Western blotting tests revealed that the following cellular proteins did not show increased phosphorylation following pre-treatment: P-IκBα, P-P38, and P-ERK. There was no difference in the bands between pre-treated infected cells and infected non-pretreated cells. suggesting that NF-κB and these MAPK pathways were not differentially amplified under primed conditions. However, the blots showed increased phosphorylation of JNK MAP kinase in pretreated infected cells compared to infected cells. This effect was observed at 10-, 20-, and 30-minutes following infection, after which it subsided. indicating a possible role for improved AP-1–mediated transcription in the observed increase in TNF-α secretion. Given the rapid kinetics of NF-κB activation and the likelihood of ceiling effects during infection (equal phosphorylation in infected cells and pre-treated infected cells). The lack of difference in P-IκBα does not exclude NF-κB involvement but suggests that augmented TNF-α production is more likely driven by JNK-dependent signalling rather than enhanced NF-κB activation. Or that LPS may increase TNF-α through another added factor to NF-κB, which is AP-1[261].

The increased phosphorylation of JNK in pre-treated infected cells may also suggest that immediate signalling through these factors overrides or precedes the TNF-α promoter methylation.

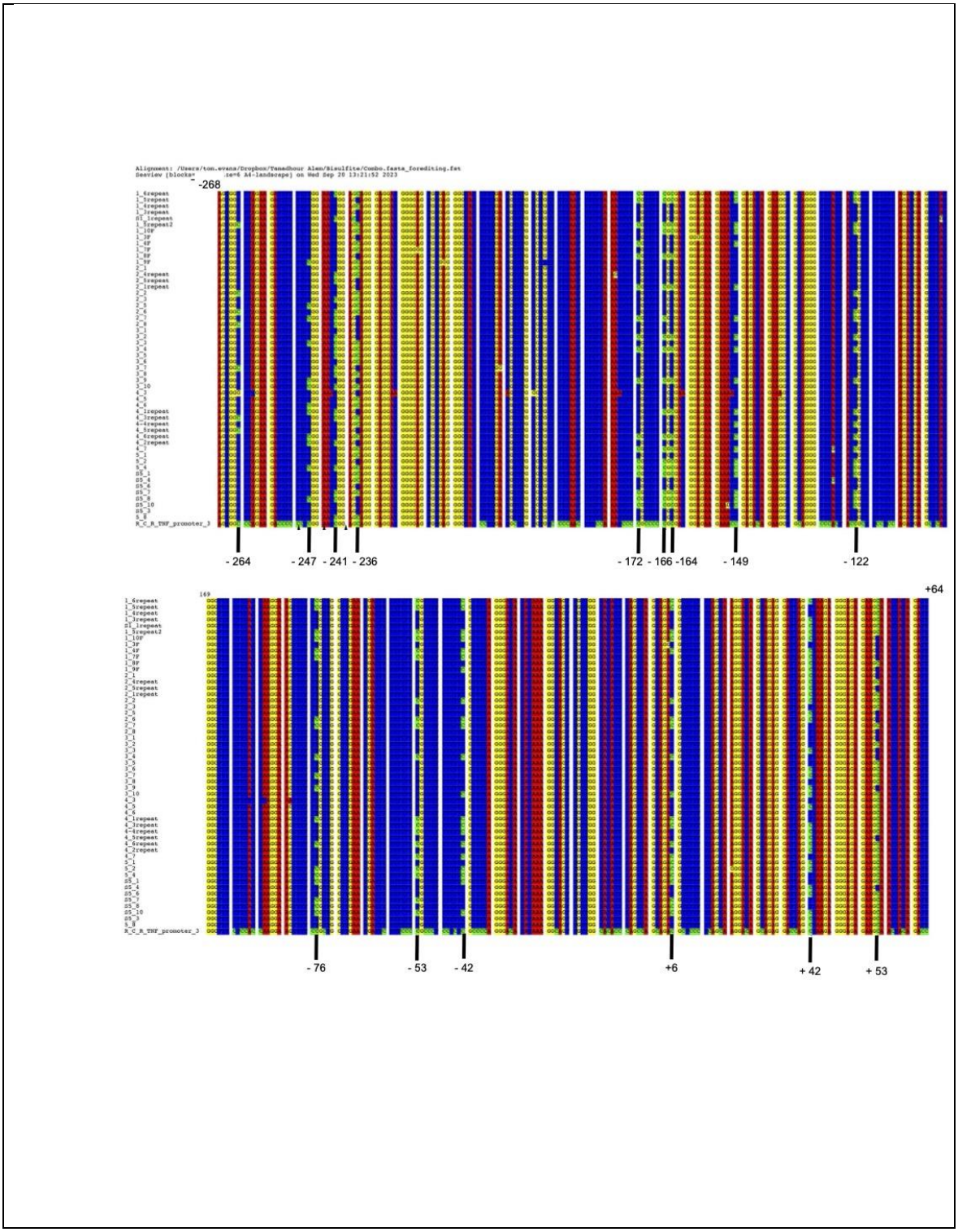
7.1 Future Work

This thesis investigated innate immunity against *Streptococcus pneumoniae,* particularly the phenomenon of trained immunity. It was noted that in U937 cells, pre-treatment with a low dose of LPS resulted in increased TNF-α secretion. In the future, this could be examined in a primary cell line, with additional resources, or in blood samples obtained from a single donor to eliminate donor variability that may have contributed to inconsistent experimental results in primary cells. If such an effect is observed in primary cells, it could serve as a basis for further work in therapeutics as a potential treatment for infections and some cancers. It is essential to involve longer resting periods between the initial stimulus and secondary challenge in these experiments to ensure that the observed effects are attributable to trained immunity rather than an adjuvant stimulatory effect. Additionally, examination of epigenetic changes such as histone modifications and DNA methylation is needed to support the "trained immunity effect", perhaps with the use of epigenetic inhibitors such as histone deacetylases and DNA methyltransferases.

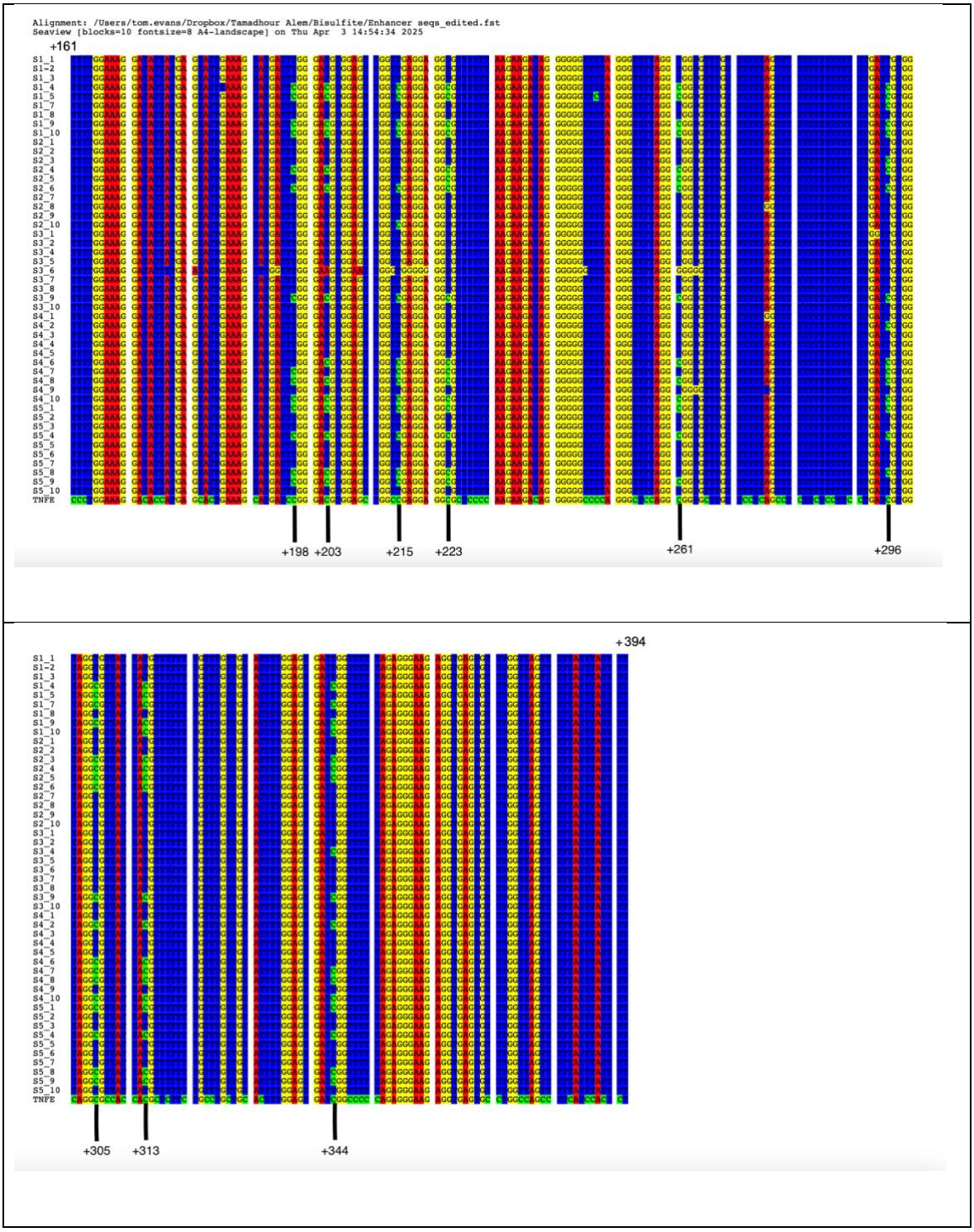
Another aspect for future exploration is to sequence longer segments of the TNF-α gene to determine the methylation levels in its segments following LPS pre-treatment and infection, and to compare these results to LPS pre-treatment without infection. By doing so, hopefully, we'll gain a better and broader understanding of the effect of LPS pre-treatment on TNF-α gene methylation. Additionally, I would like to explore the transcription factors that bind to the TNF-α gene and cause the increased TNF-α mRNA expression and TNF-α secretion following pre-treatment. Hopefully, combining these results could provide a better understanding of the relationship between TNF-α gene methylation and its expression and translation.

Lastly, more experiments can be carried out to determine the intracellular proteins involved in the TNF-α signalling cascade following LPS, and to repeat the western blotting experiments for phosphorylated proteins with a longer serum deprivation time, using primary cells instead of a cell line, more time points such as 2 minutes, as that would probably provide a more accurate picture of the proteins involved. To confirm AP-1 transcription factor involvement, it might be helpful to repeat the experiments with a JNK inhibitor.

Appendices



Appendix 1 TNF a Amplicon 1 Samples Sequences
S1= Negative control: U937 cells without pretreatment or infection
S2=Positive control: U937 cells with LPS (1 µg/ml)
S3=TIGR4: U937 cells infected with *Streptococcus pneumoniae* TIGR4 without prior LPS pretreatment
S4=LPS pretreatment (0.1 µg/ml): U937 cells are pretreated with LPS (0.1 µg/ml) without infection
S5=TIGR4 + LPS pretreatment (0.1 µg/ml): U937 cells are pretreated with LPS (0.1 µg/ml) and infected with *Streptococcus pneumoniae* TIGR4



Appendix 2 TNF a Amplicon 2 Samples Sequences
S1= Negative control: U937 cells without pretreatment or infection
S2=Positive control: U937 cells with LPS (1 µg/ml)
S3=TIGR4: U937 cells infected with *Streptococcus pneumoniae* TIGR4 without prior LPS pretreatment
S4=LPS pretreatment (0.1 µg/ml): U937 cells are pretreated with LPS (0.1 µg/ml) without infection
S5=TIGR4 + LPS pretreatment (0.1 µg/ml): U937 cells are pretreated with LPS (0.1 µg/ml) and infected with *Streptococcus pneumoniae* TIGR4

List of References

1. Ashurst., C.F.D.J.V., *Streptococcus pneumoniae*. 2023, StatPearls Publishing.
2. Nishimoto, A.T., J.W. Rosch, and E.I. Tuomanen, *Pneumolysin: Pathogenesis and Therapeutic Target.* Front Microbiol, 2020. **11**: p. 1543.
3. Chiu, F.F., et al., *Domain 4 of pneumolysin from Streptococcus pneumoniae is a multifunctional domain contributing TLR4 activating and hemolytic activity.* Biochem Biophys Res Commun, 2019. **517**(4): p. 596–602.
4. Kadioglu, A., et al., *Upper and lower respiratory tract infection by Streptococcus pneumoniae is affected by pneumolysin deficiency and differences in capsule type.* Infect Immun, 2002. **70**(6): p. 2886–90.
5. Mitchell, A.M. and T.J. Mitchell, *Streptococcus pneumoniae: virulence factors and variation.* Clin Microbiol Infect, 2010. **16**(5): p. 411–8.
6. Scotland, P.H., *Immunisation and vaccine-preventable diseases quarterly report*
. 2024.
7. Darkwah, S., et al., *Pneumococcal serotypes and their association with death risk in invasive pneumococcal disease: a systematic review and meta-analysis.* Front Med (Lausanne), 2025. **12**: p. 1566502.
8. Hilleringmann, M., et al., *Molecular architecture of Streptococcus pneumoniae TIGR4 pili.* Embo j, 2009. **28**(24): p. 3921–30.
9. Ghaffar, F., I.R. Friedland, and G.H. McCracken, Jr., *Dynamics of nasopharyngeal colonization by Streptococcus pneumoniae.* Pediatr Infect Dis J, 1999. **18**(7): p. 638–46.
10. Wróbel-Pawelczyk, I., et al., *Pneumococcal carriage in unvaccinated children at the time of vaccine implementation into the national immunization program in Poland.* Scientific Reports, 2022. **12**(1): p. 5858.
11. Løvlie, A., et al., *Changes in pneumococcal carriage prevalence and factors associated with carriage in Norwegian children, four years after introduction of PCV13.* BMC Infect Dis, 2020. **20**(1): p. 29.
12. Mitsi, E., et al., *Nasal Pneumococcal Density Is Associated with Microaspiration and Heightened Human Alveolar Macrophage Responsiveness to Bacterial Pathogens.* Am J Respir Crit Care Med, 2020. **201**(3): p. 335–347.
13. CDC. *Pneumococcal Diseases*. Available from: https://www.cdc.gov/pneumococcal/clinicians/clinical-features.html.
14. Østergaard, C., H.B. Konradsen, and S. Samuelsson, *Clinical presentation and prognostic factors of Streptococcus pneumoniae meningitis according to the focus of infection.* BMC Infect Dis, 2005. **5**: p. 93.
15. CDC. *pneumococcal disease surveillance*. Available from: https://www.cdc.gov/pneumococcal/surveillance.html.
16. Jones, R.N., et al., *Update on antimicrobial susceptibility trends among Streptococcus pneumoniae in the United States: report of ceftaroline activity from the SENTRY Antimicrobial Surveillance Program (1998-2011).* Diagn Microbiol Infect Dis, 2013. **75**(1): p. 107–9.
17. Henriques-Normark, B. and E.I. Tuomanen, *The pneumococcus: epidemiology, microbiology, and pathogenesis.* Cold Spring Harb Perspect Med, 2013. **3**(7).

18. Urvya Iyer, M., MPH. *Pneumococcal Infections (Streptococcus pneumoniae) Medication*. 2023; Available from: https://emedicine.medscape.com/article/225811-medication?form=fpf.
19. CDC, *DRUG-RESISTANT STREPTOCOCCUS PNEUMONIAE*. 2019.
20. ECDC, *Annual Epidemiological Report for 2019*. 2019.
21. Pletz, M.W., et al., *Pneumococcal vaccines: mechanism of action, impact on epidemiology and adaption of the species.* International Journal of Antimicrobial Agents, 2008. **32**(3): p. 199–206.
22. Rappuoli, R., E. De Gregorio, and P. Costantino, *On the mechanisms of conjugate vaccines.* Proc Natl Acad Sci U S A, 2019. **116**(1): p. 14–16.
23. Timens, W., et al., *Immaturity of the human splenic marginal zone in infancy. Possible contribution to the deficient infant immune response.* J Immunol, 1989. **143**(10): p. 3200–6.
24. CDC. *Adult Immunization Schedule by Age*. 2024; Available from: https://www.cdc.gov/vaccines/schedules/hcp/imz/adult.html.
25. CDC, *Child and Adolescent Immunization Schedule by Age.* 2024.
26. WHO. *Pneumococcal Disease*. Available from: https://www.who.int/teams/health-product-policy-and-standards/standards-and-specifications/vaccine-standardization/pneumococcal-disease#:~:text=Diseases%20caused%20by%20Streptococcus%20pneumoniae,of%20pneumococcal%20disease%20every%20year.
27. Wasserman, M.D., et al., *Clinical and Economic Burden of Pneumococcal Disease Due to Serotypes Contained in Current and Investigational Pneumococcal Conjugate Vaccines in Children Under Five Years of Age.* Infect Dis Ther, 2021. **10**(4): p. 2701–2720.
28. Alqahtani, A.S., et al., *Burden of clinical infections due to S. pneumoniae during Hajj: A systematic review.* Vaccine, 2018. **36**(30): p. 4440–4446.
29. Control, E.C.f.D.P.a., *Invasive pneumococcal disease - Annual Epidemiological Report for 2018.* 2018.
30. Kadambari, S., et al., *Indirect effects of the covid-19 pandemic on childhood infection in England: population based observational study.* Bmj, 2022. **376**: p. e067519.
31. Amin-Chowdhury, Z., et al., *Does the rise in seasonal respiratory viruses foreshadow the return of invasive pneumococcal disease this winter?* Lancet Respir Med, 2022. **10**(1): p. e1–e2.
32. Abel-Santos, E., et al., *List of Contributors*, in *Molecular Medical Microbiology (Second Edition)*, Y.-W. Tang, et al., Editors. 2015, Academic Press: Boston. p. xiii–xviii.
33. Akira, S., S. Uematsu, and O. Takeuchi, *Pathogen recognition and innate immunity*, in *Cell*. 2006: United States. p. 783–801.
34. Mahla, R.S., et al., *Sweeten PAMPs: Role of Sugar Complexed PAMPs in Innate Immunity and Vaccine Biology.* Front Immunol, 2013. **4**: p. 248.
35. Bowie, A. and L.A. O'Neill, *The interleukin-1 receptor/Toll-like receptor superfamily: signal generators for pro-inflammatory interleukins and microbial products.* J Leukoc Biol, 2000. **67**(4): p. 508–14.
36. Tsukidate, T., C.W. Hespen, and H.C. Hang, *Small molecule modulators of immune pattern recognition receptors.* RSC Chem Biol, 2023. **4**(12): p. 1014–1036.
37. Erridge, C., *Endogenous ligands of TLR2 and TLR4: agonists or assistants?* J Leukoc Biol, 2010. **87**(6): p. 989–99.
38. Shimazu, R., et al., *MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4.* J Exp Med, 1999. **189**(11): p. 1777–82.

39. Werts, C., et al., *Leptospiral lipopolysaccharide activates cells through a TLR2-dependent mechanism.* Nat Immunol, 2001. **2**(4): p. 346–52.
40. Echchannaoui, H., et al., *Toll-like receptor 2-deficient mice are highly susceptible to Streptococcus pneumoniae meningitis because of reduced bacterial clearing and enhanced inflammation.* J Infect Dis, 2002. **186**(6): p. 798–806.
41. Espinoza VE, E.P., *StatPearls*. 2024: StatPearls Publishing LLC.
42. Nichols , B.A., D.F. Bainton , and M.G. Farquhar *DIFFERENTIATION OF MONOCYTES : Origin, Nature, and Fate of Their Azurophil Granules.* Journal of Cell Biology, 1971. **50**(2): p. 498–515.
43. Monie, T.P., *Section 2 - Immune Cells and the Process of Pattern Recognition*, in *The Innate Immune System*, T.P. Monie, Editor. 2017, Academic Press. p. 41–82.
44. Ovchinnikov, D.A., *Macrophages in the embryo and beyond: much more than just giant phagocytes.* Genesis, 2008. **46**(9): p. 447–62.
45. Doan, T., *Immunology*. 2008: Wolters Kluwer Health/Lippincott Williams & Wilkins.
46. Guerriero, J.L., *Chapter Three - Macrophages: Their Untold Story in T Cell Activation and Function*, in *International Review of Cell and Molecular Biology*, L. Galluzzi and N.-P. Rudqvist, Editors. 2019, Academic Press. p. 73–93.
47. Julius M Cruse, R.E.L., Huan Wang, *13- MICROBIAL IMMUNITY*, in *Immunology Guidebook*, J.M. Cruse, R.E. Lewis, and H. Wang, Editors. 2004, Academic Press: San Diego. p. 413–420.
48. Sallusto, F. and A. Lanzavecchia, *The instructive role of dendritic cells on T-cell responses.* Arthritis Research & Therapy, 2002. **4**(3): p. S127.
49. Fitzgerald-Bocarsly, P. and D. Feng, *The role of type I interferon production by dendritic cells in host defense.* Biochimie, 2007. **89**(6-7): p. 843–55.
50. Black, R.A., et al., *A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells.* Nature, 1997. **385**(6618): p. 729–33.
51. Probert, L., *TNF and its receptors in the CNS: The essential, the desirable and the deleterious effects.* Neuroscience, 2015. **302**: p. 2–22.
52. van Loo, G. and M.J.M. Bertrand, *Death by TNF: a road to inflammation.* Nature Reviews Immunology, 2023. **23**(5): p. 289–303.
53. Smith, I., *Mycobacterium tuberculosis pathogenesis and molecular determinants of virulence.* Clin Microbiol Rev, 2003. **16**(3): p. 463–96.
54. Flynn, J.L., et al., *Tumor necrosis factor-alpha is required in the protective immune response against Mycobacterium tuberculosis in mice.* Immunity, 1995. **2**(6): p. 561–72.
55. Jang, D.I., et al., *The Role of Tumor Necrosis Factor Alpha (TNF-α) in Autoimmune Disease and Current TNF-α Inhibitors in Therapeutics.* Int J Mol Sci, 2021. **22**(5).
56. Zheng, D., T. Liwinski, and E. Elinav, *Inflammasome activation and regulation: toward a better understanding of complex mechanisms.* Cell Discovery, 2020. **6**(1): p. 36.
57. Evavold, C.L., et al., *The Pore-Forming Protein Gasdermin D Regulates Interleukin-1 Secretion from Living Macrophages.* Immunity, 2018. **48**(1): p. 35–44.e6.
58. Dinarello, C.A., *Interleukin-1 in the pathogenesis and treatment of inflammatory diseases.* Blood, 2011. **117**(14): p. 3720–32.
59. Lopez-Castejon, G. and D. Brough, *Understanding the mechanism of IL-1β secretion.* Cytokine Growth Factor Rev, 2011. **22**(4): p. 189–95.

60. Ben Aribia, M.H., et al., *rIL 2-induced proliferation of human circulating NK cells and T lymphocytes: synergistic effects of IL 1 and IL 2.* J Immunol, 1987. **139**(2): p. 443–51.
61. Kaneko, N., et al., *The role of interleukin-1 in general pathology.* Inflamm Regen, 2019. **39**: p. 12.
62. Dembic, Z., *Chapter 6 - Cytokines of the Immune System: Interleukins*, in *The Cytokines of the Immune System*, Z. Dembic, Editor. 2015, Academic Press: Amsterdam. p. 143–239.
63. Platanias, L.C., *Mechanisms of type-I- and type-II-interferon-mediated signalling.* Nat Rev Immunol, 2005. **5**(5): p. 375–86.
64. Parkin, J. and B. Cohen, *An overview of the immune system.* Lancet, 2001. **357**(9270): p. 1777–89.
65. LeMessurier, K.S., et al., *Type I interferon protects against pneumococcal invasive disease by inhibiting bacterial transmigration across the lung.* PLoS Pathog, 2013. **9**(11): p. e1003727.
66. Shahangian, A., et al., *Type I IFNs mediate development of postinfluenza bacterial pneumonia in mice.* J Clin Invest, 2009. **119**(7): p. 1910–20.
67. Cao, J., et al., *Activation of IL-27 signalling promotes development of postinfluenza pneumococcal pneumonia.* EMBO Mol Med, 2014. **6**(1): p. 120–40.
68. Kudva, A., et al., *Influenza A inhibits Th17-mediated host defense against bacterial pneumonia in mice.* J Immunol, 2011. **186**(3): p. 1666–1674.
69. Lee, B., et al., *Influenza-induced type I interferon enhances susceptibility to gram-negative and gram-positive bacterial pneumonia in mice.* Am J Physiol Lung Cell Mol Physiol, 2015. **309**(2): p. L158–67.
70. Pandey, S., T. Kawai, and S. Akira, *Microbial sensing by Toll-like receptors and intracellular nucleic acid sensors.* Cold Spring Harb Perspect Biol, 2014. **7**(1): p. a016246.
71. Pomerantz, J.L. and D. Baltimore, *NF-kappaB activation by a signaling complex containing TRAF2, TANK and TBK1, a novel IKK-related kinase.* Embo j, 1999. **18**(23): p. 6694–704.
72. Kawai, T. and S. Akira, *The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors.* Nat Immunol, 2010. **11**(5): p. 373–84.
73. Ablasser, A. and Z.J. Chen, *cGAS in action: Expanding roles in immunity and inflammation.* Science, 2019. **363**(6431).
74. Hao, W., et al., *The odyssey of cGAS: From cytosol to nucleus.* Cytokine Growth Factor Rev, 2023. **74**: p. 29–39.
75. Ishikawa, H. and G.N. Barber, *STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling.* Nature, 2008. **455**(7213): p. 674–8.
76. Motwani, M., S. Pesiridis, and K.A. Fitzgerald, *DNA sensing by the cGAS-STING pathway in health and disease.* Nat Rev Genet, 2019. **20**(11): p. 657–674.
77. McWhirter, S.M., et al., *A host type I interferon response is induced by cytosolic sensing of the bacterial second messenger cyclic-di-GMP.* J Exp Med, 2009. **206**(9): p. 1899–911.
78. Koppe, U., et al., *Streptococcus pneumoniae stimulates a STING- and IFN regulatory factor 3-dependent type I IFN production in macrophages, which regulates RANTES production in macrophages, cocultured alveolar epithelial cells, and mouse lungs.* J Immunol, 2012. **188**(2): p. 811–7.
79. Liang, Q., et al., *Crosstalk between the cGAS DNA sensor and Beclin-1 autophagy protein shapes innate antimicrobial immune responses.* Cell Host Microbe, 2014. **15**(2): p. 228–38.

80. Gui, X., et al., *Autophagy induction via STING trafficking is a primordial function of the cGAS pathway.* Nature, 2019. **567**(7747): p. 262–266.
81. Watson, R.O., P.S. Manzanillo, and J.S. Cox, *Extracellular M. tuberculosis DNA targets bacteria for autophagy by activating the host DNA-sensing pathway.* Cell, 2012. **150**(4): p. 803–15.
82. Watson, R.O., et al., *The Cytosolic Sensor cGAS Detects Mycobacterium tuberculosis DNA to Induce Type I Interferons and Activate Autophagy.* Cell Host Microbe, 2015. **17**(6): p. 811–819.
83. Svenning, S. and T. Johansen, *Selective autophagy.* Essays Biochem, 2013. **55**: p. 79–92.
84. Paterson, G.K. and C.J. Orihuela, *Pneumococci: immunology of the innate host response.* Respirology, 2010. **15**(7): p. 1057–63.
85. Tercan, H., et al., *Trained Immunity.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2021. **41**(1): p. 55–61.
86. Divangahi, M., et al., *Trained immunity, tolerance, priming and differentiation: distinct immunological processes.* Nature Immunology, 2021. **22**(1): p. 2–6.
87. Netea, M.G., et al., *Defining trained immunity and its role in health and disease.* Nat Rev Immunol, 2020. **20**(6): p. 375–388.
88. Mitroulis, I., et al., *Modulation of Myelopoiesis Progenitors Is an Integral Component of Trained Immunity.* Cell, 2018. **172**(1-2): p. 147–161.e12.
89. Kaufmann, E., et al., *BCG Educates Hematopoietic Stem Cells to Generate Protective Innate Immunity against Tuberculosis.* Cell, 2018. **172**(1-2): p. 176–190.e19.
90. Allis, C.D. and T. Jenuwein, *The molecular hallmarks of epigenetic control.* Nature Reviews Genetics, 2016. **17**(8): p. 487–500.
91. Wu, C. and J.R. Morris, *Genes, genetics, and epigenetics: a correspondence.* Science, 2001. **293**(5532): p. 1103–5.
92. Smale, S.T., A. Tarakhovsky, and G. Natoli, *Chromatin contributions to the regulation of innate immunity.* Annu Rev Immunol, 2014. **32**: p. 489–511.
93. Dominguez-Andres, J. and M.G. Netea, *Long-term reprogramming of the innate immune system.* J Leukoc Biol, 2019. **105**(2): p. 329–338.
94. Bekkering, S., et al., *Trained Immunity: Reprogramming Innate Immunity in Health and Disease.* Annu Rev Immunol, 2021. **39**: p. 667–693.
95. Netea, M.G., et al., *Trained immunity: A program of innate immune memory in health and disease.* Science, 2016. **352**(6284): p. aaf1098.
96. Katzmarski, N., et al., *Transmission of trained immunity and heterologous resistance to infections across generations.* Nat Immunol, 2021. **22**(11): p. 1382–1390.
97. Kleinnijenhuis, J., et al., *Bacille Calmette-Guerin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes.* Proc Natl Acad Sci U S A, 2012. **109**(43): p. 17537–42.
98. Drummer, C., et al., *Trained Immunity and Reactivity of Macrophages and Endothelial Cells.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2021. **41**(3): p. 1032–1046.
99. van Puffelen, J.H., et al., *Trained Immunity: RoadMap for drug discovery and development.* eLife, 2025. **14**: p. e108465.
100. Quintin, J., et al., *Candida albicans infection affords protection against reinfection via functional reprogramming of monocytes.* Cell Host Microbe, 2012. **12**(2): p. 223–32.
101. Ifrim, D.C., et al., *Trained Immunity or Tolerance: Opposing Functional Programs Induced in Human Monocytes after Engagement of Various*

Pattern Recognition Receptors. Clinical and Vaccine Immunology, 2014. **21**(4): p. 534–545.
102. Hetland, G., et al., *Protective effect of beta-glucan against systemic Streptococcus pneumoniae infection in mice.* FEMS Immunol Med Microbiol, 2000. **27**(2): p. 111–6.
103. Di Luzio, N.R. and D.L. Williams, *Protective effect of glucan against systemic Staphylococcus aureus septicemia in normal and leukemic mice.* Infect Immun, 1978. **20**(3): p. 804–10.
104. Moerings, B.G.J., et al., *Continuous Exposure to Non-Soluble β-Glucans Induces Trained Immunity in M-CSF-Differentiated Macrophages.* Front Immunol, 2021. **12**: p. 672796.
105. Park, H.J., et al., *In Vivo Zymosan Treatment Induces IL15-Secreting Macrophages and KLRG1-Expressing NK Cells in Mice.* Molecules, 2023. **28**(15).
106. Seeley, J.J. and S. Ghosh, *Molecular mechanisms of innate memory and tolerance to LPS.* J Leukoc Biol, 2017. **101**(1): p. 107–119.
107. Novakovic, B., et al., *β-Glucan Reverses the Epigenetic State of LPS-Induced Immunological Tolerance.* Cell, 2016. **167**(5): p. 1354–1368.e14.
108. Redelman-Sidi, G., M.S. Glickman, and B.H. Bochner, *The mechanism of action of BCG therapy for bladder cancer--a current perspective.* Nat Rev Urol, 2014. **11**(3): p. 153–62.
109. Giamarellos-Bourboulis, E.J., et al., *Activate: Randomized Clinical Trial of BCG Vaccination against Infection in the Elderly.* Cell, 2020. **183**(2): p. 315–323.e9.
110. Cardillo, F., et al., *Bacillus Calmette–Guérin Immunotherapy for Cancer.* Vaccines, 2021. **9**(5): p. 439.
111. Mulder, W.J.M., et al., *Therapeutic targeting of trained immunity.* Nat Rev Drug Discov, 2019. **18**(7): p. 553–566.
112. Biering-Sørensen, S., et al., *Early BCG-Denmark and Neonatal Mortality Among Infants Weighing <2500 g: A Randomized Controlled Trial.* Clin Infect Dis, 2017. **65**(7): p. 1183–1190.
113. Ostadrahimi, A., et al., *Effect of beta glucan on white blood cell counts and serum levels of IL-4 and IL-12 in women with breast cancer undergoing chemotherapy: a randomized double-blind placebo-controlled clinical trial.* Asian Pac J Cancer Prev, 2014. **15**(14): p. 5733–9.
114. Liu, Z., C. Han, and Y.X. Fu, *Targeting innate sensing in the tumor microenvironment to improve immunotherapy.* Cell Mol Immunol, 2020. **17**(1): p. 13–26.
115. Zahalka, S., et al., *Trained immunity of alveolar macrophages requires metabolic rewiring and type 1 interferon signaling.* Mucosal Immunol, 2022. **15**(5): p. 896–907.
116. Muñoz, N., et al., *Mucosal administration of flagellin protects mice from Streptococcus pneumoniae lung infection.* Infect Immun, 2010. **78**(10): p. 4226–33.
117. Yang, J. and H. Yan, *TLR5: beyond the recognition of flagellin.* Cellular & Molecular Immunology, 2017. **14**(12): p. 1017–1019.
118. Lafaille, J.J. and M.A. Curotto de Lafaille, *IgE antibodies : generation and function*. Current Topics in Microbiology and Immunology. Vol. 388. 2015: Springer, Berlin/Heidelberg.
119. Moore, K.J., F.J. Sheedy, and E.A. Fisher, *Macrophages in atherosclerosis: a dynamic balance.* Nat Rev Immunol, 2013. **13**(10): p. 709–21.
120. Carpenter, S., et al., *Post-transcriptional regulation of gene expression in innate immunity.* Nat Rev Immunol, 2014. **14**(6): p. 361–76.

121. Medzhitov, R. and T. Horng, *Transcriptional control of the inflammatory response.* Nat Rev Immunol, 2009. **9**(10): p. 692–703.
122. www.yourgenome.org. *DNA Transcription*. Available from: https://www.yourgenome.org/theme/what-is-rna-splicing/.
123. Elkon, R., A.P. Ugalde, and R. Agami, *Alternative cleavage and polyadenylation: extent, regulation and function.* Nat Rev Genet, 2013. **14**(7): p. 496–506.
124. Wang, E.T., et al., *Alternative isoform regulation in human tissue transcriptomes.* Nature, 2008. **456**(7221): p. 470–476.
125. Wells, C.A., et al., *Alternate transcription of the Toll-like receptor signaling cascade.* Genome Biol, 2006. **7**(2): p. R10.
126. Stevenson, B.J., et al., *Use of transcriptome data to unravel the fine structure of genes involved in sepsis.* J Infect Dis, 2003. **187 Suppl 2**: p. S308–14.
127. Iwami, K.I., et al., *Cutting edge: naturally occurring soluble form of mouse Toll-like receptor 4 inhibits lipopolysaccharide signaling.* J Immunol, 2000. **165**(12): p. 6682–6.
128. Jaresová, I., et al., *Kinetics of Toll-like receptor-4 splice variants expression in lipopolysaccharide-stimulated antigen presenting cells of healthy donors and patients with cystic fibrosis.* Microbes Infect, 2007. **9**(11): p. 1359–67.
129. Gray, P., et al., *Identification of a novel human MD-2 splice variant that negatively regulates Lipopolysaccharide-induced TLR4 signaling.* J Immunol, 2010. **184**(11): p. 6359–66.
130. Rao, N., et al., *A novel splice variant of interleukin-1 receptor (IL-1R)-associated kinase 1 plays a negative regulatory role in Toll/IL-1R-induced inflammatory signaling.* Mol Cell Biol, 2005. **25**(15): p. 6521–32.
131. Rabani, M., et al., *Metabolic labeling of RNA uncovers principles of RNA production and degradation dynamics in mammalian cells.* Nature Biotechnology, 2011. **29**(5): p. 436–442.
132. Elkon, R., et al., *Major role for mRNA stability in shaping the kinetics of gene induction.* BMC Genomics, 2010. **11**: p. 259.
133. Lelouard, H., et al., *Regulation of translation is required for dendritic cell function and survival during activation.* J Cell Biol, 2007. **179**(7): p. 1427–39.
134. Kimball, S.R., *Eukaryotic initiation factor eIF2.* Int J Biochem Cell Biol, 1999. **31**(1): p. 25–9.
135. Donnelly, N., et al., *The eIF2α kinases: their structures and functions.* Cell Mol Life Sci, 2013. **70**(19): p. 3493–511.
136. López-Pelaéz, M., et al., *Cot/tpl2-MKK1/2-Erk1/2 controls mTORC1-mediated mRNA translation in Toll-like receptor-activated macrophages.* Mol Biol Cell, 2012. **23**(15): p. 2982–92.
137. Jaramillo, M., et al., *Leishmania repression of host translation through mTOR cleavage is required for parasite survival and infection.* Cell Host Microbe, 2011. **9**(4): p. 331–41.
138. Ivanov, S.S. and C.R. Roy, *Pathogen signatures activate a ubiquitination pathway that modulates the function of the metabolic checkpoint kinase mTOR.* Nat Immunol, 2013. **14**(12): p. 1219–28.
139. Crawford, E.K., et al., *The role of 3' poly(A) tail metabolism in tumor necrosis factor-alpha regulation.* J Biol Chem, 1997. **272**(34): p. 21120–7.
140. Swanson, B.J., et al., *RANTES production by memory phenotype T cells is controlled by a posttranscriptional, TCR-dependent process.* Immunity, 2002. **17**(5): p. 605–15.

141. Hayden, M.S. and S. Ghosh, *NF-κB in immunobiology.* Cell Res, 2011. **21**(2): p. 223–44.
142. Bricker, A.L. and A. Camilli, *Transformation of a type 4 encapsulated strain of Streptococcus pneumoniae.* FEMS Microbiology Letters, 1999. **172**(2): p. 131–135.
143. Padilla, A., et al., *Effects of α-conotoxin ImI on TNF-α, IL-8 and TGF-β expression by human macrophage-like cells derived from THP-1 pre-monocytic leukemic cells.* Scientific Reports, 2017. **7**.
144. di Giovine, F.S., J.A. Symons, and G.W. Duff, *Kinetics of IL1 beta mRNA and protein accumulation in human mononuclear cells.* Immunol Lett, 1991. **29**(3): p. 211–8.
145. Dinarello, C.A., *Proinflammatory cytokines.* Chest, 2000. **118**(2): p. 503–8.
146. Arthur, J.S. and S.C. Ley, *Mitogen-activated protein kinases in innate immunity.* Nat Rev Immunol, 2013. **13**(9): p. 679–92.
147. Stothers, C.L., et al., *β-Glucan Induces Distinct and Protective Innate Immune Memory in Differentiated Macrophages.* J Immunol, 2021. **207**(11): p. 2785–2798.
148. Moorlag, S., et al., *β-Glucan Induces Protective Trained Immunity against Mycobacterium tuberculosis Infection: A Key Role for IL-1.* Cell Rep, 2020. **31**(7): p. 107634.
149. Ciarlo, E., et al., *Trained Immunity Confers Broad-Spectrum Protection Against Bacterial Infections.* The Journal of Infectious Diseases, 2019. **222**(11): p. 1869–1881.
150. Kang, A., et al., *LPS-induced lung tissue-resident trained innate immunity provides differential protection against pneumococci and SARS-CoV-2.* Cell Rep, 2024. **43**(10): p. 114849.
151. Systems, R.D. *TNF a ELISA Duo Set Kit*. Available from: https://www.rndsystems.com/products/human-tnf-alpha-duoset-elisa_dy210#product-datasheets.
152. Systems, R.D., *IL-1 b ELISA Duo Set KIt.*
153. Systems, R.D., *IFN b ELISA Duo Set Kit.*
154. Scientific™, T. *EpiJET Bisulfite Conversion Kit*. Available from: https://www.thermofisher.com/order/catalog/product/K1461?ef_id=Cj0KCQjwkZm_BhDrARIsAAEbX1F8IGIKdsPtjIUzv1ouuCUECLa-LUa_nC_A8sWsOEW-ZPNYOEX0c2kaAnhjEALw_wcB:G:s&s_kwcid=AL!3652!3!606515189508!!!g!!!16893189581!143415109412&cid=bid_sap_rst_r01_co_cp1362_pjt0000_bid00000_0se_gaw_dy_awa_con&gad_source=1&gclid=Cj0KCQjwkZm_BhDrARIsAAEbX1F8IGIKdsPtjIUzv1ouuCUECLa-LUa_nC_A8sWsOEW-ZPNYOEX0c2kaAnhjEALw_wcB.
155. Agilant. *StrataClone PCR Cloning Kits*. Available from: https://www.agilent.com/en/product/polymerase-chain-reaction-%28pcr%29/cloning-pcr-detection-kits/cloning-kits/strataclone-pcr-cloning-kits-785866.
156. Qiagen. *QIAprep Spin Miniprep Kit – Plasmid Purification*. Available from: https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/plasmid-dna/qiaprep-spin-miniprep-kit.
157. Yang, F., et al., *IKKβ Plays an Essential Role in the Phosphorylation of RelA/p65 on Serine 536 Induced by Lipopolysaccharide1.* The Journal of Immunology, 2003. **170**(11): p. 5630–5635.

158. Wang, W., et al., *Role of TLR4-p38 MAPK-Hsp27 signal pathway in LPS-induced pulmonary epithelial hyperpermeability.* BMC Pulmonary Medicine, 2018. **18**(1): p. 178.
159. Guha, M., et al., *Lipopolysaccharide activation of the MEK-ERK1/2 pathway in human monocytic cells mediates tissue factor and tumor necrosis factor α expression by inducing Elk-1 phosphorylation and Egr-1 expression: Presented in abstract form at the 42nd annual meeting of the American Society of Hematology, December 1-5, 2000, San Francisco, CA.* Blood, 2001. **98**(5): p. 1429–1439.
160. Qin, L.-h., et al., *LPS Induces Occludin Dysregulation in Cerebral Microvascular Endothelial Cells via MAPK Signaling and Augmenting MMP-2 Levels.* Oxidative Medicine and Cellular Longevity, 2015. **2015**(1): p. 120641.
161. Scientific™, T. *Pierce BCA Protein Assay Kit* Available from: https://www.thermofisher.com/order/catalog/product/23225.
162. Thermofisher. *PureLink RNA Mini Kit*. Available from: https://www.thermofisher.com/sa/en/home/life-science/dna-rna-purification-analysis/rna-extraction/rna-types/total-rna-extraction/purelink-rna-mini-kit.html.
163. Biosystems™, A. *High-Capacity RNA-to-cDNA™ Kit*. Available from: https://www.thermofisher.com/order/catalog/product/4387406.
164. Biosystems™, A. *PowerUp™ SYBR™ Green Master Mix for qPCR*. Available from: https://www.thermofisher.com/order/catalog/product/A25741.
165. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. **25**(4): p. 402–8.
166. Richardson, J.T., *The analysis of 2 × 2 contingency tables--yet again.* Stat Med, 2011. **30**(8): p. 890; author reply 891–2.
167. Campbell, I., *Chi-squared and Fisher-Irwin tests of two-by-two tables with small sample recommendations.* Stat Med, 2007. **26**(19): p. 3661–75.
168. Szylar, G., et al., *A novel Streptococcus pneumoniae human challenge model demonstrates Treg lymphocyte recruitment to the infection site.* Scientific Reports, 2022. **12**(1): p. 3990.
169. Damjanovic, D., et al., *Type 1 interferon gene transfer enhances host defense against pulmonary Streptococcus pneumoniae infection via activating innate leukocytes.* Molecular Therapy Methods & Clinical Development, 2014. **1**.
170. Nakamura, S., K.M. Davis, and J.N. Weiser, *Synergistic stimulation of type I interferons during influenza virus coinfection promotes Streptococcus pneumoniae colonization in mice.* J Clin Invest, 2011. **121**(9): p. 3657–65.
171. Liu, Y.J., *IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors.* Annu Rev Immunol, 2005. **23**: p. 275–306.
172. Pestka, S., et al., *INTERFERONS AND THEIR ACTIONS.* Annual Review of Biochemistry, 1987. **56**(Volume 56, 1987): p. 727–777.
173. McNab, F., et al., *Type I interferons in infectious disease.* Nat Rev Immunol, 2015. **15**(2): p. 87–103.
174. Auerbuch, V., et al., *Mice lacking the type I interferon receptor are resistant to Listeria monocytogenes.* J Exp Med, 2004. **200**(4): p. 527–33.
175. Manca, C., et al., *Virulence of a Mycobacterium tuberculosis clinical isolate in mice is determined by failure to induce Th1 type immunity and is*

associated with induction of IFN-alpha /beta. Proc Natl Acad Sci U S A, 2001. **98**(10): p. 5752–7.
176. Watanabe, T., et al., *NOD1 contributes to mouse host defense against Helicobacter pylori via induction of type I IFN and activation of the ISGF3 signaling pathway.* J Clin Invest, 2010. **120**(5): p. 1645–62.
177. Mancuso, G., et al., *Type I IFN signaling is crucial for host resistance against different species of pathogenic bacteria.* J Immunol, 2007. **178**(5): p. 3126–33.
178. Parker, D., et al., *Streptococcus pneumoniae DNA initiates type I interferon signaling in the respiratory tract.* mBio, 2011. **2**(3): p. e00016–11.
179. Weigent, D.A., et al., *Role of interferon in streptococcal infection in the mouse.* Microb Pathog, 1986. **1**(4): p. 399–407.
180. Kelly-Scumpia, K.M., et al., *Type I interferon signaling in hematopoietic cells is required for survival in mouse polymicrobial sepsis by regulating CXCL10.* J Exp Med, 2010. **207**(2): p. 319–26.
181. Li, W., B. Moltedo, and T.M. Moran, *Type I interferon induction during influenza virus infection increases susceptibility to secondary Streptococcus pneumoniae infection by negative regulation of γδ T cells.* J Virol, 2012. **86**(22): p. 12304–12.
182. Hsi, E.D. and D.G. Remick, *Monocytes are the major producers of interleukin-1 beta in an ex vivo model of local cytokine production.* J Interferon Cytokine Res, 1995. **15**(1): p. 89–94.
183. Grahames, C.B., et al., *Pharmacological characterization of ATP- and LPS-induced IL-1beta release in human monocytes.* Br J Pharmacol, 1999. **127**(8): p. 1915–21.
184. Houldsworth, S., P.W. Andrew, and T.J. Mitchell, *Pneumolysin stimulates production of tumor necrosis factor alpha and interleukin-1 beta by human mononuclear phagocytes.* Infect Immun, 1994. **62**(4): p. 1501–3.
185. Zangari, T., et al., *Type I Interferon Signaling Is a Common Factor Driving Streptococcus pneumoniae and Influenza A Virus Shedding and Transmission.* mBio, 2021. **12**(1): p. 10.1128/mbio.03589–20.
186. Bahl, A., et al., *Infection-induced trained immunity: a twist in paradigm of innate host defense and generation of immunological memory.* Infection and Immunity, 2025. **93**(1): p. e00472–24.
187. Monticelli, S. and G. Natoli, *Short-term memory of danger signals and environmental stimuli in immune cells.* Nat Immunol, 2013. **14**(8): p. 777–84.
188. Biswas, S.K. and E. Lopez-Collazo, *Endotoxin tolerance: new mechanisms, molecules and clinical significance.* Trends Immunol, 2009. **30**(10): p. 475–87.
189. Foster, S.L., D.C. Hargreaves, and R. Medzhitov, *Gene-specific control of inflammation by TLR-induced chromatin modifications.* Nature, 2007. **447**(7147): p. 972–8.
190. NIH. *FPR1 formyl peptide receptor 1 Homo sapiens (human)*. Available from: https://www.ncbi.nlm.nih.gov/gene/2357#gene-expression.
191. Fan, Y., et al., *Serum amyloid A3 confers protection against acute lung injury in Pseudomonas aeruginosa-infected mice.* Am J Physiol Lung Cell Mol Physiol, 2020. **318**(2): p. L314–l322.
192. Feinberg, A.P., *The Key Role of Epigenetics in Human Disease Prevention and Mitigation.* N Engl J Med, 2018. **378**(14): p. 1323–1334.
193. Alaskhar Alhamwe, B., et al., *Histone modifications and their role in epigenetics of atopy and allergic diseases.* Allergy Asthma Clin Immunol, 2018. **14**: p. 39.

194. Lyakh, L., et al., *Regulation of interleukin-12/interleukin-23 production and the T-helper 17 response in humans.* Immunol Rev, 2008. **226**: p. 112–31.
195. Smale, S.T., *Selective transcription in response to an inflammatory stimulus.* Cell, 2010. **140**(6): p. 833–44.
196. Sen, R. and D. Baltimore, *Inducibility of kappa immunoglobulin enhancer-binding protein Nf-kappa B by a posttranslational mechanism.* Cell, 1986. **47**(6): p. 921–8.
197. Zhang, A., et al., *Mechanisms that lead to the regulation of NLRP3 inflammasome expression and activation in human dental pulp fibroblasts.* Molecular Immunology, 2015. **66**(2): p. 253–262.
198. Mogensen, T.H., et al., *Streptococcus pneumoniae stabilizes tumor necrosis factor α mRNA through a pathway dependent on p38 MAPK but independent of Toll-like receptors.* BMC Immunology, 2008. **9**(1): p. 52.
199. Wallner, S., et al., *Epigenetic dynamics of monocyte-to-macrophage differentiation.* Epigenetics & Chromatin, 2016. **9**(1): p. 33.
200. Jhamat, N., et al., *LPS-treatment of bovine endometrial epithelial cells causes differential DNA methylation of genes associated with inflammation and endometrial function.* BMC Genomics, 2020. **21**(1): p. 385.
201. Vandenbon, A., et al., *Waves of chromatin modifications in mouse dendritic cells in response to LPS stimulation.* Genome Biology, 2018. **19**(1): p. 138.
202. Willeaume, V., et al., *Tumor necrosis factor-alpha production induced by viruses and by lipopolysaccharides in macrophages: similarities and differences.* J Inflamm, 1995. **46**(1): p. 1–12.
203. Suzuki, M.M. and A. Bird, *DNA methylation landscapes: provocative insights from epigenomics.* Nature Reviews Genetics, 2008. **9**(6): p. 465–476.
204. Klose, R.J. and A.P. Bird, *Genomic DNA methylation: the mark and its mediators.* Trends Biochem Sci, 2006. **31**(2): p. 89–97.
205. Heard, E. and C.M. Disteche, *Dosage compensation in mammals: fine-tuning the expression of the X chromosome.* Genes Dev, 2006. **20**(14): p. 1848–67.
206. Mohandas, T., R.S. Sparkes, and L.J. Shapiro, *Reactivation of an inactive human X chromosome: evidence for X inactivation by DNA methylation.* Science, 1981. **211**(4480): p. 393–6.
207. Venolia, L., et al., *Transformation with DNA from 5-azacytidine-reactivated X chromosomes.* Proc Natl Acad Sci U S A, 1982. **79**(7): p. 2352–4.
208. Hellman, A. and A. Chess, *Gene body-specific methylation on the active X chromosome.* Science, 2007. **315**(5815): p. 1141–3.
209. Laird, P.W. and R. Jaenisch, *The role of DNA methylation in cancer genetic and epigenetics.* Annu Rev Genet, 1996. **30**: p. 441–64.
210. Brenet, F., et al., *DNA methylation of the first exon is tightly linked to transcriptional silencing.* PLoS One, 2011. **6**(1): p. e14524.
211. Chevalier-Mariette, C., et al., *CpG content affects gene silencing in mice: evidence from novel transgenes.* Genome Biology, 2003. **4**(9): p. R53.
212. Fazzari, M.J. and J.M. Greally, *Epigenomics: beyond CpG islands.* Nat Rev Genet, 2004. **5**(6): p. 446–55.
213. Josse, J., A.D. Kaiser, and A. Kornberg, *Enzymatic synthesis of deoxyribonucleic acid. VIII. Frequencies of nearest neighbor base sequences in deoxyribonucleic acid.* J Biol Chem, 1961. **236**: p. 864–75.
214. Gardiner-Garden, M. and M. Frommer, *CpG islands in vertebrate genomes.* J Mol Biol, 1987. **196**(2): p. 261–82.

215. Takai, D. and P.A. Jones, *Comprehensive analysis of CpG islands in human chromosomes 21 and 22.* Proc Natl Acad Sci U S A, 2002. **99**(6): p. 3740–5.
216. Larsen, F., et al., *CpG islands as gene markers in the human genome.* Genomics, 1992. **13**(4): p. 1095–1107.
217. Bird, A., *DNA methylation patterns and epigenetic memory.* Genes Dev, 2002. **16**(1): p. 6–21.
218. Zhang, Y., et al., *DNA methylation analysis of chromosome 21 gene promoters at single base pair and single allele resolution.* PLoS Genet, 2009. **5**(3): p. e1000438.
219. Meissner, A., et al., *Genome-scale DNA methylation maps of pluripotent and differentiated cells.* Nature, 2008. **454**(7205): p. 766–770.
220. Ioshikhes, I.P. and M.Q. Zhang, *Large-scale human promoter mapping using CpG islands.* Nat Genet, 2000. **26**(1): p. 61–3.
221. Sullivan, K.E., et al., *Epigenetic regulation of tumor necrosis factor alpha.* Mol Cell Biol, 2007. **27**(14): p. 5147–60.
222. Bannister, A.J. and T. Kouzarides, *Regulation of chromatin by histone modifications.* Cell Research, 2011. **21**(3): p. 381–395.
223. Bernstein, B.E., et al., *Genomic maps and comparative analysis of histone modifications in human and mouse.* Cell, 2005. **120**(2): p. 169–81.
224. Ramirez-Carrozzi, V.R., et al., *Selective and antagonistic functions of SWI/SNF and Mi-2beta nucleosome remodeling complexes during an inflammatory response.* Genes Dev, 2006. **20**(3): p. 282–96.
225. Heintzman, N.D., et al., *Distinct and predictive chromatin signatures of transcriptional promoters and enhancers in the human genome.* Nat Genet, 2007. **39**(3): p. 311–8.
226. Motif, A.; Available from: https://www.activemotif.com/catalog/695/bisulfite-conversion.
227. Rhoades, K.L., S.H. Golub, and J.S. Economou, *The regulation of the human tumor necrosis factor alpha promoter region in macrophage, T cell, and B cell lines.* J Biol Chem, 1992. **267**(31): p. 22102–7.
228. Medzhitov, R., P. Preston-Hurlburt, and C.A. Janeway, *A human homologue of the Drosophila Toll protein signals activation of adaptive immunity.* Nature, 1997. **388**(6640): p. 394–397.
229. Yin, Y., et al., *Impact of cytosine methylation on DNA binding specificities of human transcription factors.* Science, 2017. **356**(6337).
230. Hu, S., et al., *DNA methylation presents distinct binding sites for human transcription factors.* eLife, 2013. **2**: p. e00726.
231. Connie Rye, R.W., Vladimir Jurukovski, Jean DeSaix, Jung Choi, Yael Avissar, *RNA Processing in Eukaryotes*, in *Biology*. 2016, OpenStax.
232. Swantek, J.L., L. Christerson, and M.H. Cobb, *Lipopolysaccharide-induced tumor necrosis factor-alpha promoter activity is inhibitor of nuclear factor-kappaB kinase-dependent.* J Biol Chem, 1999. **274**(17): p. 11667–71.
233. Kontoyiannis, D., et al., *Impaired on/off regulation of TNF biosynthesis in mice lacking TNF AU-rich elements: implications for joint and gut-associated immunopathologies.* Immunity, 1999. **10**(3): p. 387–98.
234. Han, J., et al., *A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells.* Science, 1994. **265**(5173): p. 808–11.
235. Swantek, J.L., M.H. Cobb, and T.D. Geppert, *Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) is required for lipopolysaccharide stimulation of tumor necrosis factor alpha (TNF-alpha) translation: glucocorticoids inhibit TNF-alpha translation by blocking JNK/SAPK.* Mol Cell Biol, 1997. **17**(11): p. 6274–82.

236. Vaure, C. and Y. Liu, *A comparative review of toll-like receptor 4 expression and functionality in different animal species.* Front Immunol, 2014. **5**: p. 316.
237. Jiang, D., et al., *Regulation of lung injury and repair by Toll-like receptors and hyaluronan.* Nature Medicine, 2005. **11**(11): p. 1173–1179.
238. Tsukamoto, H., et al., *Lipopolysaccharide (LPS)-binding protein stimulates CD14-dependent Toll-like receptor 4 internalization and LPS-induced TBK1-IKKε-IRF3 axis activation.* J Biol Chem, 2018. **293**(26): p. 10186–10201.
239. Shen, H., et al., *Dual Signaling of MyD88 and TRIF Is Critical for Maximal TLR4-Induced Dendritic Cell Maturation1.* The Journal of Immunology, 2008. **181**(3): p. 1849–1858.
240. Pålsson-McDermott, E.M. and L.A.J. O'Neill, *Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4.* Immunology, 2004. **113**(2): p. 153–162.
241. Macflan75. *TLR4 signaling pathways* 2024; Available from: https://en.wikipedia.org/wiki/File:TLR4_signaling_pathways_V2.pdf#file.
242. Aggarwal, B.B. and R.K. Puri, *Human cytokines: their role in disease and therapy.* (No Title), 1995.
243. DeFranco, A.L., et al., *The role of tyrosine kinases and map kinases in LPS-induced signaling.* Progress in clinical and biological research, 1998. **397**: p. 119–136.
244. Whitmarsh, A. and R. Davis, *Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways.* Journal of molecular medicine, 1996. **74**: p. 589–607.
245. Tsai, E.Y., et al., *A lipopolysaccharide-specific enhancer complex involving Ets, Elk-1, Sp1, and CREB binding protein and p300 is recruited to the tumor necrosis factor alpha promoter in vivo.* Mol Cell Biol, 2000. **20**(16): p. 6084–94.
246. Baeuerle, P.A. and D. Baltimore, *NF-kappa B: ten years after.* Cell, 1996. **87**(1): p. 13–20.
247. Pearson, G., et al., *Mitogen-Activated Protein (MAP) Kinase Pathways: Regulation and Physiological Functions*.* Endocrine Reviews, 2001. **22**(2): p. 153–183.
248. Yu, J., et al., *Regulation of Host Immune Responses against Influenza A Virus Infection by Mitogen-Activated Protein Kinases (MAPKs).* Microorganisms, 2020. **8**(7).
249. Segalés, J., E. Perdiguero, and P. Muñoz-Cánoves, *Regulation of Muscle Stem Cell Functions: A Focus on the p38 MAPK Signaling Pathway.* Front Cell Dev Biol, 2016. **4**: p. 91.
250. Kim, E.K. and E.J. Choi, *Pathological roles of MAPK signaling pathways in human diseases.* Biochim Biophys Acta, 2010. **1802**(4): p. 396–405.
251. Huang, H., et al., *Distinct patterns of dendritic cell cytokine release stimulated by fungal beta-glucans and toll-like receptor agonists.* Infect Immun, 2009. **77**(5): p. 1774–81.
252. Jiang, W.G., M.C. Puntis, and M.B. Hallett, *U937 cells stimulated with opsonised zymozan particles provide a convenient laboratory source of tumour necrosis factor alpha.* J Immunol Methods, 1992. **152**(2): p. 201–7.
253. McNeela, E.A., et al., *Pneumolysin Activates the NLRP3 Inflammasome and Promotes Proinflammatory Cytokines Independently of TLR4.* PLOS Pathogens, 2010. **6**(11): p. e1001191.
254. Kanjan, P., et al., *Immune effects of β-glucan are determined by combined effects on Dectin-1, TLR2, 4 and 5.* Journal of Functional Foods, 2017. **37**: p. 433–440.

255. Levy, E., et al., *Distinct Roles of TLR4 and CD14 in LPS-Induced Inflammatory Responses of Neonates.* Pediatric Research, 2009. **66**(2): p. 179–184.
256. Kelly, M.M., et al., *The Lung Responds to Zymosan in a Unique Manner Independent of Toll-Like Receptors, Complement, and Dectin-1.* American Journal of Respiratory Cell and Molecular Biology, 2008. **38**(2): p. 227–238.
257. Cedar, H. and Y. Bergman, *Programming of DNA methylation patterns.* Annu Rev Biochem, 2012. **81**: p. 97–117.
258. Goldfeld, A.E., J.L. Strominger, and C. Doyle, *Human tumor necrosis factor alpha gene regulation in phorbol ester stimulated T and B cell lines.* J Exp Med, 1991. **174**(1): p. 73–81.
259. Smale, S.T. and G. Natoli, *Transcriptional control of inflammatory responses.* Cold Spring Harb Perspect Biol, 2014. **6**(11): p. a016261.
260. Pacis, A., et al., *Bacterial infection remodels the DNA methylation landscape of human dendritic cells.* Genome Res, 2015. **25**(12): p. 1801–11.
261. Hargreaves, D.C., T. Horng, and R. Medzhitov, *Control of inducible gene expression by signal-dependent transcriptional elongation.* Cell, 2009. **138**(1): p. 129–45.